Journal of Thermal Biology 46 (2014) 40–46

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Dietary lecithin potentiates thermal tolerance and cellular stress protection of milk fish (*Chanos Chanos*) reared under low dose endosulfan-induced stress

Neeraj Kumar [a,*], P.S. Minhas [a], K. Ambasankar [b], K.K. Krishnani [a], R.S. Rana [c]

[a] National Institute of Abiotic Stress Management (NIASM), Malegaon, Baramati, Pune 413115, India
[b] Central Institute of Brackishwater Aquaculture (CIBA), Chennai 600028, India
[c] Central Institute of Fisheires Education (CIFE), Mumbai 400061, India

ARTICLE INFO

Article history:
Received 13 July 2014
Received in revised form
10 October 2014
Accepted 14 October 2014
Available online 18 October 2014

Keywords:
Lecithin
Thermal tolerance
Endosulfan
Antioxidative enzymes
Chanos chanos

ABSTRACT

Endosulfan is an organochlorine pesticide commonly found in aquatic environments that has been found to reduce thermal tolerance of fish. Lipotropes such as the food additive, Lecithin has been shown to improve thermal tolerance in fish species. This study was conducted to evaluate the role of lipotropes (lecithin) for enhancing the thermal tolerance of *Chanos chanos* reared under sublethal low dose endosulfan-induced stress. Two hundred and twenty-five fish were distributed randomly into five treatments, each with three replicates. Four isocaloric and isonitrogenous diets were prepared with graded levels of lecithin: normal water and fed with control diet (En0/L0), endosulfan-treated water and fed with control diet (En/L0), endosulfan-treated water and fed with 1% (En/L1%), 1.5% (En/L 1.5%) and 2% (En/L 2%) lecithin supplemented feed. The endosulfan in treated water was maintained at the level of 1/40th of LC_{50} (0.52 ppb). At the end of the five weeks, critical temperature maxima (CTmax), lethal temperature maxima (LTmax), critical temperature minima (CTmin) and lethal temperature minima (LTmin) were Determined. There was a significant ($P < 0.01$) effect of dietary lecithin on temperature tolerance (CTmax, LTmax, CTmin and LTmin) of the groups fed with 1, 1.5 and 2% lecithin-supplemented diet compared to control and endosulfan-exposed groups. Positive correlations were observed between CT max and LTmax ($R^2 = 0.934$) as well as between CTmin and LTmin ($R^2 = 0.9313$). At the end of the thermal tolerance study, endosulfan-induced changes in cellular stress enzymes (Catalase, SOD and GST in liver and gill and neurotansmitter enzyme, brain AChE) were significantly ($p < 0.01$) improved by dietary lecithin. We herein report the role of lecithin in enhancing the thermal tolerance and protection against cellular stress in fish exposed to an organochlorine pesticide.

1. Introduction

Lecithin is a methyl donor and is needed for many metabolic reactions which transfer CH_3 groups to DNA, RNA, protein, carnitine, creatine, lipid and many other important CH_3-containing metabolic compounds (Smolin and Benevega, 1989; Frontiera et al., 1994). Research on methyl group metabolism and regulation has shown a significant relationship between methyl donors, metabolism and disease. However, deficiency of methyl groups in animals may lead to under-methylation of DNA and concomitant activation of oncogenes (Newberne, 1993). Lecithin is also a source of phospholipids (PL) in the diet, acts as a non-protein energy source, feed attractant, increases resistance to stress (Kumar et al., 2014a) and can reduce the oxidation (antioxidant) of vitamin A, C and E, as well as enhance the utilization of these vitamins in aquaculture species (ADM, 2003). Methyl groups are of vital importance for terrestrial and aquatic animals. However, animals cannot synthesize methyl groups and thus need to be supplemented 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).

An organochlorine pesticide; endosulfan 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). 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. However, in presence of any kinds of contamination/pesticide the

* Corresponding author. Fax: +91 2112 254056.
E-mail address: neeraj_journal@live.in (N. Kumar).

http://dx.doi.org/10.1016/j.jtherbio.2014.10.004
0306-4565/

thermal tolerance of fish has been reduced by 2.6–6 °C (Patra et al., 2007).

Milkfish (*Chanos chanos*) is an important tropical brackishwater fish and is cultured in the Philippines, Indonesia, India and Taiwan (Rabanal, 1988). The production of *C. chanos* was estimated to be 943,259 t (FAO, 2012). Milk fish has been the subject of numerous studies by investigators in relation to biology, aquaculture, hatchery context and toxicity (Bagarinao, 1994; Magesh and Kumaraguru, 2006). 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 (Ficke et al., 2007). Fish being an ectothermal organism, any alterations in the water temperature would 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 tolerance over which it survives and has a narrower range for optimum growth to occur (Katersky and Carter, 2007). However, temperatures beyond optimum limits of a particular species adversely affects the health of aquatic animal due to metabolic stress and increases oxygen demand (Wedemeyer et al., 1999). Temperature tolerance differs with species, acclimation temperature, acclimation duration and salinity (Ficke et al., 2007; Das et al., 2004; Diaz et al., 2007; Manush et al., 2004). Hence, thermal stress studies have gained importance in comprehending the impact of global warming on animals including fish. In our previous studies (Kumar et al., 2011a, 2012, 2014a), we observed that dietary lecithine mitigates endosulfan-induce stress and modulates immunity in *Labeo rohita* fingerlings. In this study, an attempt was made to evaluate the effect of supplementation of lecithin on the thermal tolerance of Milk fish, reared under low doses endosulfan-induced stress.

2. Materials and methods

2.1. Experimental animals and experimental design

Milk fish fingerlings were obtained from the Central Institute of Brackishwater Aquaculture (CIBA), Muttukudu Experimental Station, Chennai, India. The experimental animals were maintained in fiber reinforced plastic (FRP) tanks (circular, 500 L) for a period of 15 days prior to the experiment for acclimatization after which fish were randomly distributed into 15 FRP tanks of 150 L capacity and reared for five weeks. Fifteen fish of uniform size (12.65 ± 1.25 g) were stocked in each tank with three replicates for each treatment, following a completely randomized design. The fish were fed with the experimental diet to satiation twice daily (10:00 h and 17:00 h) for five weeks. Continuous aeration was provided using a compressed air pump and manual water exchange (two third) was carried out on alternate days. Four iso-nitrogenous and iso-caloric diets were formulated i.e control diet (without supplementation of lecithin L0), lecithin (L1) diet 1% (10 g/kg), lecithin (L2) 1.5% supplemented diet (15 g/kg) and lecithin (L3) 2% supplemented diet (20 g/kg). The experimental setup consisted of normal water (without endosulfan) and fed with (negative control) control diet (En0/L0), endosulfan-treated water and fed with (positive control) control diet (En/L0), endosulfan-treated water and fed with lecithin 1% supplemented (En/L 1%), endosulfan-treated water and fed with lecithin 1.5% supplemented (En/L 1.5%) and endosulfan-treated water and fed with 2% lecithin supplemented feed (En/L 2%). The endosulfan treatment was made at a level of 1/40th (0.52 ppb) of 96 h LC_{50} (21.47 ppb, *C. chanos*, wt. 110 g, data communicated) for all the treatment groups using technical grade endosulfan (99% pure; α:β ratio of 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) and added to the water (Kumar et al., 2014b). Water quality parameters were analyzed weekly using the methods of APHA (1998) and were found to be within the recommended range for fish rearing.

2.2. Experimental conditions

Water in the tanks was continuously aerated and water temperature was recorded and was in the range of 26.4–28.8 °C. The animals were fed with experimental diet (35% crude protein) at 3% of their body weight. 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 (1998), total hardness (carbonate hardness test kit, Merck, Germany), ammonia (at 635 nm by phenate method APHA (1998), nitrite and nitrate (543 nm wave length APHA (1998) were recorded.

2.3. Experimental diet

Four iso-caloric and iso-nitrogenous diets viz. control diet and three supplemented diets: 1, 1.5 and 2.0% lecithin diets were prepared using soylecithin as a source of lecithin. Soylecithin was procured from M/S HIMEDIA. Soylecithin was mixed in oil before incorporating in the feed mix. The other ingredeints used in the formulations viz., fish meal, soybean meal, sunflower meal, wheat flour, wheat bran and sunflower oil were procured from local market and used. The dough was thoroughly mixed, pelleted and kept in a hot air oven at 60 °C to arrive at a moisture content of less than 10% and was subsequently stored at 4 °C until required.

2.4. 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 muffle furnace at 600 °C for 6 h. Total carbohydrate was calculated by difference, i.e. total carbohydrate % 100 − (CP% + EE% + Ash%). The digestible energy value of experimental diets was calculated by Halver (1976).

2.5. Tissue homogenate preparation

Gill, liver and brain tissues of fish from all the groups were dissected carefully and 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 in ice to avoid denaturation of the enzymes during 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 after Lowry et al. (1951) using bovine serum albumin as a standard. The supernatants were collected in glass vials and stored at −20 °C until further analysis.

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	Control	Lecithin Supplemented diet		
		Lecithin 1%	Lecithin 1.5%	Lecithin 2%
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	15.47	15.47	15.47
Wheat bran[a]	10	10	10	10
Sunflower oil[a]	4.5	3.5	3	2.5
Cod liver oil[a]	2	1.5	1.5	1.5
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
Soylecithin[b]	0	1	1.5	2
Total	100	100	100	100
Proximate analysis of feed				
CP	35.87 ± 0.78	35.55 ± 0.65	35.08 ± 1.11	35.85 ± 0.88
EE	11.23 ± 0.34	12.16 ± 0.81	10.99 ± 0.67	11.59 ± 0.95
ASH	9.48 ± 0.13	9.73 ± 0.08	10.11 ± 0.23	9.78 ± 0.19
TC	43.42 ± 0.98	42.56 ± 0.19	43.82 ± 0.96	42.78 ± 0.68
OM	90.52 ± 0.13	90.27 ± 0.08	89.89 ± 0.23	90.22 ± 0.19
DM	92.90 ± 0.62	93.44 ± 0.58	93.60 ± 0.73	92.16 ± 0.20
DE	418.24 ± 2.08	421.87 ± 4.19	414.52 ± 4.13	418.87 ± 4.89

[c]Composition of vitamin mineral mix (quantity/250 g starch powder):
Vitamin A 550,000 IU; Vitamin D_3 110,000 IU;VitaminB_1:20mg, Vitamin B_2 200 mg; Vitamin E 75 mg; Vitamin K 100mg; Vitamin B_{12} 0.6 μg; Calcium Pantothenate 250 mg; Nicotinamide 1000mg; pyridoxine:100mg; Mn 2,700 mg; I 100mg; Fe750 mg; Zn 500 mg; Cu 200 mg; Co 45 mg; Ca 50 g; P 30g; Selenium 0.5 ppm.
Digestible energy (DE) (K cal/100 g)=(%CPx4)+(%EEx9)+(TCx4).
CP—crude protein; EE—ether extract; TC—total carbohydrate; OM—organic matter, DM—dry matter; DE—digestible energy.
Data expressed as mean ± SE, $n=3$.

[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.

2.6. Thermal tolerance experiment

Thermal tolerance was assessed at the end of feeding trial using the critical thermal methodology (CTM) as described previously (Beitinger et al., 2000; Dalvi et al., 2009). The fish were depurated for a 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 tank for a particular treatment group. Fish from each treatment group were shifted to separate thermo static water bath aquaria (Suan Scientific Instruments & Equipment's, Kolkata, India, 52 L water capacity, sensitivity ± 0.2 °C) for temperature tolerance study. Endosulfan concentration of water in the thermostatic aquaria were similar to those in the experimental groups. Dissolved oxygen concentration was maintained at 6.5 ± 0.5 mg/L throughout the temperature tolerance study by continuous aeration using a 2-HP centralized air blower. Water temperature in the aquarium was increased/decreased at a constant rate of 0.30 °C/min, until loss of equilibrium (LOE) was reached, which was designated as the CTmax/CTmin (Beitinger et al., 2000; Paladino et al., 1980). The lethal thermal maxima (LTmax)/lethal thermal minima (LTmin) were determined by increasing/decreasing the temperature until the opercular movement ceased (Tsuchida, 1995; Kita et al., 1996). This technique has been critically evaluated by Das et al., 2004 and Sarma et al., 2010 and it has been well established as a powerful tool for studying the thermal tolerance in fish (Paladino et al., 1980).

2.7. Antioxidant enzymes

2.7.1. Superoxide dismutase (SOD)

The SOD (EC 1.15.1.1) activity was measured by the method of Misra and Fridovich (1972) and was expressed in units/mg protein. The assay was 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 (freshly prepared) were mixed well and immediately read at 480 nm for 3 min in a Shimadzu-UV spectrophotometer.

2.7.2. Catalase (CAT)

The CAT (EC 1.11.1.6) activity was measured by the method of Takahara et al. (1960) and expressed as units/mg protein. The reaction mixture of 2.45 ml phosphate buffer (50 mM; pH-7), 50 μl tissue homogenate and 1 ml of hydrogen peroxide substrate solution (freshly prepared) were mixed well and decrease in absorbance was read at 240 nm for 3 min.

2.7.3. Glutathione-S-transferase (GST)

The GST (EC 2.5.1.18) was measured spectrophotometrically after Habing et al. (1974) using S-2, 4-dinitrophenyl glutathione (CDNB) as the substrate and expressed as units/mg protein. The method was based on the principle of formation of adduct of CDNB, S-2, 4-dinitrophenyl glutathione was monitored by measuring the increase in absorbance at 340 nm against blank.

2.8. Acetylcholine esterase (AChE)

AChE (EC. 3.1.1.7) activity as measured by the change in OD at 540 nm after Hestrin (1949) modified by Augustinsson (1957) and expressed as nmol/min/mg protein. The activity was spectrophotometrically measured as the increase in absorbance of the sample at 412 nm. Acetyl choline iodide and dithiobisnitrobenzoic acid were used as substrate the activity being measured against a blank.

2.9. Statistics

The data were analyzed by Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS, Chicago, IL), using 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.

3. Results and discussion

3.1. CTmax, LTmax and CTmin and LTmin

In the present study, lecithin significantly ($P < 0.01$) enhanced the thermal tolerance of *C. chanos* as evident by the decreased CTmin and LTmin (Fig. 1) and increased CTmax and LTmax values (Fig. 2). The CTmax and LTmax values were significantly ($P < 0.01$) higher in the group fed of lecithin supplemented compared to control and endosulfan-exposed group. The CTmax (41.83 °C) and LTmax (43.43 °C) values of the group fed with control diet also showed significant increase ($p < 0.01$) compared to endosulfan-exposed group fed with control diet. Positive correlations were observed between CTmax and LTmax (R^2:0.934) and between CTmin and LTmin (R^2: 0.931). The LTmax was found to be maximum in group fed with 2% lecithin supplemented diet (46.40 °C) followed by 1.5% (45.10 °C) and 1% (44.53 ± 0.15 °C) lecithin. Similarly, dietary lecithin significantly ($P < 0.01$) reduced the lower thermal tolerance limits (CTmin and LTmin) (Fig. 1). Maximum reduction (both in CTmin and LTmin) was observed in group fed with 2% followed by 1.5%. Earlier studies have demonstrated that the thermal tolerance of fish largely depends on their acclimation temperature (Debnath et al., 2006; Das et al., 2004; Sarma et al., 2010), toxic chemical (Beitinger et al., 2000), species (Das et al., 2004), size and condition factor (Baker et al., 1990). There is no literature available to corroborate our results of increased CTmax and LTmax values in *C. chanos* fingerlings fed with diet containing lecithin. Gupta et al. (2010) and Akhtar et al. (2011) reported increased CTmax and LTmax after feeding of 1% and 1.25% levan and pyridoxine in *L. rohita*, respectively. Similarly, CTmin and LTmin values of positive control group were similar to the findings of Chatterjee et al. (2004). However, we observed significant reduction in CTmin and LTmin in *C. chanos* fed with 1.5% and 2% lecithin. This may possibly be due to the hypothermic (reduction in core body temperature) effect of lecithin. There is no report of the beneficial effect of lecithin on thermal tolerance of fish.

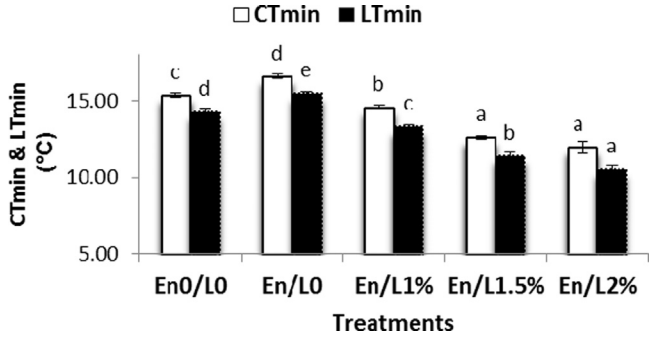

Fig. 1. Effect of dietary lecithin on critical temperature minima (CTmin) and lethal temperature minima (LTmin) of *C. chanos* fingerlings under low dose endosulfan exposed for five weeks.
Abbreviations for exposure/diet treatments of fish: En0/L0, fish group reared in normal/control water and fed control feed (negative control); En/L0, low dose endosulfan-exposed (En) and control feed (L0) fed group (positive control); En/L 1%, (10 g/kg) low dose endosulfan exposed and supplemental lecithin fed group; En/L 1.5 % (15g/kg) low dose endosulfan exposed and supplemental lecithin fed group; En/L 2% (20g/kg), low dose endosulfan exposed and supplemental lecithin fed group. Values in the same series with different superscript (a, b, c, d, e) differ significantly ($P < 0.01$). Values are expressed as mean ± SE, n=6

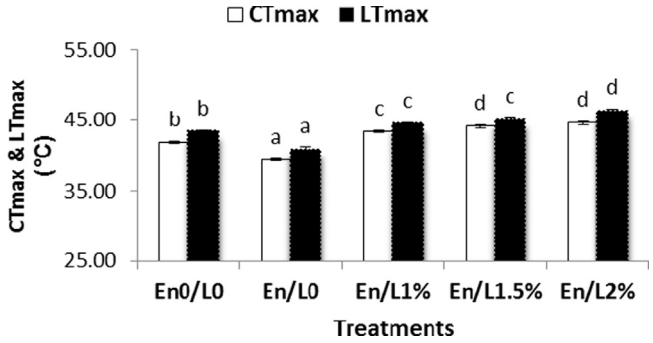

Fig. 2. Effect of dietary lecithin on critical temperature maxima (CTmax) and lethal temperature maxima (LTmax) of *C. chanos* fingerlings under low dose endosulfan exposed for five weeks.
For abbreviations see figure 1.

Lecithin is a phosphatidylcholine methyl donor's compound and commercially, it refers to a natural mixture of neutral and polar lipids. Choline itself is the precursor for the synthesis of the neurotransmitter acetylcholine, the methyl donor betaine and phospholipids, including phosphatidylcholine and sphingomyelin among others. Lecithin plays an important role by multiracial interaction through DNA Methylation and expression regulation and heat shock protein (HSP) production for providing membrane stability and proper folding. While membrane damage caused during the thermal stress needs raw material for the repair of biomembrane, lecithins act as a polar moiety raw material maintaining membrane fluidity and stability. It is well documented that lecithin acts in synergy with other antioxidants like ascorbic acid and vitamin E and plays a role in oxidative protection of membrane against lipo-peroxide and free radicals (Halliwell and Gutteridge, 1999). However, when organisms are subjected to thermal stress and concurrent xenobiotic compounds, the rate of production of reactive free oxygen (ROS) such as superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and peroxyl radical (ROO^-) exceeds their scavenging capacity. All organisms have their own cellular antioxidative defense systems, comprising enzymatic as well as non-enzymatic components. Enzymatic pathway consists of SOD, CAT and GPX, O_2^- and are dismutated by SOD to H_2O_2 which is then reduced to water and molecular oxygen by CAT or is neutralized by GPX that catalyzes the reduction of H_2O_2 to water and organic peroxide to alcohols using GSH as a source of reducing equivalent. Glutathione reduce (GR) regenerates Glutathione (GSH) from oxidized glutathione (GSSG), which is a scavenger of ROS as well as a substrate for the other enzymes. The GST conjugates xenobiotics with GSH for excretion. The non-enzymatic component consists of small organic molecules such as β-carotene, GSH, 3 vitamin E and vitamin C. The most notable success in the oxidative stabilization has been studied in fish oil by Vitamin E, C and lecithin. The stabilization was achieved with a tertiary mixture that contained α or ϒ tocopherol, ascorbyl palmitate and lecithin (Pongracz, 1973; Loliger et al., 1996; Chang and Wu, 1991). Synergy between δ-tocopherol, lecithin, and ascorbic acid has also been reported when ascorbic acid is present in an aqueous micro emulsion in fish oil (Han et al., 1991).

It is also possible that dietary lecithin may have enhanced the expression of heat shock proteins (HSPs). Inside the cell, HSPs have been found to repair and prevent damage from cellular stress associated with protein denaturation at high and low temperatures (Nakano and Iwama, 2002; Werner et al., 2005). Expression of HSP70 has been previously reported in *L. rohita* after thermal acclimation (Das et al., 2004) and endosulfan-exposure (Kumar et al., 2014a).

Table 2
Effect of dietary lecithin on catalase, SOD and GST activity in liver as well as gill after critical temperature minima (CTmin) and lethal temperature minima (LTmin) of *C. chanos* fingerlings under low dose endosulfan exposed for five weeks.

Treatments	Catalase		SOD		GST	
	Liver	Gill	Liver	Gill	Liver	Gill
En0/L0	$68.11^{c} \pm 2.11$	$65.88^{b} \pm 2.04$	$36.21^{b} \pm 2.75$	$37.91^{b} \pm 2.51$	$2.32^{a} \pm 0.08$	$3.60^{a} \pm 0.45$
En/L0	$80.44^{d} \pm 2.07$	$76.18^{b} \pm 6.13$	$47.17^{c} \pm 3.03$	$46.64^{c} \pm 2.19$	$3.38^{b} \pm 0.19$	$5.04^{b} \pm 0.53$
En/L 1%	$52.36^{b} \pm 1.29$	$45.09^{a} \pm 4.65$	$20.30^{a} \pm 1.62$	$22.29^{a} \pm 0.94$	$2.00^{a} \pm 0.44$	$3.84^{a} \pm 0.18$
En/L 1.5%	$49.48^{ab} \pm 2.27$	$38.03^{a} \pm 0.60$	$20.52^{a} \pm 1.72$	$23.73^{a} \pm 1.79$	$1.96^{a} \pm 0.08$	$2.95^{a} \pm 0.13$
En/L 2%	$44.19^{a} \pm 3.38$	$34.00^{a} \pm 1.55$	$24.08^{a} \pm 3.61$	$26.12^{a} \pm 2.59$	$1.79^{a} \pm 0.29$	$2.76^{a} \pm 0.37$
P-value	$P < 0.001$	$P < 0.003$	$P < 0.001$	$P < 0.007$	$P < 0.009$	$P < 0.010$

Abbreviations for exposure/diet treatments of fish: En0/L0, fish group reared in normal/control water and fed control feed (negative control); En/L0, low dose endosulfan-exposed (En) and control feed (L0) fed group (positive control); En/L 1%, (10 g/kg) low dose endosulfan exposed and supplemental lecithin fed group; En/L 1.5% (15 g/kg) low dose endosulfan exposed and supplemental lecithin fed group; En/L 2% (20 g/kg), low dose endosulfan exposed and supplemental lecithin fed group. Values in the same series with different superscripts (a, b, c, d, e) differ significantly ($P < 0.01$). Values are expressed as mean ± SE, $n = 6$.

Gupta et al. (2010) reported an increased expression of HSP70 and improved thermal tolerance of *L. rohita* when fed a diet containing 1.25% levan. In the present study, it could be possible that the enhanced thermal tolerance of *C. chanos* fingerlings fed diet supplemented with 1, 1.5 and 2% lecithin might have resulted in the establishment of homeostasis with increased expression of some functional proteins like HSPs or cold shock proteins. Our previous investigations also (Kumar et al., 2011a, 2012, 2014a) revealed improved values of total leukocytes, respiratory burst activity, lysozyme activity, hemoglobin, serum albumin, globulin, lactate dehydrogenase (LDH), malate dehydrogenases (MDH), alanine transferees (ALT), aspartate transferase (AST), acetylcholine esterases (AChE), anti-oxidative enzymes and other stress biomarkers (caspase, vitellogenin, cortisol and methyl transferase) in *L. rohita* fingerlings reared under endosulfan-stress and fed lecithin supplemented diets. It is therefore, likely that in the present study, enhanced thermal tolerance of *C. chanos* fingerlings may also be due to the stimulation of nonspecific defense mechanism by lecithin. However, further elaborate investigations are required to comprehend the actual mechanism of action of dietary lecithin in enhancing thermal tolerance in fish.

3.2. Antioxidative enzymes

At the end of the thermal tolerance study (maximum and minimum temperature) the activities of CAT, SOD and GST in liver and gill of *C. chanos* fingerlings were studied and are presented in Tables 2 and 3. The activities of catalase, SOD and GST in liver and gill were significantly ($P < 0.01$) higher in lecithin supplemented groups than the positive and negative control groups. In the maximum and minimum temperature tolerance study, the supplementation of lecithin protected the cell from damage against cellular stress. In both the cases, 1.5 and 2% lecithin supplementation exhibited a positive influence ($P < 0.01$) on the activities of catalase, SOD and GST in liver as well as in gills. In the case of minimum and maximum temperature tolerance, the values of catalase were higher during CTmax and LTmax (maximum temperature tolerance) in both liver and gills. However, the SOD and GST values were higher in CTmin and LTmin (minimum temperature tolerance) in both the organs.

Fish being thermal dependent organisms, are subjected to fluctuations in temperature. Thermal tolerance of animal is linked to the aerobic capacity. Cooling as well as warming temperatures influence the balance of oxygen supply versus demand and may critically lower oxygen levels in animal tissues. Adaptation to change in temperature involves adjustments of both density and functional properties of the mitochondria, thus affecting ROS (Reactive oxygen species) generation and antioxidant defenses (for review see Portner, 2002). This explains the high liver SOD activity of active fish, a value close to that found in mammalian species. The level of SOD could be a safeguard against changes in temperature to which these species are naturally exposed (Filho et al., 1993). Abele and Puntarulo (2004) indicated that life under permanent cold water conditions in polar habitat 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 a common feature of cold water adaptation in polar invertebrates and fishes, these mitochondria might actually produce more ROS under stress.

Endosulfan-induced oxidative tissue damage results from the release of oxygen free radicals (OFRs) (Hincal et al., 1995). Due to high reactivity of OFRs, most components of the cellular structure

Table 3
Effect of dietary lecithin on catalase, SOD and GST activity in liver as well as gill after critical temperature maxima (CTmax) and lethal temperature maxima (LTmax) of *C. chanos* fingerlings under low dose endosulfan exposed for five weeks.

Treatments	Catalase		SOD		GST	
	Liver	Gill	Liver	Gill	Liver	Gill
En0/L0	$70.12b^{c} \pm 1.36$	$70.02^{bc} \pm 5.34$	$27.38^{b} \pm 1.76$	$26.12^{b} \pm 2.59$	$0.43^{b} \pm 0.01$	$0.45^{b} \pm 0.01$
En/L0	$77.06^{c} \pm 1.81$	$79.68^{c} \pm 4.40$	$33.22^{c} \pm 1.23$	$33.12^{c} \pm 1.40$	$0.57^{c} \pm 0.05$	$0.55^{c} \pm 0.03$
En/L 1%	$66.42^{abc} \pm 3.67$	$61.84^{ab} \pm 2.88$	$21.87^{a} \pm 2.03$	$21.39^{a} \pm 0.68$	$0.39^{ab} \pm 0.04$	$0.38^{ab} \pm 0.02$
En/L 1.5%	$54.29^{a} \pm 6.85$	$46.27^{a} \pm 7.78$	$19.48^{a} \pm 1.09$	$18.55^{a} \pm 1.00$	$0.36^{ab} \pm 0.02$	$0.32^{a} \pm 0.01$
En/L 2%	$58.76^{a} \pm 2.42$	$53.08^{a} \pm 2.43$	$20.36^{a} \pm 0.82$	$18.91^{a} \pm 0.77$	$0.32^{a} \pm 0.02$	$0.33^{a} \pm 0.04$
P-value	$P < 0.012$	$P < 0.005$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Abbreviations for exposure/diet treatments of fish: En0/L0, fish group reared in normal/control water and fed control feed (negative control); En/L0, low dose endosulfan-exposed (En) and control feed (L0) fed group (positive control); En/L 1%, (10 g/kg) low dose endosulfan exposed and supplemental lecithin fed group; En/L 1.5% (15 g/kg) low dose endosulfan exposed and supplemental lecithin fed group; En/L 2% (20 g/kg), low dose endosulfan exposed and supplemental lecithin fed group. Values in the same series with different superscripts (a, b, c, d, e) differ significantly ($P < 0.01$). Values are expressed as mean ± SE, $n = 6$.

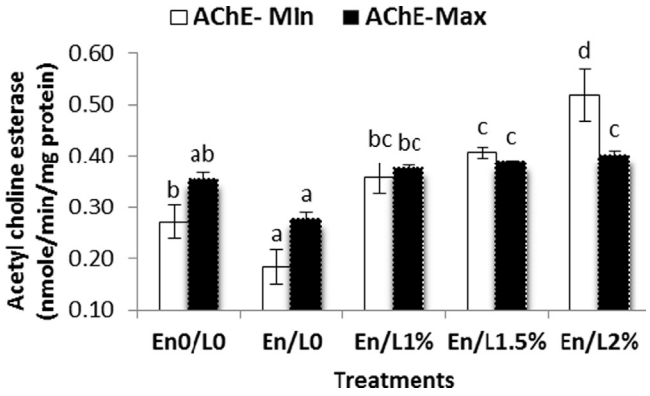

Fig. 3. Effect of dietary lecithin on brain AChE activity after critical temperature minima (CTmin), lethal temperature minima (LTmin), critical temperature maxima (CTmax) and lethal temperature maxima (LTmax) of *C. chanos* fingerlings under low dose endosulfan exposed for five weeks.
For abbreviations see figure 1.

and function may become potential targets of oxidative damage. The OFRs may also induce protein damage (Bainy et al., 1996). To neutralize the impact of OFRs, both enzymatic and non-enzymatic antioxidants are activated (Lopez–Torres et al., 1993). Liver is the principal organ that detoxifies endosulfan to endosulfan ether (Rao et al., 1980, 1981). Thus, anti-oxidant enzyme activities that protect liver from oxidative damage are sensitive indicators of endosulfan toxicity. In our earlier studies (Kumar et al., 2011b) reported that after exposure to endosulfan the activities of catalase, SOD and GST were higher in Tilapia whereas in another study (Kumar et al., 2014a), reduced catalase, SOD and GST activities were reported in *L. rohita* fed diets supplemented with lecithin. Similar results relating to reduced antioxidative enzyme activity (SOD and catalase) have been reported by Gupta et al. (2013) through different level of levan supplementation in common carp fry exposed to fipronil.

3.3. Acetylcholine esterase (AChE) activity

At the end of thermal tolerance minima as well as maxima, the brain AChE activity was analyzed and presented in Fig. 3. The brain AChE activities of *C. chanos* during minima as well as maxima were significantly higher ($P<0.01$) in lecithin supplemented groups compared to positive and negative control group. The group fed with 1.5 and 2% lecithin supplemented diet showed more prominent AChE activity and to minima and maxima thermal tolerance temperature, inspite of five weeks of exposure to endosulfan. In case of CTmin LTmin (minimum and maximum thermal tolerance) as well as CTmax LTmax, the AChE activities were similar in all lecithin supplemented diets. However, the 2% lecithin supplemented diet exhibited significant higher activity compared to CTmax LTmax.

It is a well-known fact that AChE inhibition occurs in organophosphate toxicity (Guilhermino et al., 1996). The mechanism of AChE inhibition during organochlorine (endosulfan) pesticide toxicity is worth exploring. Our previous study (Kumar et al., 2011b) found reduction in brain AChE activity in *Oreochromis mossambicus* when exposed to endosulfan for 96 h and also Kumar et al., 2012 reported that supplementation of lecithin in the diet of *L. rohita* resulted in higher AChE activity. To corroborate our finding Muthappa et al. (2013) reported that exposure to endosulfan resulted in a significant reduction in AChE activity in *L. rohita* and supplementation of lecithin resulted in significantly higher AChE activity. Gupta et al. (2014) reported that levan supplementation in the diet improved AChE activities in common carp exposed to fipronil.

4. Conclusion

The role of lecithin in enhancing the thermal tolerance and protection against cellular stress is being reported. Further, this study indicated that supplementation of lecithin at 1.5% (15 g/kg) and 2% (20 g/kg) in the diet could be an effective strategy to enhance the thermal tolerance of *C. chanos* fingerlings through protection of antioxidative status and neurotransmitter enzyme. This data could prove useful in formulating specialised feeds for culture of *C. chanos* in different agro climatic conditions. However, further studies are required to elucidate the mode of action of lecithin on enhancing thermal tolerance and protection against antioxidative status and neurotransmitter enzyme.

Acknowledgements

The first author is grateful to Dr. P.S. Minhas, Director, National Institute of Abiotic Stress Management, (ICAR) Baramati, Pune and Director, Central Institute of Brackishwater Aquaculture, Chennai, India for providing supports for this study. We also thank Mr. D. Srinivasan for the technical assistance rendered by him during the experimental trial.

References

Abele, D., Puntarulo, S., 2004. Formation of reactive species and induction of antioxidant defense systems in polar and temperate marine invertebrates and fish. Comp. Biochem. Physiol. 138A, 405–415.
ADM, 2003. Lecithin in aquaculture. Available at ⟨www.admworld.com/mktcolpdf/eu⟩ Lecithins In Aquaculture.pdf. (Retrieved on 01.07.07).
Akhtar, M.S., Pal, A.K., Sahu, N.P., Alexander, C., Meena, D.K., 2011. Dietary pyridoxine enhances thermal tolerance of *Labeo rohita* (Hamilton) fingerlings reared under endosulfan stress. J. Therm. Biol. 36, 84–88.
AOAC, 1995. 16th edn. In: Cunnif, P.A. (Ed.), Official Methods of Analysis of the Association of Official Analytical Chemists, Vol. 1. AOAC International, Arlington, pp. 31–65.
APHA-AWWA-WEF, 1998. In: Clesceri, L.S., Greenberg, A.E., Eaton, A.D. (Eds.), Standard Methods for the Estimation of Water and Waste Water, 20th edn. American Public Health Association, American Water Works Association, Water Environment Federation, Washington, DC..
Bagarinao, T., 1994. Systematics, distribution, genetics and life history of Milkfish *Chanos chanos*. Environ. Biol. Fish. 39, 25–41.
Bainy, A.C.D., Saito, E., Carvalho, P.S.M., Junqueira, V.B.C., 1996. Oxidative stress in gill, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. Aquat. Toxicol. 34, 151–162.
Beitinger, T.L., Bennett, W.A., MCCauley, R.W., 2000. Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. Environ. Biol. Fish. 58, 237–275.
Bender, D.A., 1992. Nutritional Biochemistry of the Vitamins. Cambridge University Press, New York.
Cengiz, E.I., Unlu, E., 2002. Histological changes in the gill of mosquito fish, Gambusia affinis exposed to endosulfan. Bull. Environ. Contam. Toxicol. 68, 290–296.
Chang, S.S., Wu, K., 1991. Composition of Natural Antioxidants for the Stabilization of Polyunsaturated Oils, U.S. Patent, 5,077,069.
Chatterjee, N., Pal, A.K., Manush, S.M., Das, T., Mukherjee, S.C., 2004. Thermal tolerance and oxygen consumption of *Labeo rohita* and *Cyprinus carpio* early fingerlings acclimated to three different temperatures. J. Therm. Biol. 29, 265–270.
Dalvi, R.S., Pal, A.K., Tiwari, L.R., Das, T., Baruah, K., 2009. Thermal tolerance and oxygen consumption rates of the catfish *Horabagrus brachysoma* (Gunther) acclimated to different temperatures. Aquaculture 295, 116–119.
Das, T., Pal, A.K., Chakraborty, S.K., Manush, S.M., Chatterjee, N., Mukherjee, S.C., 2004. Thermal tolerance and oxygen consumption of Indian Major Carps acclimated to four different temperatures. J. Therm. Biol. 23, 157–163.
Debnath, D., Pal, A.K., Sahu, N.P., Baruah, K., Yengkokpam, S., Das, T., Manush, S.M., 2006. Thermal tolerance and metabolic activity of yellow catfish *Pangasius pangasius* (Hamilton, 1822) advanced fingerlings with emphasis on their culture potential. Aquaculture 258, 606–610.
Diaz, F., Re, A.D., Gonzalez, R.A., Sanchez, L.N., Levya, G., Valenzuela, F., 2007. Temperature preference and oxygen consumption of the largemouth bass *Micropterus salmoides* (Lacepede) acclimated to different temperatures. Aqua. Res 38, 1387–1394.
FAO, 2012. Yearbook Fishery and Aquaculture Statistics: pp. 30.
Ficke, A.D., Myrick, C.A., Hansen, L.J., 2007. Potential impacts of global climate change on freshwater fisheries. Rev. Fish Biol 17, 581–613.

Filho, D.W., Giulivi, C., Boveris, A., 1993. Antioxidant defences in marine fish-I. Teleosts. Comp. Biochem. Physiol 106C, 409–413.
Frontiera, M.S., Stabler, S.P., Kolhouse, J.F., Allen, R.H., 1994. Regulation of methionine metabolism: effect of nitrous oxide and excess methionine. J. Nutr. Biochem. 5, 28–38.
Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Inhibition of acetyl cholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere 32 (47), 27–738.
Gupta, S.K., Pal, A.K., Sahu, N.P., Dalvi, R.S., Akhtar, M.S., Jha, A.K., Baruah, K., 2010. Dietary microbial levan enhances tolerance of *Labeo rohita* (Hamilton) juveniles to thermal stress. Aquaculture 306, 398–402.
Gupta, S.K., Pal, A.K., Sahu, N.P., Jha, A.K., Akhtar, M.S., Mandal, S.C., Das, P., Prusty, A. K., 2013. Supplementation of microbial levan in the diet of *Cyprinus carpio* fry (Linnaeus, 1758) exposed to sublethal toxicity of fipronil: effect on growth and metabolic responses. Fish Physiol. Biochem. 39, 1513–1524. http://dx.doi.org/10.1007/s10695-013-9805-7.
Gupta, S.K., Pal, A.K., Sahu, N.P., Saharan, N., Mandal, S.C., Prakash, C., Akhtar, M.S., Prusty, A.K., 2014. Dietary microbial levan ameliorates stress and augments immunity in *Cyprinus carpio* fry (Linnaeus, 1758) exposed to sublethal toxicity of fipronil. Aqua. Res. 45, 893–906.
Habing, W.H., Pabst, M.N., Bjacoby, W., 1974. Glutathione-S- transferase, the first enzymatic step in mercatpopunc acid formation. J. Biol. Chem. 249, 7130–7138.
Halliwell, B., B. Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine,. Clarendon Press, Oxford.
Halver, J.E., 1976. The nutritional requirements of cultivated warm water and cold water fish species. In: Proceedings of the FAO Technical Conference on Aquaculture, Kyoto, Japan, 26 May–2 June, 1976. FAO Fisheries Report no. 188 FI/R188 (En), p. 9.
Han, D., Yi, O.S., Shin, H.K., 1991. Solubilization of vitamin C in fish oil and synergistic effect with vitamin E in retarding autoxidation. IBID 68, 740–743.
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. Biol. Chem. 180, 249–261.
Hincal, F., Gurbay, A., Giray, B., 1995. Induction of lipid peroxidation and alteration of glutathione redox status by endosulfan. Biol. Trace Elem. Res. 47, 321–326.
Jonassen, T.M., Imsland, A.K., Stefansson, S.O., 1999. The interaction of temperature and fish size on growth of juvenile halibut. J. Fish Biol. 54, 556–572.
Katersky, R.S., Carter, C.G., 2007. High growth efficiency occurs over a wide temperature range for juvenile barramundi *Lates calcarifer* fed a balanced diet. Aquaculture 272, 444–450.
Kidd, M.T., Ferket, P.R., Garlich, J.D., 1997. Nutritional and osmoregulatory functions of betaine. Poult. Sci. 53, 125–139.
Kita, J., Tsuchida, S., Setoguma, T., 1996. Temperature preference and tolerance and oxygen consumption of the marbledrock fish *Sebastiscus marmoratus*. Mar. Biol. 125, 467–471.
Kumar, N., Gupta, S., Chandan, N.K., Aklakur, M., Pal, A.K., Jadhao, S.B., 2014a. Lipotropes protect against pathogen-aggravated stress and mortality in low dose pesticide-exposed fish. PLoS One 9 (4), e93499. http://dx.doi.org/10.1371/journal.pone.0093499.
Kumar, N., Sharma, R., Tripathi, G., Kumar, K., Dalvi, R.S., Krishna, G., 2014b. Cellular metabolic, stress and histological response on exposure to acute toxicity of endosulfan in Tilapia (*Oreochromis mossambicus*). Environ. Toxicol. , http://dx.doi.org/10.1002/tox.22026.
Kumar, N., Jadhao, S.B., Chandan, N.K., Akhlak, M., Rana, R.S., 2012. Methyl donors potentiates growth, metabolic status and neurotransmitter enzyme in *Labeo rohita* fingerlings exposed to endosulfan and temperature. Fish Physiol. Biochem. 38 (5), 1343–1353. http://dx.doi.org/10.1007/s10695-012-9622-4.
Kumar, N., Jadhao, S.B., Chandan, N.K., Rana, R.S., 2011a. Dietary choline, betaine and lecithin mitigates endosulfan induced stress in *Labeo rohita* fingerlings. Fish Physiol. Biochem 38, 989–1000. http://dx.doi.org/10.1007/s10695-011-9584-y.
Kumar, N., Prabhu, A.J., Pal, A.K., Remya, S., Aklakur, M., Rana, R.S., Gupta, S., Raman, R.P., Jadhao, S.B., 2011b. Anti-oxidative and immuno-hematological status of Tilapia (*Oreochromis mossambicus*) during acute toxicity test of endosulfan. Pest. Biochem. Physiol. 99, 45–52. http://dx.doi.org/10.1016/j.pestbp.2010.10.003.
Loliger, J., Lambelet, P., Aeschbach, R., Prior, E.M., 1996. Natural Antioxidants: From Radical Mechanisms to Food Stabilization. In: McDonald, R.E., Min, D.B. (Eds.), Food Lipids and Health. Marcel Dekker, New York, pp. 315–344.
Lopez-Torres, M., Perez-Campo, R., Cadenas, S., Rojas, Barja,, C.G., 1993. A comparative study of free radicals in vertebrate. II. Non-enzymatic antioxidants and oxidative stress. Comp. Biochem. Physiol. 105B, 757–763.
Lowry, O.H., Ronebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.
Magesh, S., Kumaraguru, A.K., 2006. Acute toxicity of endosulfan to milk fish, *Chanos chanos*, of the southeast coast of India. Bull. Environ. Contam. Toxicol. 79, 622–628.
Manush, S.M., Pal, A.K., Chatterjee, N., Das, T., Mukherjee, S.C., 2004. Thermal tolerance and oxygen consumption of Macrobrachium rosenbergii acclimated to three temperatures. J. Therm. Biol. 29, 15–19.
Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247, 3170–3175.
Muthappa, N.A., Gupta, S., Yengkokpam, S., Debnath, D., Kumar, N., Pal, A.K., Jadhao, Lipotropes, S.B., 2013. Promote immunobiochemical plasticity and protect fish against low-dose pesticide-induced oxidative stress. Cell Stress Chaperone 19 (1), 61–81.
Nakano, K., Iwama, G.K., 2002. The 70-kD a heat shock protein response in two intertidal sculpins, *Oligocottus maculosus* and O. snyderi: relationship of hsp70 and thermal tolerance. Comp. Biochem. Physiol A133, 79–94.
Newberne, P.M., 1993. Themethyl deficiency model: history characteristic and research direction. J. Nutr. Biochem. 4, 618–624.
NRC, (National Research Council), 1993. Nutrient Requirements of Fish. National Academy Press, Washington Bender DA (1992) Nutritional Biochemistry of the Vitamins. Cambridge University Press, New York).
Paladino, F.V., Spotlia, J.R., Schubauer, J.P., Kowalski, K.T., 1980. The critical thermal maximum: a technique used to elucidate physiological stress and adaptation in fish. Rev. Can. Biol 39, 115–122.
Patra, R.W., Chapman, J.C., Lim, R.P., Gehrke, P.C., 2007. The effects of three organic chemicals on the upper thermal tolerances of four freshwater fishes. Env. Toxicol. Chem 26 (7), 1454–1459.
Pongracz, G., 1973. Antioxidant mixtures for use in foods. Int. J. Vit. Nutr. Res. 43, 517–525.
Portner, H.O., 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comp. Biochem. Physiol 132A, 739–761.
Rabanal, H.R., 1988. Development of the aquaculture industry in Southeast Asia. In: Juario, J.V., Benitez, L.V. (Eds.), in: Perspectives in Aquaculture Development in Southeast Asia and Japan. SEAFDEC Aquaculture Department, Tigbauan, pp. 3–37.
Rao, D.M.R., Devi, A.P., Murty, A.S., 1980. Relative toxicity of endosulfan its isomers, and formulated products to the freshwater fish, *Labeo rohita*. J. Toxicol. Environ. Health 6, 825–834.
Rao, D.M.R., Devi, A.P., Murty, A.S., 1981. Toxicity and metabolism of endosulfan and its effect on oxygen consumption and total nitrogen excretion of the fish *Macrognathus aculeatum*. Pest. Biochem. Physiol. 15, 282–287.
Sarma, K., Pal, A.K., Ayyapan, S., Das, T., Manush, S.M., Debnath, D., Baruah, K., 2010. Acclimation of *Anabas testuideneus* (Bloch) to three test temperatures influences thermal tolerance and oxygen consumption. Fish Physiol. Biochem. 36, 85–90.
Smolin, L.A., Benevega, N.J., 1989. Methionine, homocysteine, cysteine metabolic Interrelationships. In: Mendel-Friedman, Z. (Ed.), in: Absorption and Utilization of Amino Acids, Vol. 1. CRC Press, Boca Raton, pp. 155–187.
Takahara, S., Hamilton, B.H., Nell, J.V., Kobra, T.Y., Ogura, Y., Nishimura, E.T., 1960. Hypocatalesemia, a new generis carrier state. J. Clin. Investig. 29, 610–619.
Tsuchida, S., 1995. The relationship between upper temperature tolerance and final preferendum of Japanese marine fish. J. Therm. Biol. 20, 35–40.
Wedemeyer, G.A., Meyer, F.P., Smith, L., 1999. Environmental Stress and Fish Diseases. Narendra Publication House, Delhi.
Werner, I., Smith, T.B., Feliciano, J., Johnson, M.L., 2005. Heat shock proteins in juvenile steelhead reflect thermal conditions in the Navarro river watershed, California. Trans. Am. Fish. Soc. 134, 399–410.