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Pyrroloquinoline quinone supplemented diet enhances metabolism, feed intake and growth in common carp (*Cyprinus carpio* Linnaeus, 1758) reared at low temperature

GOPIKA RADHAKRISHNAN[1], ASHUTOSH D. DEO[*1], N. SHAMNA[1], ANAKHY MONDAL[1], AKLAKUR M. D.[1], ELDHO VARGHESE[2] AND NAROTTAM PRASAD SAHU[1]

[1]*Fish Nutrition, Biochemistry and Physiology Division, ICAR-Central Institute of Fisheries Education, Panch Marg Off Yari Road, Versova - 400 061, Mumbai, India*

[2]*ICAR-Central Marine Fisheries Research Institute, Kochi - 682 018, Kerala, India*

e-mail: ashutosh.cife19@rediffmail.com, ashutosh@cife.edu.in

ABSTRACT

A pilot study was conducted to delineate the potential of dietary pyrroloquinoline quinone (PQQ) on *Cyprinus carpio* Linnaeus, 1758, at water temperature of 12-14°C using a recirculatory system with water flow rate of 1.8 l min^{-1}. Six isonitrogenous (32% CP) and iso-caloric (16.7 MJ kg^{-1}) diets were prepared with varying levels of PQQ *viz*. control (0 mg kg^{-1}), T1 (0.2 mg kg^{-1}), T2 (0.4 mg kg^{-1}), T3 (0.6 mg kg^{-1}), T4 (0.8 mg kg^{-1}) and T5 (1.0 mg kg^{-1}). Two hundred and sixteen common carp juveniles were distributed randomly into control and five treatment groups in triplicates with 12 fish per tub and fed twice daily at satiation level for 30 days. Results indicated that dietary quinone at 0.8 mg kg^{-1} significantly ($p<0.05$) increased the feed intake and weight gain, specific growth rate (SGR), feed conversion ratio (FCR) as well as protein efficiency ratio (PER). The thyroid hormone levels as well as digestive and metabolic enzyme activities were significantly higher ($p<0.05$) in treatments than in control. The superoxide dismutase (SOD) activity in liver was significantly higher ($p<0.05$) in T1, T2 and T5. Catalase activity in gill was significantly higher ($p<0.05$) in control and lowest in T3 and T4. Therefore, quinone can be used as a feed additive for enhancing feed intake, enzymatic activities and growth during winter.

Keywords: Feed additive, Feed intake, Metabolism, Pyrroloquinoline quinone, Winter feeding

Introduction

Aquaculture is considered the most efficient way to feed the growing population. Many people around the globe depend on fisheries and aquaculture for food, essential nutrients and livelihoods. External factors like environmental temperature are significant parameters which affect fish ethology and physiology (Crawshaw *et al.,* 1977) and can strongly influence fish feed intake, growth, metabolic rate and energy expenditure (Brett *et al.,* 1969; Andrews and Stickney, 1972; Andrews *et al.*, 1978). Seasonal changes like winter periods or conditions of low temperature can create a stressful or critical condition (Reimers, 1963; Hunt, 1969; Whitworth and Strange, 1983).

During the conditions of low temperature, the animal remains at the bottom rather than at the surface; hence there is much possibility of accumulation of toxic gases, thus making immune system weak and vulnerable to diseases, affecting fish growth, metabolism, feed intake and production. Therefore, for profitable farming of fish during winter, it would be prudent to follow a winter-feeding program (Afzal Khan *et al*., 2004). A planned study and proper feeding strategy can improve growth and cold stress mitigation in fish. Reported strategies focused on nutritional approaches, which include feeding fishes with a high-energy diet in winter in catfish (Tackett *et al.,* 1987), but the same is not ideal for carps. A few experiments on carps involved feeding and rearing fish with a compound diet in a polyhouse to maintain the warm temperature during winter periods (Afzal Khan *et al.,* 2004). This method however involves expenses to build the polyhouse and is not feasible for open waters or large water bodies. Therefore, approaches involving diet manipulation by adding functional feed additives into the diet can be more effective in solving the problem encountered by fish during low temperatures.

Mitochondrial modifications to enhance the tolerance of lower temperature exposure can improve the cold stress in various tissues like muscle (Johnston *et al.,* 1998; Miranda and Hazel, 2002). This property can be exploited in improving the nutritional status of fish especially under low-temperature conditions or extended winter periods. Pyrroloquinoline quinine is one such compound that is known to increase the mitochondrial number and its efficiency within the biological system (Rucker *et al.*, 2005) with less or no side effects. It is a water-soluble,

heat-stable compound having reducing property (Aizenman *et al.,* 1992), usually present as disodium salt in "trihydrate (12.7% water) or pentahydrate (22.9% water)" form. It can carry out around 20,000 redox reactions before degradation (Anthony, 2001; Laura, 2013). Several studies revealed the antioxidant potential of PQQ both *in vivo* and *in vitro* (Harris *et al.*, 2013). The dietary antioxidant di-sodiated PQQ, is reported to be safe in humans, rodents, chickens and pigs (Steinberg *et al.,* 1994; Samuel *et al.,* 2015; Wang *et al*., 2015) and it is approved to use in human food and drug (Health Canada, 2012).

The main hypothesis of the work is to use a metabolic modifier that can help in enhancing the metabolism of fish at low temperature. Increasing metabolism creates increased energy demand. Therefore, it was speculated that this compound can be a potent counter-measure for enhancing fish metabolism, at low temperatures. Enzymes associated with various metabolic pathways such as citrate synthase, isocitrate dehydrogenase and glucose 6 phosphate dehydrogenase, address the stimulation of mitochondrial biogenesis (Chowanadisai *et al*., 2010), reprogramming of the TCA cycle (Friedman *et al.,* 2018) and assess the production of nicotinamide adenine dinucleotide (NADPH) respectively, that are important for the protection against oxidative damages (Berg *et al.,* 2002). The common carp *Cyprinus carpio* is an ideal fish species to evaluate the PQQ effects in fish, especially at low temperatures as it has high temperature tolerance. Therefore, the present study seeks to determine the effects of dietary PQQ on metabolic enzymes, intake of feed, growth, digestive enzymes, the status of antioxidant enzymes and thyroid hormones of *C. carpio* exposed to low temperature.

Materials and methods

Diet preparation

Six iso-caloric (16.67 MJ kg^{-1}), iso-lipidic (7%) and iso-nitrogenous (32% CP) practical diets were made with proposed doses of PQQ *viz*. 0.0 (C), 0.2 (T1), 0.4 (T2), 0.6 (T3), 0.8 (T4) and 1 mg kg^{-1} (T5) (Table 1). Common practical feed ingredients used in aquafarms, such as defatted soybean meal, de-oiled rice bran, groundnut oil cake, wheat flour, sunflower oil and fish oil were used to prepare the experimental diets. All the selected ingredients were sieved and powdered to get a fine particle size. This powder was then properly mixed with optimum amount of water (70% W/V) to form dough. The dough was steamed in a pressure cooker for 20 min, then cooled and the remaining ingredients *viz*. betaine, BHT, vitamin-mineral premix, oil, choline chloride and PQQ were added and mixed properly. The feed was pelletised (2 mm size) using a hand pelletiser and air dried. The airtight packed pellets were stored at 4°C until use. The PQQ·Na_2 (purity≥98%) was procured from Herb Nutritionals and synthesised by Wuxi Cima Science Co., Ltd. (Cima Science Co., Ltd. WuXi Jiangsu, China). The PQQ mixture of 1 mg kg^{-1} was made by diluting with distilled water before mixing with the diet.

Feeding trial

The experimental feeding trial was conducted with fingerlings of common carp, *C. carpio* procured from Prem Fisheries Consultancy, Ankleshwar, Gujarat for the experimental purpose and was taken to the wet laboratory of the ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Mumbai. The fishes were acclimatised to the rearing conditions for one week and fed a commercial carp diet containing 32% crude protein before initiation of the experiment. After acclimatisation for one week, all fish were collected and kept in a circular cement tank. About 216 individuals with an average initial weight of 4.05 g were selected and randomly distributed to 18 nos. of 80 l experimental plastic tubs (80 l water volume with 37 cm dia × 50 cm height) in a recirculatory aquaculture system (RAS) which was thermo regulated by an online chiller (2 t), observing a flow rate retained at 1.8 l min^{-1}, with a stocking density of 12 fish per tub. A slight and gradual decrease in ambient water temperature in the tub was done every two days with the help of the chiller to attain a stable final experimental temperature of 13±1^{0}C in the tub. This uniform reduction in temperature was carried out to reduce the stress in fish and to acclimatise the fish to experimental temperature. Insulating sheets were used to cover each tub to minimise the heat exchange and maintain the temperature. Once the water in the tubs reached the experimental temperature, the fish were manually fed daily at 09:00 hrs and 17:00 hrs *ad libitum* for 30 days with their respective experimental diets. Feed intake was monitored daily and the uneaten feed left at the bottom of the tub was removed, dried and the weight was recorded. The feed consumed by fish was calculated as the difference from the feed given. The following parameters were assessed for each treatment for 30 days:

Weight gain (WG) (%) = (Final weight (g) - Initial weight (g) x 100/Initial weight (g)

Specific growth rate (SGR, % day^{-1}) = (Ln Final weight - Ln Initial weight (g)/Duration of feeding) x 100

Feed Intake (FI, % BW day^{-1}) = Feed fed (g) / [(Initial tank biomass + Final tank biomass)/2 x Days of trial duration] x100

Feed conversion ratio (FCR) = Feed given [(g dry weight)/ Bodyweight gain (g wet weight)]

Protein efficiency ratio (PER) = Weight gain (g)/Protein intake (g)

Survival (%) = (Total number of fish harvested/Total number of fish stocked) ×100

Table 1. Formulation and proximate composition of the experimental diets fed to common carp juveniles

Composition	Control	T1	T2	T3	T4	T5
Ingredients (g kg^{-1})						
DSBM [2]	265	265	265	265	265	265
GNOC [2]	285	285	285	285	285	285
DORB [2]	336	336	336	336	336	336
Wheat flour[2]	32.8	32.8	32.8	32.8	32.8	32.8
Fish oil[2]	20	20	20	20	20	20
Sunflower oil[2]	20	20	20	20	20	20
Vitamin mineral mix[2]	20	20	20	20	20	20
BHT[1]	0.2	0.2	0.2	0.2	0.2	0.2
Betaine[1]	5	5	5	5	5	5
Choline chloride[1]	1	1	1	1	1	1
CMC[1]	15	15	15	15	15	15
PQQ-Na_2[3]	-	0.0002	0.0004	0.0006	0.0008	0.001
Total	1000	1000	1000	1000	1000	1000
Proximate composition of the diet (g kg^{-1}; on dry matter basis; mean of triplicates)						
Moisture	65.1	70.4	67.0	65.0	50.3	61.2
Crude protein	317.3	317.7	315.5	316.8	317.7	319.3
Crude lipid	76.2	71.5	74.5	72.5	71.4	71.9
Crude fibre	50.9	53.3	55.0	51.5	56.0	56.1
Total ash	51.5	59.9	56.1	54.2	54.6	54.8
GE (MJ kg^{-1})	16.71	16.51	16.50	16.61	16.50	16.54
P/E ratio (g MJ^{-1})	19.0	19.2	19.1	19.0	19.2	19.3

[1]Procured from HImedia Ltd., India.
[2]Procured from the local market, India.
[3]Pyrroloquinoline quinone disodium salt (PQQ•Na_2) procured from Herb Nutritionals.
DSBM: Defatted soybean meal, GNOC: Groundnut oil cake, DORB: Deoiled rice bran, BHT: Butylated hydroxytoluene, CMC: Carboxymethyl cellulose.
Proximate composition: GNOC (CP- 436 g kg^{-1}, CL- 35.6 g kg^{-1}, TA- 106 g kg^{-1}, CF- 65 g kg^{-1}) and DSBM (CP- 520 g kg^{-1}, CL- 8 g kg^{-1}, TA- 65 g kg^{-1}, CF- 43 g kg^{-1}).
Composition of vitamin mineral mix (Premix Plus) (quantity per 2 kg): Vitamin A - 55,00,000 IU; Vitamin D_3 - 11,00,000 IU; Vitamin B_2 - 2000 mg; Vitamin E - 750 mg; Vitamin K - 1,000 mg; Vitamin B6 - 1,000 mg; Vitamin B_{12} - 6 ug; Calcium pantothenate - 2,500 mg; Nicotinamide - 10 g; Choline chloride - 150 g; Mn - 27,000 mg; I - 1,000 mg; Fe - 7,500 mg; Zn - 5,000 mg; Cu - 2000 mg; Co - 450 mg; L-lysine - 10 g; DL-methionine - 10 g; Selenium -125 mg; Vitamin C - 2500 mg.

Physico-chemical parameters of water

Water temperature, pH and dissolved oxygen was monitored every day and ammonia-N was monitored every week. The water temperature was checked four times a day (at 06:00, 13:00, 19:00 and 21:00 hrs) with the help of a digital thermometer (MERCK, Germany). A digital pH meter (HANNA Instruments, Singapore) was used to estimate the water pH of all the experimental tubs every day. The dissolved oxygen level was measured using a digital DO probe (Lab Junction LJ-810, Kingston Lab Solutions, Haryana). Ammonia-nitrite Test Kit (Spectroquant NOVA-MERCK, Germany) was used to measure the concentration of Ammonia-N.

Sampling

Sampling of fish was done at the beginning and end of the trial for calculating the growth parameters. Fish were not fed for 24 h before sampling. Wet weight of fish was taken using an electronic weighing balance. For estimating the carcass proximate composition, three fish from each tank was sampled and weight was recorded. Then 6 fish per tub was sampled and anesthetised using clove oil (50 µl l^{-1}). Amongst them, two fish were dissected for collecting liver. Liver samples were used for the determination of different metabolic enzymes activity. Two fish were dissected, for obtaining the intestine to prepare tissue homogenate for estimation of digestive enzymes activity. The rest two fishes were dissected to remove the liver and gill for evaluating the antioxidant enzymes activity. The samples were homogenised with chilled 0.25 M sucrose solution in a mechanical tissue homogeniser (Teflon-coated) to make 5% tissue homogenate. The homogenates were centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant obtained were later stored at -20°C until use. Additionally, two fish from each tub was taken for determining the thyroid hormone concentrations of blood plasma. Blood was collected using a 1 ml hypodermic syringe (without anticoagulant) from the caudal vein in Eppendorf vials. Part of the collected blood was allowed to clot at room temperature for about 2 h. After clotting, centrifugation

(6000 g) was done for 10 min for collecting the serum. The remaining quantity of blood was taken in an EDTA containing Eppendorf vials and shaken slightly to prevent haemolysis and used for further analysis.

Proximate analysis of diets and the whole body of fish

Proximate composition of the diet and the entire body of fish was determined in triplicate using standard AOAC (1995) methods. Samples were dried to a constant weight in a 105°C hot air furnace to determine the moisture content. Crude protein content (N % x 6.25) was analysed using the micro-Kjeldahl method (Kelplus, PELICAN, India), while crude lipid content was estimated using the Soxhlet extraction method (SOCS plus, SAS-AS 08, PELICAN, India). The ash content was obtained by combustion in a muffle furnace at 550°C for 5 h and fibre estimation was done in Fibertech (Tulin Equipment, India) apparatus. The gross energy content of the experimental diets was determined in an adiabatic bomb calorimeter (Model- 5E-AC/PL, Changsha Kaiyuan Instruments Co., Ltd., China).

Enzyme assays

Determination of tissue protein

Tissue protein quantification was done by Lowry's method (Lowry *et al.*, 1951). Bovine serum albumin was used as the standard to prepare the standard curve. This standard curve was used to estimate the protein concentration of different tissue samples. The obtained protein value was used for calculating the activity of enzymes in the samples.

Intestinal digestive enzyme assays

The amylase activity was assessed based on the reducing sugars formed due to the action of glucoamylase and α-amylase on carbohydrates using the dinitrosalicylic acid (DNS) method (Rick and Stegbauer, 1974). The activity of amylase was expressed as the mole of maltose released from starch min^{-1} mg $protein^{-1}$ at 37°C. The protease activity was calculated using 1% casein as a substrate using the method described by Drapeau (1974). The reaction mixture comprised a base, tris-phosphate buffer of 0.05 M (pH 7.8) and a sample of 100 µl. Ten percent of trichloroacetic acid (TCA) was applied after 10 min to stop the reaction and filtered. One unit of enzyme activity was described as the amount of enzyme required to release acid-soluble fragments at 280 nm min^{-1} and pH 7.8 equivalent to 0.001. Lipase activity was assessed as per Cherry and Crandall (1932). The reaction mixture was prepared and incubated with distilled water, the sample, phosphate buffer solution (pH 7) and olive oil emulsion. This reaction mixture was then titrated against 0.05 N NaOH until a permanent pink colour was obtained. The enzyme activity was estimated as milli-equivalent of the alkali ingested and expressed as unit mg $protein^{-1}$.

Metabolic enzyme assays

The test for citrate synthase was performed using a colourimetric test based on the reaction between 5,5'-Disulfanediylbis (2-nitrobenzoic acid) (DTNB) and CoA-SH to form TNB which shows maximum absorption at 412 nm (Morgunov and Srere, 1998). The absorbance strength is proportional to the activity of the citrate synthase and was expressed as nanomoles mg $protein^{-1}$ min^{-1}.

The activity of isocitrate dehydrogenase was assessed as per Bergmeyer *et al*. (1974). The reaction process comprised oxidation to oxalosuccinate (a ketone) of isocitrate (secondary alcohol), followed by the decarboxylation to ketone of the carboxyl group beta, producing alpha-ketoglutarates. Phosphate buffer was added to the enzyme, along with magnesium chloride, NADP and isocitrate. The activity was measured in a spectrophotometer at 340 nm and was expressed in nanomoles mg $protein^{-1}$ min^{-1}.

The glucose-6-dehydrogenase phosphate (G6PDH) was analysed according to DeMoss *et al.* (1953). A total of 3 ml reaction volume comprising 1.5 ml of 0.1 M tris buffer (pH 7.8), 0.2 ml of 2.7 mM NADP, 0.1 ml of homogenous tissue, 1.05 ml of distilled water and 0.1 ml of 0.02 M glucose-6-phosphate (G6P) was prepared. The OD was observed at intervals of 15 s against distilled water at 340 nm for 3 min and enzyme activity was expressed as mg $protein^{-1}$ min^{-1} units.

The method described by Wootton (1964) was used to assess alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The compound α-ketoglutarate was the substrate used for AST and DL-aspartic acid was used for ALT. This homogeneous sample and substratum together form the reaction mixture that was incubated at 37°C for 1 h. To stop the reaction, 2, 4-dinitrophenylhydrazine (DNPH) was used. Having added 0.4 NaOH (5 ml), the absorbance was taken at 540 nm. The enzyme activity was expressed as nanomoles of oxaloacetate formed min^{-1} mg $protein^{-1}$ at 37°C.

Oxidative stress enzymes assay

Superoxide dismutase (SOD) was evaluated as per the method defined by Misra and Fridovich (1972). Absorption due to the enzyme transition oxidation of epinephrine-adrenochrome was estimated at 480 nm for 3 min. The activity of one SOD unit was expressed as the amount of protein needed for 50% inhibition of epinephrine autooxidation. Catalase (CAT) behaviour was assessed based on a technique developed by Takahara *et al*. (1960). This process uses a phosphate buffer (50 mM, pH

7.0) and H_2O_2 solution was applied to start the reaction. The decreased absorbance was calculated at 240 nm. One unit of action of CAT was the amount of protein required to decompose H_2O_2.

Thyroid hormone assay

The thyroid hormones of serum thyroxine and triiodothyronine assay, were performed using T4 Elisa kit 2nd generation-96 T assay kit (Anand Brothers/AB Diachem Systems Pvt. Ltd., India) without any procedural modifications as suggested by Agharanya (1990).

Statistical analysis

The data obtained were initially subjected to one-way ANOVA using a GLM setup and to find out the best treatment, multiple comparisons of treatment effects were done using Tukey's HSD test ($p<0.05$) wherever significant effects were noted. It was seen that dietary supplementation of PQQ was non-significant for the parameters such as triiodothyronine, whereas other estimated parameters such as feed intake, weight gain, specific growth rate (SGR), protein efficiency ratio (PER), feed conversion ratio (FCR), SOD, catalase, thyroxine, AST, ALT, citrate synthase, isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase were found to be significant. Contrast analysis was conducted using both linear and quadratic orthogonal polynomials and a model of second-order was fitted to the responses wherever quadratic effects were significant.

$$f(x_u) = \beta_0 + \beta_1 x_u + \beta_2 x_u^2 + e_u$$

where $u = 1, 2, \ldots, N$; x_u is the level of the PQQ in the diet in the u^{th} treatment; $f(x_u)$ denotes the response obtained from u^{th} treatment; e_u is the random error associated with the u^{th} observation that is independently and normally distributed with mean zero and common variance σ^2; β_0 is the intercept; β_1 is the linear regression coefficient and β_2 is the quadratic regression coefficient. Adequacy of the fitted model was assessed by R^2, coefficient of determination and its statistical significance was tested by F-test. Both statistical analyses were performed using Windows SAS 9.4 (SAS Institute Inc.) software (SAS, 2013).

Results and discussion

Water temperature is one of the most crucial environmental factors (Fry, 1947), which influence the physiological processes like feed intake, growth and metabolic activity in fish (Richard *et al.,* 2016) as they are poikilothermic. The low water temperature around 14°C almost limits the production in aquaculture ponds for about 4-5 months (O'Gorman *et al.*, 2016). During winter conditions or lower water temperature, fish becomes inactive and prefer to stay at the pond bottom to conserve energy (Donaldson *et al.*, 2008). However, this can expose them to low oxygenic conditions and a non-conducive environment (Dunham and Smitherman, 1981). This unfitting environment adversely affects the metabolism, feed intake and immune systems, which finally leads to poor growth and reduced production (Bly and Clem, 1992). Hence, it is essential to understand the feed intake metabolism and antioxidant status of tropical fishes during low-temperature exposure and counter-strategies for increasing the metabolism through dietary intervention for higher growth and better immunity. The current study was conducted to evaluate feed intake, metabolism, thyroid hormone activities, antioxidant status and growth performances of common carp reared at low temperature through dietary manipulation using a metabolic modifier, Pyrroloquinoline quinone (PQQ).

The responses of common carp fingerlings to increasing levels of PQQ in the practical diet are summarised in Tables 2, 3, 4 and 5. External damage or pathological symptoms have not been found in fish for the entire experimental period. The responses such as feed intake, SOD (gill), catalase (gill and liver), AST and ALT were both linearly and quadratically significant due to supplementation of PQQ. On the other hand, no quadratic significance was observed in the rest of the parameters. The statistical significance (quadratic) of the calculated parameters and model fitness is shown in Table 6.

Water quality parameters

Water quality parameters such as water temperature, dissolved oxygen, pH and ammonia were found in the range of 12-14°C, 8-10 mg l^{-1}, 7.8-8.1 g l^{-1} and 0.006-0.007 mg l^{-1}, respectively, over the entire experimental period.

Proximate composition of diets and the whole body of fish

Proximate composition of the experimental diets (Table 1) showed no significant difference ($p>0.05$) between the experimental groups. The crude protein content in the diets ranged from 31.55 to 31.93%, whereas the crude fat content varied from 7.14 to 7.62%. The ash content of the diets varied between 5.15 to 5.99%. The crude fibre of the diets was estimated in the range of 5.09 to 5.61%. The nitrogen-free extract was calculated within the range of 50.18 to 50.90%. The estimated digestible energy (GE) varied from 16.5 to 16.71 MJ kg^{-1}. Therefore, the diets were isonitrogenous (around 32%) and isocaloric (16 MJ kg^{-1}) and isolipidic (7%). The total body composition of the fish (Table 3) did not vary significantly ($p>0.05$) between different experimental groups. Moisture, protein, ether extract, total carbohydrate and total ash remained unchanged ($p>0.05$).

Growth performance and nutrient utilisation

The growth and nutrient utilisation parameters *i.e.,* feed intake, WG%, SGR, FCR and PER, are given in Table 2. The WG%, SGR, FCR and PER were found to be linearly significant ($p<0.05$), whereas feed intake was found to be both linearly and quadratically significant ($p<0.05$) as given in Table 6. The feed intake values ranged from 3.23 to 4.53 and significantly higher feed intake was detected in the T4 group, which was fed with 0.8 mg kg^{-1} PQQ in the diet and displayed maximum optimum response at 0.75 mg of PQQ (Fig. 1) as per the equation of the second-order model. This was also as per the experiment with weaned pigs and broiler chicks wherein the latter fed with 0.2 mg PQQ kg^{-1} enhanced feed intake, carcass yield and growth performance (Wang *et al.*, 2015; Yin *et al.*, 2019). At the same time, Stites *et al.* (2000) and Steinberg *et al*. (1994) suggested that feed intake, growth, development and reproductive parameters in rodent models were improved when complemented by 0.1-0.2 mg PQQ kg^{-1} purified diets. Dietary PQQ deprivation leads to impairment of growth, resistance against pathogens and reproductive performance in rodent models (Steinberg *et al.,* 1994; 2003; Yin *et al*., 2019). To the best of our knowledge, no studies have been conducted in fish so far with PQQ. However, the present study provides sustenance for the usage of PQQ as a fish feed additive at low temperatures. The use of PQQ as a feed additive is also supported in commercial swine production (Yin *et al.,* 2019) and dietary additive for promoting nutrient utilisation in broiler chicks (Samuel *et al*., 2015). A significantly lower value was seen in Control, T1 and T2 groups. However, feed intake was reduced when fed the highest level of PQQ in the diet (T5 group). Bodyweight gain (%) of fish in different experimental groups ranged from 20.44 to 65.56 and showed a maximum value of 65.56 at 0.8 mg kg^{-1} of the PQQ treatment group and a significantly lower value in the control and T1 group.

SGR values ranged from 0.62 to 1.68 and showed a significantly higher value in the T4 group and less value was observed in the control and T1 group. This indicated that feeding of 0.8 mg kg^{-1} PQQ increased the weight gain in common carp because PQQ supplementation in the diet enhances the mitochondrial number with an associated increase in the mitochondria-related functions *in vivo* which eventually increases energy demand thereby increasing feed intake (Bauerly *et al*., 2011). The higher growth rate in the current study might be because of the PQQ compounds, as suggested by Naito *et al.* (1993) and Stites *et al*. (2006), who observed higher weight gain in mice fed with the PQQ compound. PQQ has

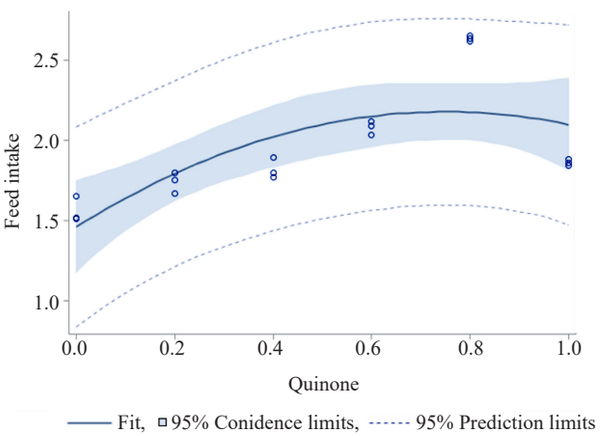

Fig. 1. Relationship of feed intake with a graded level of PQQ

Table 2. Growth indices and nutrient utilisation of common carp fingerlings fed diets containing graded PQQ levels

Parameters	Control	T1	T2	T3	T4	T5
FI (% day^{-1})	3.23±0.16 ^{a}	3.34±0.03 ^{a}	3.45±0.04 ^{a}	3.92±0.07^{b}	4.53±0.01^{c}	3.98±0.08 ^{b}
WG (%)	20.44±0.09^{a}	20.98±0.01^{a}	26.98±0.01^{b}	46.42±0.01^{c}	65.56±0.13^{e}	55.45±0.01^{d}
SGR (% day^{-1})	0.62±0.01^{a}	0.62±0.01^{a}	0.79±0.01^{b}	1.27±0.01^{c}	1.68±0.01^{e}	1.47±0.01^{d}
FCR	3.91±0.18^{d}	4.02±0.04^{d}	3.16±0.04^{c}	2.08±0.04^{b}	1.71±0.01^{a}	1.78±0.04^{a}
PER	0.88±0.01^{a}	0.89±0.01^{b}	0.89±0.01 ^{ab}	0.90±0.01^{bc}	0.91±0.01^{cd}	0.92±0.01^{d}
Survival (%)	97.2	100	100	97.2	100	100

Data expressed as mean±SE (n=3). Mean values in each row with different superscripts differ significantly ($p<0.05$).
FI(% BW day^{-1}) - Feed intake; WG(%) - Weight gain%; SGR - Specific growth rate; FCR - Feed conversion ratio; PER - Protein efficiency ratio.

Table 3. Carcass composition (g kg^{-1} wet weight basis) of common carp fingerlings fed diets containing graded levels of PQQ

Parameters	Control	T1	T2	T3	T4	T5
Moisture	766.3±0.95	765.7±1.29	768.4±0.91	754.5±0.49	762.8±1.13	760.1±0.48
Crude protein	131.2±0.54	140.1±0.77	139.5±0.56	146.8±0.26	143.1±0.68	131.2±0.54
Crude lipid	26.9±0.31	27.9±0.27	30.6±0.24	26.0±0.38	26.9±0.32	25.7±0.35
Total carbohydrates	45.4±0.12	31.8±0.41	36.9±0.38	29.3±0.29	34.2±0.24	45.4±0.12
Ash	30.2±0.36	35.2±0.068	28.9±0.23	40.1±0.3	35.3±0.32	30.2±0.36

Data expressed as mean±SE (n=3). Mean values in each row with different superscripts differ significantly ($p<0.05$).

been demonstrated to be an unique growth stimulator in animals (Killgore *et al.*, 1989). Unlike these reports, the dose was found to be higher in fish. This might be due to the variation in the habitat of the animal and leaching of PQQ in water as PQQ is reported to be soluble in water (>1 g PQQ per litre of water) and is readily absorbed at dietary concentrations (Smidt *et al.,*1991). The protein requirement of common carp ranged from 30-45% (Anwar and Jafri, 2001) and ether extract from 7.14 to 7.62% (Inayat and Salim, 2005). The estimated digestible energy (DE) varied from 16.5 to 16.7 MJ kg^{-1} feed.

A significantly higher ($p<0.05$) FCR was seen in the control and T1 groups whereas low feed utilisation was observed in T4 and T5 groups. The better feed efficiency or FCR observed due to the inclusion of dietary PQQ was probably linked with the variation of mitochondrial function (Chowanadisai *et al.*, 2010). Various studies have shown that mitochondrial function is highly associated with feed performance in broiler chicks (Bottje *et al.,* 2006). PQQ stimulates cAMP and enhances the biogenesis of mitochondria (Goffart and Wiesner*,* 2003). Feed efficiency in the control and T1 groups was not very much affected but greatly improved in T2, T3, T4 and T5 groups, which indicate that a much higher dose is required for PQQ to promote growth in fishes. The PER of fish in different treatments ranged from 0.88 to 0.92, with a maximum value observed in the T5 (1 mg kg^{-1}) group, compared to T4 (0.8 mg kg^{-1} PQQ) group. The control and T2 groups registered a significantly lower ($p<0.05$) PER value compared to the other groups. The survival rate was similar ($p>0.05$) among all the groups and was observed to be within the range of 97.2-100%.

Digestive enzymes

Digestive enzymes (protease, lipase and amylase) activities are good indicators of digestive capacity. Examination of these enzymes helps us to understand the activities at low temperatures and how these activities are altered due to the presence of PQQ. It also describes the adaptation of the body to dietary change (Gisbert *et al.*, 2009; Shamna *et al.,* 2015). The nutrient composition has a major role in digestive enzyme activity (Kawai and Ikeda, 1972). Dietary PQQ supplementation enhances the barrier integrity in the intestine (Farkas *et al.,* 2001), which improves the performance of digestive enzymes (Yin *et al.,* 2019). The protease, amylase and lipase activities were significantly different ($p<0.05$) among all the treatments. All three enzymes presented a progressive increase with the increase in concentration of PQQ. The digestive enzyme activity in the intestine of fish is reported in Table 4. The activity of enzymes was found to be linearly significant ($p<0.05$) when fed with dietary PQQ. All three digestive enzymes exhibited an increasing trend with increasing dietary concentration of PQQ. The higher amylase activity was noted in the group fed with 0.8 and 1 mg kg^{-1} PQQ (T4 and T5 group respectively), whereas the highest lipase and protease activities in the intestine were recorded in the T5 (1 mg kg^{-1} PQQ) group. Among all the treatments, the lower value was detected in the control group in all three enzymes. This progressive increase may be due to high digestive amylase and proteolytic activity in carp (Hidalgo *et al.,* 1999) which is speculated to be due to the quinone. The results of the present study are close to those of previous studies in rats, as indicated by many authors (Holmes *et al*., 1999; Winder *et al.*, 2000). Other causes for increased enzyme activity may be due to the availability of substrate for the action of enzymes. This might be due to improved feed intake stimulated by PQQ, as the compound is known to increase the energy demand in the biological system (Canto *et al.,* 2009).

Metabolic enzymes

Metabolic enzymes such as citrate synthase, glucose 6-phosphate dehydrogenase and isocitrate dehydrogenase activity (Fig. 2) were found to be linearly significant ($p<0.05$). The value in each treatment group increased when compared to control. Citrate synthase exhibited significantly higher activity in the T4 group. The values of all treatment groups were similar ($p>0.05$) except for the T4 group and the lowest value was recorded for the control group. The exposure of animals to the PQQ-rich diet has shown stimulated mitochondrial biogenesis, which is similar to the studies conducted by Saihara *et al.* (2017). Citrate synthase is the initial regulatory enzyme in Kreb's cycle (Frenkal *et al.,* 1976). Citrate synthase is triggered when there is high energy demand in the system as caused

Table 4. Digestive enzymes activities of common carp fingerlings fed diets containing graded levels of PQQ

Parameters	Control	T1	T2	T3	T4	T5
Protease	3.72 ± 0.26^{a}	5.15 ± 0.23^{b}	6.04 ± 0.38^{b}	7.83 ± 0.27^{c}	11.96 ± 0.62^{d}	19.06 ± 0.63^{e}
Amylase	5.8 ± 0.08^{a}	6.82 ± 0.27^{b}	7.34 ± 0.11^{b}	10.01 ± 0.06^{c}	14.32 ± 0.33^{d}	15.02 ± 0.43^{d}
Lipase	0.14 ± 0.01^{a}	0.21 ± 0.01^{b}	0.35 ± 0.01^{c}	0.34 ± 0.01^{c}	0.42 ± 0.03^{d}	0.58 ± 0.02^{e}

Data expressed as mean±SE (n=3). Mean values in each row with different superscripts differ significantly ($p<0.05$)
Protease activity expressed as nanomoles of tyrosine released $min^{-1}g$ $protein^{-1}$.
Amylase activity expressed as moles of maltose released from starch min^{-1} mg $protein^{-1}$ at 37°C
Lipase activity expressed as units mg $protein^{-1}$

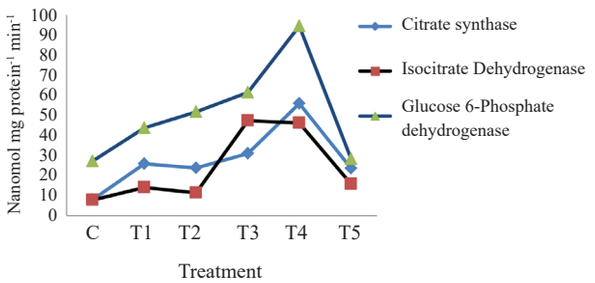

Fig. 2. Citrate synthase, Isocitrate dehydrogenase activity and Glucose 6 - phosphate dehydrogenase activity in the liver of *C. carpio* fingerlings fed with different experimental diets. C-control+0 mg kg^{-1} PQQ; T1-Basal feed+0.2 mg kg^{-1} PQQ; T2-Basal diet+0.4 mg kg^{-1} PQQ; T3-Basal feed+0.6 mg kg^{-1} PQQ; T4-Basal diet+0.8 mg kg^{-1} PQQ; T5-Basal diet+1 mg kg^{-1} (PQQ).

by growth stimulants like PQQ (Wang *et al.*, 2015). In the present study, there was an increase in hepatic citrate synthase activity in the group fed with 0.8 mg kg^{-1} of PQQ, which indicates an increased mitochondrial function and biomass stimulated by PQQ as described by Raffaella *et al.* (2008) and Hirschey *et al.* (2010). Related results were also observed by Wang *et al*. (2015), who interpreted that PQQ improved hepatic mitochondrial metabolism and feed intake in broilers when fed with PQQ. Studies in mice have supported the idea that citrate synthase activity was enhanced in hepatic (Hepa1-6) cells when exposed to PQQ for 24 or 48 h (Chowanadisai *et al.,* 2009).

Pentose phosphate pathway (PPP) aids in the production of NADPH in the system which is essential for the fatty acid synthesis and conversion of GSSG (oxidised glutathione) to GSH (reduced glutathione). This reduced glutathione (GSH) is regarded as one of the major reactive oxygen species (ROS) scavengers and its ratio to oxidised glutathione (GSSG) can be used as an oxidative stress marker for GPx (Glutathione Peroxidase) operation (Lehninger, 2002; Zitka *et al.,* 2012). The enzyme glucose 6-phosphate dehydrogenase showed significantly higher ($p<0.05$) activity in the T4 group when compared to the control group ($p<0.05$). The enzyme isocitrate dehydrogenase also showed a significantly higher value in T3 and T4 groups, which were similar ($p>0.05$). All other groups, except T3 and T4, showed similar values ($p>0.05$). In our study, the lower level of G6PDH in the control and 1 mg kg^{-1} PQQ fed group indicates that the activity of G6PDH was less in these groups, whereas the higher G6PDH activity in the 0.8 mg kg^{-1} PQQ fed group showed an efficient antioxidant capacity of PQQ with the help of NADPH produced from pentose phosphate pathway (PPP). Studies explain that the GSH/GSSG pair is ready to donate electrons when subjected to PQQ treatment in the case of rodents (Cao *et al*., 2013; Wang *et al.*, 2015).

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) catalyses the reversible transfer of an amino group from glutamate to oxaloacetate. If AST predominates over ALT, it indicates the aerobic tendency of the tissues and higher glucose utilisation as it is a mitochondrial enzyme. The protein metabolic enzymes such as AST and ALT were found to be significant both linearly and quadratically ($p<0.05$) as given in Table 5. The AST values in muscle ranged from 11.74 to 29.05 and showed a significantly higher ($p<0.05$) value in the T5 group and the lowest value in T1 group. The minimum optimum response was observed at 0.33 mg kg^{-1} PQQ as per the equation of the second-order model. The AST values in the liver ranged from 10.94 to 19.15 and showed a significantly higher ($p<0.05$) value in the T5 group and the lowest value in the control and T1 group. The optimum response was observed at 0.21 mg kg^{-1} PQQ as per the equation of the second-order model. The ALT

Table 5. Protein metabolic enzymes, oxidative stress enzymes, and thyroid hormone activities of common carp fingerlings fed diets containing different quinone levels

Parameter	C	T1	T2	T3	T4	T5
AST (Muscle)	13.17 ± 0.76^{ab}	11.74 ± 0.35^{a}	13.36 ± 1.08^{ab}	12.91 ± 0.31^{ab}	14.10 ± 0.65^{b}	29.05 ± 0.67^{c}
AST (Liver)	11.08 ± 0.44^{a}	10.94 ± 0.49^{a}	12.44 ± 0.37^{b}	12.97 ± 0.22^{b}	12.73 ± 0.35^{b}	19.15 ± 0.36^{c}
ALT (Muscle)	15.11 ± 0.44^{b}	15.67 ± 0.84^{b}	15.57 ± 0.39^{b}	11.93 ± 1.06^{a}	24.43 ± 0.53^{c}	27.16 ± 0.38^{d}
ALT (Liver)	12.39 ± 0.73^{a}	13.55 ± 1.03^{a}	14.58 ± 1.18^{a}	12.05 ± 0.68^{a}	23.23 ± 0.76^{b}	23.56 ± 0.26^{b}
SOD (Liver)	45.83 ± 0.52^{a}	74.03 ± 0.48^{b}	74.54 ± 0.33^{b}	45.45 ± 0.29^{a}	46.65 ± 0.71^{a}	74.08 ± 0.03^{b}
SOD (Gill)	43.55 ± 0.29^{a}	75.87 ± 0.42^{c}	67.1 ± 0.61^{b}	69.22 ± 1.09^{b}	83.78 ± 0.21^{d}	69.49 ± 1.19^{b}
Catalase (Liver)	12.38 ± 0.65^{d}	8.47 ± 0.79^{c}	2.02 ± 0.28^{a}	3.38 ± 0.54^{a}	8.08 ± 0.44^{c}	4.62 ± 0.8^{b}
Catalase (Gill)	11.85 ± 0.66^{d}	10.32 ± 0.08^{c}	9.18 ± 0.49^{c}	3.34 ± 0.68^{a}	3.76 ± 0.23^{a}	5.44 ± 0.06^{b}
Triiodothyronine	1.77 ± 0.02^{a}	1.50 ± 0.04^{a}	2.42 ± 0.02^{b}	2.28 ± 0.18^{b}	2.24 ± 0.11^{b}	2.21 ± 0.11^{b}
Thyroxine	1.30 ± 0.01^{a}	1.73 ± 0.73^{a}	2.25 ± 0.25^{a}	6.05 ± 0.45^{bc}	6.76 ± 0.24^{c}	5.25 ± 0.05^{b}

Data expressed as Mean±SE n=3; Mean values in the same row with different superscripts differ significantly ($p<0.05$).
AST=Aspartate aminotransferase, ALT=Alanine aminotransferase; SOD: Superoxide dismutase
AST activity expressed as nanomoles oxaloacetate released min^{-1} mg $protein^{-1}$ at 37°C; ALT activity expressed as Nanomoles Na pyruvate released min^{-1} mg $protein^{-1}$ at 37°C; Triiodothyronine and thyroxine levels expressed as pmol l^{-1}

Table 6. Parameter estimates of second-order model fitted for feed intake, VSI, SOD (gill), catalase and protein metabolic enzymes of common carp fingerlings fed diets containing graded quinone levels

Parameter	β_0	β_1	β_2	R^2	p value	Optimum dietary PQQ	Response at optimum PQQ
Feed Intake	1.46*	1.92*	-1.28*	0.53	0.04	0.75##	2.18
	(0.14)	(0.64)	(0.61)				
SOD (Gill)	48.73*	84.61*	-62.38*	0.63	<0.01	0.68##	77.42
	(4.32)	(20.34)	(19.52)				
Catalase (Gill)	12.76*	-18.19*	9.96*	0.70	<0.01	0.91#	4.46
	(0.88)	(4.14)	(3.97)				
Catalase (Liver)	12.57*	-30.79*	23.13*	0.87	<0.01	0.67#	2.32
	(0.76)	(3.57)	(3.42)				
AST (Muscle)	14.35*	-23.48*	35.78*	0.82	<0.01	0.33#	10.49
	(1.47)	(6.92)	(6.64)				
AST (Liver)	11.45*	-4.91*	11.52*	0.81	<0.01	0.21#	10.93
	(0.71)	(3.32)	(3.18)				
ALT (Muscle)	16.04*	-15.50*	27.34*	0.77	<0.01	0.28#	13.84
	(1.54)	(7.26)	(6.97)				
ALT (Liver)	12.84*	-4.51*	16.27*	0.74	<0.01	0.14#	12.54
	(1.47)	(6.93)	(6.65)				

Values in parentheses indicate SE of estimates
*Significance at p<0.05, # Optimum is minimum, ## Optimum is maximum
VSI: Viscero-somatic Index, SOD: Superoxide dismutase, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase

values in muscle ranged from 11.93 to 27.16 and showed a significantly higher (p<0.05) value in the T5 group and the lowest value in the T3 group. The optimum response was observed at 0.28 mg kg^{-1} PQQ as per the equation of the second-order model. The ALT values in the liver ranged from 12 to 23 and showed a significantly higher (p<0.05) value in T4 and T5 and lower value in the control, T1, T2 and T3 groups. In the liver, AST activity showed a significant difference among the groups and the highest activity was found in the T5 group both in the liver and muscle. ALT activity in the liver showed the highest activity in T4 and T5 groups, varied significantly among the groups. This might be because of the utilisation of the glucose-derived TCA intermediates for protein synthesis. Moreover, data on exactly how PQQ affects AST and ALT are scarce. However, the results obtained from the study conducted on rats when exposed to an endotoxin significantly improved AST and ALT by PQQ administration (Matsumoto *et al.*, 1989).

Antioxidant stress enzyme and thyroid hormone assay

Non-enzymatic antioxidants like vitamin E, GSH, selenium and vitamin C as well as enzymatic antioxidants (SOD, GSH-Px and catalase) are responsible for maintaining the redox status inside the cell. Fish during low temperatures, are vulnerable to stress, producing more free radicals and thus making the immune system weak (Crawshaw *et al.*, 1984). PQQ is a competent non-enzyme antioxidant. Either PQQ or pyrroloquinoline quinol (reduced form) can directly scavenge superoxide anion, hydrogen peroxide and lipid radicals (Rucker *et al.*, 2000). In addition, PQQ tends to improve the antioxidant defence mechanism by inducing antioxidant enzymes (Misra *et al.,* 2004), such as SOD and catalase. Inclusion of quinone compounds in the diet of common carp functions as an efficient non-enzymatic antioxidant directly scavenging superoxide anions and hydrogen peroxide molecules as supported by the studies conducted by Misra *et al.* (2004). The antioxidant enzymes such as SOD and catalase along with the activities of thyroid hormones are given in Table 5. Among these, SOD (liver), triiodothyronine and thyroxine exhibited only linear significance (p<0.05).

Gill SOD was found to be significantly higher in the T4 group relative to other treatments, since quinone can lead to the production of superoxide in the presence of a reducing agent (Aizenman *et al*., 1992) suggesting that quinone feeding could not reduce gill SOD activity. Such an increased SOD activity was observed in the intestine in pigs (Yin *et al.*, 2019). Also, a higher catalase activity was observed in 0.2 and 0.8 mg kg^{-1} quinone fed groups due to free radical production at a higher dose as mentioned above. The SOD values in the liver ranged from 45 to 74 unit activity (amount of protein required to give 50% inhibition of epinephrine auto-oxidation) and the highest SOD activity was seen in T1, T2 and T5 groups and the lowest value was seen in T3 and T4 groups. The SOD values in gills ranged from 43.55 to 83.78 unit activity and showed a significantly higher value in the T4 group (p<0.05) and the lowest value in the control group. The maximum optimum response was obtained at 0.68 mg kg^{-1}

PQQ (Fig. 3) as per the equation of the second-order model. This might be due to the free radical scavenging capacity of quinone. Inflammatory responses can be blocked by PQQ in rats (Hamagishi *et al.,* 1990).

The catalase value in the liver ranged from 2.02 to 12.38 (nanomoles H_2O_2 decomposed min^{-1} mg $protein^{-1}$ at 37°C) and showed a significantly higher ($p<0.05$) value in the control group and comparatively lower value in the rest of the group. The minimum optimum response was obtained at 0.67 mg kg^{-1} PQQ (Fig. 4) as per the equation of the second-order model. The catalase values in gill ranged from 3.34 to 11.85 and showed a significantly higher value in the control group ($p<0.05$) and the lowest value in T3 and T4 groups. The catalase values were significantly lower in all the other treatments compared to the control group. This lower activity might be due to the hydrogen peroxide neutralisation property of quinone (Tsuchida *et al*., 1993; Urakami *et al.*, 1997; Scanlon *et al.,* 1997) and further studies are required to track the exact reason for discrete variations.

All physiological functions in an animal are regulated by thyroid hormones. Any defects in the production and release of thyroid hormones can harm the biological system (Panda *et al*., 2007). Thyroid dysfunction is also related to the development of reactive oxygen species (ROS) (Erdamar *et al.,* 2008; Kumar and Kar, 2014). Since PQQ control oxidative damage and hypothyroidism has an influence on oxidative stress (Venditti *et al*., 2006; Jena *et al*., 2012), it was assumed that this compound could alter oxidative damage (Kumar and Kar, 2013). PQQ administration has the potential to improve the status of cellular antioxidants (Misra *et al.,* 2004; Ouchi *et al.,* 2009). Thyroid hormones sustain and control heat production by stimulating the expression of uncoupling

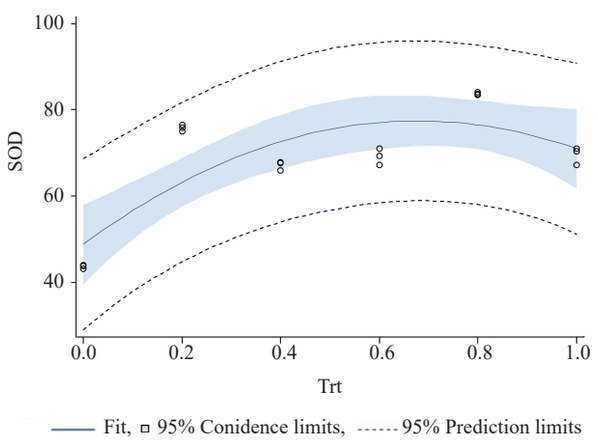

Fig. 3. Relationship of superoxide dismutase (gill) with graded levels of PQQ

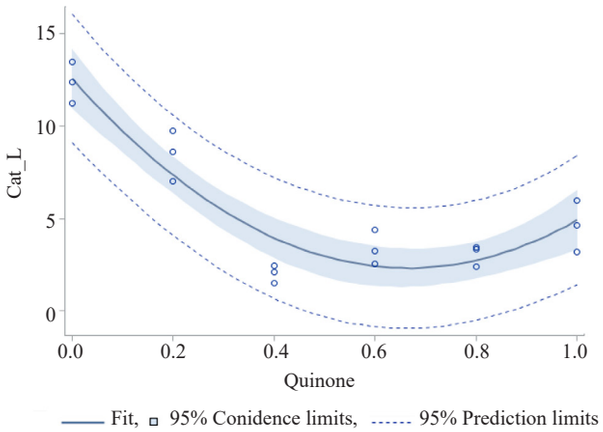

Fig. 4. Relationship of catalase (Liver) with graded levels of PQQ

proteins (UCPs) that lead to increased heat production, and therefore has a role in low-temperature adaptation (Paakkonen and Leppaluoto, 2002). The thyroxine and triiodothyronine values increased in 0.4, 0.6, 0.8 and 1 mg kg^{-1} PQQ fed groups. Triiodothyronine value showed a significantly higher response ($p<0.05$) in T2, T3, T4 and T5 when compared to the values obtained in the control and T1 groups as given in Table 5. Thyroxine values were found to be significantly higher ($p<0.05$) in the T4 group (Table 5) and the lowest value were found to be in the control, T1, T2 and T3 groups. Similar results were also observed by Larsen *et al.* (2001), who observed a correlation between higher feed intake and thyroxine values in Coho salmon. Furthermore, the estimation of PQQ in biological samples is often difficult because of its elevated reactivity. Therefore, in the present study detection of PQQ was not carried out due to this limitation.

As far as the current knowledge of authors, incorporation of pyrroloquinoline quinone (PQQ) as a feed additive in fish at low temperature is reported for the first time. In conclusion, incorporation of PQQ at 0.8 mg kg^{-1} diet in aquafeed can be beneficial in increasing the feed intake by around 1.5 times more when compared to other treatments, thereby enhancing the performance of fish during the winter period. From contrast analysis, the optimum PQQ required for enhancing feed intake and growth of common carp during low temperature is 0.75 mg kg^{-1} diet. Pyrroloquinoline quinone can modify metabolic functions, especially the vital mitochondrial enzymes like citrate synthase, iso-citrate dehydrogenase, glucose-6-phosphate dehydrogenase and thyroid hormone during low-temperature exposure. For better understanding, further study is required especially on the formulation of dependable analytical procedures to trace PQQ in compounds added into diets (plants, or animal-based material) to obtain insight into the distribution and the

pathway of biosynthesis within. Also, a more prolonged study duration is required to find out how exactly the variations in enzymes occur. Some studies related to the specific genes that are responsible for feed intake, growth and thermal adaptability would be beneficial. This study will facilitate making strategies for enhancing the growth of fish during winter months and profitability to farmers.

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