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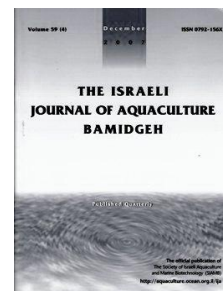
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## Effects of Dietary Pyridoxine on Growth and Physiological Responses of *Labeo rohita* Fingerlings Reared in High Water Temperature

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### Abstract

The role of dietary pyridoxine (vitamin B6) on the growth and physiological responses of *Labeo rohita* fingerlings reared in a high temperature of 33°C was examined in a 60-day trial. Two hundred and seventy fingerlings (6.71±0.32 g) were randomly distributed into six treatments in triplicate. Five iso-caloric and iso-nitrogenous purified diets were prepared with graded levels of pyridoxine: 0, 10, 50, 100, and 200 mg. The control group was given a diet containing 10 mg pyridoxine and reared at ambient temperature (26°C). Pyridoxine supplementation at 100 or 200 mg/kg diet significantly augmented the specific growth rate but there were no significant ( $p>0.05$ ) effects of pyridoxine supplementation on the feed conversion ratio. Superoxide-dismutase, catalase, blood glucose, and serum cortisol were significantly lower and acetylcholinesterase (AChE) significantly ( $p<0.05$ ) higher in the pyridoxine-fed groups. Results indicate that dietary pyridoxine supplementation at the rate of 100 mg/kg diet augments growth and helps lower stress in *L. rohita* fingerlings reared in a high water temperature.

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## Introduction

*Labeo rohita*, an Indian major carp, is one of the most important freshwater aquaculture species in India. The culture period of this species usually encounters seasonal water temperature fluctuations. During summer, the water temperature generally goes up to 34-37°C, beyond the optimum temperature for growth of this species (Das et al., 2005). Temperature has a significant influence on fish physiology and behavior (Beitinger et al., 2000) and is a pervasive factor affecting structure and function at all levels of biological organization. Temperature beyond optimum limits adversely affects fish health due to metabolic stress and increases oxygen demand and disease susceptibility (Wedemeyer et al., 1999), leading to reduced growth and biomass. It is, therefore, imperative to find ways to overcome the implications caused by high summer temperatures. In this context, we examined supplementation of high doses of dietary pyridoxine to overcome the physiological consequences of elevated temperatures in *L. rohita* fingerlings.

Pyridoxine (vitamin B6), including pyridoxal and pyridoxamine, is essential for absorption and metabolism of amino acids and the development of red blood cells (Calderón-Guzmán et al., 2004). It is rapidly taken up by circulating erythrocytes and converted into pyridoxamine and pyridoxal phosphate (Calderón-Guzmán et al., 2004). These act as coenzymes for transaminases (Rogers and Mohan, 1994) and control the biosynthesis of neurotransmitters such as gamma-aminobutyric acid (GABA), dopamine, and serotonin (5-HT). Thus, they are important for development and function of the central nervous system and have anti-stress effects (Ernährungswiss, 1996). High tissue levels of pyridoxal phosphate may work centrally and peripherally to mitigate the physiological consequences of stress (McCarty, 2000). High doses of dietary pyridoxine mitigate endosulfan induced stress and trigger immunity in fish (Akhtar et al., 2010). Pyridoxine also plays an important role in mitigating stress in vertebrate models including human beings (Lettko and Meuer, 1990; McCarty, 2000).

Thermal stress potentially affects enzyme activity and the antioxidant defense system in aquatic organisms (Pörtner, 2002; Hardewig et al., 2004). High temperatures increase reactive oxygen species release and enhance the risk of oxidative damage (Abele et al., 1998). Increased availability of anti-oxidative enzymes such as superoxide dismutase and catalase are believed to minimize oxidative stress (by directly detoxifying harmful reactive oxygen species) and oxidative damage to cellular components and, thus, could be indicators of oxidative stress (Pörtner, 2002). Acetylcholine is a neurotransmitter in the nerve synapse, and the enzyme acetylcholine esterase (AChE) breaks down the compound to prepare for new nerve conduction (Chatterjee et al., 2010). AChE activity is reduced in fish exposed to stressors (Wood et al., 1999; da Fonseca et al., 2008; Akhtar et al., 2010) and may be considered a biomarker for stress.

This experiment was conducted to elucidate the effect of dietary pyridoxine on the growth and stress responses of *L. rohita* fingerlings.

## Materials and Methods

**Fish and acclimation.** Fingerlings of *L. rohita* ( $6.71 \pm 0.32$  g) were procured from Prem Fisheries Consultancy, Gujarat, India, and transported in an aerated circular 500-l container to the experimental facilities at the Central Institute of Fisheries Education, Mumbai. The fish were acclimatized to the experimental rearing conditions for 15 days during which they were fed the control diet containing the normal pyridoxine requirement for Indian major carps, 10 mg/kg (Murthy, 2002).

**Feed and trial.** After acclimatization, 270 fish were distributed into 18 plastic tanks (80 x 57 x 42 cm) containing 150 l water, following a completely randomized design. Five isonitrogenous (35.45-35.75% crude protein) purified diets were prepared from a basal (control) diet (Table 1). Pyridoxine hydrochloride (Himedia Laboratories, Mumbai, India) was added to the control diet at graded levels of 0, 10, 50, 100, or 200 mg/kg. The five pyridoxine-supplemented groups were reared in an elevated temperature of 33°C, raised by thermostatic water heaters to impart thermal stress (Das et al., 2006). The sixth (control) group was fed the basal diet containing no supplemental pyridoxine hydrochloride and reared at ambient temperature, 26°C. The physicochemical

Table 1. Composition of the basal diet.

Ingredient	(g/kg)
Casein (vitamin free) <sup>1</sup>	30
Starch soluble <sup>1</sup>	30
Gelatin <sup>1</sup>	10
Dextrin <sup>1</sup>	10
Cellulose powder <sup>1</sup>	8
Cod liver oil <sup>2</sup>	4
Sunflower oil <sup>3</sup>	4
Carboxymethylcellulose <sup>1</sup>	2
Vitamin mineral mix <sup>4</sup>	1.96
Betaine hydrochloride <sup>1</sup>	0.02
Vitamin C <sup>5</sup>	0.01
Butylated hydroxy toluene <sup>1</sup>	0.01

<sup>1</sup> Himedia Laboratories, India<sup>2</sup> Procured from local market<sup>3</sup> Ruchi Soya Industries Ltd., Raigad, India

<sup>4</sup> Pyridoxine-free, prepared manually, components from Himedia Ltd. India or Glaxo Pharmaceuticals, Mumbai, India if indicated by asterisk; per 250 g starch powder: vitamin A\* 55,00,00 IU; vitamin D<sub>3</sub>\* 11,00,00 IU; vitamin B<sub>2</sub> 200 mg; vitamin E 75 mg; vitamin K 100 mg; vitamin B<sub>12</sub>\* 30 µg; calcium pantothenate 250 mg; nicotinamide 1000 mg; choline chloride 15 g; MnSO<sub>4</sub> 2700 mg; I (KI) 100 mg; ferric citrate 75 mg; ZnSO<sub>4</sub> 500 mg; CuSO<sub>4</sub> 200 mg; CoCl<sub>2</sub> 45 mg; Ca (CaCO<sub>3</sub> and dibasic calcium phosphate) 50 g; P (dibasic calcium phosphate) 30 g; selenium (sodium selenite) 50 ppm

<sup>5</sup> Sd Fine Chemicals Ltd., India

parameters of the water were within the optimum ranges throughout the experimental period: dissolved oxygen 5.6-7.4 mg/l, pH 7.5-8.6, ammonia nitrogen 0.14-0.27 mg/l, nitrite nitrogen 0.001-0.005 mg/l, and nitrate nitrogen 0.02-0.07 mg/l. Aeration was continuous throughout the experimental period. Fish were fed daily at 2.5% of their body weight: about two thirds of the daily ration was given at 09:00 and one third at 18:00. Uneaten feed and fecal matter were removed by siphoning and about 25% of the water was exchanged with pre-heated water (33°C) daily. At the end of the the 60-day experiment, specific growth rate and feed conversion ratio were calculated.

**Proximate analysis of feed.** Proximate compositions of the experimental diets were determined by standard methods of AOAC (1995; Table 2). Moisture was determined by drying at 105°C to a constant weight, nitrogen by the Kjeldahl method, and crude protein by multiplying the percent nitrogen by 6.25. Ether extract was measured by the solvent extraction method using diethyl ether (boiling point, 40-60°C) as a solvent and ash was determined by incinerating the samples in a muffle furnace at 600°C for 6 h. Total carbohydrate (%) was calculated as 100 - (%crude protein + %ether extract + %ash).

**Sample preparation.** At the end of the experiment, two fish from each replicate from each treatment were anesthetized with CIFEALM at 200 µl/l (CIFE, Mumbai, India). For enzyme assay, separate homogenates of gill, liver, and brain tissues were prepared. The tissues were homogenized with chilled 0.25 M sucrose solution using a mechanical tissue homogenizer. The

homogenized samples were centrifuged (5000 x g for 10 min at 4°C) and supernatants were collected and stored at -20°C for subsequent enzyme assay. Blood was collected from caudal veins using a no. 23 medical syringe rinsed with 2.7% anticoagulant EDTA solution and gently shaken to prevent hemolysis of the blood. The blood samples were used to determine blood glucose. For serum, two more fish from each replicate were anesthetized; blood was collected without an anti-coagulant, allowed to clot for 2 h, centrifuged at 3000 x g for 5 min, and kept at -80°C until use.

**Enzyme assays.** AChE (EC.3.1.1.7) activity was measured by the change in optical density (OD) at 540 nm using the method of Augustinsson (1957). Catalase (E.C.1.11.1.6) was assayed using 50 mM phosphate buffer (pH 7.0). The reaction was initiated by adding 30% H<sub>2</sub>O<sub>2</sub> as a substrate and OD was recorded at 240 nm (Claiborne, 1985). Superoxide dismutase (E.C.1.15.1.1) activity was estimated by the method of Misra and Fridovich (1972). The assay is based on the oxidation of epinephrine-adrenochrome trans-  
 my Table 2. Proximate analysis of experimental diets (% dry matter basis) fed to *Labeo rohita* fingerlings for 60 days (means±SE, n = 3).  
 The reaction mixture consisted of 50 µl of the sample, 1.5 ml phosphate buffer, and 0.5 ml epinephrine. The solution was mixed and OD was immediately read at 480 nm.

Table 2. Proximate analysis of experimental diets (% dry matter basis) fed to *Labeo rohita* fingerlings for 60 days (means±SE, n = 3).

Proximate composition (%)	Dietary supplement (mg pyridoxine hydrochloride/kg diet)				
	0	10	50	100	200
Moisture	8.38±0.20	8.04±0.06	8.71±0.25	8.42±0.65	8.48±0.41
Dry matter	91.62±0.20	91.96±0.06	91.29±0.25	91.58±0.65	91.52±0.41
Crude protein	35.70±0.24	35.45±0.29	35.68±0.42	35.56±0.13	35.75±0.28
Ether extract	8.54±0.13	8.74±0.37	8.69±0.43	8.84±0.14	8.81±0.40
Ash	9.09±0.40	8.44±0.26	9.65±0.68	9.10±0.38	9.02±0.58
Total carbohydrate	46.67±0.32	47.37±0.7	45.98±0.6	46.50±0.43	46.42±1.1

**Blood glucose and serum cortisol.** Blood glucose was estimated by the method of Nelson and Somogyi (1945). Cortisol in fish serum was estimated using a validated radioimmunoassay as described by Winberg and Lepage (1998) and expressed as ng/ml.

**Statistical analysis.** Mean values of all parameters were subjected to one-way ANOVA to study the treatment effect and Duncan's Multiple Range Tests were used to determine the significance of differences between means. Comparisons were made at the 5% probability level. Data were analyzed using statistical package SPSS (vers. 16).

## Results

Dietary pyridoxine supplementation significantly affected the specific growth rate (SGR) but not the feed conversion ratio (FCR; Table 3). AChE activity was significantly affected and there was a strong negative correlation ( $r = 0.9$ ) between AChE activity and blood glucose. Catalase activity in the liver and gill significantly differed between groups but there were no definite trends in catalase activity. In general, SOD activity in fish reared in 33°C was significantly higher than in control fish and decreased as the level of pyridoxine rose. Blood glucose positively correlated with serum cortisol ( $r = 0.79$ ). Serum cortisol demonstrated a third order polynomial relationship with dietary pyridoxine ( $Y = 6.877x^3 - 83.482x^2 + 298.25x - 134.66$ ;  $R^2 = 0.939$ ).

Table 3. Effect of dietary pyridoxine supplementation on *Labio rohita* fingerlings reared in a high water temperature of 33°C (means±SE, n = 6).

	Control	Dietary supplement (mg pyridoxine hydrochloride/kg diet)				
	0 (26°C)	0	10	50	100	200
SGR <sup>1</sup>	1.20±0.01 <sup>d</sup>	1.02±0.01 <sup>a</sup>	1.07±0.02 <sup>b</sup>	1.08±0.06 <sup>b</sup>	1.11±0.02 <sup>c</sup>	1.10±0.01 <sup>c</sup>
FCR <sup>2</sup>	1.62±0.01	1.88±0.03	1.76±0.07	1.79±0.12	1.72±0.04	1.71±0.01
Liver catalase <sup>3</sup>	6.84±2.01 <sup>a</sup>	12.26±1.35 <sup>b</sup>	5.24±0.47 <sup>a</sup>	4.42±0.48 <sup>a</sup>	6.14±0.16 <sup>a</sup>	10.82±0.70 <sup>b</sup>
Gill catalase <sup>3</sup>	0.59±0.05 <sup>d</sup>	0.43±0.05 <sup>c</sup>	0.21±0.03 <sup>a</sup>	0.31±0.02 <sup>ab</sup>	0.45±0.03 <sup>c</sup>	0.42±0.01 <sup>bc</sup>
Liver SOD <sup>4</sup>	77.30±0.80 <sup>a</sup>	93.49±0.44 <sup>c</sup>	96.17±0.35 <sup>c</sup>	82.47±4.39 <sup>ab</sup>	78.26±1.52 <sup>ab</sup>	84.19±1.08 <sup>b</sup>
Gill SOD <sup>4</sup>	19.64±1.38 <sup>b</sup>	35.06±2.18 <sup>d</sup>	25.29±1.16 <sup>c</sup>	21.65±1.66 <sup>bc</sup>	12.26±0.41 <sup>a</sup>	11.69±0.25 <sup>a</sup>
Brain AChE <sup>5</sup>	2.02±0.07 <sup>c</sup>	1.03±0.05 <sup>a</sup>	1.16±0.06 <sup>a</sup>	1.52±0.11 <sup>b</sup>	1.54±0.12 <sup>b</sup>	1.59±0.10 <sup>b</sup>
Blood glucose (g/dl)	32.37±2.93 <sup>a</sup>	40.57±1.52 <sup>c</sup>	38.58±2.54 <sup>bc</sup>	33.92±1.40 <sup>ab</sup>	32.99±0.90 <sup>ab</sup>	33.37±1.17 <sup>ab</sup>
Serum cortisol (ng/ml)	85.88±2.11 <sup>a</sup>	189.05±3.20 <sup>e</sup>	180.88±1.55 <sup>d</sup>	177.55±2.63 <sup>d</sup>	121.10±0.90 <sup>b</sup>	136.63±2.44 <sup>c</sup>

Values in a row bearing different superscripts vary significantly ( $p < 0.05$ ).

<sup>1</sup> Specific growth rate (%/day) =  $100(\text{Log}_e \text{ final wt} - \text{Log}_e \text{ initial wt})/\text{no. days}$

<sup>2</sup> Feed conversion ratio = dry wt of feed given/wet wt of body gain

<sup>3</sup> Catalase = m moles  $\text{H}_2\text{O}_2$  decomposed /min/mg protein at 37°C

<sup>4</sup> Superoxide dismutase =  $\mu$  mole/mg protein/min at 37°C

<sup>5</sup> Acetylcholine esterase =  $\mu$  moles of acetylcholine hydrolyzed/min/mg protein at 37°C

## Discussion

The present study shows that dietary pyridoxine significantly affects growth performance, anti-oxidative enzyme activity, and stress parameters. The significant reduction in SGR in groups reared in high temperature agrees with findings of Das et al. (2005), who reported a reduction in percent weight gain in *L. rohita* fingerlings reared under thermal stress (33°C). The higher SGR in groups fed the 100 and 200 mg/kg diets could be due to stress mitigating effects of dietary pyridoxine (Akhtar et al., 2010) or improved dietary protein utilization and enhanced protein synthesis (Albrektsen et al., 1993). Similar findings were reported in jian carp (He et al., 2009), gilthead sea bream fry (Baker and Davies, 1995), and *L. rohita* fingerlings (Akhtar et al., 2011).

Among the fish raised in high temperature, liver catalase activity and SOD were highest in fish that received no supplementary pyridoxine and lowest in fish fed the diet containing 100 mg supplementary pyridoxine, in agreement with Akhtar et al. (2010), indicating that oxidative stress was lowest in fish fed the 100 mg/kg diet. Among the fish raised in high temperature, AChE activity in brain tissue rose when fish were fed at least 50 mg supplementary pyridoxine, suggesting that pyridoxine counteracts stress caused by high temperature in *L. rohita* fingerlings.

Supplementation of dietary pyridoxine led to a decrease in the blood glucose level, similar to *L. rohita* exposed to endosulfan (Akhtar et al., 2010). The level of blood glucose rises in rainbow trout when stressors such as dichlorodiphenyldichloroethane are applied (Benguira et al., 2002) and in Atlantic salmon (*Salmo salar*) when the aluminum concentration is high (Ytrestøyl et al., 2001). The lower glucose levels in the pyridoxine supplemented groups may result from efficient utilization of blood glucose.

Release of cortisol is dependent on the duration and strength of a stressor (Barton and Iwama, 1991). Elevated cortisol levels result from environmental stressors in rainbow trout such as acid water (Brown et al., 1984) or mercury chloride and methylmercury (Bleau et al., 1996) and in *Cirrhinus mrigala* fingerlings after chronic crowding stress (Tejpal et al., 2009). In our study, among the fish raised in high temperature, the cortisol level was significantly highest in those fed the 0 mg diet and lowest in those fed the 100 mg diet. Hence, we conclude that dietary pyridoxine helps reduce thermal stress by maintaining the serum cortisol level.

In conclusion, dietary supplementation of pyridoxine at 100 mg/kg diet enhances growth and reduces thermal stress in *L. rohita* fingerlings reared in high water temperature.

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