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Dietary vitamin E requirement for maximizing the growth, conversion efficiency, biochemical composition and haematological status of fingerling *Channa punctatus*

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Abstract

The effect of feeding graded levels of vitamin E (E_0, E_{20}, E_{40}, E_{60}, E_{100}, E_{140}, E_{180}, E_{220}, E_{260}) in nine casein–gelatin-based isonitrogenous (450 g kg^{-1} crude protein) and isoenergetic (17.97 kJ g^{-1} gross energy) experimental diets was evaluated in fingerling *Channa punctatus* for 12 weeks. Growth, nutritional and haematological parameters were studied. Hepatic lipid peroxidation as thiobarbituric acid-reactive substances (TBARS) was also assayed. The maximum absolute weight gain (AWG g/fish, 55), best feed conversion ratio (FCR, 1.32), protein retention efficiency (PRE, 40%) and energy retention efficiency (ERE, 76%) were achieved in fish fed on a diet supplemented with 140 mg vitamin E kg^{-1} diet (E_{140}). A consistent decline in the hepatic TBARS concentration and an improvement in haematocrit (Hct) and haemoglobin (Hb) were displayed in fish fed on diets with increasing concentrations of vitamin E up to 140 mg kg^{-1} (E_0–E_{140}), beyond which (E_{180}–E_{260}) a reverse trend in these parameters was evident. Based on the broken-line regression and exponential analyses of AWG, FCR, PRE, ERE, Hb and Hct data, diets for fingerling *C. punctatus* should contain vitamin E in the range of 140–169 mg kg^{-1} to maintain satisfactory fish performance.

Keywords

vitamin E, growth, conversion efficiency, biochemical composition, fingerling *Channa punctatus*, lipid peroxidation

Introduction

Vitamins are one of the most expensive additives to prepare nutritionally complete diets for fish production (Gaylord, Rawles & Gatlin III 1998). Among these, fat-soluble antioxidants such as vitamin E have received considerable attention in fish nutrition because of their role in various biochemical and physiological processes like preventing oxidation of unsaturated lipids in fish tissues (Zhong, Lall & Shahidi 2007). Also, the role of vitamin E in signalling transduction has been looked into (Kocabas & Gatlin III 1999; Brigelius-Flohé, Kelly, Salonen, Neuzil, Zingg & Azzi 2002; Fang, Yang & Wu 2002). Vitamin E is an essential nutrient that cannot be synthesized by the fish based on its dietary intake (Peng & Gatlin III 2009). Feeding diets supplemented with vitamin E have been proved to improve growth performance (Kocabas & Gatlin III 1999), enhance immunity, increase oxidative stability and shelf-life (Gatlin III 2002; Sealey & Gatlin III 2002; Puangkaew, Kiron, Somamoto, Okamoto, Satoh, Takeuchi & Watanabe 2004; Trushenski & Kohler 2007) and restore impaired immunity (Montero, Marrero, Izquierdo, Robaina, Vergara & Tort 1999; Sahoo & Mukherjee 2002) On the other hand, vitamin E maintains the flesh quality, normal resistance of red blood corpuscles to haemolysis and the permeability of capillaries and heart muscle (Halver 2002). The deficiency of vitamin E in fish causes muscular dystrophy, exudative diathesis, anaemia, impaired erythropoiesis, erythrocyte fragility, skin decolouration and ceroid

pigment deposition (Mourente, Bell & Tocher 2007). As one of the most important free radical scavengers, vitamin E plays very important roles in the maintenance of redox potential and prevention of lipid peroxidation, which may be exacerbated by its imbalance in dietary formulations. For this reason, biochemical profiles like the levels of hepatic thiobarbituric acid (TBA)-reactive substances (TBARS) such as malonaldehyde (MDA) and haematological parameters are good indicators to ascertain the optimum vitamin E requirement (Bahmani, Kazemi & Donskaya 2001).

Hepatic TBARS analysis is one of the most popular and commonly used methods for assessing tissue peroxidation (Rosmini, Perlo, Pérez-Alvarez, Pagán-Moreno, Gago-Gago, López-Santoveña & Aranda-Catalá 1996). The products of endoperoxide reactions are ketones and various toxic aldehydes. Among these aldehydes, MDA, a secondary oxidation product of polyunsaturated fatty acids, is an important indicator of the oxidative changes of lipid (Shahidi & Hong 1991). Likewise, blood offers an important parameter to study the nutritional impact on aquatic animals. Different blood parameters are often subjected to change depending on the nutritional status of the fish. Major changes occur in the blood composition of the fish in response to stress, pollutants and nutritional conditions. Variations in the levels of blood cells have also been reported in response to dietary manipulations. Therefore, haematological studies are regarded as one of the important biological features in aquaculture (Bahmani *et al.* 2001).

Channa punctatus, commonly known as spotted murrel or soli, is a highly priced, commercially important tropical freshwater food fish (Hossain, Ehshan, Mazid, Rahman & Razzaque 2000; Sarma, Pal, Sahu, Mukharjee & Baruah 2010). It is found to be distributed throughout the south-east Asian countries and is a natural inhabitant of stagnant muddy pond waters, paddy fields, weedy derelict swamps, beels canals and reservoirs (Chondor 1999). This fish is considered to be hardy due to its tolerance to the extreme environmental conditions, crowdness and diseases and is well known for its taste, high protein content, low intramuscular spines, high nutritive value, recuperative and medicinal qualities. It is recommended in a diet during convalescence and, therefore, is a good candidate for intensive aquaculture (Haniffa, Marimuthu, Nagarajan, Jesu Arockia Raj & Kumar 2004; Bhuiyan, Afroz & Zaman 2006; Marimuthu, Haniffa & Rahman 2009). The flesh extract of *Channa striatus,* another species of the same genus, has been reported to be of pharmaceutical value and is traditionally used for post operative wound healing and reduction of post surgery pains (Wee 1982; Mat Jais, McCulloh & Croft 1994; Baie & Sheikh 2000). Although much work has been carried out in the past to optimize the dietary vitamin E concentrations in various other cultivable fish species (Murai & Andrews 1974; Watanabe, Takeuchi, Matsui, Ogino & Kawabata 1977; Wilson, Bowser & Poe 1984; NRC 1993; Hamre & Lie 1995; Bai & Lee 1998; Kocabas & Gatlin III 1999; Paul, Sarkar & Mohanty 2004; Sau, Paul, Mohanta & Mohanty 2004; Peng & Gatlin III 2009), information on the effects of dietary vitamin E levels on the growth, biochemical composition and haematological parameters of fingerling *C. punctatus* is not available. The present study was, therefore, aimed at generating data on the optimum dietary vitamin E requirement of fingerling *C. punctatus* for developing vitamin E-balanced commercial feeds for its culture.

Materials and methods

Experimental diets

Casein–gelatin-based isonitrogenous (450 g kg^{-1} crude protein) and isoenergetic (17.97 kJ g^{-1} gross energy) purified diets were formulated (Table 1). The basal diet was supplemented to achieve eight levels of vitamin E (DL-α-tocopheryl acetate): 20, 40, 60, 100, 140, 180, 220 and 260 mg kg^{-1} diet. The dietary range necessary to quantify the vitamin E requirement was fixed keeping in view the information available on other warm water fish species (Sau *et al.* 2004). Vitamin-free casein was used as an intact protein source. Gelatin, dextrin, carboxymethylcellulose and α-cellulose were ethanol extracted to remove the traces of vitamins. A blend of cod liver oil and corn oil (2:5) was used as the dietary lipid source to provide n-3 and n-6 fatty acids. Diets were analysed for DL-α-tocopheryl acetate using reverse phase high-performance liquid chromatography (Tangney, McNair & Drikell 1981). The analysed vitamin E content of the basal diet, diet E_0, was 10 mg kg^{-1}, which was taken into consideration in designing the doses of vitamin E. The rest of the amount of vitamin E was supplemented to obtain the final doses. Mineral and vitamin premixes (excluding vitamin E) were similar to those used by Halver (2002). Calculated quantities of the dietary ingredients were stirred mechanically for about 30 min in a Hobart electric mixer (Hobart, Troy, OH, USA) and prepared as reported earlier (Khan

& Abidi 2007). The final diet with a bread dough consistency was poured in Teflon plates, cut in the form of uniform-size cubes (1 × 1 × 1 cm), packed in air-tight polythene bags and kept in a refrigerator at − 20 °C till further use. Because the diets were coated with casein and gelatin and then bound with a substantial amount of carboxymethylcellulose, the water stability of the diet was found to be 97%.

Animals and experimental conditions

Fingerling *C. punctatus* were procured from a fish pond of the Department of Zoology, Aligarh Muslim University, Aligarh, UP, India, and stocked in indoor circular aqua-blue-coloured, plastic-lined (Plastic Crafts, Mumbai, India) fish tanks (1.22 m × 0.91 m × 0.91 m; water volume 600 L) and acclimated for 2 weeks by feeding (450 g kg^{-1} crude protein) a casein–gelatin-based H-440 diet (Halver-440; Halver 2002) twice a day at 07:00 and 17:30 hours. Acclimated *C. punctatus* fingerling (7.1 ± 0.6 cm; 5.0 ± 0.3 g) were then taken and stocked at the rate of 10 fish per 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water (ground and aerated water) flow-through (1–1.5 L min^{-1}) system for each dietary treatment in triplicate groups. Fish were fed test diets in the form of semi-moist cakes to apparent satiation twice daily at 07:00 and 17:30 hours from 2 March 2009 onwards. The initial and weekly mass weights were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland). Fish were deprived of feed before the fish were batch weighed. The feeding trial lasted for 12 weeks. Faecal matter was siphoned off and unconsumed feed, if any, was collected and adjusted in the feed conversion ratio (FCR) calculation. Water from each trough was analysed daily for dissolved oxygen (6.9–8.1 mgL^{-1}), free carbon dioxide (6.5–7.9 mgL^{-1}), pH (7.3–8.0), total alkalinity (70–82 mg L^{-1}), ammonia content (< 0.02 ppm) and temperature (27.5–28 °C) following standard methods (APHA 1992). A photoperiod (12 h L:12 h D in natural light; average light intensity = 50 klx) was maintained.

Composition and gross energy assay

The proximate composition of casein, gelatin, experimental diets and initial and the final body composition was estimated using standard methods (AOAC 1995) for dry matter (oven drying at 105 ± 1 °C for 22 h), crude protein (Kjeltec Tecator TM Technology 2300, Hoegenas, Sweden), crude fat (solvent extraction with petroleum ether B.P. 40–60 °C for 2–4 h using Socs Plus equipment, SCS 4, Chennai, India) and ash (oven incineration at 650 °C for 2–3 h using Muffle Furnace, S.M. Scientific Instrument (p) Jindal Company, Delhi, India). Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter-CBB 330 010 L (Gallenkamp, Loughbrough, UK). The concentrations of DL-α-tocopheryl acetate in diets were analysed using reverse phase high-performance liquid chromatography (System Gold HPLC Unit, Beckman Instruments, San Ramon, CA, USA) (Tangney *et al.* 1981). Six subsamples of a pooled sample of 10 fish were analysed for initial body composition. At the end of the experiment, the remaining four fish from each triplicate of dietary treatments ($n = 3 \times 4$) were pooled separately and analysed for the final whole body composition.

Growth parameters

The length, body weight and liver weights of three fish per triplicate ($n = 3 \times 3$) were recorded for the calculation of the hepatosomatic index (HSI) and condition factor (CF g/cm^3)

$$\text{Absolute weight gain (AWG, g/fish)} = (\text{final body weight} - \text{initial body weight})$$

$$\text{FCR} = \text{dry feed intake} / \text{wet weight gain}$$

$$\text{Protein retention efficiency (PRE, \%)} = (\text{protein gain}/\text{protein fed}) \times 100$$

$$\text{Energy retention efficiency (ERE, \%)} = (\text{energy gain}/\text{energy fed}) \times 100$$

$$\text{HSI (\%)} = (\text{liver weight})/(\text{whole body weight}) \times 100$$

$$\text{CF (g cm}^{-3}) = (\text{body weight})/(\text{body length})^3 \times 100$$

Blood assays

At the termination of the experiment, before taking the mass weight, fish were tranquilized using MS-222 (Methane sulphonic acid), blood samples were collected in the morning at 09:30 hours using a heparinized syringe from the caudal vein of three fish from each treatment in triplicate ($n = 3 \times 3$), pooled and used to determine blood characteristics. Erythrocyte count was determined using an improved Neubauer haematocytometer with Yokoyama's (1947) solution as the diluting medium. Blood haemoglobin (Hb) was estimated colorimetrically

Table 1 Composition of the basal diet used for estimating the dietary vitamin E requirement of fingerling *Channa punctatus*

Ingredients	(g kg^{-1} dry diet)
Casein (vitamin free)*	450
Gelatin†	120
Dextrin (dextrin white)‡	301.8
Corn oil§	50
Cod liver oil¶	20
Vitamin mix†† (vitamin E free)‖,**	30
Mineral mix††,‖	40
α-cellulose (cellulose microcrystalline)	11.9
Carboxymethyl cellulose	100
Total	1000
Proximate composition of the diet (g kg^{-1} dry basis)	
Analysed crude protein (g kg^{-1})	448
Analysed crude lipids (g kg^{-1})	69.9
Analysed moisture content (g kg^{-1})	321
Analysed ash content (g kg^{-1})	76.4
Analysed crude fibre (g kg^{-1})	2.5
NFE	83.1
Gross energy (kJ g^{-1}, dry weight basis)§§	17.98

*Crude protein (800 g kg^{-1}); Loba Chemei (Mumbai, India).
†Crude protein (970 g kg^{-1}); Loba Chemei.
‡Loba Chemei.
§Corn oil was obtained from Fortune, Adani Wilmar (Gujrat, India).
¶Cod liver oil from SevenSeas (Hull, UK).
‖Halver 2002.
**The analysed vitamin E contents of the diets supplemented with 20, 40, 60, 100, 140, 180, 220 and 260 mg kg^{-1} as DL-α-tocopheryl acetate were 25, 47, 70, 110, 149, 190, 235 and 270 mg kg^{-1} respectively. The analysed vitamin E content of the basal diet, diet E_0, was 10 mg kg^{-1}.
††Vitamin premix (10:20 g kg^{-1}; 9.6 g vitamin mix+19.2 g α-cellulose) choline chloride 5; inositol 2; ascorbic acid 1; niacin 0.75; calcium pantothenate 0.5; riboflavin 0.2; menadione 0.04; pyridoxine hydrochloride 0.05; thiamin hydrochloride 0.05; folic acid 0.015; biotin 0.005; vitamin B_{12} 0.0001; vitamin E as DL-α-tocopheryl acetate was supplemented to the basal diet at the expense of small amounts of α-cellulose.
‡‡Mineral mixture (g kg^{-1}) calcium biphosphate 135.7; calcium lactate 326.9; ferric citrate 29.7; magnesium sulphate 132.0; potassium phosphate (dibasic) 239.8; sodium biphosphate 87.2; sodium chloride 43.5; aluminium chloride · $6H_2O$ 0.154; potassium iodide 0.15; cuprous chloride 0.10; magnus sulphate · H_2O 0.80; cobalt chloride · $6H_2O$ 1.0; zinc sulphate · $7H_2O$ 4.0; Loba Chemie India.
§§Gross energy of the diet was estimated on a Gallenkamp ballistic bomb calorimeter.

following Wong's (1928) method. Haematocrit (Hct) value was measured by spinning the micro-Wintrobe tube containing well-mixed blood for about 5 min at 12 000 *g*. Erythrocyte osmotic fragility (EOF) of the fingerling *C. punctatus* fed various concentrations of dietary vitamin E was assessed using the method of gradient saline solutions as per Ezell, Sulya and Dodgen (1969). Six microlitre of blood was added to 2 mL of a salt (NaCl) solution each with different concentrations (0%, 0.25%, 0.45%, 0.55%, 0.65% and 0.85%). After incubation at room temperature for 30 min, the tubes were centrifuged at 3000 *g* for 10 min. The percentage of haemolysis was calculated by reading the optical density of the supernatant against salt concentrations at 540 nm using a spectrophotometer (ThermoSpectronic, Genesys-10S, Rochester, NY, USA).

TBARS assays

After blood extraction, the liver was removed from experimental fish (three fish from triplicate treatment, $n = 3 \times 3$) for assays of MDA, a secondary oxidation product of polyunsaturated fatty acids. The reaction substance was formed by the condensation of one MDA molecule with two TBA molecules. Lipid peroxidation was determined using the procedure of Utley, Bernheim and Hachslein (1967), with some modifications as adopted by Fatima, Ahmad, Sayeed, Athar and Raisuddin (2000). Briefly, the liver tissue was homogenized in a chilled 0.1 M KCl solution. The assay mixture contained 0.67% TBA, 10% chilled TCA and homogenate (10%) in a total volume of 3 mL. The rate of lipid peroxidation was expressed as nanomoles of TBARS g^{-1} liver in the form of MDA using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Statistical analyses

Data were subjected to analysis of variance (Sokal & Rohlf 1981). Differences among treatment means were determined using Duncan's multiple range test at a $P < 0.05$ level of significance (Duncan 1955). Optimum vitamin E requirement was estimated using broken-line regression analysis ($Y = a + bX$; Robbins, Norton & Baker 1979). Because the response of a limiting nutrient is non-linear, an exponential analysis of the growth, feed conversion, protein and ERE was also carried out to predict a more reliable estimate of optimum dietary vitamin E requirement.

Because the weight gain, PRE and ERE are generally found to increase in response to increasing concentrations of limiting nutrient in the diets up to a certain level, the equation used for describing the respective response variables is as follows:

$$y = y_0 + A/(W^*\mathrm{sqrt}(pi/2))^{e-2(x-x_c)2/W2}$$

where y is the dependent variable; y_0 is the offset; x_c is the centre of the curve and W is the plateau value for the curve.

Contrary to the above, because FCR shows a negative response with increasing concentrations of the dietary limiting nutrient up to a certain level, the equation used is as follows:

$$y = y_0 + A_1 e_1^{-x/t} - A_2 e_2^{-x/t}$$

where y is the dependent variable; y_0 is the offset; A_1 and A_2 are the amplitudes; and t_1 and t_2 are the decay constants.

All the statistical analyses were performed using ORIGIN (version 6.1; Origin Software, San Clemente, CA, USA) and MATLAB (version 7.1; MATLAB Software, Natick, MA, USA).

Results

Body composition

The body composition of fingerling *C. punctatus* was significantly ($P < 0.05$) affected by the dietary vitamin E concentrations (Table 2). Moisture content showed a positive correlation, whereas in contrast, the fat content decreased with an increase in the vitamin E concentrations. Body protein tended to increase significantly ($P < 0.05$) with increasing dietary vitamin E concentrations up to 140 mg kg^{-1} (E_{140}) of the diet. Body ash content was found to decrease significantly in fish fed diets with increasing levels of vitamin E up to 100 mg kg^{-1} (E_{100}), followed by significant decreased values ($P < 0.05$) that were maintained for the remaining increasing levels of vitamin E.

Growth performance

Survival was not affected by the dietary deficiency or excess of vitamin E. Fish fed the dietary vitamin E at 140 mg kg^{-1} (E_{140}) had significantly higher ($P < 0.05$) AWG, PRE, ERE and the lowest FCR (Table 3). These parameters remained almost the same for the groups fed dietary vitamin E at 180 mg kg^{-1} (E_{180}). However, further inclusion of dietary vitamin E at 220 and 260 mg kg^{-1} in diets E_{220} and E_{260} significantly depressed ($P < 0.05$) growth, feed conversion and nutrient retention efficiency.

Data related to somatic indices are summarized in Table 4. Condition factor and HSI were significantly increased and decreased, respectively, by dietary vitamin E supplementation up to 140 mg kg^{-1} (E_{140}).

Haematological parameters

The haematological parameters of the fish fed diets with different concentrations of vitamin E are pre-

Table 2 Biochemical composition of fingerling *Channa punctatus* fed graded concentrations of dietary vitamin E

	Experimental diets								
% (wet basis)	**(E_0)**	**(E_{20})**	**(E_{40})**	**(E_{60})**	**(E_{100})**	**(E_{140})**	**(E_{180})**	**(E_{220})**	**(E_{260})**
Moisture	74.5 ± 0.11^{f}	74.7 ± 0.3^{f}	74.8 ± 0.1^{f}	75.3 ± 0.8^{e}	75.9 ± 0.1^{d}	76.9 ± 0.24^{c}	77.2 ± 0.1^{c}	78.1 ± 0.2^{b}	79.2 ± 0.4^{a}
Protein	12.0 ± 0.3^{h}	12.9 ± 0.3^{g}	13.8 ± 0.3^{f}	14.7 ± 0.8^{e}	15.5 ± 0.5^{d}	18.3 ± 0.3^{a}	18.0 ± 0.2^{a}	17.4 ± 0.2^{b}	16.1 ± 0.2^{c}
Fat	6.6 ± 0.2^{a}	6.54 ± 0.13^{a}	6.4 ± 0.3^{a}	5.7 ± 0.8^{b}	4.9 ± 0.5^{c}	4.6 ± 0.4^{d}	4.51 ± 0.12^{e}	4.4 ± 0.2^{f}	4.34 ± 0.13^{g}
Ash	4.4 ± 0.3^{a}	3.9 ± 0.3^{b}	3.8 ± 0.5^{b}	3.7 ± 0.8^{b}	3.7 ± 0.5^{b}	3.1 ± 0.4^{c}	3.2 ± 0.4^{c}	3.0 ± 0.2^{c}	3.1 ± 0.4^{c}
TBARS (n mol MDA g^{-1} liver tissue)	1.79 ± 0.15^{a}	1.67 ± 0.23^{b}	1.56 ± 0.45^{b}	1.55 ± 0.28^{b}	1.53 ± 0.35^{b}	1.46 ± 0.49^{c}	1.48 ± 0.44^{c}	1.50 ± 0.21^{c}	1.52 ± 0.24^{c}

Mean values of pooled samples ($n = 3 \times 4$). Mean with different superscripts letter in a same row are significantly different ($P < 0.05$).

Table 3 Growth and conversion efficiencies of fingerling *Channa punctatus* fed graded concentrations of dietary vitamin E

	Experimental diets							
	(E_0)	(E_{20})	(E_{40})	(E_{60})	(E_{100})	(E_{140})	(E_{180})	(E_{220})	(E_{260})
Initial weight (g)	7.1 ± 0.2^{ab}	6.9 ± 0.3^{ab}	7.4 ± 0.3^{a}	7.2 ± 0.2^{ab}	7.3 ± 0.4^{ab}	7.2 ± 0.5^{ab}	7.5 ± 0.9^{a}	7.1 ± 0.2^{ab}	7.4 ± 0.6^{a}
Final weight (g)	29.2 ± 0.5^{f}	36.3 ± 0.9^{e}	41.4 ± 0.7^{d}	46.2 ± 0.8^{c}	47 ± 0.7^{c}	62.4 ± 0.9^{a}	61.5 ± 0.5^{a}	57.1 ± 0.9^{b}	42.4 ± 0.8^{d}
AWG (g/fish)	22.1 ± 2.1^{f}	29.4 ± 1.2^{e}	34.1 ± 2.3^{d}	39.3 ± 3.4^{d}	47.1 ± 2.2^{c}	55.2 ± 1.5^{a}	54.1 ± 2.4^{a}	50.2 ± 2.4^{b}	35.3 ± 1.6^{d}
FCR	2.91 ± 0.13^{a}	2.33 ± 0.15^{b}	2.01 ± 0.12^{b}	1.88 ± 0.21^{c}	1.65 ± 0.15^{d}	1.32 ± 0.15^{g}	1.33 ± 0.11^{g}	1.47 ± 0.09^{f}	1.59 ± 0.18^{e}
PRE (%)	14.1 ± 1.1^{f}	20.2 ± 2.1^{e}	28.3 ± 1.5^{d}	33.3 ± 2.1^{c}	37.1 ± 2.1^{b}	42.1 ± 2.1^{a}	40.2 ± 4.1^{a}	37.1 ± 3.2^{b}	30.4 ± 2.2^{c}
ERE (%)	45.3 ± 2.1^{f}	49.1 ± 1.4^{e}	55.3 ± 2.4^{d}	63.3 ± 3.2^{c}	71.1 ± 2.1^{b}	79.3 ± 2.3^{a}	76.1 ± 2.1^{a}	72.4 ± 1.5^{b}	61.3 ± 2.1^{c}

Mean values of pooled samples ($n = 3 \times 3$). Mean with different superscripts letter in a row are significantly different ($P < 0.05$).
AWG, absolute weight gain; FCR, feed conversion ratio; PRE, protein retention efficiency; ERE, energy retention efficiency.

sented in Table 4. The levels of vitamin E in the diet significantly affected Hct, Hb concentration and red blood cells count (RBCs). Hct and Hb concentration increased significantly with an increase in the dietary vitamin E concentrations up to 140 mg kg^{-1} (E_{140}), followed by a significant decline in fish fed diets E_{180} and E_{220}.

EOF

Erythrocyte osmotic fragility was found to decrease with an increase in the dietary vitamin E concentration up to 140 mg kg^{-1} diet, beyond which no significant differences ($P < 0.05$) in the erythrocyte fragility were evident (Table 4).

TBARS concentrations

Tissue TBARS concentrations showed a significant negative correlation with the increased supplementation of dietary vitamin E up to 140 mg kg^{-1} in diet E_{140} and ranged between 1.46 and 1.79 nmol MDA g^{-1} of liver tissue. From this concentration, the values remained almost unchanged.

Broken-line and exponential analyses

Broken-line regression analysis of AWG, FCR, PRE, ERE, Hb and Hct data (Y) against dietary vitamin E concentrations (X) yielded the break-points at approximately 155, 145, 143.9, 151, 150.72 and 154 mg kg^{-1} vitamin E of the diet. The linear equations used to calculate the optimum dietary vitamin E levels are provided in Table 5. However, the exponential fitting of AWG, FCR, PRE, ERE, Hb and Hct data (Y) to dietary concentrations of vitamin E (X) yielded the estimated values for AWG, FCR, PRE, ERE, Hb and Hct at approximately 167 (Fig. 1), 142, 166, 169, 168 and 169 mg kg^{-1} vitamin E of the diet. The exponential equations used to calculate the respective response variables are depicted in Table 5.

Discussion

Vitamin E is one of the important lipid-soluble vitamins and anti-oxidant molecules present in biological systems. It is a membrane-associated vitamin that functions as a radical scavenger inhibiting the peroxidation of lipids in cellular membranes. It is a potent antioxidant that prolongs the life of erythrocytes and plays an essential role in cellular respiration (Hung, Cho & Slinger 1981). Vitamin E deficiency

Table 4 Somatic and haematological indices of fingerling *Channa punctatus* fed diets with various concentrations of vitamin E

Experimental diets	HSI (%)	CF (g/cm^3)	Hb (g/dL)	Hct (%)	RBCs ($\times$ 10^9/mL)	EOF (%)
E_0	1.48 $\pm$ 0.05^a	0.99 $\pm$ 0.11^f	7.36 $\pm$ 0.71^d	22.93 $\pm$ 1.33^g	3.45 $\pm$ 0.13^b	85.1 $\pm$ 0.4^a
E_{20}	1.48 $\pm$ 0.03^a	1.03 $\pm$ 0.04^f	7.76 $\pm$ 0.55^d	24.77 $\pm$ 1.12^f	3.46 $\pm$ 0.12^b	73.4 $\pm$ 0.1^b
E_{40}	1.47 $\pm$ 0.15^a	1.15 $\pm$ 0.05^e	8.15 $\pm$ 0.85^c	28.18 $\pm$ 1.41^e	3.44 $\pm$ 0.15^b	61.2 $\pm$ 0.3^c
E_{60}	1.47 $\pm$ 0.14^a	1.21 $\pm$ 0.03^d	8.87 $\pm$ 0.64^b	32.07 $\pm$ 1.19^d	3.45 $\pm$ 0.17^b	50.5 $\pm$ 0.5^d
E_{100}	1.44 $\pm$ 0.35^a	1.43 $\pm$ 0.02^c	8.94 $\pm$ 0.41^b	33.96 $\pm$ 1.23^c	3.46 $\pm$ 0.14^b	30.3 $\pm$ 0.7^e
E_{140}	1.39 $\pm$ 0.07^c	1.59 $\pm$ 0.03^a	9.63 $\pm$ 0.83^a	36.33 $\pm$ 1.13^a	3.78 $\pm$ 0.12^a	16.4 $\pm$ 0.4^f
E_{180}	1.40 $\pm$ 0.04^c	1.54 $\pm$ 0.05^a	9.55 $\pm$ 0.76^a	36.15 $\pm$ 0.97^a	3.77 $\pm$ 0.15^a	15.2 $\pm$ 0.2^f
E_{220}	1.42 $\pm$ 0.02^b	1.48 $\pm$ 0.02^b	8.85 $\pm$ 0.59^b	34.11 $\pm$ 1.23^b	3.76 $\pm$ 0.17^a	16.1 $\pm$ 0.5^f
E_{260}	1.42 $\pm$ 0.01^b	1.45 $\pm$ 0.04^b	8.71 $\pm$ 0.83^b	30.82 $\pm$ 0.11^c	3.75 $\pm$ 0.13^a	15.2 $\pm$ 0.3^f

Mean value of pooled samples ($n = 3 \times 3$). Mean with different superscripts letters in the same column are significantly different ($P < 0.05$).

HSI, hepatosomatic index; CF, condition factor; Hb, haemoglobin; Hct, haematocrit; RBCs, red blood cell count; EOF, rrythrocyte osmotic fragility.

signs such as retarded growth, poor feed efficiency and low Hct have been reported (Kocabas & Gatlin III 1999). Also, lipid peroxidation in the liver and whole body (Cowey, Andron, Walton, Murray, Youngson & Knox 1981;Wilson *et al.* 1984; Tocher, Mourente, Van der Eecken, Evjemo, Diaz, Bell, Geurden, Lavens & Olsen 2002) as well as accumulation of isoprostanes (Tocher *et al.* 2002) have been reported in a number of fish species.

Dietary vitamin E levels had a significant influence on body protein content, which was found to increase up to 140 mg kg^{-1} (E_{140}), remained unaffected at 180 mg kg^{-1} (E_{180}) and then decreased at still higher levels in fish fed diets E_{220} and E_{260}. Body fat content decreased remarkably with increasing levels of dietary vitamin E, which is presumably due to the tendency of vitamin E to promote fat assimilation and transportation, thus reducing the accumulation in the body storage sites (Gabriel Mourente, Bell & Tocher 2007). The lower body protein value in fish fed vitamin E at sub-optimum levels may be due to the higher body lipid content in fish fed these experimental diets because these two constituents show an inverse relationship; an increase in one is coupled with a simultaneous decrease in the other. This is also clearly evident from the data in Table 2, in which the body fat content in *C. punctatus* attained its peak values for the groups fed vitamin E-unsupplemented and vitamin E-deficient diets, leading to a decrease in the body protein content in fish fed these diets. High moisture and low protein were observed in the muscle of the young carp fed a tocopherol-deficient diet (Watanabe, Takashima & Ogino 1970) and juvenile golden shiner, *Notemigonus chrysoleucas* (Chen, Lochmann, Goodwin, Praveen, Dabrowski & Lee 2004). The high moisture content among these was probably associated with the severity of muscular atrophy in carps (Watanabe *et al.* 1970) and juvenile golden shiner (Chen *et al.* 2004). Contrary to this, dietary vitamin E levels did not affect carcass composition in fry mrigal, *Cirrhinus mrigala* (Paul *et al.* 2004), sea bass, *Dicentrarchus labrax* (Gatta, Pirini, Testi, Vignola & Monetti 2000), and fry rohu, *Labeo rohita* (Sau *et al.* (2004)).

The intake of dietary vitamin E above the requirement level of rohu, *L. rohita*, showed positive impacts on immunocompetence (Sahoo & Mukherjee 2002). Some other workers also reported that elevated levels of dietary vitamin E in fish have been shown to stimulate the immune response in salmonids (Verlhac, N'Doye, Gabaudan, Troutaud & Deschaux 1993; Verlhac & Gabaudan 1994) and result in improved disease resistance (Navarre & Halver 1989). However, in the present study, the maximum AWG, SGR, best FCR, PRE and ERE were recorded in fish fed a diet with 140 mg kg^{-1} vitamin E. Fish fed dietary vitamin E at higher levels (> 140 mg kg^{-1}) in diets E_{220} and E_{260} resulted in a significant reduction in weight gain. The growth depression at super-optimum levels of dietary vitamin E may be because of the imbalance and accumulation of vitamin E radicals, which may act as prooxidants (Hamre, Waagbo, Berge & Lie 1997). Excess levels of dietary vitamin E have been reported to depress the bactericidal activity of phagocytes, thus lowering the immune system (Prasad 1980). Super-optimum doses of vitamin E have also been reported to reduce platelet aggregation in human subjects (Cox, Rao, Gerrard & White 1980). The growth depression during this study, at higher levels of vitamin E in the diets, may also be due to these possible reasons.

Table 5 Broken-line and exponential equations used for response variables

Response variables	Broken-line equation	Exponential equations
AWG (g/fish)	$L_1 = 22.3512 + 0.2253X$; $X < 149$ mg kg^{-1} ($r^2 = 0.912$)	$y = 5.1001y_0 + 1395.1A/(221.41W^*\text{sqrt}(\text{pi}/2)e_c^{-2(x-166.95x)2/221.41W2}$
	$L_2 = 81.2143 - 0$; $X > 149$ mg kg^{-1}	($r^2 = 0.933$)
FCR (dry wt)	L_1 $Y = 2.6891 - 0.00982X$; $X < 149$ mg kg^{-1} ($r^2 = 0.941$)	$y = 1.44y_0 + 1.24A_1e_1^{-x/36.83t} - (-0.59)A_2e_2^{-x/50.95t}$
	L_2 $Y = 0.939 + 0.00232X$; $X > 149$ mg kg^{-1}	($r^2 = 0.945$)
PRE (%)	L_1 $Y = 16.11853 + 0.191X$; $X < 149$ mg kg^{-1} ($r^2 = 0.957$)	$y = -86.33y_0 + 74642A/(463.3W^*\text{sqrt}(\text{pi}/2)^{e-2(x-166.35x)2/463.3W2}_c$
	L_2 $Y = 56.94 - 0.09235X$; $X > 149$ mg kg^{-1}	($r^2 = 0.978$)
ERE (%)	L_1 $Y = 43.64242 + 0.25X$; $X < 149$ mg kg^{-1} ($r^2 = 0.995$)	$y = 16.28y_0 + 19992A/(256.02W^*\text{sqrt}(\text{pi}/2)^{e-2(x-169.245x)2/256.02W2}_c$
	L_2 $Y = 101.63 - 0.14X$; $X > 149$ mg kg^{-1}	($r^2 = 0.991$)
Hb (g/dL)	L_1 $Y = 7.3925 + 0.0155X$; $X < 149$ mg kg^{-1} ($r^2 = 0.968$)	$y = 3.3162y_0 + 2719.5A/(349.21W^*\text{sqrt}(\text{pi}/2)e_c^{-2(x-168.42x)2/349.21W2}$
	L_2 $Y = 10.989 - 0.00855X$; $X > 149$ mg kg^{-1}	($r^2 = 0.954$)
Hct (%)	L_1 $Y = 23.0571 + 0.0971X$; $X < 149$ mg kg^{-1} ($r^2 = 0.969$)	$y = -19.761y_0 + 302891A/(^{429.23}W^*\text{sqrt}(\text{pi}/2)e_c^{-2(x-169.23x)2/429.23W2}$
	L_2 $Y = 43.83 - 0.0449X$; $X > 149$ mg kg^{-1}	($r^2 = 0.965$)

AWG, absolute weight gain; FCR, feed conversion ratio; PRE, protein retention efficiency; ERE, energy retention efficiency; Hb, haemoglobin; Hct, haemocrit.

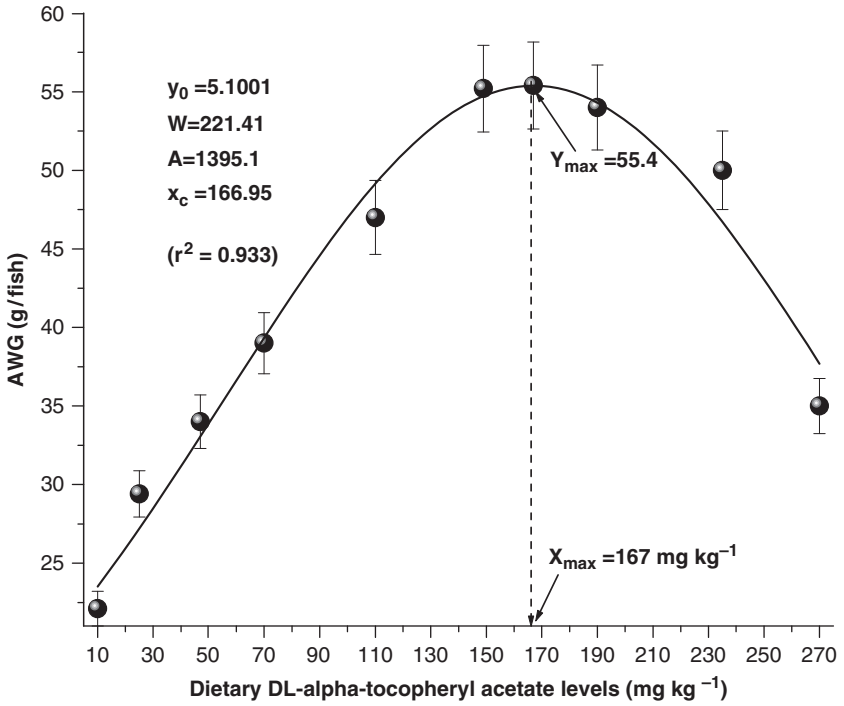

Figure 1 Exponential fit of absolute weight gain (AWG) to dietary vitamin E concentrations (each point represents the mean of a pooled sample of 10 fish/replicate; $n = 3 \times 3$).

Blood parameters like Hb content, Hct value and RBCs count were also significantly affected by the dietary vitamin E levels. These parameters were the highest for the groups receiving diets at 140 mg vitamin E kg^{-1} diet; however, no significant change in the above parameters was noted in fish

receiving dietary vitamin E beyond this level in diet E_{180}.

Erythrocyte fragility is one of the important parameters used to determine the dietary vitamin E status in fish (Halver 2002). Erythrocytes' breakdown was reported to be faster in the case of an improper membrane function (Robbins, Cotran & Kumar 1984). Dietary vitamin E concentrations were found to influence the strength of erythrocyte membranes during this study. This indicates that erythrocytes of fish fed diets up to 140 mg vitamin E kg^{-1} diet were more resistant to haemolysis in hypotonic salt solutions. The erythrocytes were more susceptible to osmotic lysis in fish fed diets with lower concentrations of vitamin E than required (E_{140} mg kg^{-1}), which is consistent with the results of Cowey, Adron and Youngson (1983), Pearce, Harris and Davies (2003) and Chen *et al.* (2004), who also have reported the symptoms of anaemia characterized by low Hct and Hb in juvenile golden shiner, *N. chrysoleucas*, fed vitamin E-unsupplemented diets. The present finding strengthens the fact that vitamin E has a direct effect on erythrocyte fragility. Because diet E_{140} contained vitamin E as per the requirement of the fish and also at the level that seems to be needed for the protection of biomembranes against oxidative damage, the erythrocytes of this fish were less susceptible to lysis compared with those fed dietary vitamin E at E_0, E_{20}, E_{40}, E_{60} and E_{100} levels. The increase in the cell wall strength with the increase in the dietary vitamin E content may also be the reason for the lower susceptibility of erythrocytes at 140 mg kg^{-1} and beyond. Sau *et al.* (2004) and Yang and Desai (1977) have also reported this positive relation of dietary vitamin E supplementation and the reduction in erythrocyte fragility due to an increase in cell wall strength in *L. rohita* and rats. This phenomenon of increased cell wall strength with the increased supplementation of dietary vitamin E up to the requirement level of the fish has also been reported by Sau *et al.* (2004) in *L. rohita* and Yang and Desai (1977) in rats.

Vitamin E-deficient fish have been reported to have impaired membrane structure and function due to lipid oxidation. Quantification of TBARS is one of the most commonly used methods for assessing tissue peroxidation in response to dietary vitamin E levels. The inadequacy of dietary vitamin E has been reported to enhance the level of fatty acid peroxidation, mainly because of the formation of considerable amounts of oxidative radicals in the tissues (Tocher *et al.* 2002). The high values of hepatic TBARS in fish fed diets without vitamin E supplementation (E_0) and those fed 20 mg kg^{-1} vitamin E (E_{20}), 40 mg kg^{-1} vitamin E (E_{40}) and 60 mg kg^{-1} vitamin E (E_{60}) suggest that lipid peroxidation in the liver of *C. punctatus* is promoted if this vitamin is not included in the diet or is at sub-optimum levels. In the present study, tissue TBARS values were inversely related to the level of vitamin E inclusion in the diet (Table 3). This is also consistent with the findings on poultry (Sheehy, Morrissey & Flynn 1991), beef (Arnold, Scheller, Arp, Williams & Schaeffer 1993), pork (Whang, Aberle, Judge & Peng 1986), veal (Engeseth, Gray, Booren & Asghar 1993) and fish species such as rainbow trout, *Oncorhynchus mykiss* (Hung & Slinger 1982; Frigg, Prabucki & Ruhdel 1990), red seabream (Murata & Yamauchi 1989), channel catfish (Gatlin, Poe, Wilson, Ainsworth & Bowser 1992), hybrid tilapia, *Oreochromis niloticus x Oreochromis aureus* (Huang & Hung 2004), and black seabream, *Acanthopagrus schlegelii* (Peng & Gatlin III 2009).

In the present study, the hepatic TBARS values, growth, HSI and CF of fingerling *C. punctatus* were significantly influenced by the dietary vitamin E levels. Fish fed vitamin E free (E_0) and the diet containing vitamin E at sub-optimal levels (E_{20}, E_{40}, E_{60}, E_{100}) tended to have higher HSI and poor CF compared with those fed dietary vitamin E at 140 mg kg^{-1} (E_{140}). This increase in HSI may be the result of increased liver size due to the accumulation of lipid peroxidates in fish fed diets free of vitamin E and at sub-optimum levels (Baker & Davies 1996). The same has been observed for African catfish, *Clarius gariepinus* (Baker and Davies (1996), and gilt head seabream, *Sparus aurata* (Mourente, Diaz-Salvago, Bell & Tocher 2002). Hepatic ceroidosis has also been reported in the liver and spleen of rainbow trout (Moccia, Hung, Slinger & Furgeuson 1984) and chinook salmon (Smith 1979) when reared on a vitamin E-deficient diet. Fish fed diets with higher concentrations of vitamin E (E_{180}, E_{220}, E_{260}) exhibited high HSI and poor CF. However, the change in these parameters for these groups of fish was small.

The nutrient requirements of fish have been estimated using different statistical and mathematical models, the most common being the broken-line model. Because this model underestimates the requirement, a non-linear model has been suggested to be more appropriate over the broken-line regression model (Mercer, May & Dodds 1989; NRC 1993; Cowey 1994; Rodehutscord & Pack 1999). As the response of an animal to a dietary increment of a limiting nutrient is not linear and is not broken at one particular point (Mercer *et al.* 1989; Cowey 1992;

Fuller & Garthwaite 1993; Rodehutscord, Jacobs, Pack & Pfeffer 1995; Schuttle & Pack 1995; Shearer 2000), the non-linear approach has been considered to be more prudent. Of all the non-linear approaches, the exponential one has been used widely in most of the requirement studies. In the present study, the vitamin E level has been determined using both the broken-line and the exponential models. Linear (broken-line regression) and non-linear (exponential) analyses of weight gain, FCR, protein and energy retention efficiencies, and haematological indices against dietary vitamin E concentration showed a strong relationship. The broken-line regression and exponential analysis of growth, feed conversion and nutrient retention efficiencies predicted the optimum level to range between 140 and 169 mg kg^{-1} of the diet, indicating that *C. punctatus* has higher requirements for dietary vitamin E.

The dietary vitamin E levels for many fish species have been established and generally fall in the range of 20–50 mg kg^{-1} dry feed (NRC 1993) but Taveekijakarn, Miyazaki, Matsumoto and Arai (1996) reported that the dietary level of this essential additive for maximum growth and prevention of gross deficiency signs varies from 20 to 300 mg kg^{-1} depending largely on the species. The optimum dietary vitamin E level worked out in this study, on the basis of broken-line and exponential fitting of the data, is higher than those reported for Atlantic salmon, *Salmo salar* 30–60 mg kg^{-1} (NRC 1981; Hamre & Lie 1995), red drum, *Sciaenops ocellatus*, 31 mg kg^{-1} (Peng & Gatlin III 2009), channel catfish, *Ictalurus punctatus*, 30–50 mg kg^{-1} (Murai & Andrews 1974; Wilson *et al.* 1984), fingerling mrigal, *C. mrigala*, 99 mg kg^{-1} (Paul *et al.* 2004), Korean rockfish, *Sebastes schlegelii*, 45 mg kg^{-1} (Bai & Lee 1998), sunshine bass, *Morone chrysops x Morone saxatilis*, 28 mg kg^{-1} (Kocabas & Gatlin III 1999), and rohu, *L. rohita*, 131.91 mg kg^{-1} (Sau *et al.* 2004) diet and is lower than the requirement reported for common carp, *Cyprinus carpio* 200–300 mg kg^{-1} diet (Watanabe *et al.* 1977).

From the above findings, it is evident that *C. punctatus* has a higher requirement for dietary vitamin E compared with other teleostean species. The higher vitamin E requirement of *C. punctatus* may be to quench the free radicals produced out of its high fat content. Vitamin E requirement depends on dietary factors such as the level of polyunsaturated fatty acids (Lin & Shiau 2005), oxidized lipid (Hung *et al.* 1981) as well as the presence and abundance of other antioxidant nutrients such as selenium and vitamin C (Sealey & Gatlin III 2002). The vitamin E requirements may also be elevated by stress or other physiological responses to environmental factors such as dissolved oxygen (Ritola, Livingstone, Peters & Lindström-Seppä 2002) and crowding (Montero, Tort, Izquierdo, Robaina & Vergara 1998), heat challenge (Chen, Lochmann, Goodwin, Praveen, Dabrowski & Lee 2003), environmental pollution and parasitism (Marcogliese, Brambilla, Gagné & Gendron 2005). These situations may increase the dietary vitamin E requirement to a considerable extent. The dietary inclusion level for vitamin E in fish is also affected by the experimental conditions (NRC 1993; Aksoy, Lim, Li & Klesius 2008). Therefore, the reported variations in dietary vitamin E needs among various finfish species may be attributed to the above possible reasons. In this sense, to assess the true dietary vitamin E requirement of *C. punctatus*, the above-mentioned influential factors have been taken care of carefully while conducting the experiment.

Elevated erythrocyte fragility and low Hct coupled with depressed growth retardation were the only signs of vitamin E deficiency observed in *C. punctatus*. Fish fed diets containing more than the optimum level of vitamin E also resulted in depressed growth and low Hct. On the basis of the broken-line and exponential fitting of AWG, FCR, PRE, ERE, Hb and Hct data, the optimum dietary vitamin E requirement for fingerling *C. punctatus* is estimated to be in the range of 140–169 mg kg^{-1} of the diet. The information generated in the present study will be useful in formulating vitamin E-balanced practical feeds for the intensive culture of this species.

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