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Title: **Influence of Calcium and Phosphorous Ratio on Hematology and Muscle Proximate Composition of *Hypophthalmichthys molitrix* Fingerlings**

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Influence of Calcium and Phosphorous Ratio on Hematology and Muscle Proximate Composition of *Hypophthalmichthys molitrix* Fingerlings

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ABSTRACT

Background. Calcium (Ca) and phosphorous (P) are essential minerals for fish growth and development, but Ca absorption from water is limited and its presence also influences P absorption. Therefore, dietary Ca and P are vital for fish to perform physiological activities efficiently.

Methodology. This study investigated the interactive effect of Ca and P ratio (Ca:P) on muscle proximate composition and hematology of *Hypophthalmichthys molitrix* fingerlings (average initial weight = 13.7 ± 0.05 g) for 90 days. Calcium lactate and sodium di-phosphate were used as Ca and P sources. A total of 9 isonitrogenous, isolipidic, and isocaloric diets were formulated by combining 3 Ca levels (0%, 1%, and 2%) with three P levels (0%, 1%, and 2%) and fed to fish twice a day at 5% body weight.

Results. The results showed that moisture and crude protein content in muscles significantly increased ($p<0.05$) the interactive effect of Ca and P except fat and ash content ($p>0.05$). Furthermore, the hematological parameters of fingerlings remained unaffected ($p>0.05$) by the individual supplementation of Ca. However, P supplementation significantly affected the MCV, MHCH, and PLT count. Moreover, the interactive supplementation of Ca and P did not show a significant effect except HCT and MHCH. Platelets count increased at 1% of Ca and 1% of P supplementation, while the RBC count increased at 2% and 1 to 2% (Ca/P). The remaining blood counts did not show considerable variation upon supplementation of Ca/P at different levels.

Conclusion. It was concluded that mineral supplementation showed promising results at 1:2 (Ca/P) level for the optimum performance of *H. molitrix*.

Keywords: Calcium, Ca/P, hematology, phosphorus, proximate composition, silver carp

Highlights

- A balanced Ca:P significantly affects the hematological parameters and muscle proximate composition of *H. molitrix* fingerlings.
- Appropriate Ca:P ratio in the diet of *H. molitrix* significantly improved the moisture and crude protein content and reduced the crude fat content in muscles.

- The optimal Ca:P ratio substantially influences HCT, and MHCH in the blood of *H. molitrix* fingerlings, indicating a critical role of Ca:P balance in maintaining healthy blood parameters.

1. INTRODUCTION

Aquaculture is a vibrant sector producing high-protein and nutritious food that any other food commodity cannot easily substitute [1]. Its contribution to human nutrition has been fully recognized. It is widely understood that nutrition-sensitive approaches must be promoted [2]. Hence, the demand for aquatic food is expected to grow even higher in the coming decades as the global population is anticipated to approach 10 billion by 2050 [3]. Fishmeal is valued in aquaculture for its high protein content and palatability [4], but its rising costs and limited availability have prompted the exploration of sustainable alternatives, particularly plant protein sources including full-fat soybean meal [5, 6]. However, the presence of anti-nutritional factors in soybean, such as phytic acids and lectins, may hinder nutrient absorption [7–9]. To mitigate these effects, mineral supplementation in a diet of fish is essential [9, 10].

Fish require minerals for the development of the skeleton, electron transfer chain, tissue development, regulation of acid-base balance, synthesis of membrane potential, and osmoregulation [11]. Fish require macromolecules *viz.* calcium (Ca), magnesium (Mg), phosphorous (P), and potassium (K) [12]. Ca and P are considered essential minerals because they contribute up to 70% of the total minerals in fish body [12]. Ca is involved in proper growth, transmission of nerve impulses, bone mineralization, physiological functions such as blood clotting, ionic regulation in fresh water, and activation of several enzymes [12, 13]. Freshwater contains a high level of Ca which aquatic animals sufficiently absorb to carry out metabolic reactions [14, 15]. Some studies have reported that water Ca is not absorbed by some fish species, such as tilapia [16], channel catfish [17], red lip mullet [18], and American cichlid [19]. So, they must be supplied with dietary Ca supplementation for proper body growth. Ca is also necessary for the uptake of P because Ca serves as a carrier for the binding of Ca and P in the intestine of fish [20].

On the other hand, P supplementation is also essential in fish diet because water has a low level of dissolved P which remains insufficient for the survival of fish, therefore, fish must obtain sufficient P from their food [21–23]. P is required as an important component of nucleic acid and the phospholipid bilayer of cell membrane which is directly involved in cell metabolism. Its deficiency causes adverse effects on fish growth, feed efficiency, bone mineralization, production of ATP, dark colouration, and cell membrane that causes anorexia [13, 24]. The supplementation of P has been found to improve the growth of snakehead [25], catfish [26], and blunt snout bream [22]. It has been reported that the range of P requirements in fish varies from 3.0 to 15.0 g/kg of diet [27]. Many fishes can maintain a balanced Ca and P ratio in their body.

Thus, appropriate Ca/P level must be optimized in the formulated diet of fish. Otherwise, an imbalanced Ca/P ratio may cause adverse effects on bone mineralization and physiological processes [12, 28]. A high level of dietary Ca interferes with P absorption in the intestine because the combination of Ca and P forms

insoluble complexes of calcium phosphate [29, 30]. This complex results in the abundant excretion of undigested nutrients, particularly P, into water bodies that raise the level of algal bloom and contribute to water pollution [30]. Moreover, a high level of Ca also causes an inhibitory effect on the absorption of trace elements, such as iron, manganese, and zinc [12]. It is recommended that the levels of Ca/P ratio for fish diet must be approximately within the range of 0.5:1 to 41:1.3 [31]. Hence, it is crucial to optimize the balanced Ca/P level in artificial diet for proper growth and physiological functions of silver carp fish.

H. molitrix, commonly known as silver carp, is a native fish of the temperate freshwaters of China and is widely cultivated in South Asian countries [32–34]. Due to its rapid growth, tempting taste, and cost-effectiveness, it is a potential candidate for aquaculture [33, 35]. In addition, it is a rich source of unsaturated fatty acids, protein content, and essential amino acids that make it a better alternative to marine fish for the processing of surimi [35]. There is scant literature available on silver carps nutritional requirement. Currently, the researchers are interested in formulating a balanced artificial diet for silver carp [36]. Therefore, there is a need to optimize the Ca/P level in the artificial diet for silver carp. Hence, the findings of the current research would help the fish farmers to manufacture a balanced and optimized diet for the excellent fish farming of silver carp.

2. MATERIALS AND METHODS

2.1. Ethical Statement, Study Species, and Site Selection

The silver carp (*H. molitrix*) fingerlings were taken from the Fish Hatchery, University of Veterinary and Animal Sciences, Ravi Campus, C Block, Pattoki. The research was conducted in the Fish Rearing Unit of the Department of Fisheries and Aquaculture, UVAS, Pattoki, following approval from the university's ethical review committee.

2.2. Preparation and Diet Formulation

The dry ingredients including fish meal, canola meal, soya bean meal, wheat flour, rice polish, and vitamin C were obtained from the local ingredients market, while fish oil was procured from Poultry vet-Co, Karachi, Pakistan. Calcium lactate (Sigma-Aldrich) was used as Ca supplement and sodium diphosphate (Sigma-Aldrich) was used as P supplement in the experimental diet. A total of 9 isolipidic, isonitrogenous, and isocaloric experimental diets were formulated from basal diet 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 named as D_1, D_2, D_3, D_4, D_5, D_6, D_7, D_8, and D_9 (Table 1). The dry ingredients were ground and screened (0.05mm) in the grinder (KENWOOD). Then, vitamin premix, mineral mixture and fish oil were added and mixed electrically to formulate the diet. Dough was formed after the addition of 20% water. Afterward, the pellets were formed by using a meat mincer (ANEX). The pellets were sun-dried to approximately 10% moisture content and stored in polythene bags [37].

2.3. Feeding Trial and Collection of Sample

Before beginning the feeding trial, the fingerlings were acclimatized to laboratory conditions after being treated with $KMnO_4$ (5g/L) for 1-2 hours to prevent them from contracting diseases. Then, 15 fish fingerlings were stocked randomly in each aquarium for trial. The fingerlings were fed with basal diet twice a day at a rate of 3% of their wet weight. Each treatment diet was

fed to triplicate tanks. After 3 hours of feeding, the uneaten diet was removed, and the water was changed regularly. The optimum temperature, dissolved oxygen, and pH were monitored constantly in tanks throughout the experimental trial [38] which continued for 90 days. After the feeding trial, the fingerlings were starved for a day and then harvested. They were anaesthetized for a minute using clove oil (3000 mg/l) [39].

Table 1. Composition of Experimental Diets (%)

Ingredients	Experimental diets[1]								
	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9
Fish meal	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00
Soya bean meal	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Canola meal	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Rice polish	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Wheat flour	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50
Fish oil	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Vitamin C	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin premix*	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Mineral mixture (Ca and P)**	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Chromic oxide	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Calcium Lactate	0.00	0.00	0.00	7.69	7.69	7.69	15.38	15.38	15.38
Sodium di phosphate	0.00	3.10	6.20	0.00	3.10	6.20	0.00	3.10	6.20
Proximate composition									
Dry Matter	92.23	92.27	93.01	92.89	91.98	92.51	93.01	91.53	92.25
Crude Protein	30.74	31.04	31.5	30.85	30.50	32.75	31.53	31.84	30.57
Crude Fat	9.23	8.78	9.21	7.99	8.82	9.12	8.89	7.89	9.32
Crude Ash	2.26	2.25	2.25	2.24	2.26	2.21	2.31	2.21	2.25

[1]Experimental diets containing Ca and P ratio: D_1 = 0:0 (Ca : P), D_2 = 0:1 (Ca:P), D_3 = 0:2 (Ca:P), D_4 = 1:0 (Ca:P), D_5 = 1:1 (Ca :P), D_6 = 1:2 (Ca : P), D_7 = 2:0 (Ca : P), D_8 = 2:1 (Ca :P), D_9 = 2:2 (Ca:P). *Each Kg of Vitamin premix contains: Vit. A1 5 M.I.U. Vit. D3 3 M.I.U. Nicotinic acid 25000mg Vit. B1 5000mg Vit. E 6000 IU. Vitamin B2 6000mg Vit. K3 4000mg Vit. B6 4000mg Folic acid 750mg Vit. B12 9000mg Vit. C 15000mg Calcium pantothenate 10,000mg.

**Mineral mixture (Ca and P): $MgSO_4$.$7H_2O$ (153 mg/g), NaCl (51 mg/g), COCl. $6H_2O$ (0.0816 mg/g), $AlCl_3.6H_2O$ (0.255 mg/g), $CuSO_4.5H_2O$ (210.67 mg/g), $FeSO_4.H_2O$ (100.67 mg/g), $MnSO_4.5H_2O$ (116.67 mg/g), $ZnSO_4.7H_2O$ (121.33 mg/g)

2.4. Analysis

2.4.1. Muscle Proximate Analysis.

2.4.1.1. Estimation of Dry Matter. The estimation of dry matter was done by following AOAC guidelines [40]. The moisture content was determined by drying the sample in the oven at 105°C for 12-24 hours. Then, the dried sample was transferred to the desiccator for 5-10 minutes. The moisture percentage was calculated by using the following formula:

$$Moisture\ (\%) = \frac{(Weight\ of\ Sample\ before\ drying - weight\ of\ sample\ after\ drying)}{Weight\ of\ sample} \times 100$$

$$Dry\ matter = 100 - moisture\ (\%)$$

2.4.1.2. Percentage Estimation of Crude Protein. The micro Kjeldahl apparatus was used to estimate the amount of nitrogen in feed and muscle samples by following the AOAC method [41]. The nitrogen percentage in the sample was calculated using the following formula:

The calculated nitrogen was multiplied by 6.25 for the estimation of crude protein percentage.

$$Nitrogen\ (\%) = \frac{(Volume\ of\ H2SO4\ used \times Normality\ of\ H2SO4 \times 0.014 \times 250)}{Weight\ of\ sample \times 10} \times 100$$

2.4.1.3. Percentage Extraction of Crude Fat and Percentage Estimation of Crude Ash. Crude fat was extracted by petroleum ether using Soxhlet apparatus, according to the guidelines of AOAC [41]. The formula to calculate the crude fat percentage is as follow:

$$Crude\ fat\ (\%) = \frac{(Weight\ before\ extraction - Weight\ after\ extraction)}{Weight\ of\ sample} \times 100$$

Following the AOAC standard method [41], the crude ash of feed and muscle samples was determined by using muffle furnace. The samples were ignited in a muffle furnace at 600°C till grey or whitish ash was obtained. Ash was placed in a desiccator for 3 minutes and its amount was recorded. The following formula was used to estimate the crude ash percentage of samples:

$$Crude\ ash\ (\%) = \frac{Weight\ of\ ash\ (g)}{Weight\ of\ sample\ (g)} \times 100$$

2.4.2. Hematology Analysis. For hematological analysis, the fingerlings were anesthetized using clove oil (3000mg/l). The blood sample (~100µl) was collected using 27G needle and 1ml syringe from the caudal vein of the fingerling (n=5/replicate) in the EDTA vial containing anti-coagulants to avoid blood clotting. Different hematological parameters including white blood cells (WBC), red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MHCH), and platelets (PLT) were analysed by automated hematology analyser (version celltac MEK-6550) [15].

2.4.3. Statistical Analysis. The normality of data was assessed by using the Kolmogrove-Smirnov test. The data were also subjected to Two-Way ANOVA to analyze the interaction of Ca and P levels.

Furthermore, a significant difference between diets was observed by applying One-Way ANOVA, followed by post hoc Tukey test of significance. The whole data were analyzed by using Co-Stat statistical software package (6.303 version) [42].

3. RESULTS

3.1. Muscle Proximate Composition

Table 2 illustrates the muscle proximate composition of *H. molitrix* fingerlings when fed with different Ca × P levels. The moisture content significantly ($p<0.05$) decreased in diets D_7, D_8, and D_9, with the corresponding increase in the levels of Ca (2%) and P (2%), demonstrating both the individual and the interactive effect of Ca and P. Crude protein significantly increased ($p<0.05$) with higher levels of Ca (2%) and P (2%), both individually and interactively. Moreover, the interactive effect of Ca and P was significant ($p<0.05$) for individual P, but no significant effect ($p>0.05$) was observed for Ca (individual) or their interaction (Ca×P). Furthermore, crude ash showed a significant effect for both Ca and P individually, but their interaction (Ca×P) showed non-significant results.

Table 2. Effect of Ca and P Levels on Muscle Composition of *H. molitrix* Fingerlings

Diets	Ca× P (%)	Moisture (%) (M±SD)*	Crude protein (%) (M±SD)*	Crude Fat (%) (M±SD)*	Crude Ash (%) (M±SD)*
D_1	(0,0)	75.53 ± 0.06^{f}	17.30 ± 0.01^{b}	4.48 ± 0.01^{e}	1.41 ± 0.01^{a}
D_2	(0,1)	75.45 ± 0.06^{d}	17.23 ± 0.006^{a}	4.31 ± 0.01^{d}	1.45 ± 0.006^{b}
D_3	(0,2)	75.50 ± 0.01^{e}	17.33 ± 0.006^{c}	4.25 ± 0.006^{b}	1.50 ± 0.01^{cd}
D_4	(1,0)	75.14 ± 0.01^{c}	17.69 ± 0.01^{d}	4.44 ± 0.02^{e}	1.46 ± 0.006^{b}
D_5	(1,1)	75.09 ± 0.01^{b}	17.70 ± 0.01^{d}	4.30 ± 0.01^{cd}	1.51 ± 0.01^{d}
D_6	(1,2)	75.09 ± 0.02^{b}	17.71 ± 0.01^{d}	4.21 ± 0.01^{a}	1.56 ± 0.006^{e}
D_7	(2,0)	74.79 ± 0.01^{a}	17.96 ± 0.02^{e}	4.46 ± 0.01^{e}	1.49 ± 0.01^{c}
D_8	(2,1)	74.80 ± 0.01^{a}	18.00 ± 0.02^{f}	4.30 ± 0.005^{cd}	1.56 ± 0.01^{e}
D_9	(2,2)	74.79 ± 0.01^{a}	18.05 ± 0.02^{g}	4.26 ± 0.05^{b}	1.61 ± 0.01^{a}

Two Way ANOVA

	Ca	P	Ca×P
Moisture	0.000	0.006	0.027
Crude protein	0.000	0.000	0.009
Crude fat	0.104	0.000	0.868
Crude ash	0.000	0.000	0.156

*M±SD (Mean±Standard deviation); Different alphabets within same column representing the significant difference ($p<0.05$).

Table 3. Effect of Ca and P Levels on Hematological Parameters of *H. molitrix* Fingerlings

Diets	Ca× P (%)	WBCs (×10^3/ UL) (M±SD)*	RBC (10^6/UL) (M±SD)*	HGB (g/ dL) (M±SD)*	HCT (%) (M±SD)*	MCV (f L) (M±SD)*	MCH (pg) (M±SD)*	MHCH (g/d L) (M±SD)*	PLT (10^3/UL) (M±SD)*
D_1	(0,0)	5.20±0.14^a	0.21±0.007^d	5.80±0.14^a	34.50±0.14^a	239.50±O.71^a	48.35±0.21^a	20.20±0.14^a	127.50±0.71^a
D_2	(0,1)	5.45±0.07^a	0.33±0.04^d	5.70±0.14^a	34.70±0.14^a	241.50±0.71^a	48.45±0.21^a	20.30±0.14^a	127.00±1.41^a
D_3	(0,2)	5.15±0.07^a	0.85±0.01^c	5.70±0.14^a	34.60±0.14^a	241.50±0.85^a	48.2±0.57^a	20.70±0.14^a	126.60±0.85^a
D_4	(1,0)	4.95±0.07^b	1.02±0.02^c	5.75±0.21^a	34.80±0.14^a	241.50±0.42^a	48.05±0.21^a	20.45±0.07^a	126.90±0.14^a
D_5	(1,1)	5.15±0.21^a	1.37±0.01^b	5.70±0.42^a	34.20±0.14^a	241.50±0.21^a	48.05±0.70^a	19.95±0.07^a	128.95±0.07^a
D_6	(1,2)	5.20±0.14^a	1.32±0.007^b	5.70±0.14^a	34.35±0.07^a	240.25±0.35^a	48.35±0.70^a	20.35±0.07^a	125.70±0.99^a
D_7	(2,0)	5.15±0.21^a	2.36±0.02^a	5.45±0.07^a	34.60±0.14^a	239.60±0.85^a	48.30±0.14^a	20.45±0.21^a	127.40±0.14^a
D_8	(2,1)	5.20±0.14^a	2.34±0.01^a	5.65±0.21^a	34.35±0.21^a	241.55±0.78^a	48.55±0.07^a	20.10±0.14^a	127.15±0.35^a
D_9	(2,2)	5.10±0.14^a	2.33±0.02^a	5.80±0.14^a	34.50±0.21^a	241.45±0.78^a	48.20±0.14^a	20.35±0.07^a	125.95±1.34^a

Two Way ANOVA

	Ca	P	Ca×P
WBCs (10^3/ UL)	0.174	0.174	0.389
RBC (10^6/UL	0.484	0.178	0.849
HGB (g/ dL)	0.673	0.844	0.580
HCT (%)	0.237	0.087	0.053
MCV (f L)	0.985	0.019	0.082
MCH (pg)	0.318	0.668	0.385
MHCH (g/d L)	0.170	0.003	0.026
PLT (10^3/UL)	0.770	0.021	0.171

*M±SD (Mean±Standard deviation); Different alphabets within same column representing the significant difference ($p<0.05$).

3.2. Hematological Analysis

The results of hematological parameters are shown in Table 3. The statistical results depicted that there was no significant individual and interactive effect of Ca and P on hematological parameters. Briefly, WBCs, RBCs, HGB, HCT, and MHCH showed non-significant results, both individually and based on the interactive effect of Ca and P. Moreover, MCV and PLT showed non-significant results at Ca (individual) and based on the interactive effect (Ca × P), while the individual effect of P showed significant results. Meanwhile, MHCH showed a significant effect both at P (individual effect) and based on their interaction (Ca × P). It was noted that RBC count significantly increased on 2% Ca and 0 to 2% P, while PLT count significantly increased on 1% Ca and 1% P.

4. DISCUSSION

In the current study, the fingerlings fed with 0% Ca supplement did not show significant symptoms of Ca deficiency, rather they utilised water-borne Ca to fulfil their requirements for growth. On the other hand, artificial diets with 0% P supplementation reduced protein content more than P supplemented feed. This trend suggests that P supplementation is highly required for the better performance of fish, as studied in bighead carp juveniles [43], rainbow trout [44], Chinese mitten crab [45], and silver carp juveniles [46]. It was reported that Ca/P ratio (1:1) improved the performance and growth of carp [47], as observed in the current carp.

The individual Ca supplementation significantly affected the moisture, protein, and ash content, while P supplementation showed a significant effect on all the parameters of muscle proximate composition. In contrast, the individual supplementation of dietary P was found ineffective previously against the orange spotted grouper [48]. In another study, the increasing P supplementation above 6.5 g/kg significantly reduced ($p < 0.05$) the feed intake of silver carp [49]. The moisture content in muscles was not in line of the results of other studies[31, 50]. Interactive supplementation enhanced the protein content but the fat content did not vary considerably in the current study, as observed by other researchers in case of bighead carp [15]. The enhanced protein content was due to P supplementation because it contributes to the synthesis of proteins by providing the source of high-energy yielding nucleotides [50]. Moreover, the fat content was found to be significantly reduced in other studies on abalone [50] and catfish [51] that disagree with the current findings. The fat content was reduced due to the synthesis of fatty Acyl-CoA. Furthermore, citric acid cycle inhibited the fatty acid synthesis from amino acids that resulted in the reduction of fat content in juvenile haddock [52]. In case of ash content, an insignificant variation was recorded, similar to the reported data on *Orechromis aureus* [53] and juvnile *H. molitrix* [46]. In this regard, dissimilar results were reported on grouper juveniles which showed that ash content increased significantly [31]. These contradictions occurred due to the different diet compositions, geographical variations in the selection of experimental fish, experimental duration, and feeding trial conditions.

Blood parameters are considered as positive indicators of the physiological conditions and health status of fish, in response to dietary supplements [54]. The results of hematological parameters revealed that Ca supplemention had an insignificant effect and P supplementation

showed a significant effect on MCV, MHCH, and PLT. While, combined supplementation showed significant results only on HCT and MHCH. Overall, blood parameters did not show considerable variation in the supplementation of Ca/P in the current findings. Relatable results were reported on Ca/P supplementation of juvenile *Oreochromis niloticus* and *Ctenopharyngodon idella* [55]. A negative and linear relationship between the amount of phosphorous and dietary calcium content was reported in carps [47]. Previously, dietary Ca supplementation indicated an insignificant effect on RBCs, HCT, HGB, and WBC count of bighead carp, almost parallel to the current research [15]. Selenium is a trace element that showed significant impact on hematological parameters of common carp that is inconsistent with the current mineral supplementation [56]. However, there is scarce availability of data on the dietary effect of Ca/P supplementation on the blood parameters of carp.

4.1. Conclusion

Ca is required for the absorption of P, while individual Ca supplementation retards the growth and performance of silver carp. Hence, it was concluded that the combined supplementation of Ca (1%) and P (1% to 2%) showed promising results on muscle proximate composition and hematological parameters, rather than individual supplementation. So, the best Ca/P ratio (1:2) can be used to attain the optimum performance of silver carp.

CONFLICT OF INTEREST

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILTY STATEMENT

The data associated with this study will be provided by the corresponding author upon request.

FUNDING DETAILS

No funding has been received for this research.

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