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Effects of dietary iron oxide nanoparticles on the growth performance, biochemical constituents and physiological stress responses of the giant freshwater prawn *Macrobrachium rosenbergii* post-larvae

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Abstract
This study was performed to assess the growth promoting potential of dietary Fe_2O_3 NPs in *Macrobrachium rosenbergii* PL. Fe_2O_3 NPs was supplemented at 0, 10, 20, 30, 40 and 50 mg kg^{-1} with the basal diet (containing 5.45 mg Iron kg^{-1}). The concentration of Fe in Fe_2O_3 NPs supplemented diet was increased correspondingly. Fe_2O_3 NPs supplemented diets were fed to *M. rosenbergii* PL (initial weight, 0.11±0.04 g) for a period of 90 days. In the carcass of experimental prawns, the content of Fe was found to be elevated significantly when compared with control. Similarly, Cu, Zn, Ca, Mg, Na and K levels were also elevated. Therefore, the supplemented Fe_2O_3 NPs were taken up and incorporated into the body of the prawns, which facilitates absorption of other minerals as well. Significant ($P < 0.05$) improvements were observed in survival, growth, activities of digestive enzymes (protease, amylase and lipase), concentrations of basic biochemical constituents (total protein, amino acid, carbohydrate and lipid, profiles of amino acids and fatty acids) and population of haemocytes [total and differential (hyalinocytes, semigranulocytes and granulocytes)] at 10 and 20 mg kg^{-1} Fe_2O_3 NPs supplemented feeds fed prawns, whereas, 30-50 mg kg^{-1} Fe_2O_3 NPs supplementations showed negative performance. The polynomial regression analysis revealed that a dietary requirement of 24.56 mg kg^{-1} Fe_2O_3 NPs for optimal growth of prawns. SDS-PAGE revealed separation of 14 polypeptide bands in the muscle of prawns. Among these, the staining intensity of 116, 99, 50, 26, 20, 18, 15, 14 and 13 kDa polypeptide bands were found to be higher in 20 mg kg^{-1} Fe_2O_3 NPs supplemented feed fed prawn when compared with control. The lowest food conversion ratio (FCR) recorded in 20 mg kg^{-1} of Fe_2O_3 NPs supplemented diet fed PL reflects the superior quality of the diet prepared. Therefore, up to optimum level supplementation of Fe_2O_3 NPs, the general health of the experimental prawns was improved due to its incorporation in the body. But beyond the optimum level it turned towards toxic. Therefore significant ($P<0.05$) elevations in activities of antioxidant enzymes (superoxide dismutase and catalase), lipid peroxidation, and metabolic enzymes (glutamic oxaloacetic transaminase and glutamic pyruvic transaminase) were recorded in 30-50 mg kg^{-1} Fe_2O_3 NPs supplemented diets fed prawns. The better profiles of protein, amino acids, fatty acids, biochemical constituents and haemocytes helped to attain appreciable survival and growth of *M. rosenbergii* PL. Thus, this study recommends Fe_2O_3 NPs supplementation up to 20 mg kg^{-1} for its sustainable maintenance.

Keywords: Prawn, Fe_2O_3 NPs, Growth, Protein, Amino acids, Fatty acids, Hemocytes, SOD, Catalase, Lipid peroxidation, GOT, GPT.

1. Introduction
Culture of food fish and aquatic plants has grown significantly during the past half-century. There growth of aquaculture has peaked in some regions for some species of fishes, prawns, crabs, lobster, crayfish etc. Over half of all aquaculture production of fish, crustaceans and molluscs is from the freshwater, with 34% coming from mariculture and the remaining is brackish water systems [1]. Freshwater prawn culture possesses immense potential and has considerably expanded in recent years as an alternative to marine shrimp culture, which faces disease outbreak and poses severe environmental threats [2]. The giant freshwater prawn, *Macrobrachium rosenbergii* has been the principal species, adopted both under monoculture and polyculture. Under monoculture farming, the freshwater prawn production reached about 3,332 MT in India during 2012-13 [3]. It is the most popular prawn species used for commercial farming and has been transported to many parts of the world including south

America and China [4]. Environmental sustainability of freshwater prawn farming and recent technological developments in the culture methods has boosted *Macrobrachium* production [5-8].

The fisheries and aquaculture industries can be revolutionized by using nanotechnology with new tools, such as rapid diagnosis of diseases which will enhance the ability of cultivable organisms to uptake drugs like hormones, vaccines and essential nutrients etc., [9]. The metal nanoparticles (NPs), such as Fe, FeO, Se, Zn, ZnO, Cu, and MgO etc., play a crucial role in aquaculture operations. It has been reported that iron NPs when fed to young carp, *Carassius auratus* and sturgeon, *Acipenser gueldenstaedtii* showed a faster growth rate, 30% and 24% respectively [10]. Different Se sources (nano-Se and Selenomethionine) supplemented diets improved the growth, antioxidant status and muscle Se concentration of Crucian carp, *Carassius auratus gibelio* [11]. In recent reports, dietary supplementation of Zn, nano Zn and Cu have produced better survival and growth in *M. rosenbergii* PL [8, 12, 13]. Furthermore, iron-based nanoparticles (NPs) are also used for soil and groundwater remediation and water treatment processes [14, 15].

Iron is one of the most essential micronutrients in terms of its effect on the functioning of the immune system and defense against various infections [16-18]. It is an indispensable element for the functioning of organs and tissues of higher animals, including fish, because of its vital role in physiological processes such as oxygen transport, cellular respiration and lipid oxidation reactions [19-21]. Iron deficiency causes immune suppression, growth depression, changes in hematological parameters, susceptibility to diseases, poor food conversion and microcytic anemia in common carp [22-26]. In order to prevent the deteriorative oxidative reactions or to increase the oxidative stability to maintain the food quality and nutritional value, antioxidants such as iron have been added to the feed of farm animals [27].

Iron oxide nanoparticle (Fe_2O_3 NPs) is of great interest due to its unique physicochemical properties. It has a great potential in biomedical applications, as food additives, antimicrobial additives, drug carriers etc., due to its super paramagnetic properties and their potential biocompatibility [18]. Recently, it has been reported that Fe_2O_3 NPs boost bioavailability than other forms of iron nanoparticles in humans, rats and fishes [28, 29]. However, there is dearth of information that the trace element, Fe_2O_3 NPs on growth performance, haematology, immune response, tissue deposition, activities of antioxidant and metabolic enzymes in freshwater prawns. Hence, the present study was designed to optimize the dietary Fe_2O_3 NPs for assessing its effects on the survival, growth, nutritional indices [feed intake (FI), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER)], activities of digestive enzymes (protease, amylase and lipase), contents of biochemical constituents (total protein, amino acids, carbohydrate and lipid) including profiles of proteins, amino acids and fatty acids, haemocytes population, activities of antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)], status of lipid peroxidation (LPO) and activities of metabolic enzymes [glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT)] in *M. rosenbergii* PL.

2. Materials and methods

2.1. Maintenance of prawns

The post larvae (PL-10) of the freshwater prawn, *M. rosenbergii* were procured from ADAK Hatchery, Odayam, Varkala (8.733°N 76.717°E), Thiruvananthapuram, Kerala, India. They were transported to the laboratory in polythene bags filled with oxygenated water. The prawns were acclimatized to the ambient laboratory condition with ground water in cement tanks (6×3×3 feet) for 2 weeks. The ground water satisfied the required physico-chemical parameters (Temperature, 26±1.0 °C; pH, 7.10±0.28; total dissolved solids, 0.92±0.05 g L^{-1}; dissolved oxygen, 7.00±0.29 mg L^{-1}; BOD, 35.00±1.42 mg L^{-1}; COD, 127.00±4.00 mg L^{-1}; ammonia, 0.024±0.005 mg L^{-1}). During acclimatization the prawns were fed with boiled egg albumin, live *Artemia* nauplii and commercially available scampi feed. More than 75% of tank water was routinely changed every day in order to maintain a healthy environment and aeration was also provided. This ensures sufficient oxygen supply to the prawns and an environment devoid of accumulated metabolic wastes. The unfed feeds, faeces, moult and dead prawns if any were removed by siphoning without disturbing the prawns.

2.2. Preparation of diets

The experimental diets were prepared with the following locally available feed ingredients (g kg^{-1}), Fish meal (330) and soybean meal (330) as protein sources, wheat bran (100) and tapioca flour (100) as carbohydrate sources, tapioca flour and egg albumin (100) as binding agents, and Cod liver oil (20) as lipid source, using ''Pearson's square-method'' to maintain a pre determined protein value of 40%. Vitamins and minerals free of Fe_2O_3 (20) were also added in the form of Zincovit® tablets (Apex Laboratories Private Limited, Chennai, India), each tablet contains, Energy, 3 kcal; Total carbohydrate, 0.2 g; Carbohydrate (as sugar), 0.2 g; Protein and Fat 0 g each; Vitamins: Vitamin C, 40 mg; Vitamin B_3, 18 mg; Vitamin E, 10 mg; Vitamin B_5, 3 mg; Vitamin B_2, 1.6 mg; Vitamin B_1, 1.4 mg; Vitamin B_6, 1 mg; Vitamin A, 600 mcg; Folic acid, 200 mcg; Biotin,150 mcg; Vitamin B_{12},1 mcg; Vitamin D_3, 200 IU; Minerals: Zinc, 10 mg; Magnesium, 3 mg; Manganese, 250 mcg; Iodine, 100 mcg; Copper, 30 mcg; Selenium, 30 mcg; Chromium, 25 mcg; Natural extract, Grape Seed Extract, 50 mg.

The required proportion of each ingredient was taken in powder form, steam cooked and then cooled at room temperature (28 ºC). Then egg albumin, Cod liver oil, vitamins and minerals free of Fe_2O_3 were added one by one. Fe_2O_3 NPs (average size <50 nm by TEM analysis, purity ≥98% trace metals basis, Sigma Aldrich, USA) was supplemented with the basal diet at 0, 10, 20, 30, 40 and 50 mg kg^{-1}. These concentrations range of dietary iron was chosen based on its requirements for crustaceans available in the literature [30]. Diet with '0' % supplementation of Fe_2O_3 NPs was served as control. Thus six experimental diets were prepared. The dough was prepared for each formulation and pelletized separately (approximately 3.00 mm sized). The pellets were dried in a thermostatic oven (M/s. Modern Industrial, Mumbai, India) at 40 ºC until they reached constant weight, and stored in airtight plastic jars at -20 ºC and consequently used during the feeding trial.

In the present study, the proximate composition of organic matters was determined by adopting the methodology of AOAC [31]. Analysis of total nitrogen was performed after single acid digestion (con. H_2SO_4) using Kjeldahl technique, titrated against 0.1N HCl and the crude protein content was calculated (N×6.25). The crude fat was extracted with petroleum ether, the extract was dessicated and weighed. For crude fiber, sample was successively digested by boiling acid and alkali. The extract was converted into ash and the

difference was calculated. The sample was ignited in a muffle furnace and the inorganic residue was calculated as total ash. The feed sample was placed in a hot air oven at slightly >100 ºC and the loss of weight calculated as the moisture content. The basal diet formulated (control) contains 40.37% crude protein, 5.79% crude fat, 2.70% crude fibre, 8.84% total ash, 7.51% moisture and 35.42% carbohydrate (total nitrogen free extract) with energy value of 15.19 (kJ g^{-1}). These diets were freshly prepared once in every 30 days to ensure the freshness in order to maintain palatability throughout the duration of feeding trail. The water stability of the feeds formulated was checked by immersion and drying method and the leaching percentage after 8 h of immersion was found to be between 20-22%.

The levels of trace elements (Fe and other elements, Cu and Zn, and mineral salts, Ca, Mg, Na and K) in Fe_2O_3 NPs supplemented diets were analyzed using Atomic Absorption Spectrophotometer [31] by outsource service (Animal Feed Analytical and Quality Assurance Laboratory (AFAQAL), Veterinary College and Research Institute, Namakkal, Tamil Nadu, India) and are presented in table 1.

2.3. Feeding trial

Six groups of prawns (PL 30; 1.47±0.39 cm and 0.11±0.04 g of initial length and weight respectively) were assigned in triplicate experimental set up. One group was served as control and fed with '0' mg kg^{-1} Fe_2O_3 NPs supplemented diet. The remaining five groups were fed with 10, 20, 30, 40 and 50 mg kg^{-1} Fe_2O_3 NPs supplemented diets respectively. Each group consists of 40 PLs in an aquarium maintained with 40 L of ground water. The water medium was renewed every 24 h by siphoning method without severe disturbance to the PLs and aerated adequately. The PLs were fed with above prepared diets at 10% of body weight twice a day (6.00 a.m. and 6.00 p.m.) for 90 days. During feeding trial, the unfed feed, feces and moults if any were collected on a daily basis while renewing aquarium water. The similar experimental set up was maintained then and there to study different parameters.

2.4. Contents of carcass trace elements and mineral salts

The dietary and whole body mineral contents, such as Fe, Cu, Zn and (in metal forms), Ca, Mg, Na and K (in salt forms) were analyzed using the atomic absorption spectrophotometer (AAS of Perkin-Elmer; Model 2380) under air acetylene flame by adopting AOAC [31] method of triple acid digestion (H_2SO_4: HNO_3: $HClO_4$ at 9:3:1 ratio) of dry sample (10×6=60 prawns×3 (triplicate) = 180 prawns).

2.5. Survival rate and nutritional indices

At the end of the feeding trial, the survival rate [SR (No. of live prawns/ no. of prawns introduced×100)] and nutritional indices parameters, such as feed intake [Feed eaten (g)/ total number of days], length gain [Final length (cm)–initial length (cm)], weight gain [Final weight (g)–initial weight (g)], specific growth rate [*log* final weight (g)–*log* initial weight (g)/ total number of days×100], feed conversion ratio [total feed intake (g)/ total weight gain (g)] and protein efficiency ratio [total weight gain (g)/ total protein intake (g)] were calculated by adopting the formulae of Tekinay and Davies [32].

2.6. Activities of digestive enzymes

Activities of digestive enzymes, such as protease, amylase and lipase were assayed on final day of feeding trial. The digestive tract of prawn was carefully dissected out and homogenized in ice-cold distilled water and centrifuged at 9329 g under 4 ºC for 20 min. Five prawns per group were sacrificed (5×6=30 prawns × 3 (triplicate) = 90 prawns). The supernatant was used as a crude enzyme source. Total protease activity was determined by casein-hydrolysis method of Furne *et al.* [33], one unit of enzyme activity represents the amount of enzyme required to liberate 1 µg of tyrosine per minute under assay conditions. Amylase activity was determined by starch-hydrolysis method put forth by Bernfeld [34], the specific activity of amylase was calculated as milligrams of maltose liberated per gram of protein per hour (mg/g/h). Lipase activity was determined by method of Furne *et al.* [33], one unit of lipase activity was defined as the amount of free fatty acid released from triacylglycerol per unit time, estimated by the amount of NaOH required to maintain pH constant and represented as mille equivalents of alkali consumed.

2.7. Concentrations of biochemical constituents

On the final day, the concentrations of basic biochemical constituents, such as total protein, total amino acid, total carbohydrate and total lipid in the muscle of prawns were determined. Total protein was estimated by the method of Lowry *et al.* [35]. Total amino acid was estimated by the method of Moore and Stein [36]. Total carbohydrate was estimated by the method of Roe [37]. Total lipid was extracted with chloroform–methanol mixture following the method of Folch *et al.* [38] and estimated by the method of Barnes and Blackstock [39]. For each of these parameters, tissues from five prawns were pooled together from each group to constitute a single observation and three such observations were made to fulfill the triplicate analysis (5×6=30 prawns×3 (triplicate) = 90 prawns per each parameter (×4 = 360 prawns).

2.8. Profiles of proteins

SDS-PAGE analysis was done for 20 mg kg^{-1} of Fe_2O_3 NPs supplemented feed fed prawns. The muscle tissue sample was first defrosted in phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH-7.4), homogenized under ice cooled condition and centrifuged at 1500 rpm at 4 ºC for 5 min. The soluble protein content in supernatant was determined Lowery *et al.* [35]. SDS-PAGE was performed [40] on vertical slab gel with 4% stacking and 10% separating gels. Protein markers consisting of six different molecular weights (Medox-Biotech Pvt. Ltd., India), such as β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa) and lysozyme (14 kDa) was also ran simultaneously. The polypeptides banding patterns between control and test prawn was compared by using information on apparent molecular masses of bands and their intensity.

2.9. Profiles of amino acids

The profiles of amino acids were done for 20 mg kg^{-1} of Fe_2O_3 NPs supplemented feed fed prawns. The high performance thin layer chromatographic (HPTLC) method of Hess and Sherma [41] was adopted to analyze the profiles of amino acids. The prawns were dried (80 ºC for 3 h), digested with 6M aqueous hydrochloric acid and dried under vacuum. The powdered sample was dissolved in distilled water and 5 µl of sample was loaded on 8 mm thick pre-coated Silica gel 60F254 TLC plate (20 cm×15 cm) and processed in CAMAG-LINOMAT 5 instrument. The plate was developed in Butane-Ammonia-Pyridine-Water (3.9:1:3.4:2.6) mobile phase. The plate was sprayed with ninhydrin reagent prepared in propan-2-ol and

dried. The developed plate was documented using photo documentation chamber (CAMAG-11REPROSTAR 3) at UV 254 nm and UV 366 nm lights. Finally, the plate was scanned at 500 nm using CAMAG-TLC SCANNER 3. The peak area of the sample was compared with standard amino acids and quantified. Four groups of standard amino acids were also run in parallel. Group-I: lysine, asparagine, glutamine, glutamic acid and methionine; Group-II: proline, serine, cystine, tyrosine and tryptophan; Group-III: histidine, arginine, aspartic acid, threonine and leucine; Group-IV: glycine, alanine, valine, isoleucine and phenyl alanine.

2.10. Profiles of fatty acids

The profiles of fatty acids were done for 20 mg kg^{-1} of Fe_2O_3 NPs supplemented feed fed prawns. The gas chromatographic (GC) method of Nichols *et al.* [42] was adopted to analyze the profiles of fatty acids. Fatty acids were obtained from lipids by saponification using NaOH dissolved in methanol H_2O mixture (hydrolysis with alkali). They were methylated into fatty acid methyl ester using HCl and methanol mixture, which can be easily identified by gas chromatography. The fatty acid methyl ester was separated using mixture of hexane and anhydrous diethyl ether. For the organic phase aqueous NaOH was used as base wash and the upper organic layer was separated. 2 µL of sample was injected and analyzed using Chemito 8610 Gas chromatography, with BPX70 capillary column and flame ionization detector. Nitrogen was used as carrier gas. The chromatogram was used for calculation. Standard fatty acids were analyzed simultaneously. Based on the retention time and peak area of the standard fatty acids, each fatty acid in the unknown sample was identified.

2.11. Haemocyte population

At the end of the experiment, haemolymph (100 µL) was withdrawn from the ventral sinus in the first abdominal segment using a 26-gauge hypodermic needle on a 1 mL syringe. Each syringe was pre-filled with 200 µL of anticoagulant (10 mM Tris-HCl, 250 mM sucrose, 100 mM sodium citrate, pH 7.6). More anticoagulants were added to make the volume up to 1 mL in the anticoagulated haemolymph. Further, a volume of 200 µL anticoagulated haemolymph was fixed with an equal volume of formalin (10%) for 30 min. The fixed haemolymph was used to enumerate the total and differential haemocytes numbers (THC and DHC respectively). Five prawns per group were sacrificed (5×6=30 prawns × 3 (triplicate) = 90 prawns).

For THC, 100 µL of fixed haemolymph was diluted at 1:2 ratio (v/v) with ice-cold phosphate-buffered saline (PBS, 20 mM, pH 7.2). The diluted haemolymph was stained with 20 µL of Rose Bengal strain (1.2% Rose Bengal in 50% ethanol) and incubated at room temperature for 20 min. THC was determined by haemocytometer (Neubauer improved, Germany) under the light microscope at RP 1000X (Labomed, CXR2).

THC (106 cells mL^{-1}) = Counted cells × depth of chamber × dilution factor/ Number of 1^{-mm} square.

For DHC, fixed haemolymph was stained with Rose Bengal solution (10%) for 10 min and smeared on a slide. The numbers of differential haemocytes, such as hyalinocytes, semigranulocytes and granulocytes, were characterized according to Tsing *et al.* [43], and 350–400 cells from each smear were counted under a Trinocular Inverted Microscope (model number INVERSO 3000) RP 1000X.

2.12. Activities of enzymatic antioxidants and lipid peroxidation

Tissues of muscle, and hepatopancreas (100 mg each) was homogenized (10% w/v) in ice-cold 50 mM Tris buffer (pH 7.4), centrifuged at 9329 g for 20 min at 4 ºC and the supernatant was used to assay the activities superoxide dismutase (SOD) and catalase (CAT).

SOD activity was measured by pyrogallol (10 mM) autoxidation in Tris buffer (50 mM, pH 7.0) by adopting the method of Marklund and Marklund [44]. The reaction was initiated by the addition of NADH. The mixture was incubated at 30 °C for 90 s and arrested by the addition of glacial acetic acid. The reaction mixture was then shaken with n-butanol and the intensity of the chromogen in the butanol layer was measured at 560 nm using spectrophotometer. The specific activity of the enzyme was expressed in unit/ mg protein.

CAT activity was measured by using H_2O_2 as the substrate in phosphate buffer by adopting the method of Sinha [45]. The reaction was initiated by the addition of phosphate buffer (0.01 M, pH 7.1), H_2O_2 (0.2 M). After 60 s the reaction was stopped by the addition of dichromate acetic acid reagent. The absorbance of the chromophore was read at 620 nm. The activity of CAT was expressed as µM of hydrogen peroxide consumed/ minute/ mg protein.

Lipid peroxidation (LPO) in tissue homogenate was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA) by adopting the method of Ohkawa *et al.* [46]. The absorbance of the supernatant was measured at 535 nm against the reagent blank. TBARS was expressed as nM of malondialdehyde (MDA)/ mg protein. Concentration of soluble proteins was determined by the method of Lowry *et al.* [35].

For each of these parameters, five prawns per group were sacrificed (5×6=30 prawns×3 (triplicate) = 90 prawns × 4 parameters = 360 prawns).

2.13. Activities of metabolic enzymes

The metabolic enzymes such as glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were analyzed by the method of Reitman and Frankel [47] using a med source kit (Medsource Ozone Biomedicals Pvt. Ltd. Haryana, India). Each 100 mg of hepatopancreas, and muscle tissues were homogenized in 0.25 M sucrose and centrifuged at 3300 g for 20 min in a high speed cooling centrifuge at 4 ºC. The supernatant was used as the enzyme source. Five prawns per group were sacrificed (5×6=30 prawns × 3 (triplicate) = 90 prawns).

GOT analysis, the substrate solution, L-aspartic acid (500 µL; pH, 7.4) was added to a 100 µL sample and incubated at 37 ºC for 1 h. Further, 500 µL of 2, 4-dinitrophenyl hydrazine was added and allowed to stand for 20 min at room temperature. Then 3 mL of freshly prepared 4 N sodium hydroxide solution was added to the above solutions. The color development was read at 505 nm using spectrophotometer within 15 min. Sodium pyruvate (160 U L^{-1}) was used as a calibrator. The activity of GOT was expressed as Unit L^{-1}.

GPT analysis, buffered L-alanine, 2-oxoglutarate substrate (500 µL; pH, 7.4) was added to a 100 µL sample and incubated at 37 ºC for 20 min. With this, 500 µL of 2, 4-dinitrophenyl hydrazine was added and allowed to stand at room temperature for 30 min followed by the addition of 3 mL of freshly prepared 4 N sodium hydroxide solution. The color development was read at 505 nm using a spectrophotometer within 15 min. Sodium pyruvate (170 U L^{-1}) was used as a calibrator. The activity of GPT was expressed as Unit L^{-1}.

2.14. Statistical analysis

The data were expressed as mean ± S.D, and analyzed by one-way analysis of variance (ANOVA) using SPSS (version-20), followed by Duncan's multiple range test (DMRT) to compare the significant differences among treatments at $P < 0.05$. The data obtained for profiles of amino acids and fatty acids were analyzed by student *t*-test. The actual requirement of dietary Fe_2O_3 NPs for optimal growth of *M. rosenbergii* PL was calculated by the polynomial regression method based on the weight gain [48].

3. Results

3.1. Contents of trace elements and mineral salts in diets and prawns carcass

In the control diet, the contents of trace elements (mg kg^{-1}), Fe, Cu and Zn, and mineral salts (g kg^{-1}), Ca, Mg, Na and K presented were 5.45, 2.10 and 4.52, and 0.40, 0.95, 0.32 and 2.19 respectively. In the experimental diets supplemented with 10-50 mg kg^{-1} of Fe_2O_3 NPs, the level of Fe alone was elevated gradually based on the supplementation of Fe_2O_3 NPs, whereas, Cu, Zn, Ca, Mg, Na and K levels were not fluctuated much, because any forms of these trace elements and minerals were not supplemented (Table 1).

At the end of feeding trial, the content of Fe was gradually increased significantly ($P < 0.05$) in the carcass of prawns fed with 10-50 mg kg^{-1} Fe_2O_3 NPs supplemented feeds, based on the original contents present in different experimental diets when compared with control (Table 2). The contents of other dietary minerals, such as Cu, Zn, Ca, Mg, Na and K were also showed elevation in the carcass of prawns, but only up to the optimum level of Fe_2O_3 NPs, that was 20 mg kg^{-1}, beyond that the levels of other minerals showed gradual decrease in absorption (Table 2).

Table 1: Concentrations of trace elements and mineral salts in Fe_2O_3 NPs supplemented diets

Parameters		Concentrations of Fe_2O_3 NPs (mg kg^{-1})						*F*-value
		0 (Control)	Experimental					
			10	20	30	40	50	
Trace elements (mg kg^{-1})	Fe	$5.45±0.67^f$	$15.07±1.54^e$	$24.56±1.72^d$	$34.81±1.89^c$	$44.49±1.53^b$	$54.77±2.0^a$	390.40
	Cu	$2.10±0.15^a$	$2.19±0.21^a$	$2.11±0.11^a$	$2.17±0.18^a$	$2.10±0.15^a$	$2.15±0.13^a$	0.18
	Zn	$4.52±0.80^a$	$4.65±0.86^a$	$4.58±0.94^a$	$4.55±0.74^a$	$4.83±0.43^a$	$4.60±0.63^a$	0.08
Mineral salts (g kg^{-1})	Ca	$0.40±0.03^a$	$0.41±0.02^a$	$0.42±0.02^a$	$0.41±0.04^a$	$0.43±0.01^a$	$0.42±0.04^a$	0.39
	Mg	$0.95±0.02^a$	$0.96±0.02^a$	$0.95±0.05^a$	$0.97±0.03^a$	$0.95±0.04^a$	$0.96±0.03^a$	0.17
	Na	$0.32±0.02^a$	$0.33±0.04^a$	$0.32±0.02^a$	$0.32±0.01^a$	$0.34±0.03^a$	$0.33±0.02^a$	0.31
	K	$2.19±0.10^a$	$2.21±0.08^a$	$2.20±0.06^a$	$2.19±0.04^a$	$2.21±0.03^a$	$2.22±0.04^a$	0.11

Each value is mean ± SD; *n*=3; Mean values within the same row sharing the different alphabetical superscripts are statistically significant at $P < 0.05$ (one way ANOVA and subsequent *post hoc* multiple comparison with DMRT).
Mean values within the same row sharing the same alphabetical superscripts are not statistically significant at $P > 0.05$.
Fe, iron; Cu, copper; Zn, zinc; Ca, calcium; Mg, magnesium; Na, sodium; K, potassium.

Table 2: Concentrations of trace elements and mineral salts in the carcass of *M. rosenbergii* PL fed with Fe_2O_3 NPs supplemented diets

Parameters		Concentrations of Fe_2O_3 NPs (mg kg^{-1})						*F*-value
		0 (Control)	Experimental					
			10	20	30	40	50	
Trace elements (µg g^{-1})	Fe	$42.08±1.35^f$	$61.70±3.02^e$	$86.23±2.43^d$	$101.65±2.01^c$	$122.81±1.76^b$	$132.57±2.82^a$	690.69
	Cu	$35.05±2.13^e$	$74.33±2.73^b$	$81.75±1.36^a$	$73.40±3.77^b$	$65.57±1.55^c$	$58.03±3.19^d$	122.58
	Zn	$37.51±1.58^e$	$85.21±2.07^b$	$96.28±2.65^a$	$82.53±3.02^b$	$76.52±3.18^c$	$71.96±2.29^d$	190.98
Mineral salts (µg g^{-1})	Ca	$73.73±3.17^e$	$132.29±2.51^b$	$152.68±3.02^a$	$128.51±3.47^b$	$116.25±2.80^c$	$98.42±3.56^d$	239.34
	Mg	$102.14±3.72^f$	$183.18±3.55^b$	$198.43±4.12^a$	$175.82±3.04^c$	$155.10±4.52^d$	$126.74±3.93^e$	272.06
	Na	$118.69±2.78^d$	$175.77±3.27^a$	$181.10±3.46^a$	$159.44±2.92^b$	$143.67±3.81^c$	$106.05±2.67^e$	243.79
	K	$145.27±2.55^f$	$197.45±4.36^b$	$212.61±3.50^a$	$182.05±3.19^c$	$169.21±4.73^d$	$157.03±3.45^e$	139.14

Each value is mean ± SD; *n*=3; Mean values within the same row sharing the different alphabetical superscripts are statistically significant at $P < 0.05$ (one way ANOVA and subsequent *post hoc* multiple comparison with DMRT). Fe, iron; Cu, copper; Zn, zinc; Ca, calcium; Mg, magnesium; Na, sodium; K, potassium.

Table 3: Survival rate, nutritional indices, concentrations of biochemical constituents and activities of digestive enzymes in *M. rosenbergii* PL fed with Fe_2O_3 NPs supplemented diets (initial length and weight: 1.47±0.39 cm and 0.11±0.04 g respectively)

Parameters		Concentrations of Fe_2O_3 NPs (mg kg^{-1})						*F*-value
		0 (Control)	Experimental					
			10	**20**	**30**	**40**	**50**	
Survival rate (%)		$80.03±3.81^{cd}$	$87.50±2.50^{ab}$	$90.83±1.44^a$	$85.83±2.88^b$	$83.33±1.44^{bc}$	$76.66±2.88^d$	10.92
Nutritional indices	Length (cm)	$4.83±0.77^e$	$5.62±0.41^b$	$5.87±0.64^a$	$5.51±0.52^c$	$5.03±0.38^d$	$4.47±0.66^f$	651.97
	LG (cm)	$3.36±0.03^e$	$4.15±0.05^b$	$4.40±0.07^a$	$4.04±0.05^c$	$3.56±0.03^d$	$3.00±0.07^f$	293.63
	Weight (g)	$0.85±0.08^e$	$1.53±0.07^b$	$1.72±0.08^a$	$1.42±0.09^c$	$1.12±0.08^d$	$0.74±0.05^f$	1485.85
	WG (g)	$0.74±0.02^e$	$1.42±0.01^b$	$1.61±0.02^a$	$1.31±0.02^c$	$1.01±0.01^d$	$0.63±0.03^f$	1298.84
	FI (g d^{-1})	$0.44±0.01^c$	$0.57±0.03^b$	$0.61±0.01^a$	$0.54±0.02^b$	$0.46±0.02^c$	$0.39±0.01^d$	60.41
	DM (NM d^{-1})	$2.67±0.09^{cd}$	$3.18±0.06^a$	$3.26±0.05^a$	$2.96±0.07^b$	$2.78±0.10^c$	$2.54±0.07^d$	42.84
	SGR (%)	$0.99±0.06^c$	$1.25±0.05^a$	$1.30±0.05^a$	$1.22±0.05^a$	$1.11±0.05^b$	$0.93±0.07^c$	20.51
	FCR (g)	$1.34±0.06^a$	$0.90±0.04^c$	$0.86±0.02^c$	$0.92±0.02^c$	$1.04±0.06^b$	$1.41±0.11^a$	46.27
	PER (g)	$1.85±0.08^c$	$2.77±0.13^a$	$2.89±0.08^a$	$2.70±0.07^a$	$2.2.39±0.13^b$	$1.76±0.14^c$	60.14
Digestive enzymes	Protease	$1.15±0.12^c$	$1.72±0.10^{ab}$	$1.85±0.16^a$	$1.59±0.11^b$	$1.33±0.15^c$	$0.92±0.11^d$	23.62
	Amylase	$0.68±0.05^c$	$1.11±0.12^b$	$1.34±0.09^a$	$0.97±0.15^b$	$0.75±0.10^c$	$0.58±0.08^c$	23.42

(Unit mg^{-1} protein) *(×10^2 Unit mg^{-1} protein)	Lipase*	0.23±0.04^e	0.43±0.02^b	0.51±0.04^a	0.35±0.03^c	0.29±0.01^d	0.20±0.03^e	46.57
Biochemical constituents (mg g^{-1}wet wt.)	Protein	168.63±4.13^{cd}	201.72±3.30^b	224.68±2.86^a	195.24±3.97^b	173.96±2.26^c	164.28±4.79^d	121.46
	Amino acid	101.39±3.64^{ef}	122.04±2.61^b	137.76±2.19^a	114.84±2.43^c	105.35±2.08^d	97.88±2.14^f	101.51
	Carbohydrate	38.31±2.37^c	47.72±1.50^{ab}	51.07±2.84^a	44.11±1.71^b	39.40±2.10^c	35.91±3.19^c	18.60
	Lipid	18.53±1.09^c	24.76±1.31^{ab}	26.92±1.08^a	23.61±2.06^b	19.03±2.21^c	17.55±2.13^c	15.08
	Moisture (%)	74.87±2.30^a	67.20±1.96^{bc}	65.53±1.53^c	69.40±1.92^b	74.56±2.50^a	75.17±2.03^a	12.89
	Ash (%)	13.33±0.51^d	16.33±4.47^{ab}	17.17±0.40^a	15.80±0.35^b	14.37±0.31^c	12.80±0.79^d	36.46

Each value is mean ± SD; n=3; Mean values within the same row sharing the different alphabetical superscripts are statistically significant at P < 0.05 (one way ANOVA and subsequent *post hoc* multiple comparison with DMRT). LG, length gain; WG, weight gain; FI, feeding intake; DM, daily moult; NM, no. of moults; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio.

3.2. Survival, nutritional indices, digestive enzymes and biochemical constituents

The survival rate (SR), nutritional indices (length gain (LG) and weight gains (WG), feed intake (FI), specific growth rate (SGR) and protein efficiency ratio (PER), activities of digestive enzymes (protease, amylase and lipase) and concentrations of basic biochemical constituents (total protein, amino acids, carbohydrate, lipid and ash) were found to be increased in PL fed with 10 and 20 mg kg^{-1} Fe_2O_3 NPs supplemented diets with a maximum better performance in 20 mg kg^{-1} Fe_2O_3 NPs (P < 0.05) when compared with control (Table 3). Whereas, this trend was turned towards reverse in other concentrations (30-50 mg kg^{-1} of Fe_2O_3 NPs) with maximum decrease (below control level) in 50 mg kg^{-1} of Fe_2O_3 NPs supplemented diet fed prawns. In contrast, the food conversion ratio (FCR) and tissue moisture content were just opposite in 10 and 20 mg kg^{-1} Fe_2O_3 NPs supplemented diets fed PL with a very lowest rate at 20 mg kg^{-1} of Fe_2O_3 NPs when compared with the control (P < 0.05). Whereas, in the cases of 30-50 mg kg^{-1} Fe_2O_3 NPs supplemented feed fed PL the FCR and moisture content were found to be increased (Table 3). The polynomial (cubic order) regression analysis revealed that a breakpoint dietary concentration of 24.56 mg kg^{-1} Fe_2O_3 NPs was actually required for optimal growth (weight gain) of test prawn PL (Fig. 1). This analysis was approximating the relationship between maximum weight gain and essential nutrient (Fe_2O_3 NPs) intake. Further, this is defined as the maximum concentration of dietary nutrient (Fe_2O_3 NPs) required for attaining maximum growth and beyond which growth would be depressed nutrient [48, 49].

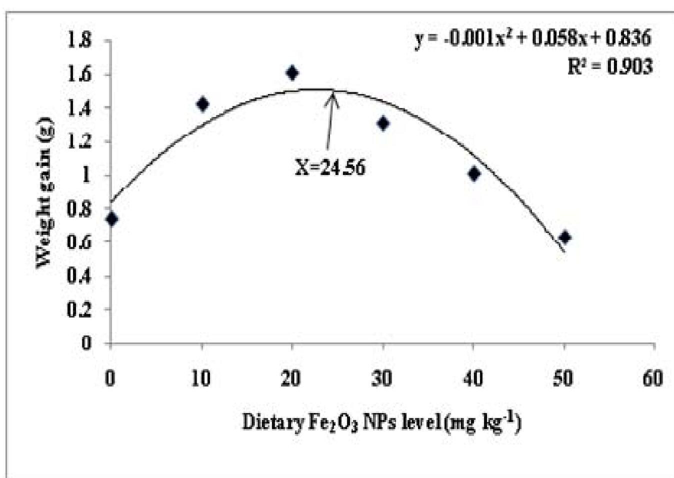

Fig 1: Relationship between weight gain (g) and dietary Fe_2O_3 NPs levels for *M. rosenbergii*. Each point represents the mean of triplicates per treatment. The optimal requirement is 24.56 mg kg^{-1} Fe_2O_3 NPs diet by using the breakpoint analysis of polynomial regression.

3.3. Profiles of proteins

Polypeptide bands of molecular weight between 116-14 kDa were resolved in the muscle tissue of test prawns (Fig. 2). Generally, there were fourteen Coomassie blue stained protein bands (116, 99, 66, 60, 50, 47, 43, 41, 26, 20, 18, 15, 14 and 13 kDa) calculated against the standard markers of 116, 66, 45, 29, 20 and 14 kDa, which represent β-galactosidase, Bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme respectively. SDS-PAGE revealed that the staining intensity of 116, 99, 50, 26, 20, 18, 15, 14 and 13 kDa polypeptide bands were found to be higher in 20 mg kg^{-1} Fe_2O_3 NPs supplemented feed fed prawn when compared with control.

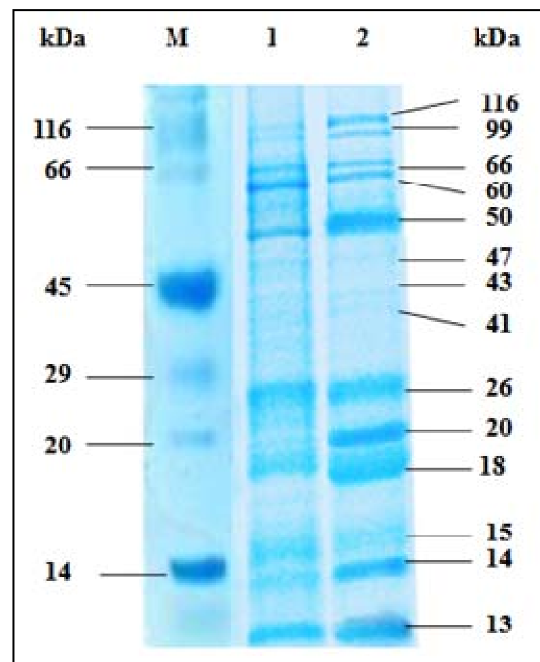

Fig 2: SDS-PAGE pattern of muscle protein of *M. rosenbergii* PL fed with Fe_2O_3 NPs supplemented diet. M, Marker; Lane 1, Control diet fed prawn; Lane 2, Fe_2O_3 NPs (20 mg kg^{-1}) supplemented diet fed prawn.

3.4. Profiles of amino acids

In the present study, there were eighteen amino acids detected in the muscle of prawn PL. Of which ten were essential amino acids (EAA), arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, and eight were non-essential amino acids (NEAA), alanine, aspartic acid, cystine, glutamic acid, glutamine, glycine, proline and tyrosine (Table 4). Generally the content of all the EAA and NEAA were found to be significantly higher (P < 0.05) in 20 mg kg^{-1} Fe_2O_3 NPs supplemented diet fed prawn PL when compared with control.

Table 4: Profiles of amino acids (g/ 100 g dry wt.) in *M. rosenbergii* PL fed with Fe_2O_3 NPs supplemented diets

Amino acids		Concentrations of Fe_2O_3 NPs (mg kg^{-1})		*t*-value
		0	Experimental	
		Control	20 mg kg^{-1} Fe_2O_3 NPs	
EAA	Arginine	2.90±0.12	3.02±0.15	-6.92
	Histidine	7.54±0.45	7.78±0.38	-5.93
	Isoleucine	2.23±0.11	2.50±0.14	-15.58
	Leucine	3.31±0.17	3.60±0.15	-25.11
	Lysine	2.57±0.15	2.77±0.18	-11.54
	Methionine	2.42±0.09	2.61±0.12	-10.97
	Phenylalanine	2.88±0.14	3.29±0.20	-11.83
	Threonine	3.58±0.17	3.77±0.25	-5.19
	Tryptophan	6.34±0.44	7.30±0.39	-33.25
	Valine	3.72±0.21	3.94±0.22	-38.10
NEAA	Alanine	2.71±0.15	2.86±0.20	-5.19
	Aspartic acid	8.62±0.73	9.11±0.76	-28.29
	Cystine	2.30±0.12	2.40±0.15	-5.77
	Glutamic acid	4.80±0.20	5.09±0.29	-5.58
	Glutamine	2.83±0.16	4.73±0.22	-54.84
	Glycine	2.03±0.11	2.35±0.15	-13.85
	Proline	5.04±0.31	5.24±0.27	-8.66
	Tyrosine	2.70±0.15	3.22±0.21	-15.01
ΣAA		68.47±3.98	75.58±4.43	-27.36
ΣEAA		37.44±2.05	40.58±2.18	-41.83
ΣNEAA		31.03±1.93	35.00±2.25	-21.48

Each value is mean ± SD; *n*=3; Values are significant (*P* < 0.05) by paired-samples '*t*' test.
EAA, essential amino acids; NEAA, non essential amino acids

3.5. Profiles of fatty acids

In the present study, eleven fatty acids were detected in the muscle of prawn PL. Of which five were saturated fatty acids (SFA), lauric acid, myristic acid, palmitic acid, stearic acid and arachidic acid; two were mono unsaturated fatty acids (MUFA), palmitoleic acid and oleic acid; four were poly unsaturated fatty acids (PUFA), linoleic acid (n-6), arachidonic acid (n-6), EPA (n-3) and DHA (n-3) (Table 5). The content of all the SFA, MUFA and PUFA were found to be significantly higher (*P*<0.05) in 20 mg kg^{-1} Fe_2O_3 NPs supplemented diet fed prawn PL when compared with control. In addition SFA and MUFA were found to be predominant than that of PUFA in both control and 20 mg kg^{-1} Fe_2O_3 NPs supplemented diet fed prawn PL.

Table 5: Profiles of fatty acids (%) in M. rosenbergii PL fed with Fe_2O_3 NPs supplemented diets

Fatty acids		Concentrations of Fe_2O_3 NPs (mg kg^{-1})		t-value
		0	Experimental	
		Control	20 mg kg^{-1} Fe_2O_3 NPs	
SFA	C12:0 (Lauric acid)	0.05±0.01	0.09±0.03	-7.794
	C14:0 (Myristic acid)	0.12±0.03	0.22±0.04	17.321
	C16:0 (Palmitic acid)	1.17±0.10	5.41±0.16	-122.398
	C18:0 (Stearic acid)	0.44±0.07	3.72±0.11	-94.685
	C20:0 (Arachidic acid)	0.20±0.04	0.79±0.07	-34.064
MUFA	C16:1 (Palmitoleic acid)	0.11±0.02	0.50±0.04	-33.775
	C18:1 (Olecic acid)	2.81±0.09	9.63±0.15	-196.876
PUFA	C18:2 n-6 (Linoleic acid)	0.17±0.03	0.20±0.02	-5.196
	C20:4 n-6 (Arachidonic acid)	0.04±0.01	0.13±0.03	-7.794
	C20:5 n-3 (EPA)	0.10±0.02	0.27±0.04	-14.722
	C22:6 n-3 (DHA)	0.07±0.01	0.18±0.03	-9.526
ΣFA		5.28±0.53	21.19±0.72	-145.03
ΣSFA		1.98±0.35	10.28±0.41	-239.60
ΣMUFA		2.92±0.11	10.13±0.19	-156.10
ΣPUFA		0.38±0.07	0.78±0.12	-13.856
n-3		0.17±0.03	0.45±0.07	-12.12
n-6		0.21±0.04	0.33±0.05	-20.78
n-3/n-6		0.81±0.01	1.36±0.01	-54.65

Each value is mean ± SD; n=3; Values are significant (P < 0.05) by paired-samples 't' test.
SFA, saturated fatty acids; MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids

3.6. Haemocytes populations

The haemocytes populations (total and differential, hyalinocytes, semigranulocytes and granulocytes) were found to be increased in *M. rosenbergii* PL fed with 10 and 20 mg kg^{-1} Fe_2O_3 NPs supplemented diets with a maximum better performance in 20 mg kg^{-1} Fe_2O_3 NPs (*P*<0.05) when compared with the control. Whereas, in the cases of 30-50 mg kg^{-1} Fe_2O_3 NPs supplemented feed fed PL the total and differential haemocytes were found to be decreased with maximum decrease (below control level) in 50 mg kg^{-1} of Fe_2O_3 NPs supplemented diets fed PL (Table 6).

Table 6: Populations of haemocytes (total and differential counts) in *M. rosenbergii* PL fed with Fe_2O_3 NPs supplemented diets

Parameters		Concentrations of Fe_2O_3 NPs (mg kg^{-1})						*F*-value
		0 (Control)	Experimental					
			10	20	30	40	50	
Haemocytes ($\times10^6$ ml^{-1})	Total Haemocytes	4.96 ± 0.22^d	7.75 ± 0.15^b	9.11 ± 0.39^a	7.25 ± 0.71^b	6.29 ± 0.55^c	4.52 ± 0.62^d	38.55
	Hyalinocytes	2.64 ± 0.37^d	4.19 ± 0.22^b	4.74 ± 0.31^a	3.81 ± 0.18^{bc}	3.35 ± 0.32^c	2.18 ± 0.25^d	34.69
	Semigranulocytes	1.23 ± 0.14^c	2.07 ± 0.09^a	2.15 ± 0.15^a	1.69 ± 0.07^b	1.52 ± 0.10^b	1.02 ± 0.06^d	52.93
	Granulocytes	0.92 ± 0.09^{cd}	1.66 ± 0.13^a	1.81 ± 0.08^a	1.43 ± 0.15^b	1.10 ± 0.04^c	0.85 ± 0.10^d	43.60

Each value is mean ± SD; *n*=3; Mean values within the same row sharing the different alphabetical superscripts are statistically significant at $P < 0.05$ (one way ANOVA and subsequent *post hoc* multiple comparison with DMRT).

3.7. Activities of antioxidant and metabolic enzymes, and lipid peroxidation

In this study, no significant alterations were observed in activities of antioxidant enzymes (SOD and CAT) and metabolic enzymes (GOT and GPT), and lipid peroxidation (LPO) in the hepatopancreas and muscle of PLs fed with 10 and 20 mg kg^{-1} Fe_2O_3 NPs supplemented diets. However, gradual and significant elevations ($P < 0.05$) were recorded in SOD, CAT, GOT and GPT activities, and LPO in PLs fed with 30, 40 and 50 mg kg^{-1} Fe_2O_3 NPs supplemented diets when compared with control (Table 7).

Table 7: Activities of antioxidant and metabolic enzymes, and lipid peroxidation in the hepatopancreas and muscle of *M. rosenbergii* PL fed with Fe_2O_3 NPs supplemented diets

Parameters		Concentrations of Fe_2O_3 NPs (mg kg^{-1})						F-value
		0 (Control)	Experimental					
			10	20	30	40	50	
Hepatopancreas	SOD (µmol min^{-1} mg^{-1} protein)	15.84 ± 1.21^c	15.87 ± 1.17^c	15.88 ± 1.26^c	19.31 ± 1.78^b	20.46 ± 2.05^b	25.40 ± 2.27^a	15.39
	CAT (Unit mg^{-1} protein)	27.35 ± 2.08^c	27.42 ± 1.69^c	27.47 ± 1.82^c	33.80 ± 2.11^b	35.69 ± 2.49^{ab}	38.24 ± 2.22^a	16.32
	LPO (nmol MDA mg^{-1} protein)	1.87 ± 0.09^d	1.88 ± 0.06^d	1.93 ± 0.05^d	3.60 ± 0.10^c	5.66 ± 0.12^b	7.38 ± 0.14^a	1681.59
	GOT (Unit L^{-1})	14.32 ± 1.14^d	14.37 ± 1.05^d	14.41 ± 0.96^d	18.51 ± 1.22^c	20.74 ± 1.16^b	23.21 ± 1.09^a	36.03
	GPT (Unit L^{-1})	14.74 ± 1.07^c	14.85 ± 1.11^c	14.87 ± 1.05^c	24.65 ± 1.36^b	26.45 ± 1.24^{ab}	28.55 ± 1.38^a	87.84
Muscle	SOD (µmol min^{-1} mg^{-1} protein)	9.02 ± 1.37^c	9.15 ± 01.02^c	9.20 ± 1.25^c	15.29 ± 1.18^b	16.73 ± 1.32^{ab}	18.16 ± 1.15^a	37.28
	CAT (Unit mg^{-1} protein)	22.96 ± 1.04^c	23.11 ± 1.39^c	23.17 ± 1.33^c	27.55 ± 1.21^b	30.04 ± 1.75^b	33.22 ± 1.68^a	27.81
	LPO (nmol MDA mg^{-1} protein)	0.71 ± 0.06^d	0.72 ± 0.03^d	0.74 ± 0.02^d	2.30 ± 0.07^c	3.59 ± 0.04^b	4.12 ± 0.06^a	2879.32
	GOT (Unit L^{-1})	9.38 ± 1.18^c	9.43 ± 0.72^c	9.48 ± 0.65^c	15.31 ± 2.05^b	16.79 ± 1.68^{ab}	18.86 ± 2.31^a	22.54
	GPT (Unit L^{-1})	10.56 ± 1.05^b	10.58 ± 1.12^b	10.60 ± 1.08^b	16.69 ± 1.96^a	17.42 ± 1.71^a	19.44 ± 1.83^a	21.99

Each value is mean ± SD; *n*=3; Mean values within the same row sharing the different alphabetical superscripts are statistically significant at $P < 0.05$ (one way ANOVA and subsequent *post hoc* multiple comparison with DMRT).
SOD, superoxide dismutase; CAT, catalase; LPO, lipid peroxidation; MDA, malondialdehyde; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase.

4. Discussion

4.1. Carcass minerals

Iron and iron associated protein is essential for human health [50]. Consequently, muscle protein and seafood are dependable dietary sources of the minerals [51]. In this study, the level of Fe was found to be elevated in test prawns based on Fe_2O_3 NPs incorporation in diets. Iron is readily absorbed through the gastro-intestinal tract, gills and skin of fishes and crustaceans. Dietary iron availability and absorption is usually depressed by high dietary intakes of phosphate, calcium, phytates, copper and zinc. In general, inorganic sources of iron are more readily absorbed than organic sources; the ferrous iron being more available for absorption than ferric iron [52]. Up to the optimum concentration of Fe_2O_3 NPs (20 mg kg^{-1}), the absorption of other minerals, such as Cu, Zn, Ca, Mg, Na and K were up regulated in test prawns, beyond that the levels of other minerals showed gradual decrease in absorption. Therefore, beyond 20 mg kg^{-1} the Fe_2O_3 NPs may be acted as toxic to *M. rosenbergii* PL and the test prawn itself regulates further uptake of other minerals. Moreover, no supplementations of any forms of these other minerals were done in experimental diets. Therefore, the carcass mineral contents may depend on their dietary concentrations. Though the absorption is continuous in all five levels (10-50 mg kg^{-1} of Fe_2O_3 NPs), only 10 and 20 mg kg^{-1} of Fe_2O_3 NPs showed positive responses/regulation on overall growth and survival of *M. rosenbergii* PL, whereas, the other levels (30-50 mg kg^{-1} of Fe_2O_3 NPs) showed some adverse effects (discussed in other parameters of this study). Actually, a dietary concentration of 24.56 mg kg^{-1} Fe_2O_3 NPs was found to be required for optimal growth (to attain maximum weight gain) of test prawn PL. At the outset, this study suggested that Fe_2O_3 NPs supplementation significantly increased the absorption and bioavailability of iron. Similarly, the increase in iron has been reported in *Labeo rohita* fed with ferrous sulfate and ferrous oxide NPs supplemented diets [29], and in *M. rosenbergii* PL fed with dietary Zn and Cu, and their nano sized forms [8, 12,13]. It has also been reported that elevated in Fe, K, Mg and NaCl in *Litopenaeus vannamei* fed with dietary Fe, K, Mg and NaCl supplemented diets [30, 53], and in soft-shelled turtles, *Pelodiscus sinensis* fed with iron supplemented diets [26].

4.2. Survival and growth

In the present study, 20 mg kg^{-1} Fe_2O_3 NPs has the potency to produce the maximum enhancement in survival and growth of *M. rosenbergii* PL, whereas, 50 mg kg^{-1} of Fe_2O_3 NPs produced totally negative (below control level) survival and growth. These limits may vary by the influence of various environmental factors and life stages of the individual species. The lowest food conversion ratio (FCR) recorded in 20 mg kg^{-1} of Fe_2O_3 supplemented diet fed PL reflects the superior quality of the diet prepared. It has been reported that $FeSO_4$,

$FeC_6H_6O_7$ and Fe_2O_3 supplemented feed fed fishes (*Ictalurus punctatus*, *Oncorhynchus mykiss*, *Oreochromis niloticus*, *Oreochromis aureus, Salmo salar* and *L. rohita*), the shrimp, *Penaeus vannamei* and the freshwater prawn, *M. rosenbergii* attained significant improvement in survival, growth and immune response [29, 30, 54-60]. Optimum levels of dietary Zn and Cu, and their nano sized forms have also produced similar better survival and growth in *M. rosenbergii* PL [8, 12, 13].

4.3. Digestive enzymes

Iron is an essential element for most organisms, serving as a cofactor for various enzymes. Digestion of food is one of the most important functions in the physiology of organism to obtain nutrients for various body activities, such as growth, maintenance, motion and reproduction. In this study, increased activity of protease, amylase and lipase in 20 mg kg^{-1} Fe_2O_3 NPs supplemented feed fed PL indicates, this level of Fe_2O_3 NPs has influenced maximum on the activity of digestive enzymes in *M. rosenbergii* PL, led to the maximum digestion of the food offered. The enhanced activities of digestive enzymes recorded in test prawns led to enhanced food consumption and food conversion, which in turn ultimately led to better survival and growth of *M. rosenbergii* PL. It has been reported that dietary iron supplementations increased protease and amylase activities in hybrid tilapia *O. niloticus* and *O. aureus* [61, 62]. Optimum levels of dietary Zn and Cu, and their nano sized forms have also produced increased activities of digestive enzymes in *M. rosenbergii* PL [8, 12, 13]. It has also been reported that optimum levels of dietary NaCl supplementations increased protease, amylase and lipase activities in *M. rosenbergii,* fishes, *L. rohita, Cirrhinus mrigala* and *Cyprinus carpio* [63].

4.4. Biochemical constituents

In the present study, the higher levels of total protein, amino acid, carbohydrate and lipid recorded in 20 mg kg^{-1} Fe_2O_3 NPs supplemented feed fed PL suggest maximum influence of Fe_2O_3 NPs on the metabolism of *M. rosenbergii*. It has been reported that iron supplemented feed fed Atlantic salmon, *S. salar* has synthesized and stored protein and lipid [59]. Optimum levels of dietary Zn and Cu, and their nano sized forms have also produced similar better results on the basic biochemical constituents in *M. rosenbergii* PL [8, 12, 13]. Similarly, NaCl and Zn supplemented feed fed *M. rosenbergii*, and fishes, *L. rohita, C. mrigala, C. carpio, Gadus morhua* produced significant improvements in biochemical constituents [63, 64].

4.5. Profiles of protein

Minerals involve and regulate the protein synthesis in animals [65, 66]. Dietary supplementation of minerals, Zn, Cu, Fe, Ca, Mg, Na and K can improve the synthesis of protein in fishes, *Ctenopharyngodon idella* [67]*, Huso huso* [68]*, O. niloticus* [59, 69], and *O. mykiss* [70]; freshwater prawns, *M. rosenbergii* [8, 12, 13] and *Macrobrachium olfersii* [65]; whiteleg shrimp, *L. vannamei* [71,72]; and Chinese mitten crab, *Eriocheir sinensis* [73]. In this study, the recorded increase in the staining intensity of 116, 99, 50, 26, 20, 18, 15, 14 and 13 kDa regions in the muscle tissue of 20 mg kg^{-1} Fe_2O_3 NPs supplemented feed fed PL suggest that Fe_2O_3 NPs have influenced the protein synthesis.

4.6. Profiles of amino acids

Amino acids play a central role as the building blocks of proteins and as intermediates in metabolism and further help to maintain health and vitality [74]. They are utilized to form various cell structures, of which they are key components and they serve source of energy. The recorded higher levels of essential amino acids, such as arginine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan and valine in PL fed with 20 mg kg^{-1} Fe_2O_3 NPs supplemented feed indicate the fact that Fe_2O_3 NPs have role in maintaining the essential amino acids in prawn body. It is important to mention here that naturally *M. rosenbergii* and *L. vannamei* have rich in essential amino acids [75, 76]. The higher level of non-essential amino acids, such as alanine, aspartic acid, cystine, glutamic acid, glutamine, glycine, proline and tyrosine recorded in PL fed with 20 mg kg^{-1} Fe_2O_3 NPs supplemented feed than that of control suggested that Fe_2O_3 NPs have influence on amino acid synthesis followed by protein synthesis, which in turn led to better growth and production of *M. rosenbergii* PL.

4.7. Profiles of fatty acids

Fatty acids, both SFA (lauric, myristic, palmitic, stearic and arachidic acids), MUFA (oleic and palmitoleic acids), and PUFA (linoleic (n-6), arachidonic (n-6), EPA (n-3) and DHA (n-3) acids are not only a source of body fuel, but are also structural components of cell membranes. SFA are also associated with proteins and are necessary for their normal function. SFA can be synthesized by the body. Similarly, the fatty acid synthesis by Zn has been reported in crab, *E. sinensis* [73]. MUFA are required for many body functions. Nevertheless, MUFA can be biosynthesized from other fuel sources [77-79]. PUFA need to be supplied through food only to achieve better growth performance of fish and prawn. The role of essential micronutrient (trace elements) in lipid metabolism was a subject of nutritional investigations in fishes and crabs [73, 80-82]. In the present study, elevation was recorded in muscle fatty acid content. It was clear that SFA and MUFA were the predominant class, followed by PUFA as reported in *M. rosenbergii* and *L. vannamei* [76, 83]. This indicated that Fe_2O_3 NPs have more influence on SFA and MUFA synthesis in *M. rosenbergii* PL. However, PUFA level also been found to elevate considerably in test prawns than that of control., which indicates the fact that Fe_2O_3 NPs maintain its biosynthesis/ conversion capacity in *M. rosenbergii* PL, and therefore better survival and growth was resulted. In this study the proportion of SFA was higher followed by MUFA and PUFA as reported in *M. rosenbergii* [75, 83]. The proportion of EPA was greater than that of the DHA. EPA and DHA would facilitate stress tolerance and membrane permeability [84, 85]. Thus these are essential in growth and development throughout the life and should be included in the diet.

4.5. Haemocytes population

Hematological parameters provide an index of the physiological status of organism, more sensitive to iron supplementation and act as indicators of iron requirements [26]. In this study, the recorded increase in THC and DHC (hyalinocytes, semigranulocytes and granulocytes) in 20 mg kg^{-1} Fe_2O_3 NPs supplemented feed fed *M. rosenbergii* PL suggests, this level of Fe_2O_3 NPs has influenced maximum on production of haemocytes, which indicates better operation of non-specific immune responses. Therefore, the general health of *M. rosenbergii* PL was improved under 20 mg kg^{-1} Fe_2O_3 NPs supplementation. It has been reported that nano forms of Fe has improved red blood cell count (RBCs), hemoglobin contents and drastically reduced mortality rate, without any

sign of polychromatic anemia in fishes [29, 56, 86]. Several reports showed that a deficiency of iron may contribute to several immunodeficiency syndromes, such as impaired T cell functioning, atrophy in lymph organs, etc. in both humans and animals [87]. Optimum levels of dietary Zn and Cu, and their nano sized forms have also produced similar better results on THC and DHC in *M. rosenbergii* PL [8, 12, 13].

4.6. Activities of antioxidant and metabolic enzymes, and status of lipid peroxidation

In general, the antioxidant enzymes are responsible for scavenging superoxide radicals and are involved in protective mechanisms within tissue injury following oxidative process and phagocytosis. In the present study, as unaltered LPO status was recorded there were no requirements of activations of SOD and CAT in *M. rosenbergii* PL fed with 10 and 20 mg kg^{-1} Fe_2O_3 NPs supplemented feed. Therefore, these concentrations of Fe_2O_3 NPs were not found to be exerted any toxic responses. This was further confirmed by unaltered activities of GOT and GPT, as over activations of these enzymes are indicators of liver toxicity, and such a condition is required to generate adequate energy under toxic stress condition. It is important to mention here that the hepatopancreas of crustaceans is analogue to the liver of vertebrate, and it is known that the synthesis and secretion of all the enzymes are taking place in this vital organ. Therefore, 10 and 20 mg kg^{-1} Fe_2O_3 NPs can definitely be taken as safe dietary levels as far as *M. rosenbergii* PL is concerned. It is also important to state here that iron supplementation in the basal diet could improve the SOD and GSH activity irrespective of different iron sources [29]. It has been reported that waterborne iron nanoparticle causes oxidative damages at the early exposure period in medaka embryo, however, no terminal oxidative damage occurred in adult fish due to the self-recovering capacity [62]. Optimum levels of dietary Zn and Cu, and their nano sized forms have also produced similar better results on the activities of antioxidant and metabolic enzymes, and status of lipid peroxidation in *M. rosenbergii* PL [8, 12, 13]. It has been reported that dietary Se supplemented diets increased *M. rosenbergii* immunity and disease resistance against the pathogen, *Debaryomyces hansenii* [88].

In the present study, higher value of feed conversion ratio (FCR) recorded in 30-50 mg kg^{-1} of Fe_2O_3 NPs supplemented feed fed *M. rosenbergii* PL and the corresponding elevations recorded in activities of SOD, CAT, GOT and GPT, and LPO indicates the fact that these levels of Fe_2O_3 NPs exerted toxicity. This in turn led to poor survival, growth, activities of digestive enzymes, concentration of biochemical constituents and haemocytes population. This sate indicates the fact that the general health of test prawn was deteriorated due to Fe_2O_3 NPs induced toxic stress. It has also been reported that iron content beyond the optimum level decreases the survival and growth rate in *Penaeus vannamei* and *M. rosenbergii* [30, 60]. Dietary Zn and Cu, and their nano sized forms beyond optimum concentration have also produced adverse effects on survival and growth of *M. rosenbergii* PL [8, 12, 13].

Though 30 and 40 mg kg^{-1} of Fe_2O_3 NPs supplemented feed fed *M. rosenbergii* PL showed toxic responses, the values recorded for survival, growth, activities of digestive enzymes, contents of biochemical constituents and haemocytes population were above the control level, and the induction recorded in SOD, CAT, GOT and GPT in these two concentrations of Fe_2O_3 NPs supplemented feed fed *M. rosenbergii* PL can be taken as adaptive mechanisms. Actually, the test prawns tried to neutralize/overcome the toxic stress caused by Fe_2O_3 NPs. In 50 mg kg^{-1} of Fe_2O_3 NPs supplementation, even though, the level of SOD, CAT, GOT and GPT showed active state, but, the recorded for survival, growth, digestive enzymes, biochemical constituents and haemocytes population showed below the control level. This indicates the fact that the test prawns were in non-adaptive state due to severe toxic effect caused by 50 mg kg^{-1} Fe_2O_3 NPs.

5. Conclusion

To conclude, up to 20 mg kg^{-1} of Fe_2O_3 NPs did not produced hepatotoxic effect, which was evident from the insignificant induction of GOT and GPT in test prawns over control. Therefore, there was stable LPO, which was further evident by the insignificant induction of SOD and CAT in test prawns over control. Therefore, up to 20 mg kg^{-1} of Fe_2O_3 NPs supplementations were produced significantly higher survival, growth, activities of protease, amylase and lipase, concentrations of total protein, amino acid, carbohydrate and lipid, better profiles of protein, amino acids and fatty acids, and haemocytes population in *M. rosenbergii* PL. This indicates the fact that the general health and non-specific immunity of the test prawns were improved up to 20 mg kg^{-1} of Fe_2O_3 NPs supplementations. Therefore, up to 20 mg kg^{-1} of Fe_2O_3 NPs is considered as safe concentration as far as *M. rosenbergii* PL is concerned. Thus, this study recommends supplementation of up to 20 mg kg^{-1} of Fe_2O_3 NPs for sustainable maintenance of *M. rosenbergii* PL.

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