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Optimization of Calcium and Phosphorous Ratio in the Practical Diet of *Hypophthalmichthys molitrix*

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SI conducted the experiment. MF planned and supervised the experiment. SZHS and NK helped in analysis. MB helped in writing the manuscript. Maryam and SN helped in experiments.

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Ca:P, Calcium lactate, Disodium phosphate, Growth, Nutrient digestibility, Body chemical composition

ABSTRACT

An experiment of 90-days was performed to investigate the effects of dietary calcium (Ca), phosphorus (P) and their interaction on growth, whole-body composition, nutrient digestibility and mineralization of silver carp, *Hypophthalmichthys molitrix* (average initial weight 13.7 ± 0.05 g). Nine isonitrogenous, isocaloric and isolipidic diets with three Ca levels (0, 1 and 2%) combined with three P levels (0, 1 and 2% P) were prepared. Results showed that silver carp fed the diet supplemented with 1% Ca and 1% P level yielded the similar ($p > 0.05$) growth performance as was recorded for that fed diet containing 2% Ca and 2% P. No significant difference in survival rate was recorded except for the diet containing 1% Ca with 2% P supplement. The feed intake (FI) of juvenile remained unaffected ($p > 0.05$) by mineral supplementation. Whole body protein content increased ($p < 0.05$) slightly with Ca and P supplementation. However, moisture, fat and ash contents remained unaffected ($p > 0.05$). Dietary Ca and P supplementation improved ($p < 0.05$) the protein and fat digestibility in silver carp. Ca and P contents showed significant increase ($p < 0.05$) with increasing Ca and P levels in the whole body, bones and scales, achieving the highest ($p < 0.05$) values at 2% Ca supplemented with 2% P. Whereas, Mg and Zn contents decreased ($p < 0.05$) with increasing Ca and P supplementation. Fish fed the diet containing 2% Ca level without P supplement had higher ($p < 0.05$) Ca/P ratio in whole body and bones. However, it decreased ($p < 0.05$) at 2% Ca level in scales. Further, increase in P supplementation significantly reduced ($p < 0.05$) Ca/P ratio regardless of Ca level. Conclusively, supplementation of 1% Ca in the presence of 1% P (1:1 ratio) is required for optimum performance of silver carp.

INTRODUCTION

Calcium (Ca) and phosphorous (P) are essential minerals for optimum growth and physiology of fish. They constitute up to 70% of total mineral elements in fish body (Hossain and Yoshimatsu, 2014). Calcium has important key roles in skeletal development, muscle contraction, osmoregulation, blood clotting, neuro-transmission and enzyme activation in fish (NRC, 2011). As Ca-binding proteins act as a carrier for binding of Ca and P intestine, hence, Ca is vital for P utilization as well (Brody, 1998). Most of the aquatic species can fulfil their Ca requirements from water. However, many studies have reported insufficient water-borne Ca in channel catfish (Robinson *et al*., 1986), tiger puffer (Hossain and Furuichi, 1998), red lip mullet (Hossain and Furuichi, 2000a), scorpion fish (Hossain and Furuichi, 2000b), American cichlid (Sanchez *et al*., 2000), juvenile tilapia (Shiau and Tseng, 2007) and giant croaker (Hossain and Furuichi, 1999).

Phosphorous is considered as a limiting nutrient for the growth of fish. It actively participates in skeletal development, bioenergetics and intermediary metabolism (Fontagné *et al*., 2009). Unlike Ca, low concentration of P is present in marine and freshwater. Thus, fish depends upon the dietary P supplementation to satisfy their metabolic P requirement (Yang *et al*., 2021). Previous

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studies showed that supplementation of P improved the growth performance of blunt snout bream, (Yang *et al*., 2021), catfish (Zafar and Khan, 2018), gibel carp (Xie *et al*., 2017) and snakehead (Shen *et al*., 2017). The range of dietary P requirements in fishes varies from 3.0 to 15.0 g/kg diet (NRC, 2011).

Many fishes have a potential to maintain constant Ca/P ratio in their bones and plasma. Thus, Ca/P ratio must be optimized in the artificial diet of fish otherwise, imbalances in ratio can cause negative effects on mineralization and nutrient bioavailability of fish (Hossain and Yoshimatsu, 2014) resulting in reduced growth rate. High dietary Ca level has an antagonistic effect on P absorption (Laining *et al*., 2011). The presence of excess Ca in diet interferes with the absorption of P in fish intestine as it combines with Ca to form insoluble complexes. This calcium phosphate complex is not biological available to fish, hence results in excretion of excessive nutrients into water bodies. Accumulation of these nutrients especially undigested P stimulate the algal bloom into water bodies resulting in water pollution (Sun *et al*., 2017). In addition, high Ca level has an inhibitory effect on trace elements absorption such as zinc, manganese and iron (Hossain and Yoshimatsu, 2014). Moreover, excessive Ca level consequently increased the P requirement in fish which in turn increased the feeding cost (Ye *et al*., 2006). It is suggested that optimum Ca/P ratio ranges from 1:1 to 1:17 in different fish species (Sanchez *et al*., 2000; Ye *et al*., 2006). Thus, it is very important to optimize Ca/P ratio in silver carp diet for better growth and physiological functions of fish.

Silver carp is native to Asia and widely cultivated in China. Its production was 4.51 million tons in 2017 (Gui *et al*., 2018). Rapid growth, high availability, delicious taste and low market price make it a potential candidate for fish farming (Gui *et al*., 2018). The presence of high protein content, unsaturated fatty acids and essential amino acids make it a suitable alternative to marine fish for surimi processing (Peres *et al*., 2015). There is not much literature available on the nutritional requirements of silver carp, however, recently the researchers are focusing on the nutrition of this species. Recently, some dietary studies have been reported to improve the growth, immunity and disease resistance in this species. Harikrishnan *et al.* (2021) reported addition of 200 mg/kg glycyrrhizic acid improved the immune system of silver carp. Mushtaq *et al.* (2022) studied that addition of 0.5 mg/kg Se methionine is efficient in improving the growth and antioxidative status of silver carp. Similarly, 5 g/kg micro and nano-scale chitosan effectively enhanced growth performance, non-specific immunity, and resistance of silver carp against *Staphylococcus aureus* infection (Younus *et al*., 2020). Likewise, there is scarce knowledge regarding the optimization of Ca/P ratio in silver carp. The outcomes of this study will help the feed manufacturers to formulate a cost effective and well-balanced feed for silver carp.

MATERIALS AND METHODS

Ethical statement

This research was performed in Fish Seed Rearing Unit, Department of Fisheries and Aquaculture, UVAS Ravi Campus, C Block Pattoki after the approval of ethical committee of university.

Experimental design and diets

Nine isonitrogenous, isocaloric and isolipidic experimental diets were formulated by supplementing Ca and P at (0,0), (0,1), (0,2), (1,0), (1,1), (1,2), (2,0), (2,1) and (2,2) % levels, respectively in basal diet. They were named as Ca0P0, Ca0P1, Ca0P2, Ca1P0, Ca1P1, Ca1P2, Ca2P0, Ca2P1 and Ca2P2. Calcium lactate (Sigma-Aldrich) and disodium phosphate (Sigma-Aldrich) were used as a source of Ca and P, respectively (Table I). The ingredients were analyzed for proximate composition (AOAC, 1995) before feed formulation. Fishmeal, soya bean and canola meal, fish oil was used as lipid source and wheat flour were used in basal diet. Mineral and vitamin mixture was prepared by following NRC (2011). The basal diet contained 31.04% crude protein and 9.0% crude lipid. All feed ingredients were dried, weighed, ground, sieved and mixed with other feed ingredients including wheat flour, vitamin mixture, mineral mixture and chromic oxide by using an electric mixture. The pre-weighted fish oil was added with continuous mixing followed by the addition of distilled water for dough formation. A dough of even consistency was pelleted to 2mm diameter die by using a hand pelletizer. Pellets were oven-dried at 80°C until moisture was reduced to 10%. The dried pellets were packed in labelled zip lock bags and frozen (-20°C) for feeding trial.

Feeding trial

Healthy *Hypophthalmichthys molitrix* juveniles were taken from Hatchery of UVAS and acclimated in flow through system (0.4L/min) for 02 weeks. Juveniles were hand fed with basal diet during acclamation period. For feeding trial, 405 juveniles were randomly kept into 27 tanks (58 x 30 x 49 cm) at a density of 15 fish (average initial weight 13.7 ± 0.05 g) per tank. Three tanks were allotted randomly to each diet for 90 days. Juveniles were fed twice to apparent satiation per day. Uneaten pellets were collected for the estimation of feed conversion ratio (FCR) and feed intake (FI) after each feeding session.

Table I. Composition of experimental diets (%).

Ingredients	Diets								
	Ca0P0	Ca0P1	Ca0P2	Ca1P0	Ca1P1	Ca1P2	Ca2P0	Ca2P1	Ca2P2
Fish meal[1]	25	25	25	25	25	25	25	25	25
Soya bean meal[1]	20	20	20	20	20	20	20	20	20
Canola meal[1]	15	15	15	15	15	15	15	15	15
Rice polish[1]	15	15	15	15	15	15	15	15	15
Wheat flour[1]	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Fish oil[2]	6	6	6	6	6	6	6	6	6
Vitamin C	2	2	2	2	2	2	2	2	2
Vitamin premix[3]	2	2	2	2	2	2	2	2	2
Mineral mixture[4]	2	2	2	2	2	2	2	2	2
Disodium phosphate	-	3.1	6.2	-	3.1	6.2	-	3.1	6.2
Calcium lactate	-	-	-	7.69	7.69	7.69	15.38	15.38	15.38
Chromic oxide	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Proximate composition									
Moisture	8.42	8.61	8.52	8.38	8.53	8.44	8.4	8.52	8.58
Crude protein	30.13	30.42	30.24	30.36	30.4	30.27	30.22	30.33	30.39
Crude fats	9.1	8.95	8.99	9.16	9.2	9.14	9.2	9.17	9.17

[1]Fishmeal, soybean meal, canola meal, rice polish and wheat flour were purchased from local market. [2]Fish oil was purchased from Poultry-vet Co, Nazimabad, Karachi, Pakistan. [3]Vitamin premix (kg^{-1} diet): Vitamin A 15 M.I.U; Vitamin D3 3 M.I.U; Nicotinic acid 25000mg; Vitamin B1 5000 mg; Vitamin E 6000 IU 9000 mg; Vitamin B2 6000 mg; Vitamin K3 4000 mg; Vitamin B6 4000 mg; Folic acid 750 mg; Vitamin B12 9000 mg; Vitamin C 15000mg; Calcium pantothenate 10000mg. [4]Mineral premix (kg^{-1} diet): $MgSO_4.7H_2O$ 153mg; NaCl 51mg; $COCl.6H_2O$ 0.0816mg; $AlCl_3.6H_2O$ 0.255mg; $CuSO_4.5H_2O$ 210.67mg; $FeSO_4.H_2O$ 100.67mg; $MnSO_4.5H_2O$ 116.67mg; $ZnSO_4.7H_2O$ 121.33 mg.

After 2h interval, the fecal matter from each replicate tank was collected for further analysis. Water quality parameters such as water temperature (27.85- 29.33°C), dissolved oxygen (5.56-6.65 mg/L), water total ammonia (0.16 to 0.2 mg/L) and pH (8.04-8.30) were monitored daily throughout the feeding trial.

Data collection

At the end of 90th experimental day, fish was starved and anesthetized by using clove oil (3000 mg/L) for 60 seconds (Khajepour and Hosseini, 2012). The growth performance and feed utilization were determined by using following formulas.

$$WG(\%) = \frac{\text{Final body weight(g)} - \text{Initial body weight(g)}}{\text{Initial body weight (g)}} \times 100$$

$$FI(g) = \frac{\text{Total feed intake per fish (g)}}{\text{number of days}}$$

$$FCR = \frac{Feed\ intake\ (g)}{Weight\ gain\ (g)}$$

$$SR(\%) = 100 \times \frac{\text{Final fish number}}{\text{Initial fish number}}$$

$$SGR(\%/day) = 100 \times \frac{\text{Ln(Final body weight, g)} - \text{Ln(Initial body weight, g)}}{\text{Time (days)}}$$

Proximate composition analysis

Five fish (from each replicate) were chosen for proximate composition analysis. Samples (fish, feed and feces) were pooled and ground. The standard method of AOAC (1995) was followed for proximate composition analysis. Dry matter was determined by oven-drying method (Model UNB-400, Memmert, Germany); crude protein (N×6.25) was analyzed by micro Kjeldahl method; crude fat was estimated by Soxhlet system while crude ash by igniting the samples at 550°C in muffle furnace (Ney VULCAN 3-550).

Mineral contents analysis

Four fish from each replicate were chosen for mineral content analysis. Scales were scrapped, pooled, oven-dried and stored till analysis. Fish carcass were dipped into hot water until the flesh was stripped off. Samples were rinsed, oven-dried and ether extracted through Soxhlet apparatus for fat removal. Defatted samples were ground and digested through wet digestion method (AOAC, 1995). Phosphorus analysis was performed on UV-Vis Spectrophotometer, while mineral estimation was done through atomic absorption spectrophotometry (Irmeco, Model U2020).

Nutrient digestibility

Feces from each experimental tank were siphoned out, pooled and oven-dried daily throughout the feeding trial of 90-days (AOAC, 1995). Chromic oxide was added as an inert marker of nutrient digestibility in the experimental diets. The dried fecal samples were weighed and frozen at −20°C in labelled bags. Digestion of feces was done by the method of Czarnocki *et al*. (1961). The apparent digestibility coefficient (ADC%) was determined by using the given formula (NRC, 2011).

$$ADC(\%) = 100 - 100 \times \frac{\text{Percent marker in diet} \times \text{Percent nutrient in feces}}{\text{Percent marker in feces} \times \text{Percent nutrient in the diet}}$$

Statistical analysis

The data were subjected to two-way analysis of variance (ANOVA) to determine the interaction between Ca and P. CoStat statistical software package (Version 6.303) was used for all statistical analysis. Tukey's Honestly Significant Difference Test was used for comparing means at 5% significance level.

RESULTS

Growth performance

Dietary supplementation of Ca and P individually as well as in interaction significantly improved the final weight (FW), weight gain% (WG%), specific growth rate (SGR) and FCR of silver carp (Table II). No significant variation in survival rate was recorded except for the diet containing 1% Ca with 2% P supplement. However, feed intake (FI) remained unaffected ($p > 0.05$) by their supplementation. Results showed that increase in Ca supplementation from 1 to 2% did not cause further improvement ($p > 0.05$) in the FW, WG% and SGR of fish, however, increase in P supplementation from 1 to 2% at 0 and 2% Ca level, significantly improved these parameters. Moreover, 1% Ca at 1% P fed silver carp yielded similar ($p > 0.05$) growth performance as was exhibited by fish fed diet containing 2% Ca and 2% P.

Whole-body composition

Supplementation of Ca and P enhanced ($p < 0.05$) the whole body protein content while moisture, fat and ash content remained unaffected ($p > 0.05$) by their supplementation. The protein content increased with P without Ca supplement, however, remained non-significant at 1 and 2% Ca level. Similarly, increase in Ca supplementation from 1 to 2% did not improve ($p > 0.05$) the body protein level (Table III).

Nutrient digestibility

Results of nutrient digestibility are depicted in Table IV. The highest ($p < 0.05$) protein and fat digestibility was observed in fish fed 2% Ca supplemented diet but it was similar ($p < 0.05$) to those fed 1% supplemented diet. Considering the individual element, increase in P from 0 to 1% enhanced ($p < 0.05$) protein digestibility at 1% Ca level, however, further increase in its supplementation did not improve ($p > 0.05$) the protein digestibility. Moreover, dry matter remained unaffected ($p > 0.05$) by Ca, P and their interaction.

Table II. Effect of different experimental diets on growth performance of *H. molitrix* juveniles.

Diets	FW (g)	WG%	SGR (%/day)	FI (g)	FCR	SR%
Ca0P0	49.06±0.91^{e}	272.51±7.33^{e}	1.60±0.02^{d}	60±2.64	1.67±0.1^{a}	100±0^{a}
Ca0P1	51.74±0.08^{d}	291.72±0.64^{d}	1.66±0.02^{cd}	58.33±3.78	1.51±0.1^{ab}	100±0^{a}
Ca0P2	53.1±0.12^{bc}	306±3.04^{bc}	1.70±0.01^{bc}	56±2.64	1.39±0.07^{b}	100±0^{a}
Ca1P0	52.70±0.49^{cd}	298.71±5.56^{cd}	1.68±0.01^{c}	55.16±0.76	1.39±0.02^{b}	100±0^{a}
Ca1P1	55.92±0.15^{a}	326.51±4.63^{a}	1.76±0.01^{ab}	60±1.32	1.4±0.02^{b}	100±0^{a}
Ca1P2	53.03±0.09^{bc}	304.92±2.85^{bc}	1.70±0.02^{bc}	56.33±1.52	1.41±0.03^{b}	98.3±1^{b}
Ca2P0	54.05±0.08^{b}	312.83±3.95^{b}	1.72±0.01^{abc}	56.33±1.52	1.37±0.03^{b}	100±0^{a}
Ca2P1	53.65±0.39^{bc}	307.58±4.47^{bc}	1.71±0.01^{bc}	56±2.05	1.38±0.04^{b}	100±0^{a}
Ca2P2	57.03±0.26^{a}	335.58±2.42^{a}	1.79±0.00^{a}	59±1.03	1.34±0.02^{b}	100±0^{a}
Two-way ANOVA (p-value)						
Ca	< 0.05	< 0.05	< 0.05	> 0.05	< 0.05	< 0.05
P	< 0.05	< 0.05	< 0.05	> 0.05	< 0.05	< 0.05
Ca*P	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

Mean values of triplicates ± Standard deviation mean; Means within same column sharing dissimilar letters differ significantly ($p < 0.05$). FW, Final weight; WG%, weight gain%; SGR, Specific growth rate; FI, Feed intake; FCR, Feed conversion ratio; SR%, Survival rate%.

Table III. Effect of different experimental diets on whole-body composition (%) of *H. molitrix* juveniles.

Diets	Moisture	Protein	Fat	Ash
Ca0P0	78.83±3.23	16.1±0.15^{b}	4.33±0.1	2.21±0.11
Ca0P1	75.26±0.68	18.68±0.41^{a}	4.35±0.11	2.16±0.01
Ca0P2	74.75±0.44	18.1±0.48^{a}	4.27±0.07	2.29±0.18
Ca1P0	74.81±0.76	18.1±0.19^{a}	4.22±0.07	2.16±0.08
Ca1P1	73.83±0.7	18.14±0.21^{a}	4.25±0.06	2.22±0.03
Ca1P2	74.18±0.7	18.21±0.21^{a}	4.25±0.10	2.16±0.03
Ca2P0	73.79±0.99	18.24±0.29^{a}	4.25±0.02	2.23±0.07
Ca2P1	74.8±1.15	18.14±0.18^{a}	4.29±0.02	2.27±0.03
Ca2P2	74.3±0.43	18.26±0.03^{a}	4.26±0.06	2.18±0.01
Two-way ANOVA (p-value)				
Ca	> 0.05	< 0.05	> 0.05	> 0.05
P	> 0.05	< 0.05	> 0.05	> 0.05
Ca*P	> 0.05	< 0.05	> 0.05	> 0.05

Mean values of triplicates ± Standard deviation mean; Means within same column sharing dissimilar letters differ significantly (p < 0 .05).

Mineralization

Results indicated that Ca and P contents showed significant increase with the increase in Ca and P levels in the body, bones and scales, achieving the highest values at 2% Ca supplemented with 2% P (Tables V-VII). Whereas, Mg content showed significant reduction with increasing Ca supplementation. With regard to the effect of P, maximum (p < 0.05) Mg content was recorded at 1% P in bones and 2% P in body and scales. Body and bone Zn level also tend to decrease with an increase in Ca and P supplementation (p < 0.05). However, an opposite trend (p < 0.05) was noticed in scales where the highest (p < 0.05) Zn content was recorded at 2% Ca level supplemented with 2% P. The Ca/P ratio was improved (p < 0.05) by Ca, P and their interaction. Fish fed the diet containing 2% Ca level without P supplement had higher Ca/P ratio in body and bones compared to those fed with other diets (p < 0.05). Whereas, scales Ca/P ratio tend to decrease at 2% Ca level. Further, increase in P supplementation significantly reduced (p < 0.05) Ca/P ratio regardless of Ca level.

Table IV. Effect of different experimental diets on nutrient digestibility (%) of *H. molitrix* juveniles.

Diets	Digestibility of DM	Digestibility of protein	Digestibility of fat
Ca0P0	61.34±0.99	61.85±0.77^{c}	64.66±0.35^{b}
Ca0P1	61.6±1.09	63.21±1.76^{c}	65.61±0.46^{ab}
Ca0P2	62.25±0.71	63.28±1.61^{c}	65.53±4.77^{ab}
Ca1P0	61.95±1.11	63.51±0.74^{bc}	64.01±0.47^{b}
Ca1P1	63.07±1.6	68.27±2.26^{ab}	68.59±2.78^{ab}
Ca1P2	61.7±0.35	68.76±0.47^{a}	68.62±2.34^{ab}
Ca2P0	61.88±0.75	70.34±0.47^{a}	68.56±0.87^{ab}
Ca2P1	61.04±0.62	70.72±0.86^{a}	68.96±0.35^{ab}
Ca2P2	63.15±0.26	71.13±0.65^{a}	71.01±2.05^{a}
Two-way ANOVA (p-value)			
Ca	> 0.05	< 0.05	< 0.05
P	> 0.05	< 0.05	< 0.05
Ca*P	> 0.05	> 0.05	> 0.05

Mean values of triplicates ± Standard deviation mean; Means within same column sharing dissimilar letters differ significantly (p < 0.05).

Table V. Effect of different experimental diets on levels of minerals in whole-body of *H. molitrix* juveniles.

Diets	Ca (mg/g)	P (mg/g)	Mg (mg/g)	Zn (µg/g)	Ca/P
Ca0P0	8.64±0.02^{h}	5.81±0.02^{h}	0.28±0.01^{c}	38.91±0.03^{a}	1.48±0.01^{d}
Ca0P1	8.70±0.01^{g}	6.11±0.01^{e}	0.38±0.00^{ab}	37.93±0.03^{b}	1.42±0.00^{f}
Ca0P2	8.93±0.02^{f}	6.35±0.01^{c}	0.41±0.01^{a}	37.1±0.04^{e}	1.4±0.00^{g}
Ca1P0	8.97±0.02^{f}	5.91±0.01^{g}	0.23±0.01^{d}	37.84±0.07^{b}	1.51±0.00^{c}
Ca1P1	9.04±0.00^{e}	6.23±0.01^{d}	0.35±0.01^{b}	37.69±0.04^{c}	1.45±0.00^{e}
Ca1P2	9.11±0.01^{d}	6.41±0.02^{b}	0.39±0.01^{a}	37.55±0.02^{d}	1.42±0.00^{f}
Ca2P0	9.49±0.03^{c}	5.99±0.02^{f}	0.18±0.01^{e}	36.95±0.04^{f}	1.58±0.00^{a}
Ca2P1	9.68±0.01^{b}	6.19±0.01^{d}	0.23±0.01^{d}	36.78±0.03^{g}	1.56±0.00^{b}
Ca2P2	9.82±0.03^{a}	6.60±0.01^{a}	0.29±0.01^{c}	36.57±0.02^{h}	1.48±0.00^{d}
Two-way ANOVA (p-value)					
Ca	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
P	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Ca*P	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

Mean values of triplicates ± Standard deviation mean; Means within same column sharing dissimilar letters differ significantly (p < .05).

Table VI. Effect of different experimental diets on levels of minerals in bones of *H. molitrix* juveniles.

Diets	Ca (mg/g)	P (mg/g)	Mg (mg/g)	Zn (µg/g)	Ca/P
Ca0P0	158.54±0.05^{i}	82.23±0.07^{i}	3.33±0.05^{e}	155.21±0.29^{e}	1.92±0.00^{e}
Ca0P1	166.93±0.73^{h}	93.93±0.1^{f}	3.51±0.01^{bc}	155.18±0.13^{e}	1.77±0.00^{g}
Ca0P2	188.82±0.07^{g}	100.21±0.1^{d}	3.43±0.01^{d}	154.13±0.22^{d}	1.88±0.00^{f}
Ca1P0	192.47±0.02^{f}	89.06±0.1^{h}	3.62±0.01^{a}	158.88±0.2^{a}	2.16±0.00^{c}
Ca1P1	199.99±0.06^{e}	90.3±0.02^{g}	3.56±0.01^{ab}	158.02±0.1^{b}	2.21±0.00^{b}
Ca1P2	204.14±0.03^{d}	104.15±0.04^{b}	3.51±0.01^{bc}	154.96±0.13^{e}	1.96±0.00^{d}
Ca2P0	217.19±0.02^{c}	97.27±0.03^{e}	3.41±0.01^{d}	150.12±0.13^{f}	2.23±0.00^{a}
Ca2P1	222.53±0.16^{b}	100.94±0.38^{c}	3.47±0.02^{cd}	152.57±0.41^{e}	2.2±0.00^{b}
Ca2P2	240.91±0.2^{a}	111.94±0.11^{a}	3.44±0.01^{d}	148.79±0.04^{g}	2.15±0.00^{c}
Two-way ANOVA (p-value)					
Ca	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
P	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Ca*P	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

Mean values of triplicates ± Standard deviation mean; Means within same column sharing dissimilar letters differ significantly ($p < .05$).

Table VII. Effect of different experimental diets on levels of minerals in scales of *H. molitrix* juveniles.

Diets	Ca (mg/g)	P (mg/g)	Mg(mg/g)	Zn (µg/g)	Ca/P
Ca0P0	123.15±0.27^{i}	44.85±0.08^{i}	2.41±0.02^{d}	142.34±0.08^{f}	2.74±0.00^{d}
Ca0P1	128.01±0.1^{h}	51.84±0.05^{d}	2.51±0.01^{bc}	142.42±0.11^{f}	2.46±0.00^{g}
Ca0P2	132.05±0.28^{g}	57.3±0.01^{c}	2.63±0.02^{a}	150.08±0.19^{b}	2.3±0.00^{h}
Ca1P0	143.96±0.13^{f}	46.6±0.2^{h}	2.35±0.02^{e}	148.5±0.17^{c}	3.08±0.01^{a}
Ca1P1	145.19±0.01^{e}	46.99±0.11^{g}	2.27±0.01^{g}	148.44±0.39^{c}	3.08±0.00^{a}
Ca1P2	147.69±0.04^{d}	59.41±0.02^{b}	2.55±0.02^{b}	152.73±0.26^{a}	2.48±0.00^{f}
Ca2P0	152.49±0.03^{c}	50.07±0.04^{f}	2.29±0.01^{fg}	139.96±0.16^{g}	3.04±0.00^{b}
Ca2P1	153.14±0.03^{b}	51.19±0.02^{e}	2.34±0.02^{ef}	143.45±0.22^{e}	2.99±0.00^{c}
Ca2P2	156.41±0.02^{c}	61.08±0.09^{a}	2.48±0.01^{c}	145.28±0.03^{d}	2.56±0.00^{e}
Two-way ANOVA (p-value)					
Ca	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
P	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Ca*P	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

Mean values of triplicates ± Standard deviation mean; Means within same column sharing dissimilar letters differ significantly ($p < .05$).

DISCUSSION

In the contemporary study, fish fed the diets without Ca supplement showed no symptoms of Ca deficiency indicating that silver carp might absorb water-borne Ca rather than from artificial feed to satisfy their Ca requirement for optimum performance. Moreover, P unsupplemented diets showed significantly lower FW, WG% and SGR than P supplemented diets. It implies that dietary P supplementation is very necessary for the optimum growth performance of silver carp. Similar phenomenon was observed in yellowtail (Sarker *et al*., 2009), European sea bass (Oliva-Teles and Pimentel-Rodrigues, 2004), rainbow trout, (Koko *et al*., 2010) haddock (Roy and Lall, 2003) and Chinese mitten crab (Lei *et al*., 2021). The poor growth performance due to P deficiency corresponds to the impaired metabolic functions in fish (Vielma *et al*., 2002).

Fish fed the diet containing 1% Ca with 1% P yielded the similar growth performance as exhibited by that fed 2% Ca with 2% P (Ca/P=1), in this study. This ratio was in accordance to the findings of earlier researchers who also reported Ca/P ratio of 1:1 improved the growth, feed utilization and mineralization in fish (Nakamura and Yamada, 1980; Nose, 1975; Phillips, 1959; Satoh *et al*.,

1991; Sakamoto, 1973). In addition, Yang *et al.* (2006) observed that Ca/P ratio of 1:1 found to be optimum in terms of growth and mineral deposition. Sanchez *et al.* (2000) reported that Ca/P ratio of 1:3 was important for the normal growth of in American cichlid. Moreover, Laining *et al.* (2011) reported a significant improvement in the growth performance of tiger puffer at 0:5 Ca/P ratio. In African catfish, Ca/P ratio of 2:1.5 was documented as effective ratio for the optimum growth of fish (Nawanna and Oni, 2018). Further, 0.6:1 ratio improved the growth, feed utilization, whole body composition and nutrient digestibility in Nile tilapia (Hassaan *et al.*, 2013). On the contrary, no optimum Ca/P ratio was documented in Pacific white shrimp (Davis *et al.*, 1993), black tiger shrimp (Peñaflorida, 1999), catfish (Andrews *et al.*, 1973) and juvenile abalone (Coote *et al.*, 1996; Tan *et al.*, 2001).

The analysis of whole-body proximate composition can be used for estimating the nutrient utilization and vigorous health of fish (Fernández-Gimenez *et al.*, 2009). Increased body protein as a results of increased dietary P level, in our study, coincides with the results of Tan *et al.* (2001) and Eya and Lovell (1997). This may be attributed to the availability of high-energy yielding nucleotides by high P levels for protein synthesis (Tan *et al.*, 2001). In this study, Ca and P supplemental levels had a non-significant effect on whole body fat content. On the contrary, significant reduction in body fat content was observed in African catfish (Nwanna and Oni, 2018), grouper (Ye *et al.*, 2006), abalone (Tan *et al.*, 2001) and discus (Liu *et al.*, 2021) in response to Ca and P supplemented diets. The findings of these studies indicated that supplemental P plays a vital role in fat reduction in fish by yielding fatty Acyl-CoA through enhanced esterification of the free fatty acid with extra-mitochondrial CoA. Moreover, it inhibits the fatty acids formation via TCA cycle from amino acids and resulted in reduced fat deposition in fish body (Roy and Lall, 2003). The increased ash content in whole body and bone corresponds to P retention in body tissues and supply of P from feed (Ye *et al.*, 2006). No significant variations in ash content was recorded for Ca and P supplementation in our study. Similar trend was narrated by Robinson *et al.* (1987) in tilapia. Contrarily, Ye *et al.* (2006) observed significantly increased ash content in response to Ca and P supplemented diets. These discrepancies in the results may owe to the variations in fish species, diet composition, experimental conditions and trial duration. Moreover, moisture content in whole body remained unaffected by Ca, P levels and their interaction in this study. This result was in line to the findings of other researchers who also documented a non-significant influence of Ca and P supplementation on moisture content (Tan *et al.*, 2001; Ye *et al.*, 2006).

Significantly improved protein digestibility was recorded at 1% Ca feeding which was not significantly different from that of 2% Ca level, in this experiment. Similarly, Hassaan *et al.* (2013) reported significant improvement in protein digestibility for the supplementation of Ca/P of 0.6:1 with 1000 U/kg phytase in Nile tilapia. In addition, Laining *et al.* (2011) concluded that Ca/P ratio of 0.5 combined with 2000 FTU phytase per kg significantly improved the protein digestibility. This showed that phytase supplementation with low Ca/P ratio improved the growth of fish.

Bones, scales, and whole body are considered reliable indicators for evaluating the mineral utilization especially P in fish (Ye *et al.*, 2006). Scales are the major sites of Ca storage and metabolism in fish. Moreover, exchange rate of Ca is three folds in scales than that of bone which tended to decrease during stressful conditions (Liu *et al.*, 2021). Whole body, bones and scales were chosen to compare the response of these samples to different Ca and P levels in this study. The comparison will help in the indication of P utilization in tissues. The lower mineral contents in the whole body, bones and scales as a result of without P supplementation diet. Feeding, in this study, showed that P is essential element for mineral deposition in fish. These findings are in consistent with the results of other researchers who also reported un-supplemented P diet caused a marked reduction of mineral deposition in fish (Roy and Lall, 2003; Baeverfjord *et al.*, 1998; Andrews *et al.*, 1973; Ogino and Takeda, 1978; Lovell, 1978; Brown *et al.*, 1993; Wilson *et al.*, 1982; Shim and Ho, 1989; Sakamoto and Yone, 1978; Watanabe *et al.*, 1980; Skonberg *et al.*, 1997). In the current study, the maximum Ca and P concentration in body, bone and scales was observed in 2% Ca with 2% P fed fish, indicating that uptake of Ca from the diet or surrounding water with adequate P was sufficient for bone mineralization. Similar phenomenon was observed in discus fish (Liu *et al.*, 2021), juvenile grouper (Ye *et al.*, 2006) and African catfish (Andrews *et al.*, 1973). Magnesium is present in scales and hard tissues of dermal and skeletal bones of fish. In this study, significant reduction in Mg content was recorded with the increasing Ca and P levels in diets in whole body and scales. The result was in accordance to the findings of earlier researchers who reported that excess Ca and P level had the antagonistic effects on the deposition and metabolism of Mg content in fish body (Andrews *et al.*, 1973; Cheng *et al.*, 2006; Hardy and Shearer, 1985; Porn-Ngam *et al.*, 1993; Vielma and Lall, 1998). The reduction in Mg concentration might be attributed to increase in sodium and potassium ions in scales due to enhanced membrane permeability by increased Ca and P levels (Hossain and Yoshimatsu, 2014; Peñaflorida, 1999). This

increase in Na/K^+ ions caused the negative effects on the Mg concentration that plays key roles in cell metabolism, osmoregulation, mineralization and neuromuscular transmission (NRC, 2011). Zinc influenced the bone mineralization by acting as a divalent cation, cofactor for nucleation and mineral deposition (Ye *et al*., 2006). Zinc deposition showed a negative correlation with increased Ca and P supplementation in this study. This result is in concurrent to the findings of Hossain and Furuichi (2000b) who observed a significant reduction in Zn content from 0.162 to 0.076 mg/g in vertebrae in response to 2.5% tricalcium phosphate. Likewise, Apines *et al*. (2003) also reported an inhibitory effect of 4% tricalcium phosphate on Zn content in rainbow trout. This is because high Ca and P levels interfere with the absorption of trace elements and Mg, resulting in poor mineralization in bones. In the present experimental study, Ca/P ratio was improved by Ca, P and their interaction. This showed that silver carp has a great potential to stabilize Ca/P ratio through absorption from surrounding water in response to Ca and P fluctuations in diet. This finding is supported by Liu *et al*. (2021) who also observed significant positive effect of Ca and P supplementation on Ca/P ratio in discus.

CONCLUSION

In summary, silver carp appear to require no more than 1% Ca in the diet. With respect to P, 1% P yielded the optimum growth performance of fish. Although, increase in P supplementation up to 2% is not found detrimental for fish. Thus, dietary supplementation of 1% Ca with 1% P (Ca/P =1) is recommended for better growth performance of silver carp. However, Ca should not added be supplemented in excessive amount as it negatively affects the mineralization in fish.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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