Dietary copper requirement of fingerling *Channa punctatus* (Bloch) based on growth, feed conversion, blood parameters and whole body copper concentration

Nassr-Allah H Abdel-Hameid[1], Seemab Zehra[2] & Mukhtar A Khan[2]

[1]Department of Zoology, Faculty of Science, Benha University, Benha, Egypt

[2]Fish Nutrition Research Laboratory, Department of Zoology, Aligarh Muslim University, Aligarh 202 002, India

Correspondence: M A Khan, Fish Nutrition Research Laboratory, Department of Zoology, Aligarh Muslim University, Aligarh 202 002, India. E-mail: khanmukhtar@yahoo.com

Abstract

A 12-week feeding trial was conducted to estimate the dietary copper requirement of fingerling *Channa punctatus*. Six casein−gelatin-based test diets (450 g kg^{-1} crude protein; 18.81 kJ g^{-1} gross energy) with graded levels of copper as copper sulphate (3.7, 4.7, 5.7, 6.7, 7.7 and 8.7 mg copper equivalent kg^{-1} diet) were formulated and fed to triplicate groups of fish (7.25 ± 0.81 cm; 5.21 ± 0.27 g) near to satiation. Fish fed diet with 6.7 mg kg^{-1} copper had highest absolute weight gain (AWG; 51.63 g fish^{-1}), protein efficiency ratio (PER; 1.42 g fish^{-1}), protein gain (PG; 8.34 g fish^{-1}), haemoglobin (Hb; 9.68 g dL^{-1}), haematocrit (Hct; 31.18%) and RBCs (3.24 × 10^6 × mm^{-3}). Feed conversion ratio (FCR) was found to be best (1.57) at above level of dietary copper. Whole body copper concentration was found to increase with the increasing levels of dietary copper. Hepatic thiobarbituric acid-reactive substances concentration was found to decrease with increasing dietary concentrations of copper up to 6.7 mg kg^{-1} beyond which a reverse trend in this parameter was noted. Broken-line regression analysis of AWG, FCR and PG concentrations against varying levels of dietary copper yielded the requirement in the range of 6.66–6.78 mg kg^{-1}. Data generated during this study would be useful in formulating copper-balanced commercial feeds for the intensive culture of this fish.

Keywords: copper requirement, growth, fingerling, *Channa punctatus*

Introduction

Practical feeds for fish must contain all nutrients and sufficient energy for satisfactory growth and proper health. For achieving maximum growth potential of fish, provision of balanced feed that meet their nutritional is must. A formula for balanced fish diets must include an energy source plus sufficient indispensable amino acids, essential fatty acids, specific vitamins and minerals to support life and to promote adequate growth (Halver 2002; NRC 2011). Minerals are inorganic substances, required for the maintenance of certain physicochemical processes in fish. To formulate mineral-balanced practical feeds for intensive aqua-farming of a candidate fish, species-specific understanding of the minerals needs is essential. One mineral that has been known as an essential trace element is copper. It is one of the most important trace metals for survival, development and growth in fish (Lall 2002). Copper has important functions in haematopoiesis and in copper-dependent enzymes including lysyl oxidase, cytochrome oxidase, ferroxidase, tyrosinase and superoxide dismutase (Lall 2002; NRC 2011). In addition to the physiological functions of copper, high levels of dietary copper may be toxic (Berntssen, Kundebye & Moage 1999; NRC 2011). Exposure to waterborne copper does not increase tissue copper accumulation as much as feeding elevated dietary copper (Bielmyer, Gatlinb, Iselyc, Tomassod & Klained 2005; Hoyle, Shaw & Handy 2007). Therefore, supplementation of copper to fish feed is a balance between fulfilling the copper

requirement and avoiding copper toxicity (Tan, Luo, Liu & Xie 2011).

Dietary requirements for copper have been quantified for several fish species such as common carp *Cyprinus carpio*, rainbow trout *Salmo gairdneri*, Atlantic salmon *S. salar*, channel catfish *Ictalurus punctatus*, hybrid tilapia *Oncorhynchus niloticus* × *Oncorhynchus aureas*, Malabar grouper *Epinephelus malabaricus* (NRC 2011); yellow catfish *Pelteobagrus fulvidraco* (Tan *et al.* 2011); olive Flounder *Paralichthys olivaceus* (Mohseni, Park, Lee, Okorie, Browdy, Bharadwaj & Bai 2012), beluga *Huso huso* (Mohseni, Pourkazemi & Bai 2014), Nile tilapia *Oreochromis niloticus* (Damasceno, Fleuri, Sartori, Amorim, Pezzato, Silva, Carvalho & Barros 2016), Russian sturgeon *Acipenser gueldenstaedtii* (Wang, Li, Zhu, Du, Qin & Chen 2016).

Channa punctatus, commonly known as murrel, is an important freshwater food fish species. It is found to be distributed throughout the Southeast Asian countries and is a natural inhabitant of stagnant muddy pond waters, paddy fields, weedy derelict swamps, beels, canals and reservoirs (Chondor 1999). Its air-breathing characteristics and general hardiness allow it to be cultured in areas that are not suitable for the culture of Indian major carp and other carp species. It is recommended in diet during convalescence and, therefore, is a good candidate for intensive aquaculture (Marimuthu, Arokiaraj & Haniffa 2009). Yaakob and Ali (1992) also noted the importance of murrels for hastening the healing of wounds and internal injuries due to the presence of certain fatty acids such as prostaglandin and thromboxane. Hence, this species is gaining attention as a cultured freshwater fish for medicinal purposes in the Asian market. Although it is very popular and highly demanded fish in India, the production of this fish is not organized due to the lack of nutritionally balanced feeds. Although, little information on some nutrition aspects and culture of *C. punctatus* is available (Bhuiyan, Afroz & Zaman 2006; Marimuthu *et al.* 2009; Jindal, Yadava, Jain & Gupta 2010; Saikia & Das 2010; Nassr, Khan & Abidi 2012; Zehra & Khan 2012), no information is available on dietary copper requirement of this fish. Therefore, this study was aimed at determining the dietary copper requirement for developing copper-balanced feeds to maximize the growth of fingerling *C. punctatus*.

Materials and methods

Preparation of the experimental diets

Casein−gelatin-based isonitrogenous (450 g kg^{-1} crude protein) and isoenergetic (18.81 kJ g^{-1} gross energy) diets (D1, D2, D3, D4, D5 and D6) with six levels of copper (0, 1, 2, 3, 4 and 5 mg kg^{-1} diet) were formulated by adding copper sulphate (0, 3.93, 7.86, 11.79, 15.72 and 19.65 mg kg^{-1} diet). The copper concentration in the basal diet (D1) was found to be 3.7 mg kg^{-1} dry diet. The resulting copper levels in different experimental diets were 3.7, 4.7, 5.7, 6.7, 7.7 and 8.7 mg kg^{-1} dry diet. The amount of copper sulphate ($CuSO_4 \cdot 5H_2O$) was increased at the expense of dietary cellulose to attain the intended concentrations of copper in the experimental diets. The experimental diets were analysed for copper and found to contain 3.72, 4.62, 5.71, 6.65, 7.63 and 8.72 mg kg^{-1} dry diet. The doses of copper in the experimental diets were taken on the basis of the information available on other warm water species (NRC 2011). The copper concentration in rearing water was monitored during the feeding period and ranged from 1.1 to 1.7 µg L^{-1}. The composition of the basal diet used is given in Table 1. The dietary protein level was fixed at 450 g kg^{-1} of the diet, reported optimum for the growth of fingerling *C. punctatus* (Zehra & Khan 2012). Mineral and vitamin premixes excluding the test mineral copper were prepared as per Halver (2002). 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. For making soft cake, calculated quantities of dry ingredients were thoroughly mixed and stirred in 30 mL of hot water (80°C) in a steel bowl attached to a Hobart electric mixer (K5SS; Hobart Corporation, Troy, OH, USA). Gelatin powder was dissolved separately in 20 mL of water with constant heating and stirring and then transferred to the above mixture. Vitamins, minerals and oil premixes were added to the lukewarm bowl one by one with constant mixing at 50°C. Carboxymethyl cellulose was added last, and the speed of the blender was gradually increased as the diet started to harden. The final diet with the bread dough consistency was poured into a Teflon-coated pan and placed in a refrigerator. The prepared diets were in the form of semi-moist cake from which cubes were cut and stored at −20°C in sealed polythene bags until used.

Experimental design and feeding trial

Induced bred *C. punctatus* were procured from a fish pond of the Department of Zoology, Aligarh Muslim University, Aligarh, UP, India. These were transported to the wet laboratory in oxygen filled polythene bags, given a prophylactic dip in $KMnO_4$ solution (1:3000) and stocked in indoor circular aqua-blue coloured, plastic lined (Plastic Crafts Corp, Mumbai, India) fish tanks (1.22 m in diameter, 0.91 m in height; water volume 600 L) for 2 weeks. During this period, fish were acclimatized

Table 1 Composition of the basal diet

Ingredients	g kg^{-1} dry diet
Casein* (vitamin-free)	450
Gelatin†	93.8
Dextrin	243.6
Corn oil	50
Cod liver oil	20
Mineral mix‡·¶ (copper-free)	40
Vitamin mix§·¶	30
α- Cellulose¶	22.6
Carboxy methyl cellulose	50
Total	1000
Analysed crude protein	450.8
Analysed copper (mg kg^{-1} dry diet)	3.72
Analysed lipid	68.8
Analysed gross energy (kJ g^{-1})**	18.84
Digestible energy (kJ g^{-1})††	14.52

*Crude Protein (800 g kg^{-1}).

†Crude Protein (960 g kg^{-1}).

‡Mineral mixture (g kg^{-1} of diet) calcium biphosphate 5.42; calcium lactate 13.1; ferric citrate 1.19; magnesium sulphate 5.28; potassium phosphate (dibasic) 9.59; sodium biphosphate 3.48; sodium chloride 1.74; aluminium chloride. 6H2O 0.006; potassium iodide 0.006; copper sulphate 0; manganese sulphate monohydrate 0.032; cobalt chloride. 6H2O 0.04; zinc sulphate heptahydrate 0.12; α-cellulose 0.004; Loba Chemie, Mumbai, India.

§Vitamin mixture (g kg^{-1} diet) choline chloride 5.0; meso-inositol 2.0; L-ascorbyl-2-polyphosphate 1.0; calcium D- pantothenate 0.5; riboflavin 0.2; menadione 0.04; pyridoxine hydrochloride 0.05; thiamin hydrochloride 0.05; copper hydrochloride 0.05; folic acid 0.015; nicotinic acid 0.75; alpha-tocopherol acetate 0.4; vitamin B_{12} (Cyanocobalamin) 0.0001; α-cellulose 20.0; Loba Chemie, India.

¶Halver (2002).

**Calculated on the basis of fuel values 23.08, 20.199, 16.02 and 37.64 kJ g^{-1} for casein, gelatin, dextrin, and fat, respectively, as estimated on Gallenkamp ballistic bomb calorimeter and Estimated value of the basal diet on Gallenkamp ballistic bomb calorimeter.

††Digestible energy was calculated on the basis of physiological fuel values 18.83, 14.64 and 35.56 kJ g^{-1} for protein, carbohydrate and fat, respectively (Jauncey, 1982).

on casein–gelatin-based (450 g kg^{-1} CP) H-440 diet (Halver 2002).

C. punctatus fingerlings (7.25 ± 0.81 cm; 5.21 ± 0.27 g) were taken from the above acclimated fish lot and stocked in triplicate groups in 70 L circular polyvinyl troughs (water volume 55 L) fitted with a continuous water flow-through (1–1.5 L min^{-1}) system at the rate of 20 fish per trough for each dietary treatment level. Adequate amount of encoating of the dietary copper by gelatin, casein and carboxymethyl cellulose was done in order to prevent the leaching of copper. Fish were hand-fed test diets in the form of semi-moist cake (32% moisture) near to satiation thrice daily at 0700, 1200 and 1730 h. During feeding, particular attention was given to avoid feed wastage. The unconsumed feed, if any, was collected soon after active feeding, dried, weighed and taken into account for calculating the actual feed intake. Initial and weekly weights of fish were recorded on a top-loading balance (Precisa 120A; 0.1 mg sensitivity, Oerlikon AG, Zurich, Switzerland) after anaesthetizing the fish with 0.01% aqueous solution of tricaine methane sulphonate (MS-222; Sigma, St Louis, MO, USA). Fish were deprived of feed on the day they were weighed. The feeding trial lasted for 12 weeks. Faecal matter was siphoned before every feeding. Water quality indices were monitored daily during the feeding trials and were recorded following standard methods (APHA 1992). The range of water temperature, dissolved oxygen, free carbon dioxide, pH and total alkalinity based on daily measurements, were 26.5–28.3°C, 6.5–7.2 mg L^{-1}, 4.9–9.2 mg L^{-1}, 7.1–7.4 and 66.8–78.4 mg L^{-1} respectively.

Sample collection and chemical analysis

Fishes were fasted for 24 h to empty their guts before sampling. At the beginning of the feeding trial, 30 fish were randomly sampled, killed and pooled together. Six subsamples of a pooled sample were analysed for the initial carcass composition. At the end of the experiment, nine fish from each replicate of dietary treatments were collected, killed and pooled separately. Five subsamples of the pooled samples of each replicate were analysed for final whole body composition. Another five fish from each replicate were anaesthetized with MS-222 and liver and viscera of each specimen were carefully removed. Weight of fish, viscera and liver were recorded to calculate viscerosomatic index (VSI),

hepatosomatic index (HSI) and condition factor (CF). Proximate composition of experimental diets, and initial and final whole body were estimated using standard methods (AOAC 2005) for dry matter (oven drying at $105 \pm 1°C$ for 22 h using thermostat, Yorko Instruments, New Delhi, India), crude protein (Kjeldhal nitrogen $\times$ 6.25 using Kjeltec Tecator™ Technology 2300, Hoganas, Sweden), crude fat (solvent extraction with petroleum ether B.P 40–60°C for 2–4 h using Socs Plus, SCS 4, Pelican equipments, Chennai, India) and ash (oven incineration at 650°C for 2–4 h using muffle furnace, S.M. Scientific Instrument (p). Jindal Company, New Delhi, India). Gross energy content was determined on a Gallenkamp Ballistic Bomb Calorimeter-CBB 330 010L (Gallenkamp, Loughbrough, UK). Copper contents of rearing water, test diets, casein, gelatin, initial and final whole body were determined using atomic absorption spectrophotometer Model A- Analyst 300, Perkin Elmer, Australia (AOAC 2005). Briefly, one gram of sample was placed in a 250 mL digestion tube and 10 mL of concentrated nitric acid was added. The mixture was boiled gently for 30–45 min to oxidize all easily oxidizable matter. After cooling, 5 mL of 70% perchloric acid was added and the mixture was boiled gently until dense white fumes appeared. After cooling, 20 ml of distilled water was added and the mixture was boiled further to release any fumes. The solution was cooled, further filtered through Whatman No. 42 filter paper and <0.45 μm Millipore filter paper and transferred to volumetric flasks containing 25 mL of deionized water (Hseu 2004). The concentration of copper in the final solutions was determined by atomic absorption spectrometry. Rearing water samples of 5 mL were collected and mineralized in the presence of nitric acid at 150°C for 4 h, and then diluted to 10 mL with deionized water (Luszczek-Trojnar, Sionkowski, Drąg-Kozak & Popek 2015). Samples prepared in this way were analysed for the concentrations of copper using atomic absorption spectrophotometer.

Haematological analysis

At the termination of the feeding trial, blood samples were collected in heparinized vials through cardiac puncture of the fish. The blood of five fish from each replicate of the treatment group was pooled to obtain enough samples for haematological analysis. Haematocrit levels were determined by drawing fresh blood into tubes and centrifuging in a microhaematocrit centrifuge (RM 12 C, Micro Centrifuge, Remi, RemiMotors, Bombay, India) at 3600 g for 6 min. Red blood cell counts (RBCs) and haemoglobin (Hb) were analysed as per the method adopted by Vani, Saharan, Roy, Ranjan, Pal, Siddaiah and Kumar (2012). In brief, 20 μL of blood was mixed with 3,980 μl of red blood cell diluting fluid (Dacies fluid) in a clean glass vial. The mixture was shaken well to suspend the cells uniformly in the solution. The cells were counted using a Neubauer haemocytometer. The blood haemoglobin was analysed following the cyanmethaemoglobin method using Drabkins Fluid. An amount of 20 μL of blood was mixed with 5 mL of Drabkins working solution. The absorbance was measured using a spectrophotometer at wavelength of 540 nm. The final concentration was calculated after comparing with the standard.

Thiobarbituric acid-reactive substances (TBARS) assays

After blood extraction, the liver was removed from five fish of each replicate of treatments for the assay of Malondialdehyde (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}.

Evaluation of growth performance

Growth performance of fish fed experimental diets was measured by calculating following parameters:

Absolute weight gain (g fish^{-1}) = Final body weight (g fish^{-1})−Initial body weight (g fish^{-1}).

Feed conversion ratio = Dry feed fed (g)/Wet weight gain (g).

Protein efficiency ratio (PER) = Wet weight gain (g)/Protein intake (g).

Protein gain (PG; g fish^{-1}) = Final body weight (g fish^{-1}) x final body protein − Initial body weight (g fish^{-1}) x initial body protein.

Hepatosomatic index (HSI%) = Liver weight (g)/Body weight (g) × 100.

Viscerosomatic index (VSI%) = Viscera weight (g)/Body weight (g) × 100.

Condition factor (CF) = Body weight (g)/Body length (cm)^3 × 100.

Survival Rate% = Final number of fish/Initial number of fish ×100.

Statistical analyses

All growth data were subjected to one-way analysis of variance (Sokal & Rohlf 1981). Differences among treatment means were determined by Tukey's honestly significant difference (HSD) test at a $P < 0.05$ level of significance. Dietary copper requirement for fingerling *C. punctatus* was estimated by broken-line regression analysis to the dose−growth responses relationship (Robbins, Saxton & Southern 2006). Statistical analysis was done using Origin (version 6.1; Origin Software, San Clemente, CA, USA).

Results

Growth performance

Growth parameters including absolute weight gain (AWG), feed conversion ratio (FCR), protein efficiency ratio (PER) and protein gain (g fish^{-1}) for this investigation are presented in Table 2. Growth performance of fish in terms of above parameters improved with the increase in dietary copper concentrations up to 6.7 mg kg^{-1} of the dry diet (D4) and, thereafter, a significant decline was recorded.

Whole body proximate composition and copper concentration

Data pertaining to whole body composition and copper concentration in fish fed varying levels of dietary copper are depicted in Table 3. Whole body protein was found to improve with the elevation of dietary copper concentrations up to 6.7 mg kg^{-1} (D4) and then levelled off. Whole body fat showed a significant decline with the increasing levels of dietary copper. Moisture content exhibited a reverse trend to that of whole body fat. Ash content was found to be positively correlated with the incremental levels of dietary copper. Whole body copper concentration showed a positive correlation with the increasing levels of dietary copper.

Haematological indices

Haematological indices including haematocrit value, haemoglobin and RBCs counts responded positively with the increasing levels of dietary copper up to 6.7 mg kg^{-1} (D4). However, fish fed higher levels of dietary copper showed significant decline in these parameters (Table 4).

Thiobarbituric acid-reactive substances (TBARS) concentrations and survival

Data on liver TBARS concentrations are depicted in Table 4. Liver TBARS concentrations showed a significant negative correlation with the increasing levels of dietary copper from 3.7 (D1)-6.7 mg kg^{-1} (D4). Further inclusion of dietary copper at 7.7 (D5) and 8.7 mg kg^{-1} (D6) resulted in significant increase in TBARS concentration. Survival of fish fed varying levels of dietary copper was significantly affected and found to be in the range of 74–100% in all the groups (Table 4).

Table 2 Growth performance of fingerling *Channa punctatus* fed diets containing varying levels of copper*,†

| | Varying levels of copper (mg kg^{-1} dry diet) | | | | | |
	3.7 (D1)	4.7 (D2)	5.7 (D3)	6.7 (D4)	7.7 (D5)	8.7 (D6)
Average initial weight (g)	5.21 ± 0.02	5.24 ± 0.03	5.18 ± 0.02	5.21 ± 0.06	5.21 ± 0.02	5.18 ± 0.07
Average final weight (g)	19.38 ± 0.07[e]	29.76 ± 0.12[d]	42.05 ± 0.24[c]	56.84 ± 0.28[a]	51.25 ± 0.21[b]	44.09 ± 0.19[c]
Absolute weight gain (g fish^{-1})	14.17 ± 0.34[e]	24.52 ± 0.51[d]	36.87 ± 0.41[c]	51.63 ± 0.56[a]	46.04 ± 0.49[b]	38.91 ± 0.41[c]
Protein efficiency ratio	0.64 ± 0.71[f]	0.76 ± 0.92[e]	1.05 ± 0.76[d]	1.42 ± 0.11[a]	1.35 ± 0.09[b]	1.29 ± 0.11[c]
Feed conversion ratio	3.46 ± 0.05[a]	2.94 ± 0.08[b]	2.11 ± 0.05[c]	1.57 ± 0.06[f]	1.65 ± 0.04[e]	1.72 ± 0.02[d]
Protein gain (g fish^{-1})	1.74 ± 0.04[f]	3.29 ± 0.06[e]	5.55 ± 0.08[d]	8.34 ± 0.11[a]	7.41 ± 0.09[b]	6.08 ± 0.06[c]

*Mean values of three replicates ± SEM.
†Mean values sharing the same superscripts in the same row are insignificantly different ($P > 0.05$).

Table 3 Whole body proximate composition (wet basis) and copper concentration of fingerling *Channa punctatus* fed diets containing varying levels of copper*,†

	Varying levels of copper (mg kg^{-1} dry diet)						
	Initial	3.7 (D1)	4.7 (D2)	5.7 (D3)	6.7 (D4)	7.7 (D5)	8.7 (D6)
Moisture (%)	78.11 ± 5.21	74.24 ± 3.2[b]	74.86 ± 3.7[b]	75.34 ± 4.2[b]	75.53 ± 3.8[ab]	75.91 ± 2.5[a]	76.21 ± 3.9[a]
Crude protein (%)	12.14 ± 0.18	12.26 ± 0.19[d]	13.21 ± 0.14[c]	14.69 ± 0.14[b]	15.79 ± 0.21[a]	15.71 ± 0.16[a]	15.21 ± 0.14[a]
Crude fat (%)	5.16 ± 0.08	6.23 ± 0.08[a]	5.91 ± 0.09[b]	5.47 ± 0.07[c]	5.11 ± 0.05[d]	4.94 ± 0.08[e]	4.68 ± 0.06[f]
Ash (%)	3.14 ± 0.05	2.11 ± 0.03[f]	2.37 ± 0.02[e]	2.69 ± 0.01[d]	2.98 ± 0.04[c]	3.27 ± 0.02[b]	3.42 ± 0.03[a]
Whole body copper concentrations (mg kg^{-1} of dry matter)	4.17 ± 0.06	2.11 ± 0.08[f]	7.28 ± 0.06[e]	11.19 ± 0.05[d]	12.94 ± 0.05[c]	15.63 ± 0.11[b]	21.48 ± 0.21[a]

*Mean values of three replicates±SEM.
†Mean values sharing the same superscripts in the same row are insignificantly different (*P* > 0.05).

Table 4 Haematological, somatic indices, thiobarbituric acid-reactive substances (TBARS) concentrations and per cent survival of fingerling *Channa punctatus* fed diets containing varying levels of copper*,†

	Varying levels of copper (mg kg^{-1} dry diet)					
	3.7 (D1)	4.7 (D2)	5.7 (D3)	6.7 (D4)	7.7 (D5)	8.7 (D6)
Haematocrit (%)	18.91 ± 0.29[e]	21.97 ± 0.31[d]	25.74 ± 0.36[c]	31.18 ± 0.31[a]	30.11 ± 0.29[a]	28.74 ± 0.21[b]
RBCs (10^6 x mm^{-3})	1.31 ± 0.02[e]	1.82 ± 0.05[d]	2.79 ± 0.06[c]	3.24 ± 0.04[a]	2.81 ± 0.03[b]	2.72 ± 0.03[c]
Haemoglobin (g dL^{-1})	6.27 ± 0.09[e]	7.51 ± 0.07[d]	8.21 ± 0.11[c]	9.68 ± 0.14[a]	9.17 ± 0.17[a]	8.41 ± 0.12[b]
Hepatosomatic index (%)	1.51 ± 0.09[a]	1.53 ± 0.09[a]	1.52 ± 0.09[a]	1.50 ± 0.09[a]	1.41 ± 0.09[b]	1.34 ± 0.09[c]
Viscrosomatic index (%)	4.27 ± 0.15[a]	4.22 ± 0.18[a]	4.19 ± 0.17[a]	4.14 ± 0.16[a]	3.81 ± 0.19[b]	3.61 ± 0.21[c]
Condition factor	0.91 ± 0.02[e]	1.26 ± 0.04[d]	1.42 ± 0.07[c]	1.61 ± 0.05[a]	1.53 ± 0.04[b]	1.45 ± 0.03[c]
TBARS (n mol MDA g^{-1} iver tissue)	1.89 ± 0.01[a]	1.71 ± 0.02[b]	1.54 ± 0.04[c]	1.38 ± 0.03[f]	1.49 ± 0.02[e]	1.65 ± 0.02[d]
Survival (%)	86	100	100	100	83	74

*Mean values of three replicates±SEM.
†Mean values sharing the same superscripts in the same row are insignificantly different (*P* > 0.05).

Somatic indices

Data pertaining to HSI, VSI and CF in response to varying levels of dietary copper are given in Table 4. Fish fed diets containing 3.7–6.7 mg kg^{-1} copper did not show significant differences in HSI values. However, fish fed diet containing more than 6.7 mg kg^{-1} of copper resulted in significant reduction in the values of HSI indicating the shrinkage of liver due to copper toxicity. A similar trend of HSI was also noted in VSI. Condition factor was also influenced by the varying levels of dietary copper and improved with the increase in dietary copper from 3.7 to 6.7 mg kg^{-1}. Fish fed higher levels of dietary copper exhibited significant decline in this parameter.

Copper requirement

In order to obtain more accurate information on optimum dietary copper requirement of fingerling *C. punctatus*, absolute weight gain (Fig. 1), feed conversion ratio (Fig. 2) and protein gain (Fig. 3) were subjected to broken-line regression analysis and the break points were obtained at 6.77, 6.66 and 6.78 mg copper kg^{-1} of the dry diet, respectively.

Discussion

The results indicate that fish cannot meet their physiological needs for copper from rearing water and a dietary source is required for maximum growth and tissue mineralization of *C. punctatus*.
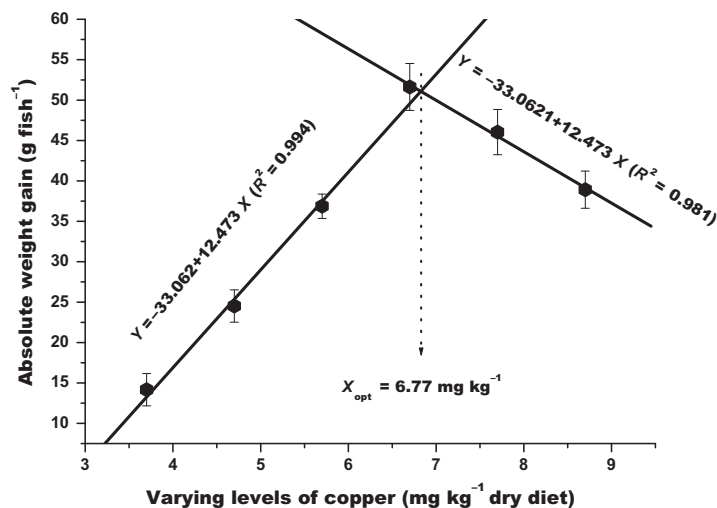
Figure 1 Broken-line relationship of dietary copper levels to absolute weight gain. Each point represents the mean of three replicates per treatment.
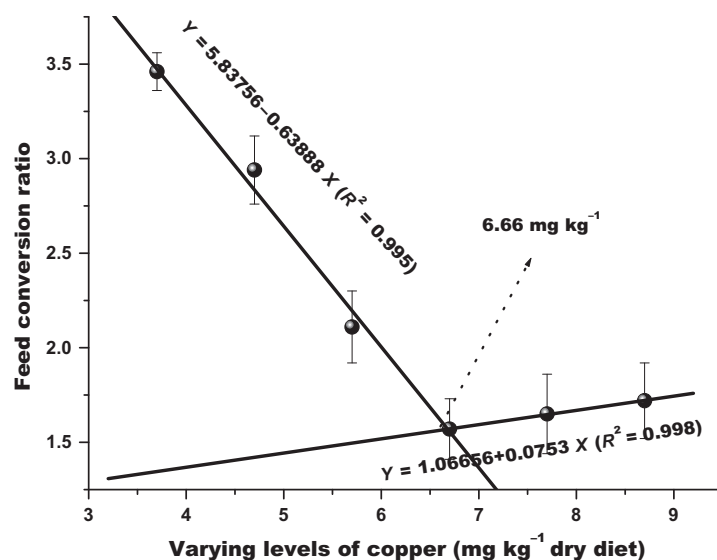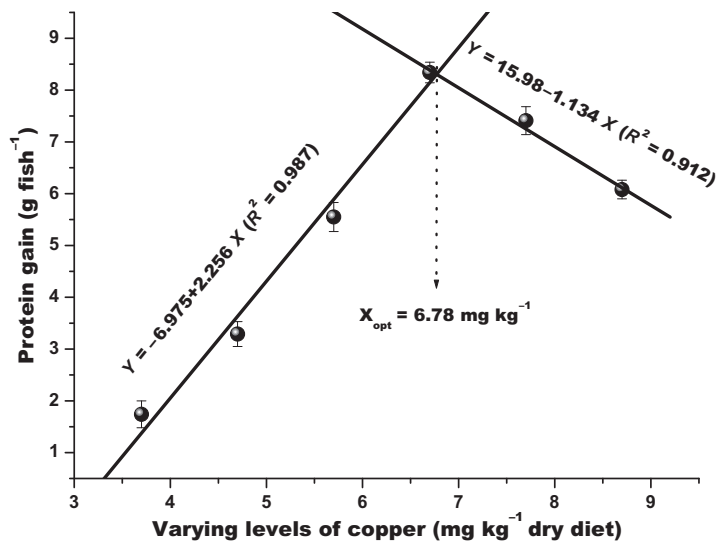
Figure 2 Broken-line relationship of dietary copper levels to feed conversion ratio. Each point represents the mean of three replicates per treatment.

The growth, feed conversion ratio, protein efficiency ratio and protein gain of fingerling *C. punctatus* fed diets with different levels of copper improved significantly up to 6.7 mg kg^{-1} indicating that the requirement of this fish was found to be 6.7 mg kg^{-1} of the dry diet. However, broken-line regression analysis of above parameters against varying levels of dietary copper indicated the optimum requirement of *C. punctatus* in the range of 6.66–6.78 mg kg^{-1} of the dry diet which is higher than that reported for 3 mg kg^{-1} diet in rainbow trout, *Oncorhynchus mykiss*, and common carp (Ogino & Yang 1980); 5 mg kg^{-1} diet in channel catfish (Gatlin & Wilson 1986); 5 mg kg^{-1} diet in Atlantic salmon (Lall & Hines 1987); 4 mg kg^{-1} diet in hybrid tilapia, *Oreochromis niloticus x*

O. aureus (Shiau & Ning 2003), 3.13–4.24 mg kg^{-1} diet in yellow catfish, *P. fulvidraco* (Tan *et al.* 2011) but lower than that of 10.3 mg kg^{-1} diet in beluga, *H. huso* (Mohseni *et al.* 2014) and comparable to the requirement of 4–6 mg kg^{-1} diet in grouper, *Epinephelus malabaricus* (Lin, Shie & Shiau 2008).

Fingerling *C. punctatus* fed higher levels of dietary copper (D5–D7) resulted in significant fall in weight gain which is probably due to copper toxicity hampering the normal physiological processes for maximizing growth. Similar pattern of growth in fish fed diets containing higher levels of copper was also noted in channel catfish (Murai, Andrews & Amith 1981); yellow catfish (Tan *et al.* 2011) and beluga (Mohseni *et al.* 2014).

Figure 3 Broken-line relationship of dietary copper levels to protein gain. Each point represents the mean of three replicates per treatment.

Copper is not only an essential but also a potentially toxic trace metal depending on its concentration (NRC 2011). It affects various blood parameters, growth, behaviour, enzyme activity and reproduction (Nussey, Van Vuren & Preez 1995). The requirement and the maximum tolerable levels of dietary copper vary widely for different studies on fish (NRC 2011). Murai *et al.* (1981) found reduced growth and feed conversion in channel catfish fingerlings exposed to 16 mg copper kg^{-1} diet for 16 weeks. In contrast, Gatlin and Wilson (1986) found no growth inhibition in channel catfish fingerlings exposed to 40 mg copper kg^{-1} diet for 13 weeks. Higher levels of dietary copper in fish cause toxic syndromes which include growth depression, increased mortality (Shiau & Ning 2003), oxidative stress (Berntssen, Lundebye & Hamre 2000) and reduced immune response (Berntssen *et al.* 1999; Lundebye, Berntssen, Wendelaar Bonga & Maage 1999). Similarly, this study showed reduced growth, anorexia and increased mortality in fish due to toxicity of copper.

Baker (1986) and Cowey (1992) have emphasized that growth is not a sufficient indicator of element status in requirement studies but should be followed by element analyses of tissues. The copper status of the experimental fish was assessed by measuring whole body, liver and serum copper concentrations (Lorentzen, Maage & Julshamn 1998; Mohseni *et al.* 2012, 2014). In this study, the whole body copper concentrations responded to dietary copper and reflected significant increment with the increasing levels of dietary copper indicating that higher levels of dietary copper could not be eliminated and accumulated in tissues. One goal of aquaculture is to produce fish fillets for human consumption and hence care should be taken to maintain the residual mineral content within safe levels for human consumption (Cinier, Petit-Ramel, Faure, Garin & Bouvet 1999). Whole body copper concentration at 6.7 mg kg^{-1} of dietary copper was found to be 12.94 mg kg^{-1} of dry matter which is good enough to meet the physiological needs of *C. punctatus* and may be the safe concentration for human consumption. However, whole body copper concentration at 7.7 (D5), and 8.7 mg kg^{-1} (D6) of dietary copper led to reduce growth, anorexia and anaemic condition in fish. The results suggest that the above concentrations of whole body copper may be toxic to fish. A similar response of whole body copper concentrations in fish fed varying levels of dietary copper has been reported in other studies (Tan *et al.* 2011; Mohseni *et al.* 2012, 2014).

Thiobarbituric acid reaction substances (TBARS) analysis is one of the most important indicators of tissue peroxidation (Rosmini, Perlo, Perez-Alvarez, Pagan-Moreno, GagoGago, LopezSantovena & Aranda-Catala 1996). Tissue TBARS concentrations responded negatively with the incremental levels of dietary copper from 3.7 (D1)–6.7 mg kg^{-1} (D4) of the dry diet. Fish fed diet with lowest concentration of copper exhibited highest value of TBARS indicating that insufficient dietary copper-induced oxidative stress in this group. The copper-induced lipid peroxidation in the tissues can be explained

by *in vivo* oxidative damage due to tissue copper accumulation and/or the intake of oxidative products from rancid feed caused by the supplementation of copper salts (Baker, Handy, Davies & Snook 1998). Similar results of TBARS in fish fed varying levels of dietary copper have been reported for grey mullet (Baker *et al.* 1998), Atlantic salmon (Berntssen *et al.* 2000), grouper (Lin *et al.* 2008) and beluga (Mohseni *et al.* 2014).

In the present study, fish fed diet with lowest level of dietary copper resulted in highest lipid deposition in body. However, whole body protein content was found to increase with the increasing levels of dietary copper up to 6.7 mg kg^{-1}. The documentation of highest whole body lipid and lowest whole body protein in fish fed diet with lowest level of copper may be because of the lower utilization of lipid as an energy source. This indicates that fish utilized protein for energy purposes instead of lipid.

Condition factor has been used as an indicator of health in fish. It provides information on the variation of fish physiological status and may be used for comparing populations living in certain feeding, climate and other conditions (Ighwela, Ahmed & Abol-Munafi 2011). Therefore, condition factor can be used to determine the feeding activity of a species to determine whether it is making good use of its feeding source (Lizama & Ambrosio 2002; Ighwela *et al.* 2011). In this study, fish fed diet with 6.7 mg kg^{-1} of copper possessed significantly higher condition factor which indicates that fish fed on this diet were in good condition. Since hepatosomatic index is an important role in the metabolism of fish, HSI is a useful biomarker to detect the deleterious effects of the environmental stressors (Pait & Nelson 2003). The lower values of HSI were obtained in *C. punctatus* fed higher levels of dietary copper suggesting the shrinkage of liver due to the adverse effects of copper at these concentrations which is similar to that reported in yellow catfish (Tan *et al.* 2011).

Assessment of the physiological and health status of fish may also be assessed by examining haematological indices. Since copper is essential to haematopoiesis (Wu, Ricker & Muench 2006), haematological indices in response to dietary copper could be considered as important tools in assessing the health status of the fish. In this study, haematological variables including RBCs, haemoglobin (Hb) and haematocrit (Ht%) values were found to significantly improve by the increasing levels of copper up to 6.7 mg kg^{-1} of the dry

diet (D4). However, fish fed diets containing deficient levels of dietary copper exhibited lower values of Hb, RBCs and haematocrit indicating that copper deficiency led to anaemic condition in *C. punctatus*.

Based on the results of this study, the dietary copper requirement of fingerling *C. punctatus* determined from the broken-line regression analysis of absolute weight gain, feed conversion ratio, protein efficiency ratio and protein gain against varying levels of dietary copper is found to be in the range of 6.66–6.78 mg kg^{-1} of the dry diet. Data generated in this study would be useful in formulating copper-balanced commercial feeds for the intensive culture of *C. punctatus*.

Acknowledgments

References

AOAC, Association of Official Analytical Chemists. (2005) Animal Feed. In: *Official methods of analysis of official analytical chemists international* (18th edn) (ed. by W. Horwitz & G.W. Latimer Jr), pp. 1–52. Association of Official Analytical Chemists, Arlington, VA.

APHA, American Public Health Association. (1992) *Standard Methods for the Examination of Water and Wastewater*, 18th editor, APHA, Washington DC, p. 1268.

Baker D.H. (1986) Problems and pitfalls in animal experiments designed to establish dietary requirements for essential nutrients. *Journal of Nutrition* **116**, 2339–2349.

Baker R.T.M., Handy R.D., Davies S.J. & Snook J.C. (1998) Chronic dietary exposure to copper affects growth, tissue lipid peroxidation, and metal composition of the grey mullet, *Chelon labrosus*. *Marine Environmental Research* **45**, 357–365.

Berntssen M.H.G., Kundebye A.K. & Moage A. (1999) Effects of elevated dietary copper concentrations on

growth, feed utilization and nutritional status of Atlantic salmon (*Salmo Solar* L.) fry. *Aquaculture* **174**, 167–181.

Berntssen M.H.G., Lundebye A.K. & Hamre K. (2000) Tissue lipid peroxidative responses in Atlantic salmon (*Salmo salar* L.) parr fed high levels of dietary copper and cadmium. *Fish Physiology and Biochemistry* **23**, 35–48.

Bhuiyan A.S., Afroz S. & Zaman T. (2006) Food and feeding habit of the juvenile and adult snake head, *Channa punctatus* (Bloch). *Life Earth Science* **1**, 53–54.

Bielmyer G.K., Gatlinb D., Iselyc J.J., Tomassod J. & Klained S.J. (2005) Responses of hybrid striped bass to waterborne and dietary copper in freshwater and saltwater. *Comparative Biochemistry and Physiology C* **140**, 131–137.

Chondor S.L. (1999) *Biology of finfish and shell fish*, pp. 422–434. SCSC publishers, India.

Cinier C.D., Petit-Ramel M., Faure R., Garin D. & Bouvet Y. (1999) Kinetics of cadmium accumulation and elimination in carp *Cyprinus carpio* tissues. *Comparative Biochemistry and Physiology C* **122**, 345–352.

Cowey C.B. (1992) Nutrition: estimating requirements of rainbow trout. *Aquaculture* **100**, 177–189.

Damasceno F.M., Fleuri L.F., Sartori M.M.P., Amorim R.L., Pezzato L.E., Silva R.L., Carvalho P.L.P.F&Barros M.M. (2016) Effect of dietary inorganic copper on growth performance and hematological profile of Nile tilapia subjected to heat-induced stress. *Aquaculture* **454**, 257–264.

Fatima M., Ahmad I., Sayeed I., Athar M. & Raisuddin S. (2000) Pollutant- induced over-activation of phagocytes is concomitantly associated with peroxidative damage in fish tissues. *Aquatic Toxicology* **49**, 243–250.

Gatlin D.M. III & Wilson R.P. (1986) Dietary copper requirement of fingerling channel cattish. *Aquaculture* **54**, 277–285.

Halver J.E. (2002) The vitamins. In: *Fish nutrition* (ed. by J.E. Halver & R.W. Hardy), pp. 61–141. Academic Press, San Diego, CA, USA.

Hoyle I., Shaw B.J. & Handy R.D. (2007) Dietary copper exposure in the African walking catfish, *Clarias gariepinus*: transient osmoregulatory disturbances and oxidative stress. *Aquatic Toxicology* **83**, 62–72.

Hseu Z.Y. (2004) Evaluating heavy metal contents in nine composts using four digestion methods. *Bioresource Technology* **95**, 53–59.

Ighwela K.A., Ahmed A.B. & Abol-Munafi A.B. (2011) Condition factor as an indicator of growth and feeding intensity of Nile Tilapia fingerlings (*Oreochromis niloticus*) feed on different levels of maltose. *American-Eurasian Journal of Agriculture & Environmental Science* **11**, 559–563.

Jauncey K. (1982) The effects of varying dietary protein level on the growth, food conversion, protein utilization and body composition of juvenile tilapias (Sarotherodon mossambicus). *Aquaculture* **27**, 43–54.

Jindal M., Yadava N.K., Jain K.L. & Gupta R.K. (2010) Effect of two dietary protein levels on body weight and composition in *Channa punctatus* (Bloch) fingerlings. *Turkish Journal of Fisheries and Aquatic Science* **10**, 203–208.

Lall S.P. (2002) The minerals. In: *Fish Nutrition* (ed. by J.E. Halver & R.W. Hardy), pp.260–308, 3rd edn. Academic Press,New York, USA.

Lall S.P. & Hines J.A. (1987) *Iron and copper requirement of Atlantic salmon (Salmo salar) grown in sea water*, pp. 23–27. International Symposium on Feeding and Nutrition of Fish, Bergen, Norway.

Lin Y.H., Shie Y.Y. & Shiau S.Y. (2008) Dietary copper requirements of juvenile grouper, *Epinephelus malabaricus*. *Aquaculture* **274**, 161–165.

Lizama M.A.P. & Ambrosio A.M. (2002) Condition factor in nine species of fish of the characidae family in the upper parana river floodplain, brazil. *Brazilian Journal of Biology* **62**, 113–124.

Lorentzen M., Maage A. & Julshamn K. (1998) Supplementing copper to a fish meal based diet fed to Atlantic salmon parr affects liver copper and selenium concentrations. *Aquaculture Nutrition* **4**, 67–77.

Lundebye A.K., Berntssen M.H.G., Wendelaar Bonga S.E. & Maage A. (1999) Biochemical and physiological responses in Atlantic Salmon (*Salmo salar*) following dietary exposure to copper and cadmium. *Marine Pollution Bulletin* **39**, 137–144.

Luszczek-Trojnar E., Sionkowski J., Drąg-Kozak E. & Popek W. (2015) Copper and lead accumulation in common carp females during long-term dietary exposure to these metals in pond conditions. *Aquaculture Research*. doi:10.1111/are.12689.

Marimuthu K., Arokiaraj A.J. & Haniffa M.A. (2009) Effect of diet quality on seed production of the spotted snakehead *Channa punctatus* (Bloch). *American-Eurasian Journal of Sustainable Agriculture* **3**, 344–347.

Mohseni M., Park G.H., Lee J.H., Okorie O.E., Browdy C., Bharadwaj A. & Bai S.C. (2012) Evaluation of toxicity of dietary chelated copper in juvenile olive flounder, *Paralichthys olivaceus*, Based on growth and tissue copper concentration. *Journal of the World Aquaculture Society* **43**, 548–559.

Mohseni M., Pourkazemi M. & Bai S.C. (2014) Effects of dietary inorganic copper on growth performance and immune responses of juvenile beluga, *Huso huso*. *Aquaculture Nutrition* **20**, 547–556.

Murai T., Andrews J.W. & Amith R.G. Jr (1981) Effects of dietary copper on channel catfish. *Aquaculture* **22**, 353–357.

Nassr A.A.H., Khan M.A. & Abidi S.F. (2012) Effects of dietary vitamin E levels on growth, conversion efficiency, biochemical composition and haematological parameters of fingerling *Channa punctatus* (Bloch). *Aquaculture Research* **43**, 226–238.

NRC. (2011) *Nutrient requirements of fish and shrimp*. The National Academies Press, Washington, DC, USA. 376 + XVI pp, ISBN: 978-0-309-16338-5.

Nussey G., Van Vuren J.H.J. & Preez H.H. (1995) Effect of copper on the haematology and osmoregulation of the Mozambique tilapia, *Oreochromis mossambicus* (Cichlidae). *Comparative Biochemistry and Physiology C* **111**, 369–380.

Ogino C. & Yang G.Y. (1980) Requirements of carp and rainbow trout for dietary manganese and copper. *Bulletin of the Japanese Society of Scientific Fisheries* **46**, 455–458.

Pait A.S. & Nelson J.O. (2003) Vitellogenesis in male *Fundulus heteroclitus* (Killifish) induced by selected estrogenic compounds. *Aquatic Toxicology* **64**, 331–342.

Robbins K.R., Saxton A.M. & Southern L.L. (2006) Estimation of nutrient requirements using broken-line regression analysis. *Journal of Animal Science* **84**, 155–165.

Rosmini M.R., Perlo F., Perez-Alvarez J.A., Pagan-Moreno M.J., GagoGago A., LopezSantovena F. & Aranda-Catala V. (1996) TBA test by an extractive method applied to 'Paté'. *Meat Science* **42**, 103–110.

Saikia A.K. & Das D.N. (2010) Survival and growth of *Channa punctatus* (Bloch) under controlled condition in cemented cistern. *Sibcoltejo* **5**, 78–81.

Shiau S.Y. & Ning Y.C. (2003) Estimating of dietary copper requirements for juvenile tilapia, *Oreochromis niloticus × O. aureus. Animal Science* **77**, 287–292.

Sokal R.R. & Rohlf F.J. (1981) *Biometry*. Freeman and Company, New York, W.H p.859

Tan X.Y., Luo Z., Liu X. & Xie C.X. (2011) Dietary copper requirement of juvenile yellow catfish *Pelteobagrus fulvidraco. Aquaculture Nutrition* **17**, 170–176.

Utley H.C., Bernheim F. & Hachslein P. (1967) Effect of sulfhydryl reagent on peroxidation in microsome. *Archives of Biochemistry and Biophysics* **260**, 521–531.

Vani T., Saharan N., Roy S.D., Ranjan R., Pal A.K., Siddaiah G.M. & Kumar R. (2012) Alteration in haematological and biochemical parameters of *Catla catla* exposed to sub-lethal concentration of cypermethrin. *Fish Physiology and Biochemistry* **38**, 1577–1584.

Wang H., Li E., Zhu H., Du Z., Qin J. & Chen L. (2016) Dietary copper requirement of juvenile Russian sturgeon *Acipenser gueldenstaedtii. Aquaculture* **454**, 118–124.

Wu J., Ricker M. & Muench J. (2006) Copper deficiency as cause of unexplained hematologic and neurologic deficits in patient with prior gastro intestinal surgery. *Journal of the American Board of Family Medicine* **19**, 191–194.

Yaakob W.A.A. & Ali A.B. (1992) Simple method for backyard production of snakehead (*Channa striata* Bloch) fry. *Naga* **15**, 22–23.

Zehra S. & Khan M.A. (2012) Dietary protein requirement for fingerling *Channa punctatus* (Bloch), based on growth, feed conversion, protein retention and biochemical composition. *Aquaculture International* **20**, 383–395.