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Effects of Dietary Phospholipids on Growth, Biochemical Composition, Fatty Acid Profiles, Blood Metrics, and Sex Steroid Hormone of Male *Pangasius nasutus* Broodstock

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Abstract

This study investigates the effects of phospholipids (PLs) supplemented diets on the growth, blood metrics, reproductive indices, and fatty acid profiles of male *Pangasius nasutus* (shark catfish) broodstock, a commercially valuable freshwater fish species. Over a 90-day feeding trial, four groups of fish were placed in a large open earthen pond within 4 cages with a control group. Each cage housed 15 fish, with an initial average weight of 643±0.34g and an average length of 43.3±0.12 cm. These fish were fed different levels of PLs (0, 1.5, 2, and 2.5%) in their diets twice daily, at 7:00 am and 6:00 pm, to satiation. The results showed that PLs supplementation significantly improved some of the biological indices, such as hepatosomatic index, coelomic fat index, feed conversion ratio, and survival rate. PLs supplementation also influenced the blood metrics, such as red blood cell count, haematocrit, haemoglobin, and white blood cell count. PLs supplementation significantly improved reproductive indices, including GSI, elevated 11-ketotestosterone production, and altered fatty acid profiles in male *P. nasutus*. These findings suggest PLs potential to enhance reproductive performance and fish nutrition, both of which are critical to aquaculture production.

Introduction

Pangasius nasutus, commonly known as shark catfish or patin buah, is a native species in peninsular Malaysia, primarily found in the Pahang Rivers and its tributaries (Abdul Halim et al., 2023), precisely in Maran and Pekan district (Asdari et al., 2011). This species is also found in the Mekong and Chao Phraya River basins, as well as river systems in Thailand, Cambodia, Laos, and Vietnam (Fauzi et al., 2023). *P. nasutus* holds significant cultural and economic value, it is often considered a delicacy in Malaysia, commanding a high market price estimated at RM 70-300/kg, which is three times higher than that of local black pangasius catfish species such as *Pangasius micronemus* and *Pangasius hypopthalmus* (Sani et al., 2023). The primary source of *P. nasutus* is still the wild population, primarily because of the challenges associated with its captive breeding, largely attributed to its high vulnerability to environmental stressors (Zulkiflee et al., 2020). This situation has led to a decline in the abundance of *P. nasutus* in the local rivers of Maran and Pekan, areas known for their dominance, resulting in its categorization as a moderately threatened species in Malaysia.

Overharvesting and habitat degradation are key contributors, supported by recent catch data (Baharuddin et al., 2014).

An understanding of the nutritional requirements and dietary influences on the growth, health, and reproductive performance of aquaculture species is paramount for sustainable fish farming practices (Hernandez de-Dios et al., 2022; Izquierdo et al., 2001). Phospholipids serve as fundamental structural elements in cell membranes, contribute to lipid metabolism, and are involved in numerous cellular signalling pathways (Dong et al., 2023; Lin et al., 2021; Martins et al., 2020; Mian et al., 2020). In addition to their structural role, phospholipids are known to influence nutrient digestion, absorption, and utilization (Murota, 2020). Phospholipids serve as essential reservoirs for fatty acids and play a crucial role in maintaining proper lipid metabolism (Qin et al., 2022). Studies on the effects of phospholipids supplementation on different parameters in fish including European sea bass *Dicentmrchus labrax L*. and turbot *Scophthalmus maximus L.* juveniles (Geurden et al., 1997), Atlantic salmon *Salmo salar* (De Santis et al., 2015), larval and juvenile large yellow croaker, *Larimichthys crocea* (Feng et al., 2017), Malaysian mahseer *Tor tambroides* (Mian et al., 2020) have been conducted.

Blood metrics, such as haematological and biochemical parameters, provide important information about the health and physiological condition of fish (Arthanari & Dhanapalan, 2016; Casanovas et al., 2021; Sebastião et al., 2011). Findings have indicated that dietary phospholipids can influence blood lipid profiles, antioxidant capacity, and immune responses (Lahnsteiner, 2022; Parrino et al., 2018). Phospholipid supplementation plays a critical role in hormone synthesis and regulation in male fish by providing essential phospholipids like phosphatidylcholine and phosphatidylinositol. These compounds serve as precursors for steroidogenesis and facilitate cell signalling pathways essential for hormone production, ultimately supporting reproductive functions and overall endocrine health (Martins et al., 2020; Torsabo et al., 2023). Although the effects of dietary phospholipids have been extensively studied in various fish species, covering the larvae and juvenile stages, their specific impacts on blood metrics, reproductive indices, and fatty acid profiles of male *P. nasutus* broodstock have not been examined. investigating the effects of phospholipid-supplemented diets on reproductive indices, such as gonadosomatic index, gonadal development, hormone profiles, and fatty acid profiles is essential for understanding the potential impact on the reproductive success of male *P. nasutus*. Therefore, this article examines the effects of phospholipid-supplemented diet on growth, blood metrics, reproductive indices, biochemical composition, and fatty acid profiles of male broodstock of *P. nasutus*. Understanding the outcomes of phospholipid supplementation in these key aspects will contribute to the knowledge base of *P. nasutus* aquaculture, providing valuable insights for optimizing broodstock nutrition and enhancing the production and sustainability of this economically important fish species.

Materials and Methods

Experimental Design

P. nastus male broodstocks were purchased from Three Ocean Fish Pond & Trading Sdn. Bhd. in Selangor, Malaysia, and moved to the experimental station within the same farm. Before the experiment, a three-week acclimatisation period was provided for the fish to adjust to the basal feed. During this phase, the fish were fed twice daily to satiation with a commercial feed TP 2 (Charoen Pokphand Foods, Malaysia) containing 32% protein and 4% lipid. After acclimatisation, fish were divided into four treatment groups: control (0%) and phospholipid-treated groups (1.5%, 2.0%, and 2.5%), with 15 fish distributed across four cages measuring (4×4×3 m) situated within an open earthen pond measuring (91.44×48.8 m). Replication was based on each fish within the tank due to space and the high price of broodstock procurement. The broodstock had an average initial weight of 643±0.34g and an average total length of 43.3±0.12 cm. During the entire experimental period, the fish were fed until satiation with the experimental diets at 9 a.m. and 5 p.m. daily. The water quality parameters were monitored daily and were within acceptable ranges throughout the experiment, maintaining a water temperature of 27.83°C and dissolved oxygen levels at 6.7 mg/L.

Diets Preparation/Coating

A commercial diet, TP 2 (Charoen Pokphand Foods, Malaysia) 4mm containing 32% protein and 4% lipid content was coated for this study. The treatment feeds were top coated with 0, 1.5, 2.0, and 2.5% of the phospholipid (Soy phospholipids, 11145, Sigma-Aldrich), resulting in total crude lipid levels of 5.62, 6.32, and 6.98%. These levels of PL were informed by prior studies that have reported beneficial effects of dietary phospholipids within similar ranges (Tocher et al., 2008). The commercial reference diet only received the manufacturer's top coating resulting in a 4% crude lipid level in the diet. The phospholipid granules were weighed in a beaker, mixed with water, and then heated to 45°C using a water bath to improve their flow. The liquid was then placed in a compressed air sprayer and slowly sprayed on the diet to allow even coverage as it was being mixed using a planetary mixer model B20-A (food machinery, China). Agar (M0660-500G, Sigma Aldrich) was used at 2% as a gelling agent. The agar was first dissolved in 50ml hot water (45°C) and sprayed on the constituted diets and the basal diet (control) in the mixer to ensure homogeneity. Due to the added moisture, the diet was placed the oven (35°C) overnight

to achieve a moisture content less than 10%. All constituted diets were packed, sealed in plastic bags, and stored in a -20°C freezer until use. The proximate composition was analysed following standardized protocols as outlined by the Association of Official Analytical Chemists, AOAC (2006) and fatty acid composition was analysed using the procedure by Abdulkadir & Tsuchiya, (2008). Phospholipids in the diet was quantified using the Phospholipid Assay Kit (MAK122, Sigma Aldrich) following the manufacturer's guidelines. Proximate and fatty acids composition of the experimental diet is presented in Table 1 and Table 2.

Sample Collection

After 90 days of the feeding trial, we sampled 5 fish from each tank (n=20) for the determination of growth performance and biological indices calculation, while 3 fish from each tank (n=12) were used for the rest of the analysis. Initially, the fish were moved from their culture cages into small fibre tanks. Ice was introduced into the tanks to immobilize the fish. The study was conducted in accordance with the animal ethic guidelines by the Institutional Animal Care and Use Committee (IACUC) at the Research Management and Innovation Centre, University Malaysia Terengganu. Subsequently, blood samples were collected from the caudal vertebral vein using 3 mL disposable plastic syringes and a 21-gauge needle. These blood samples were placed into ethylene diamine tetra acetic acid (EDTA) tubes for haematological analysis and plain BD vacutainers for biochemistry analysis. A portion of the blood (1.5 mL) was centrifuged at 3500 rpm (650 g) for 10 minutes, and the resulting plasma was stored in a -80°C freezer for later analysis of 11-ketotestosterone. Following the blood sampling, the fish were decapitated, and samples of the liver, muscle, and gonads were collected. These samples were stored in a -80°C freezer, subsequently freeze-dried, and milled to analyse fatty acid profiles and biochemical composition.

Growth Performance and Biological Indices

After conducting the study, we collected data on growth parameters and biological indices from 15 fish in each treatment (n=20). To do this, dissection of the specimens was done and the gonads, liver, coelomic fat, and visceral organs were collected. The gonadosomatic index (GSI), hepatosomatic index (HSI), viscerosomatic index (VSI), coelomic fat index (CFI), and condition factor (K) were calculated from the collected samples.

The following formulas were used to calculate the growth and biological indices:

$$\text{Specific growth rate (SGR \%)} = 100 \times \frac{\text{(Ln Final Weight-Ln Initial Weight)}}{\text{Total Time of the Experiment}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed provided (dried weight)}}{\text{weight gain (wet weight)}}$$

$$\text{Weight gain (WG)} = \text{Final weight (g)} - \text{Initial weight (g)}$$

Table 1. Proximate composition of experimental diets (% dry weight of feed)

Parameter	PL0	PL 1.5%	PL 2 %	PL 2.5%
Lipid	4.00	5.62	6.32	6.98
Protein	32.00	35.80	35.53	34.98
Ash	6.23	5.61	5.43	5.61
Moisture	5.55	6.21	6.31	6.56
Phospholipids	1.07	1.62	2.32	2.98

PL 1.5 (1.5% percent of phospholipid), PL 2 (2% of phospholipid), and PL 2.5 (2.5% percent of phospholipid)

Table 2. Fatty acid composition % of total fatty acid methyl esters (FAME) of the experimental feeds

Treatment	PL 0	PL1.5	PL2.0	PL2.5
C14:0	2.10	2.42	2.67	2.87
C16:0	15.77	16.77	17.70	18.80
C18:0	1.30	1.30	1.74	1.91
C16:1	3.55	4.44	4.56	4.57
C18:1	21.26	22.46	23.78	23.92
C20:1	1.11	1.15	1.40	1.47
C18:2n-6	29.34	30.16	31.23	31.44
C20:4n-6	0.45	0.67	0.77	0.85
C18:3n-3	4.41	4.51	4.76	4.98
C20:5n-3	2.79	3.98	4.21	4.08
C22:6n-3	4.22	4.25	4.33	4.78
n-3	11.42	12.74	13.97	13.84
n-6	29.79	30.83	32.00	32.29
n-3/n-6	0.38	0.41	0.41	0.43
∑ SFA	19.17	20.49	22.11	23.58
∑ MUFA	25.92	28.05	29.74	29.96
∑ PUFA	41.21	43.57	45.30	46.13

PL 0 (control), PL 1.5 (1.5% phospholipid), PL 2 (2% phospholipid), PL 2.5 (2.5% phospholipid)

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

$$\text{GSI} = 100 \times \frac{\text{Gonad weight (g)}}{\text{Body weight (g)}}$$

$$\text{HSI} = 100 \times \frac{\text{Liver weight (g)}}{\text{Body weight (g)}}$$

$$\text{VSI} = 100 \times \frac{\text{Visceral weight (g)}}{\text{Body weight (g)}}$$

$$\text{CFI} = 100 \times \frac{\text{Coelomic fat weight (g)}}{\text{Body weight (g)}}$$

$$K = 100\ (W/L^3)$$

Where W is the body weight of fish in g and L is the total length of fish in cm

Biochemical Analysis

The biochemical analysis for crude lipid, crude protein, moisture, and ash was conducted using samples collected from 3 fish in each tank following standardized protocols as outlined by the Association of Official Analytical Chemists, AOAC (2006). Samples used for the proximate analysis was initially freeze dried, therefore the analysis was done based on dry matter basis. The sample was dried in an oven at 105°C for 24 hours to achieve a consistent weight to determine the moisture content. The moisture content was then calculated as the difference between the initial dry weight of sample and the final dried weight. For the determination of ash content, the sample was carefully incinerated in a muffle furnace at 550°C for 8 hours. Crude protein content was quantified using the Kjeldahl method, where nitrogen (multiplied by 6.25) was employed for calculation. Finally, the determination of crude lipid content was determined using a Soxhlet apparatus with Petroleum ether at a boiling temperature range of 60-80°C.

Haematology and Blood Biochemistry

Haematological metrics including, red blood cell (RBC), hematocrit (HCT), hemoglobin (HGB), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), white blood cell (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO), platelet (PLT), platelet distribution width (PDW), mean platelet volume (MPV), and Plateletcrit (PCT) were automatically determined using proCyte Dx haematology analyser (IDEXX Laboratories, Inc., Westbrook, Maine, USA), while blood biochemistry parameters, glucose (GLU), total protein (TP), albumin (ALB), globulin (GLOB), alanine transaminase (ALT), were determined by catalyst one chemistry analyser (IDEXX Laboratories, Inc., Westbrook, Maine, USA).

Determination of 11-ketotestosterone

11-ketotestosterone levels was quantified using a Mouse Anti-Rabbit IgG coated 96 wells plate enzyme-linked immunoassay (ELISA) kits, (Cayman chemicals, USA). To perform the assay, blood plasma stored in a -80°C freezer was thawed, and two dilutions (1:500 and 1:1000) were prepared and run in triplicate for each dilution. The assay was conducted following the manufacturer's guidelines, and absorbance was read at 412 nm on a SpectraMax iD5 multi-mode microplate reader (San Jose, California, USA).

Fatty Acid Analysis of Liver, Gonad, and Muscle

P. nasutus liver, testes, and muscle samples were collected and freeze-dried for 72 hours. Before analysis, the materials were coarsely powdered and stored at -40°C. The extraction and esterification processes were combined in a single tube using the one-step approach (Abdulkadir & Tsuchiya, 2008). The fatty acid composition of pooled samples of fish liver, gonads, and muscle was determined in triplicate. Briefly, the freeze-dried samples of liver, gonads, and muscle were homogenously pulverized into powder form. Approximately 300 mg of the samples were introduced into a 50 ml centrifuge tube in three replicates combined with 4 ml of hexane and 1 ml of internal standard solution (19:0, Nonadecanoic acid≥98% purity, Sigma-Aldrich, CAS,646-3-0, Germany). The head space of the tube was flushed with nitrogen gas after adding 2 ml of 14% of boron trifluoride (BF3) in methanol and a magnetic stirring bar, and then tightly closed with a Teflon-lined screw cap. The capped tube was heated for 120 minutes on a hot plate at 100 °C with constant stirring. The tubes were then cooled to room temperature before adding 1 ml of hexane and 2 ml of distilled water. After shaking vigorously for 1 minute, the tube was centrifuged for 3 minutes at 2500 rpm (650 g) to separate FAMEs from the solution. The FAMEs on the top-phase hexane were pipetted into a clean sample vial and injected into a gas chromatograph (GC 14-B Shimadzu) equipped with a flame ionization detector (GC-FID). Gas chromatograph (Agilent Technologies, Inc., Santa Clara CA, USA) for FAME analysis. Fatty acids were identified by comparing the relative retention times of FAMEs with those of a known standard.

Statistical Analysis

The data collected is presented as means ± standard deviation and statistical significance for all statistical tests was set at $P<0.05$. All data were tested for normal distribution using the Shapiro- Wilk test and the homogeneity of variance using Levene's equal variance test. Data obtained were analysed by one-way

ANOVA (analysis of variance) using SPSS 22.0 version. To evaluate differences between means, a post hoc Tukey's test was performed. Utilizing a second-order polynomial regression analysis, we assessed the nonlinear relationship between phospholipid diets and their effects on selected blood parameters.

Results

Growth and Biological Indices

Table 3 shows that PL supplementation does not have significant effects on growth while biological indices, such as GSI, HSI, CFI, FCR, and survival rate where significantly influenced by phospholipid supplementation levels. The table also shows that the highest level of PL supplementation (2.5%) resulted in the highest values of GSI (1.45±0.25), and survival rate (93-100%). The HSI, CFI, and FCR were lowest in the PL2.5 phospholipid-supplemented group ($P<0.05$). The lowest level of PL supplementation (1.5%) resulted in the lowest values of GSI, and survival rate.

Haematological and Blood Biochemistry Indices

Table 4 shows the haematological indices of male *P. nasutus* fed with different levels of phospholipid (PL) supplemented diet for 90 days. The results indicate that there was a significant increase in the values RBC, HCT, HGB and RDW in the PL 2.5 group. The lowest values of MCV and MCH were observed in the PL 2 and PL 2.5 groups, respectively. MCHC was observed to be significantly high in the PL 2.5 group. The highest values of WBC was observed in the PL treated groups which was significantly higher than PL 0 treatment group. The value of PLT was significantly high in the PL 2 group while the lowest value of MPV was observed in the PL 2.5 group. A second-order polynomial regression analysis reveals varying degrees of influence of different phospholipid diets on blood metrics, as evidenced by the coefficient of determination (R^2). RBC, HCT, and HGB exhibit a strong association with the diet, with approximately 87.86%, 85.55%, and 85.80% of their variation explained by dietary factors, respectively (Figure 1A). Similarly, WBC and RDW also show a strong association with the diet, with about 99.96% and 89.95%

Table 3. Growth and Biological Indices of male *Pangasius nasutus* fed phospholipid supplemented for 90 days

Treatment	PL0	PL 1.5	PL 2	PL 2.5	P-value
BW (g)	670±43.6	710±62	690±57.9	760±60.0	0.707
TL (cm)	41.2±0.97	43.2±1.42	42.86±1.00	44.78±0.95	0.193
GSI	$0.49±0.09^{b}$	$0.32±0.06^{b}$	$0.39±0.14^{b}$	$1.45±0.25^{a}$	0.000
HSI	$1.05±0.05^{a}$	$1.02±0.07^{ab}$	$1.12±0.02^{a}$	$0.84±0.04^{b}$	0.006
VSI	1.51±0.08	1.65±0.15	1.57±0.04	1.29±0.09	0.095
CFI	$4.81±0.81^{ab}$	$5.76±0.47^{a}$	$6.85±0.56^{a}$	$2.63±0.19^{b}$	0.000
K	$0.95±0.03^{a}$	$0.87±0.02^{ab}$	$0.87±0.03^{ab}$	$0.84±0.03^{b}$	0.039
SGR (%)	0.21±0.09	0.13±0.04	0.19±0.06	0.14±0.07	0.797
FCR	$1.66±0.02^{a}$	$1.54±0.02^{b}$	$1.52±0.03^{b}$	$1.38±0.02^{c}$	0.000
WG	27±60	67.4±25.1	47±42.6	27±46.5	0.835
Survival (%)	100	93	96	100	-

Means in the same row with different superscripts differ significantly ($P<0.05$) (n=20)

Table 4. Hematological Indices of male *Pangasius nasutus* fed phospholipid supplemented diet for 90 days

Treatment	PL 0	PL 1.5	PL 2	PL 2.5	P-value
RBC (10^{12}/L)	$2.13±0.02^{b}$	$1.9±0.06^{c}$	$1.91±0.04^{c}$	$2.51±0.01^{a}$	0.000
HCT (%)	$30.67±0.38^{a}$	$25.37±0.86^{b}$	$24.57±0.53^{b}$	$32.13±0.13^{a}$	0.000
HGB (g/dL)	$8.67±0.14^{a}$	$7.07±0.29^{b}$	$6.87±0.19^{b}$	$9.33±0.13^{a}$	0.000
MCV (fL)	$143.73±0.93^{a}$	$133.53±0.27^{b}$	$128.6±0.49^{c}$	$127.87±0.19^{c}$	0.000
MCH (pg)	$40.6±0.53^{a}$	$37.17±0.26^{b}$	$35.93±0.38^{b}$	$37.13±0.38^{b}$	0.000
MCHC (g/dL)	$28.23±0.18^{ab}$	$27.83±0.22^{b}$	$27.97±0.18^{b}$	$29.07±0.27^{a}$	0.014
RDW (%)	$9.67±0.07^{b}$	$9.87±0.12^{b}$	$9.97±0.03^{b}$	$11.87±0.03^{a}$	0.000
WBC (10^{9}/L)	$15.85±1.33^{b}$	$29.89±0.58^{a}$	$32.64±1.98^{a}$	$31.75±0.77^{a}$	0.000
NEU (10^{9}/L)	$10.25±1.69^{b}$	$19.9±0.3^{a}$	$18.12±1.73^{a}$	$20.01±1.13^{a}$	0.003
LYM (10^{9}/L)	$5.35±0.43^{c}$	$8.43±0.39^{b}$	$12.26±0.26^{a}$	$10.16±0.75^{ab}$	0.000
MONO (10^{9}/L)	$0.18±0.04^{c}$	$1.33±0.09^{b}$	$1.92±0.05^{a}$	$1.37±0.04^{b}$	0.000
EOS (10^{9}/L)	0.04±0.01	0.16±0.06	0.27±0.22	0.05±0.01	0.500
BASO (10^{9}/L)	$0.02±0.01^{b}$	$0.07±0.02^{ab}$	$0.08±0.04^{ab}$	$0.17±0.03^{a}$	0.030
PLT (K/µL)	$3.67±0.88^{b}$	$7±1.73^{b}$	$11.67±0.33^{a}$	$4.67±0.33^{b}$	0.002
MPV (fL)	$21.37±1.08^{ab}$	$23.23±0.41^{a}$	$21.6±0.79^{ab}$	$18.27±0.53^{b}$	0.010
PDW (fL)	13.8±0.76	8.93±1.04	12±0.97	10.87±1.82	0.107
PCT (%)	$0.01±0^{b}$	$0.02±0^{ab}$	$0.02±0^{a}$	$0.01±0^{b}$	0.015

Means in the same row with different superscripts differ significantly ($P<0.05$) (n=12)

respectively of their variation influenced by the diets (Figure 1B). An R^2 of 52.80% indicates that PLT has a moderate association with the diets (Figure 1B).

Table 5 shows the blood biochemistry indices of male *P. nasutus* fed with different levels of phospholipid (PL) supplemented diet for 90 days. The highest GLU level was observed in PL 2.5 group, which was significantly higher than the other groups. The lowest urea level was found in PL 2.5 group, which was significantly lower than PL 0, PL 1.5, and PL 2 groups. The highest ALB and GLOB levels were recorded in PL 2 and PL 2.5 groups, respectively, which were significantly higher than PL 0 and PL 1.5 groups. The lowest ALB/GLOB ratio was observed in PL 2.5 group, which was not significantly lower than the other groups. The highest ALT level was found in PL 2.5 group, which was significantly higher than PL0 and PL 1.5 groups. The coefficient of determination (R^2) obtained from the second-order polynomial regression analysis shows different levels of influence from phospholipid-supplemented diets on the blood biochemistry of male *P. nasutus*. TP, ALB, and GLOB (Figure 2A), show R^2 values of 72.37%, 87.74%, and 84.03% respectively, while GLU and ALT exhibited R^2 values of 83.97% and 94.76% accordingly (Figure 2B).

Biochemical Composition of *Pangasius nasutus* Tissues

Table 6 present the biochemical composition of the liver, gonad, and muscle of male *P.nasutus* fed phospholipid supplemented diet for 90 days. There was a significant difference in the biochemical composition of the liver across diet groups. The protein content of the liver increased significantly when phospholipids were added to the diet. In the PL1.5, PL2.0, and PL2.5 groups, the liver protein content increased from 46.68±0.30 to 60.79±0.71, 65.25±0.41, and 59.27±0.62, respectively ($P<0.05$). On the other hand, the lipid

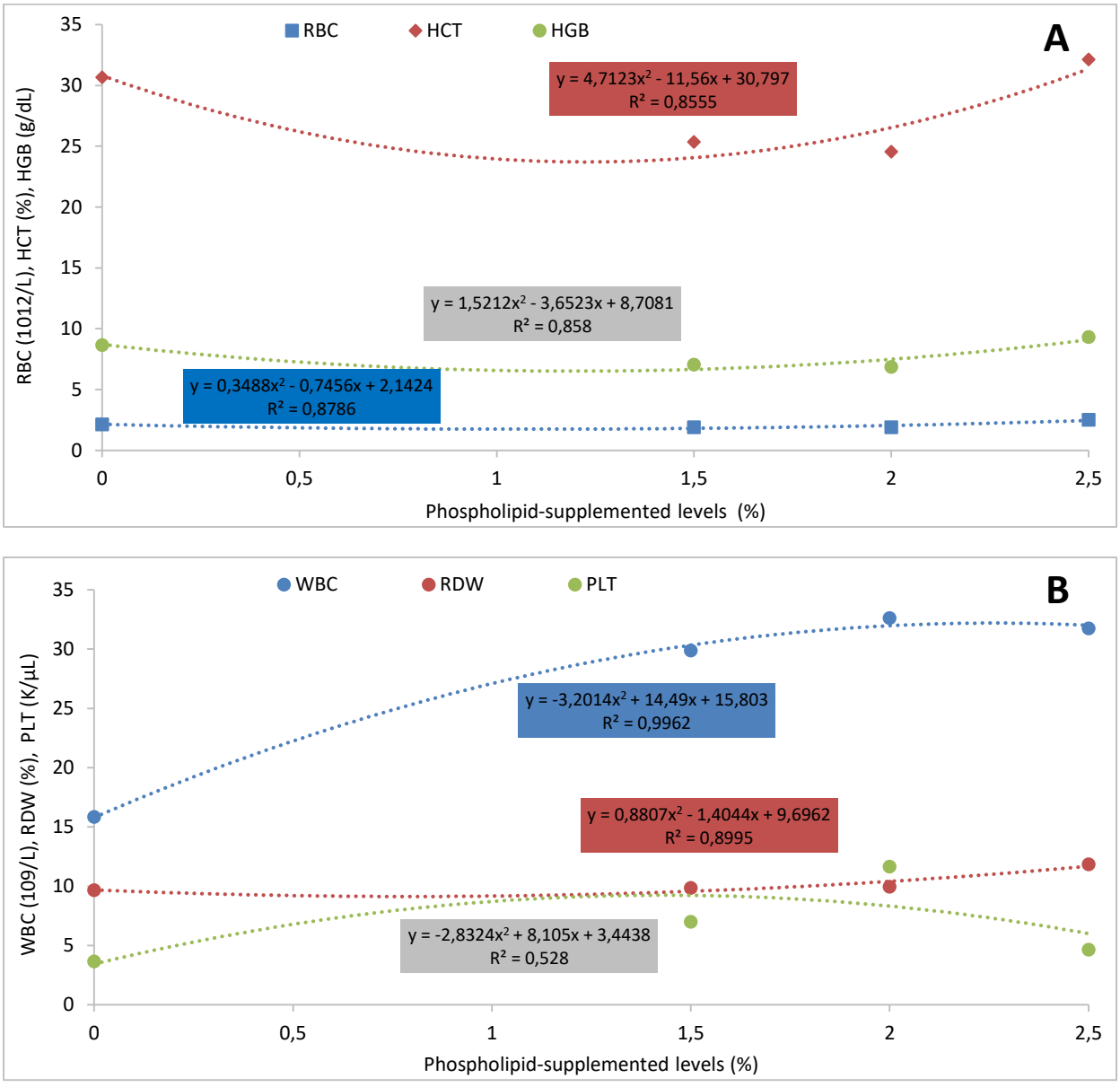

Figure 1. Second-order polynomial regression of some mean haematological metrics vs phospholipid supplementation levels.

content of the liver decreased significantly with increasing levels of phospholipid supplementation. The control group lipid content (19.12±0.19) decreased to 11.38±0.10, 13.85±0.03, and 12.69±1.41 in the PL1.5, PL2.0, and PL2.5 groups (P<0.05). Furthermore, the ash and moisture content of the liver differed significantly between diet groups (P<0.05).

There were no significant variations in protein content between diet groups in the gonads (P<0.05). The lipid content of the gonads, on the other hand, increased significantly with increasing levels of phospholipid supplementation. The starting lipid content (12.43±0.07) increased considerably (P<0.05) to 15.20±0.17, 17.97±0.50, and 19.30±0.26 in the PL1.5, PL2.0, and PL2.5 groups, respectively. Furthermore, phospholipid supplementation significantly affected ash and moisture content in the gonads (P<0.05). Similar to the liver, the muscle protein content increased significantly when phospholipids were added to the diet. The control group protein content (66.5±0.65) increased

Table 5. Blood biochemistry indices of male *Pangasius nasutus* fed phospholipid supplemented diet for 90 days.

Parameters	PL0	PL 1.5	PL 2	PL 2.5	P-value
GLU (mmol/L)	4.22±0.00^{d}	4.71±0.01^{b}	4.59±0.00^{c}	5.12±0.00^{a}	0.000
UREA (mmol/L)	2.1±0.0 0^{a}	2.1±0.00^{a}	2.2±0.00^{a}	1.15±0.05^{b}	0.000
TP (g/L)	120±0.00	118±1.00	120±0.00	120±0.00	0.107
ALB (g/L)	53±0.00^{b}	51.5±0.05^{c}	60±0.00^{a}	60±0.00^{a}	0.000
GLOB (g/L)	62.5±0.05^{c}	66±0.01^{c}	72.5±0.05^{b}	77.5±0.05^{a}	0.000
ALB/GLOB	0.8±0.00^{a}	0.8±0.00^{a}	0.8±0.00^{a}	0.7±0.00^{b}	0.000
ALT (U/L)	27.19±0.15^{b}	28.02±0.02^{b}	93.54±0.49^{a}	128.5±15.5^{a}	0.002

Means in the same row that are followed by different superscripts differ significantly (P<0.05) (n=12)

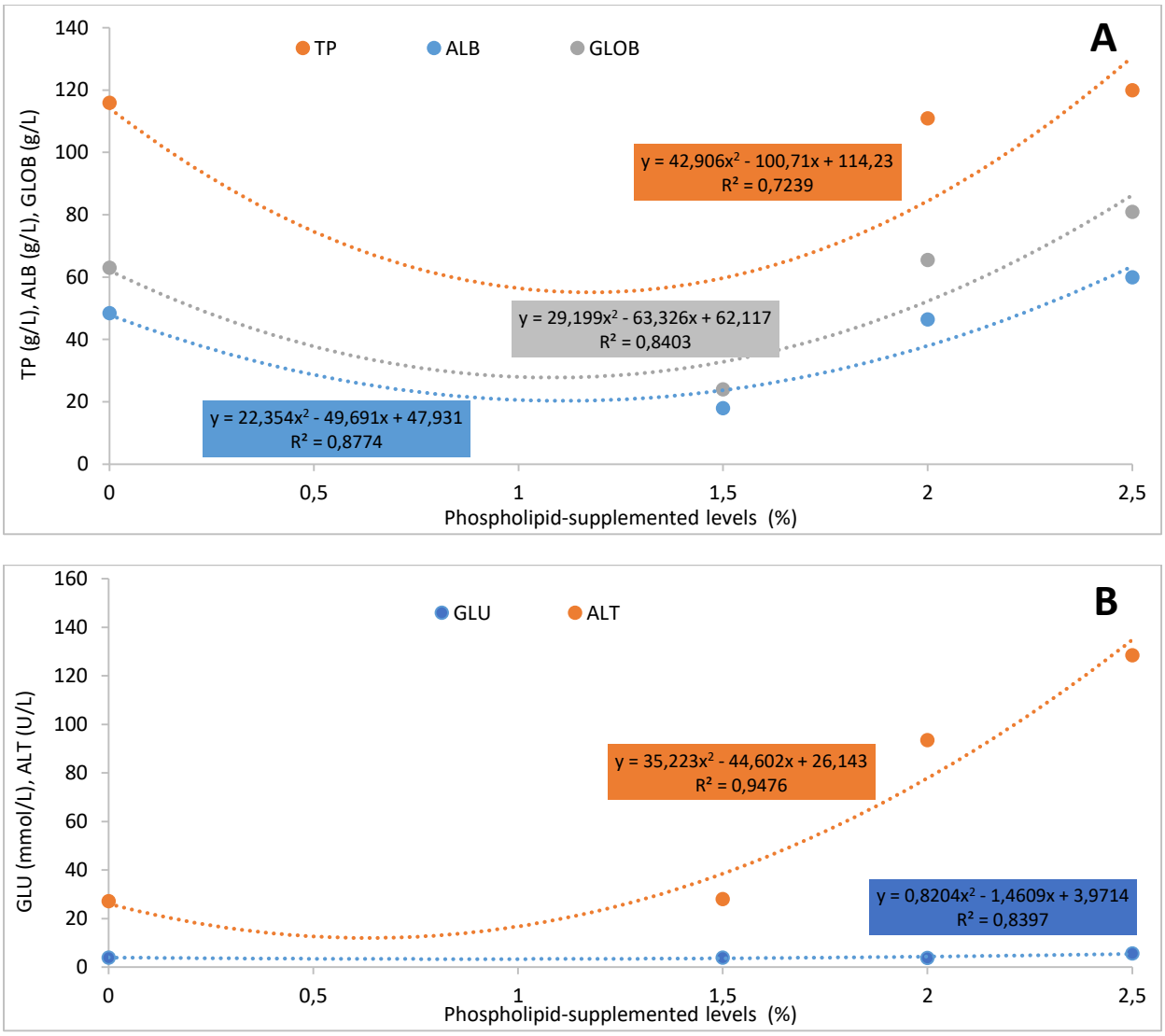

Figure 2. Second-order polynomial regression of mean Blood Biochemistry vs phospholipid supplementation levels.

to 74.18±0.36, 72.90±0.58, and 73.27±0.29, respectively, in the PL1.5, PL2.0, and PL2.5 groups (P<0.05). Furthermore, the lipid content of the muscle increased significantly with increasing amounts of phospholipid supplementation. The lipid content of the control group (9.37±0.07) increased to 12.05±0.95, 13.03±0.13, and 14.57±0.31 in the PL1.5, PL2.0, and PL2.5 groups, respectively (P<0.05). Furthermore, the moisture content in the muscle varied significantly between diet groups (P<0.05).

Fatty Acid Composition of Liver, Gonad, and Muscle

Table 7 presents the fatty acid composition of male *P. nasutus* liver after receiving varying levels of phospholipid supplementation over 90 days. Individually, specific fatty acids showed varying responses to phospholipid supplementation. For instance, C14:0, C16:0, C18:0, and C16:1 exhibited significant increases with increasing levels of phospholipid supplementation, while others such as C18:1 and C20:1 showed no consistent trend across treatment groups. The levels of saturated fatty acids (SFAs) showed a significant increase with increasing phospholipid supplementation, with the highest values observed in the PL2.0 and PL2.5 groups compared to the control and PL1.5 groups (P<0.05). Monounsaturated fatty acids (MUFAs) also exhibited significant differences among treatment groups, with the highest levels recorded in the PL2.0 and PL2.5 groups, followed by the PL1.5 group, and the lowest in the control group (P<0.05). Polyunsaturated fatty acids (PUFAs) demonstrated a similar trend, with significantly higher levels in the PL2.0 and PL2.5 groups compared to the control and PL1.5 groups (P<0.05). The ratios of n-3 to n-6 fatty acids increased significantly with higher levels of phospholipid supplementation, with the highest ratio observed in the PL2.5 group (P<0.05).

Fatty acid composition (% of total FAME) of male *Pangasius nasutus* gonad fed varying levels of

Table 6. Biochemical composition (%, based on dry weight) of liver, gonad, and muscle of male *Pangasius nasutus* fed phospholipid supplemented diets for 90 days

	Control	PL1.5	PL2.0	PL2.5
Liver				
Protein	46.68±0.30^{c}	60.79±0.71^{b}	65.25±0.41^{a}	59.27±0.62^{b}
Lipid	19.12±0.19^{a}	11.38±0.10^{d}	13.85±0.03^{b}	12.69±1.41^{c}
Ash	4.15±0.00^{d}	6.40±0.40^{a}	5.08±0.00^{c}	5.87±0.20^{b}
Moisture	4.57±0.00^{d}	6.95±0.00^{b}	7.31±0.00^{a}	6.64±0.00^{c}
Gonads				
Protein	60.37±1.54	58.95±2.36	60.62±2.44	63.79±0.98
Lipid	12.43±0.07^{d}	15.20±0.17^{c}	17.97±0.50^{b}	19.30±0.26^{a}
Ash	5.37±0.10^{d}	5.62±0.10^{c}	7.43±0.30^{a}	6.32±0.19^{b}
Moisture	4.22±0.00^{d}	5.37±0.00^{c}	6.37±0.00^{a}	5.55±0.00^{b}
Muscle				
Protein	66.5±0.65^{c}	74.18±0.36^{a}	72.90±0.58^{a}	73.27±0.29^{a}
Lipid	9.37±0.07^{d}	12.05±0.95^{bc}	13.03±0.13^{b}	14.57±0.31^{a}
Ash	5.33±0.00^{b}	5.49±0.23^{b}	5.18±0.00^{a}	5.30±0.00^{b}
Moisture	5.12±0.00^{c}	6.37±0.00^{a}	5.19±0.63^{d}	5.67±0.23^{b}

Means in the same row with different superscripts indicate significant differences (P<0.05) from one-way ANOVA; Values are means±SD (n=12)

Table 7. Fatty acid composition (% of total FAME) of male *Pangaius nasutus* liver fed varying levels of phospholipid-supplemented diets for 90 days

FAs	PL0	PL1.5	PL2.0	PL2.5
C14:0	1.26±0.01^{c}	1.43±0.22^{c}	2.26±0.01^{b}	2.75±1.29^{a}
C16:0	16.01±2.2^{c}	17.34±3.25^{a}	20.77±1.11^{c}	21.2±1.61^{a}
C18:0	1.09±0.2^{c}	1.87±0.94^{b}	1.90±0.07^{b}	2.09±0.36^{a}
C16:1	3.36±0.24^{b}	3.83±0.18^{b}	4.67±0.36^{a}	4.81±0.45^{a}
C18:1	21.15±7.92^{c}	22.92±2.16^{b}	22.52±1.36^{b}	22.76±7.84^{a}
C20:1	1.54±0.11^{c}	1.98±0.01^{c}	2.22±0.10^{c}	2.91±2.37^{b}
C18:2n-6	25.76±0.1^{d}	26.52±0.11^{c}	27.62±2.13^{b}	29.33±3.28^{a}
C20:4n-6	0.34±0.19^{c}	0.62±0.07^{c}	0.69±0.38^{a}	0.94±0.28^{b}
C18:3n-3	2.38±0.08^{d}	2.39±0.4^{c}	3.11±0.72^{b}	3.86±3.99^{a}
C20:5n-3	3.96±0.21^{c}	4.1±0.22^{b}	4.29±0.88^{b}	4.33±0.56^{a}
C22:6n-3	1.20±0.08^{c}	2.16±2.16^{b}	3.36±0.76^{a}	4.66±1.29^{a}
n-3	7.54±0.02^{d}	8.65±0.01^{c}	10.76±0.03^{b}	12.85±0.11^{a}
n-6	26.10±0.01^{c}	27.14±0.11^{c}	28.31±0.10^{b}	30.27±0.02^{a}
n-3/n-6	0.29±0.04^{d}	0.32±0.02^{c}	0.38±0.03^{b}	0.42±0.03^{a}
∑SFA	18.36±0.12^{d}	20.60±0.01^{c}	24.93±0.02^{b}	26.04±0.04^{a}
∑MUFA	26.05±0.01^{c}	28.73±0.02^{b}	29.41±0.01^{a}	30.48±0.00^{a}
∑PUFA	33.64±0.02^{d}	35.79±0.01^{c}	39.07±0.02^{b}	43.12±0.03^{a}

Results are given as mean±SD. Means in the same row with different superscripts letters are significantly different (P<0.05) (n=12)

phospholipid-supplemented diets for 90 days is presented in Table 8. Different fatty acids responded differently to phospholipid supplementation on an individual basis. As phospholipid supplementation increased, C14:0, C16:0, C18:0, and C16:1 all demonstrated notable increases; however, C18:1 and C20:1 did not show a consistent trend across treatment groups. Saturated fatty acid (SFA) levels varied significantly throughout treatment groups; the PL2.5 group had the highest amounts when compared to the control and lower phospholipid supplementation groups ($P<0.05$). Similarly, monounsaturated fatty acids (MUFAs) displayed significant differences among treatment groups, with the highest levels observed in the PL2.0 and PL2.5 groups, followed by the PL1.5 group, and the lowest in the control group ($P<0.05$). Polyunsaturated fatty acids (PUFAs) also showed significant variations across treatment groups, with the highest levels recorded in the PL2.5 group, followed by the PL2.0, PL1.5, and control groups ($P<0.05$). The ratios of n-3 to n-6 fatty acids remained relatively stable across treatment groups, with no significant differences observed ($P>0.05$).

The fatty acid composition of male *Pangasius nasutus* muscle displayed significant variations among different dietary treatments of phospholipid supplementation over a 90-day period (Table 9). Significant differences were observed in the levels of saturated fatty acids (SFAs) across treatment groups, with the highest values recorded in the PL0, PL1.5, groups compared to the PL 2.0, PL2.5 ($P<0.05$). Monounsaturated fatty acids (MUFAs) also exhibited significant differences among treatment groups, with the highest levels observed in the PL2.5 group, followed by the PL2.0, PL1.5, and control groups ($P<0.05$). Polyunsaturated fatty acids (PUFAs) showed the most pronounced variations across treatment groups, with the highest levels recorded in the PL2.5 group, followed by the PL2.0, PL1.5, and control groups ($P<0.05$).

Hormone Profiles

Plasma 11-ketotestosterone (11-keto) levels of male *P. nasutus* fed varying percentages of PL are presented in Figure 3. The concentration of 11-keto increased significantly as the PL content of the diets increased, with PL2.5 having the highest levels of 11-keto (806.86±7.10 pg/mL), followed by PL2.0 (343.14±22.30 pg/mL) and PL1.5 (124.94±8.30 pg/mL), while the control group had the lowest concentration (91.57±5.90 pg/mL).

Discussions

Growth and Biological Indices

Results from this study show that PL supplementation had no significant effects on growth parameters such as WG, while other biological indices, specifically GSI, HSI, CFI, and FCR were influenced by the levels of phospholipid supplementation. The observed poor WG is likely attributable to the preferential allocation of nutrients for reproductive purposes by the fish, given their status as broodstock which is a natural strategy to maximise their reproductive success (Carboni et al., 2015; Rijnsdorp, 1990). The metabolic demand associated with reproduction is high, and this energy demand for gamete production and spawning activities can lead to reduced somatic growth (Schreck et al., 2001). The impact of dietary phospholipids on fish growth and reproductive performance is well-documented, as demonstrated in zebrafish *(Danio rerio)* where phospholipids were found to improve reproductive success (Martins et al., 2020). Our result reveals that the highest level of PL supplementation (2.5%) resulted in the highest values of GSI and survival rate. This suggests that a higher concentration of PL in the diet may positively impact the reproductive performance and survival of male *P. nasutus.* On the other hand, the lowest level of PL supplementation (1.5%) led to the lowest values of GSI and survival rate. This aligns with a previous report, where it was observed that the dietary PL supplementation (i.e., PC and PE) benefits zebrafish reproductive condition, especially for males (Diogo et al., 2015). The observed effects on GSI, HSI, and CFI indicate that PL supplementation may have influenced the reproductive and physiological processes of male *P. nasutus.* GSI as an important indicator of reproductive activity and gonadal development showed higher values associated with PL supplementation, suggesting a potential enhancement of reproductive activities. HSI reflects the metabolic condition of the liver, and the significant effects observed in this index indicate alterations in liver functions due to PL supplementation. Additionally, variations in CFI suggest potential modifications in energy storage and utilization patterns, possibly impacting the overall health and fitness of the fish.

Evidence from Pacific white shrimp, *Litopenaeus vannamei* broodstock indicates that those fed with diet supplemented with phospholipids (PL) exhibited superior growth performance, antioxidative capacity, and innate immunity in comparison to the control group of shrimps (Liang et al., 2022). Similarly, Chinese mitten crab *Eriocheir sinesis* fed diet containing 0%, 1.2%, 2.4%, and 3.6% PL exhibited the highest higher gonadosomatic index in PL 2.4% treatment (Sui et al., 2009; Lin et al., 2021). Lower FCR values were recorded in PL supplemented groups in this study which suggests improved feed conversion, while a higher FCR was recorded in the control group which may imply reduce efficiency. This result suggests that PL supplementation may influence the metabolic processes and nutrient utilization efficiency in male *P. nasutus*. These findings are consistent with a study involving Malaysian mahseer, *Tor tambroides* that were provided with a diet enriched with 4-6% PL, leading to a significant improvement in feed conversion ratio FCR (Mian et al.,

Table 8. Fatty acid composition (% of total FAME) of male *Pangasius nasutus* gonad fed varying levels of phospholipid-supplemented diets for 90 days

FAs	PL0	PL1.5	PL2.0	PL2.5
C14:0	1.04±1.4^{b}	1.11±1.24^{b}	1.24±0.04^{b}	2.21±0.22^{a}
C16:0	10.20±1.42^{c}	11.33±0.06^{b}	12.38±1.49^{b}	14.89±1.23^{a}
C18:0	1.20±0.01^{c}	1.40±0.12^{b}	1.47±0.78^{b}	2.01±0.01^{a}
C16:1	2.05±0.14^{d}	2.77±0.64^{c}	3.23±0.01^{a}	3.07±0.01^{b}
C18:1	15.54±2.5^{a}	17.65±0.2^{b}	20.86±0.08^{b}	21.02±0.45^{a}
C20:1	1.13±0.02^{d}	1.31±0.68^{c}	1.81±0.07^{b}	1.98±0.09^{a}
C18:2n-6	15.93±0.20^{d}	18.7±0.22^{b}	23.28±0.23^{c}	28.05±0.04^{a}
C20:4n-6	0.75±1.17^{b}	0.84±0.5^{a}	0.29±0.23^{c}	0.73±0.05^{b}
C18:3n-3	4.12±1.28^{c}	4.54±0.05^{b}	5.62±1.34^{a}	5.89±0.00^{a}
C20:5n-3	2.06±0.05^{c}	2.61±0.85^{b}	2.31±0.09^{b}	2.87±0.02^{a}
C22:6n-3	2.56±0.26^{a}	3.06±0.56^{a}	4.25±0.06^{b}	6.8±1.28^{a}
n-3	8.74±0.05^{d}	10.21±0.3^{c}	12.18±0.02^{b}	15.56±0.3^{a}
n-6	16.68±0.20^{d}	19.54±0.02^{c}	23.57±0.12^{b}	28.78±0.62^{a}
n-3/n-6	0.52±0.00^{a}	0.52±0.23^{a}	0.52±0.06^{a}	0.54±0.03^{a}
∑ SFA	12.44±0.03^{d}	13.84±0.62^{c}	15.09±0.02^{b}	19.11±0.45^{a}
∑ MUFA	18.72±0.01^{d}	21.73±0.22^{c}	25.90±0.03^{b}	26.27±0.00^{a}
∑ PUFA	25.42±0.04^{d}	29.75±0.04^{c}	35.75±0.01^{b}	44.34±0.07^{a}

Results are given as mean±SD. Means in the same row with different superscripts are significantly different (P<0.05) (n=12).

Table 9. Fatty acid composition (% of total FAME) of male *Pangasius nasutus* muscle fed varying levels of phospholipid supplemented diets for 90 days

FAs	PL0	PL1.5	PL2.0	PL2.5
C14:0	1.37±0.03^{b}	1.38±0.18^{b}	2.06±0.72^{a}	2.20±0.05^{a}
C16:0	12.01±0.08^{c}	12.53±0.51^{c}	14.41±0.23^{b}	17.76±0.04^{a}
C18:0	1.3±0.16^{a}	0.24±0.07^{b}	0.34±0.04^{b}	0.20±0.06^{b}
C16:1	2.95±0.26^{d}	3.67±0.13^{c}	4.58±0.04^{b}	5.07±0.02^{a}
C18:1	18.59±1.43^{c}	22.85±0.09^{b}	22.40±4.83^{a}	23.07±0.59^{a}
C20:1	1.12±0.04^{c}	1.25±1.22^{b}	1.53±0.03^{b}	1.99±0.06^{a}
C18:2n-6	18.99±0.8^{d}	21.37±0.25^{c}	25.04±0.29^{b}	28.15±0.82^{a}
C20:4n-6	0.55±0.12^{c}	0.76±0.71^{c}	1.02±0.43^{b}	1.47±0.57^{a}
C18:3n-3	2.44±0.04^{c}	3.14±0.43^{b}	4.75±0.48^{a}	5.92±2.83^{a}
C20:5n-3	3.31±0.14^{d}	5.32±0.12^{b}	4.23±0.62^{c}	7.97±0.58^{a}
C22:6n-3	3.11±0.28^{d}	4.82±0.96^{c}	5.27±0.76^{b}	5.97±0.71^{a}
n-3	8.86±0.02^{c}	13.28±1.69^{b}	14.25±0.04^{b}	19.86±0.1^{a}
n-6	19.54±1.15^{d}	22.13±0.62^{c}	26.06±0.1^{b}	29.62±0.32^{a}
n-3/n-6	0.45±0.04^{d}	0.60±0.01^{b}	0.55±0.08^{c}	0.67±0.02^{a}
∑ SFA	14.68±0.14^{d}	14.15±0.12^{c}	16.81±0.03^{b}	20.16±0.04^{a}
∑ MUFA	22.66±3.12^{d}	27.77±0.71^{c}	28.51±0.1^{b}	30.13±0.32^{a}
∑ PUFA	28.40±0.54^{d}	35.41±0.52^{c}	40.31±0.18^{b}	49.48±0.61^{a}

Results are given as mean±SD. Means in the same row with different superscripts are significantly different (P<0.05) (n=12).

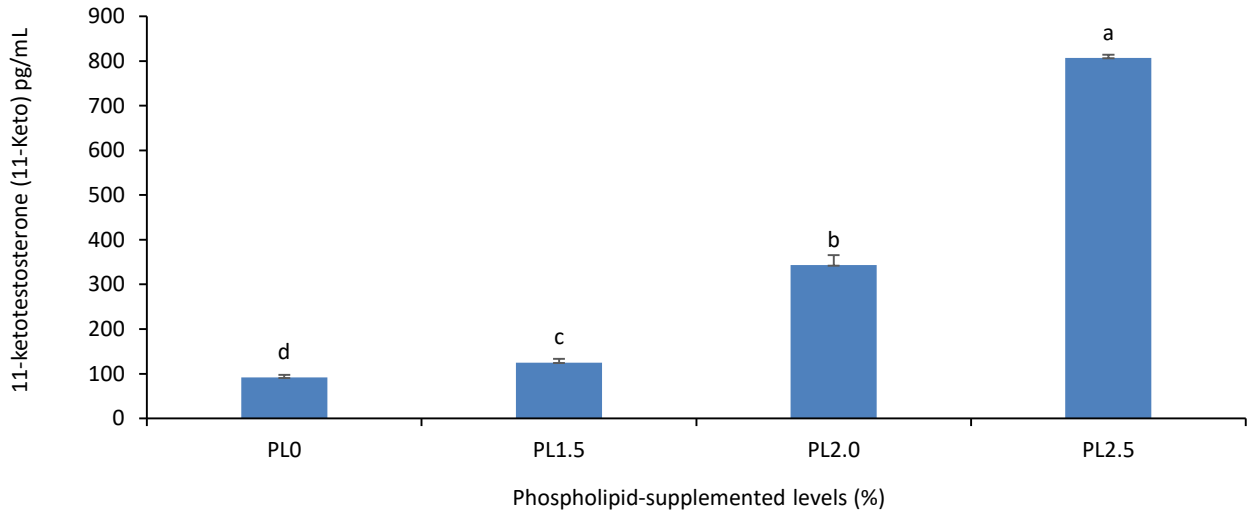

Figure 3. Plasma 11-ketotestosterone levels of male *P. nasutus* fed varying levels of phospholipid diet for 90 days. Results are presented as mean ± SD. Significant differences between treatment groups are indicated by different letters above the bars.

2020). Similarly, common carp *Cyprinus carpio* fed soybean lecithin at 10, 20, & 30 g/kg in diets showed improved FCR (Adel et al., 2017) Previous studies reported that PLs could facilitate lipid digestion and absorption in the intestine of fish larvae through enhanced emulsification (Feng et al., 2017; Poston, 1989). The observed effects of PL supplementation demonstrate its potential to enhance fish survival rates and resilience by improving their ability to withstand stressors and fight illnesses (Tocher et al., 2008).

Hematological and Blood Biochemistry

The haematological profile of a fish in aquaculture provides valuable information about its physiological condition and overall health (Rashidian et al., 2020). Previous studies revealed that PLs help improve fish immune responses (Haghparast et al., 2019). Our result shows the significant impact of PL supplementation on a range of haematological measures, indicating its ability to modify blood composition and cellular properties in male *P. nasutus*. RBC, HCT, HGB, and RDW levels in the PL 2.5 group increased significantly. This implies that a higher level of PL supplementation (2.5%) influenced red blood cell count, haematocrit, haemoglobin levels, and red cell dispersion width favourably. These results indicate that PL's capacity to support membrane fluidity and stability may be involved in enhancing erythropoiesis and blood oxygen-carrying capacity of male *P. nasutus*. The higher amount of RBC and Hb concentration could be in response to increased metabolic demand of the body, which was confirmed by the significantly higher GSI and elevated levels of 11-ketotestosterone (11-KT) in *P. nasutus* fed diets containing 2.5% PL levels which could be a sign of increased reproductive activities. Changes in haematological metrics are associated with factors such as species, temperature, stress, age, gender, and nutritional state among others (Fazio, 2019; Pavlidis et al., 2007; Vázquez & Guerrero, 2007). Furthermore, the PL 2 and PL 2.5 groups had the lowest MCV and MCH values, respectively. This implies that supplementing PL at these doses had an effect on red blood cell mean corpuscular volume and mean corpuscular haemoglobin. These modifications could be the result of variations in cell size and haemoglobin content, which could have physiological consequences for the fish. Mean corpuscular haemoglobin concentration (MCHC) was considerably elevated upon 2.5% phospholipid supplementation, suggesting a possible shift in red blood cell haemoglobin levels that could impact exchange efficiency and oxygen-carrying ability (Andreyeva et al., 2019; Harter et al., 2022).

The PL 2 group had significantly higher white blood cell counts, including neutrophils, monocytes, and basophils, compared to the other groups. This indicates that supplementing with 2% PL had a notable impact on the fish's immune response and inflammation. Additionally, the PL 2 group had a significantly higher lymphocyte count, suggesting that the 2% PL supplementation increased lymphocytes, which are important for immune defence. This implies potential improvements in immune responses and functionality. Similar results were reported for Caspian brown trout, *Salmo trutta caspius* (Haghparast et al., 2019), common carp, *Cyprinus carpio* (Adel et al., 2017), stellate sturgeon *Acipenser stellatus* (Jafari et al., 2018) fed diets containing soy bean lecithin (SBL). In terms of platelet indices, the PLT count was significantly higher in the PL 2 group, while the PL 2.5 group had the lowest mean platelet volume (MPV). This suggests that 2% PL supplementation influenced platelet count, while 2.5% PL supplementation affected platelet volume. Platelets are crucial for blood clotting and wound healing, so changes in their count and volume can impact these processes. Lastly, the PL 2 group had the highest plateletcrit (PCT) value, indicating that 2% PL supplementation affected the proportion of platelets in the blood. This highlights the influence of PL on platelet-related factors. However, there is limited literature on the effect of dietary phospholipid supplementation on blood parameters of *P. nasutus* as well as other fish species, therefore the result of this study could serve as a baseline to investigate the mechanism behind the impact on blood parameters and an appropriate inclusion level of phospholipid in the diet of *P. nasutus* broodstock.

In the present study, PL supplementation had significant effects on various blood biochemistry indices in male *P. nasutus*. The highest glucose (GLU) level was observed in the PL 2.5 group, indicating enhanced carbohydrate metabolism and energy utilization. This result agrees with Hadarabi et al. (2011) who observed that juvenile sturgeon fish, *Huso-huso* fed a 4% phospholipid diet had higher GLU levels. Similarly, juvenile stellate sturgeon *Acipenser stellatus* had GLU levels increased with increasing dietary SBL from 4-6% levels (Jafari et al., 2018). The lowest urea level was found in the PL 2.5 group, suggesting reduced protein breakdown and improved protein retention. The destabilization effect of high concentrations of urea on protein structure has been investigated in elasmobranch fishes (Yancey & Somero, 1979). Additionally, PL supplementation led to higher levels of albumin (ALB) and globulin (GLOB). The increase in ALB and GLOB is an indication of increased synthesis of plasma proteins and improved health status as reported in *Channa punctatus* (Javed & Usmani, 2015). However, the low ALB/GLOB ratio in the PL 2.5 group suggests a potential inflammatory or infectious condition that requires confirmation through additional indicators as the ratio is an index used to track changes in the composition of serum or plasma (Javed & Usmani, 2015) The highest alanine aminotransferase (ALT) level in the PL 2.5 group suggests some degree of hepatotoxicity or hepatocellular injury at high PL levels. Overall, PL

supplementation has implications for growth, health, and metabolism in *P. nasutus,* but the effects may vary with levels of inclusion. Further research is necessary to determine the optimal PL supplementation level and understand the underlying mechanisms behind these effects.

The second-order polynomial regression analysis offers valuable insights into the relationship between different phospholipid diets and various blood metrics. The coefficient of determination (R^2) is used as a measure of the strength of this association. It is evident that RBC, HCT, and HGB are strongly influenced by dietary factors, with approximately 87.86%, 85.55%, and 85.80% of their variations being explained by the diets, respectively (Figure 1A). This suggests that these blood metrics are highly responsive to changes in the phospholipid diets studied. Likewise, WBC and RDW also exhibit a strong association with the diet, with roughly 99.96% and 89.95%, respectively, of their variations being influenced by the dietary factors (Figure 1B). This indicates that these blood parameters are exceptionally responsive to dietary modifications. However, PLT shows a more moderate association with the diets, as indicated by an R^2 value of 52.80% (Figure 1B). While this association is not as strong as the aforementioned blood metrics, it still suggests a noteworthy impact of dietary choices on platelet count. Phospholipid-supplemented has been reported in *Pangasianodon hypopthalmus* to have strong to moderate associations with the same blood parameters reported in this study (Torsabo et al., 2023). For blood biochemistry, TP, ALB, and GLOB exhibit R^2 values of 72.37%, 87.74%, and 84.03%, respectively (Figure 2A). These high R^2 values suggest a strong relationship between these blood parameters and the phospholipid-supplemented diets. In other words, a substantial portion of the variation in TP, ALB, and GLOB levels can be attributed to dietary factors, indicating that these blood parameters are highly responsive to changes in the diet. Similarly, GLU and ALT show even stronger associations with the diets, as they have R^2 values of 83.97% and 94.76%, respectively (Figure 2B). These exceptionally high R^2 values imply that a large proportion of the variations in GLU and ALT levels can be explained by dietary factors. This highlights a robust connection between these parameters and the phospholipid-supplemented diets, signifying that diet plays a significant role in influencing blood biochemistry in male *P. nasutus*. The influence of phospholipid-supplemented diets on the blood biochemistry of *P. hypopthalmus* has been reported. The report shows that phospholipid diets at 2 %, 6%, and 10% supplementation levels exhibited a progressive significant impact on blood biochemistry such as TP, ALB, GLOB, GLU, and ALT (Torsabo et al., 2023). Overall, these results have important implications for understanding the dietary impact on the health and blood biochemistry of male *P. nasutus*. The strong to very strong associations between phospholipid-supplemented diets and various blood parameters suggest that dietary interventions can be a powerful tool for managing and optimizing the health of this species.

Biochemical and Fatty Acids Composition of Liver, Gonad, and Muscle

Phospholipids supplemented diet led to a significant increase in the protein content of the liver, muscle, and gonad in the current study, while the lipid content of the muscle and gonad increased with phospholipid-increasing levels, the lipid content of the liver decreased with increasing levels of phospholipids. It has been reported that phospholipids play a crucial role in protein and lipid metabolism and synthesis in fish (Dong et al., 2023; Morita & Ikeda, 2022). These findings suggest that phospholipids have a direct influence on hepatic tissue composition and function in male *P. nasutus*. Similarly, protein content was found to increase in largemouth bass *Micropterus salmoides* fed diet substituted with 1 g/kg of lysophospholipid (Lu et al., 2022). However, dietary phospholipids inclusions at 5.34% were reported to reduce the crude lipid content of whole fish body, muscle, and liver of juvenile largemouth bass *Micropterus salmoides* (Wang et al., 2022). Moisture and ash content was higher in phospholipid-treated groups in the liver, gonads, and muscles suggesting that phospholipids may influence mineral and water balance in these tissues. These disparities underline the complexity of phospholipid metabolism and its varied effects across different species, life stages, and dietary concentrations. Further research is warranted to elucidate these mechanisms comprehensively.

Fatty acids composition of liver, gonad, and muscle mirrored the dietary fatty acid profile in the present study which is similar to other observed studies previously (Feng et al., 2017; Huang et al., 2021). Fatty acids such as C14:0, C16:0, C18:0, and C16:1 showed a significant increase in PL dorse dependant manner although there was a decrease in lipid deposition in the liver at increasing levels of PL. PUFAs such as C18:2n-6 and C20:4n-6 increased with the increase of PL levels in both the liver, gonad, and muscle in the present study. The increase of C18:2n-6 has been suggested to increase lipid deposition in whole body and liver of blunt snout bream, *Megalobrama amblycephala* (Li et al., 2016), and large yellow croaker, *Larimichthys crocea* (Feng et al., 2017). The increase of C18:2n-6 in the present study also resulted in a corresponding increase in lipid deposition in both the gonad and muscle, while there was a decrease in lipid deposition in the liver as the levels of PL increased. The incident of increased lipid deposition as a result of the increase in PL levels in whole body and liver of large yellow croaker, *Larimichthys crocea* (Zhao et al., 2013), and Dojo loach, *Misgurmus anguillicaudatus* (Gao et al., 2014) has been reported. Similarly, an increase in PL levels facilitated the increase

of lipid content in organs of amberjack, *Seriola dumerili* (Uyan et al., 2009), and channel catfish, *Ictalurus punctatus* (Sink & Lochmann, 2014). The reasons for the increase in lipid content could be related to the various roles of phospholipids which include the following. Phospholipids play a significant role in lipid absorption due to their emulsification properties (Tocher et al., 2008). The crucial function of phospholipids in facilitating lipid transportation from the intestine and liver throughout the body has been validated, as they are integral in forming chylomicrons and very low-density lipoproteins, correspondingly (Huang et al., 2022). In the present study, the concentration of C20:4n-6 increased with the increasing proportion of PL levels. Bogevik et al. (2014) reported an increase in C20:4n-6 and phospholipids concurrently in the testes of male Atlantic salmon, *Salmo salar* during sexual maturation compared to immature males. This increase of C20:4n-6 proportional to PL levels might be associated with its role in enhancing cholesterol transport during steroidogenesis.

The content of PUFAs such as C18:3n-3, C20:5n-3, and C22:6n-3 in the present study increased significantly with the increase in the levels of PL. n-3 long-chain polyunsaturated fatty acids (LC-PUFA) are essential in broodstock diet for ideal gonadal and embryonic development in several fish species (Ferosekhan et al., 2021). The increase in PUFA content could be linked to the abundant presence of PUFAs in phospholipids, particularly phosphatidylcholine (PC), as reported by Ferosekhan et al. (2021) which is predominantly abundant phospholipid in soy lecithin (Wu & Wang, 2003). The elevated levels of both n-3 LC- PUFA and n-6 LC-PUFAs in response to increasing levels of PL in the liver, gonad, and muscle of *P. nasutus* might serve as a promising strategy to improve the reproductive performance of this species. The content of essential fatty acids (EFA) in the broodstock diet has been identified as one of the important dietary variables that determine effective reproduction and offspring survival. Highly unsaturated fatty acids (HUFA) with 20 or more carbon atoms, as well as their metabolites, have been shown to influence fish maturation and steroidogenesis (Brown-Vuillemin et al., 2023; Izquierdo et al., 2001). The intricate nature of fatty acid observed in various treatment groups in terms of fatty acid content by different organs in different quantities demonstrates that their functions in those organs and their demand for such fatty acids vary respectively. Further research is required to elucidate the underlying mechanisms driving these changes and their impacts on the physiology and performance of the liver, gonads, and muscles, as well as reproductive biology and the overall health of male *P. nasutus* and related species. While the effects of phospholipid dietary supplementation have been extensively studied in early-stage fish and other higher animals, including humans, there is limited knowledge regarding its impact on adult fish in this regard.

Sex Steroid Hormone (11-Ketotestosterone)

In teleost fish, 11-ketotestosterone (11-KT) is an important male-specific androgen, playing essential roles in the development of sexual characteristics, spermatogenesis, and male reproductive behaviors (Damsteegt et al., 2020; Ogino et al., 2021; Roosta et al., 2022; Soyano et al., 2022). The present study revealed that phospholipid-supplemented diets led to increased synthesis of 11-KT hormones in the blood plasma of male *P. nasutus*. Specifically, fish fed PL2.5 exhibited the highest concentration of 11-KT, followed by those fed PL2, and finally PL1.5. The significant rise in 11-KT levels among male fish in this study strongly suggests a significant advancement towards sexual maturity, achieved through the strategic accumulation of crucial reproductive hormones. The concentration of 11-KT in male fish has been reported to increase in the process of sexual maturation spermatogenesis in several wild and cultured fish species such as Siamese fighting fish, *Betta splendens* (Dzieweczynski et al., 2006), rainbow trout, *Oncorhynchus mykiss* (Thorarensen et al., 1996), *Lythrurus fasciolaris* (Schade & Stallsmith, 2012), and goldfish, *Carassius auratus* (Roosta et al., 2022). The increase in the concentration of 11-KT observed in our study could be related to nutrient transport and absorption roles of phospholipid thereby making the nutrients more bioavailable for reproductive processes. The transport role of phospholipid has been reported in several teleosts fish (Gupta et al., 2021; Hara et al., 2016; Taylor et al., 2015; Tocher et al., 2008).

This result also demonstrates the active role of phospholipids in nutrient transport and absorption by the transfer of stored liver fat to the testis, and their retention which lead to an increase in 11-KT levels in correspondence with increased PL levels in the diets. The transfer of fat from the liver and lipid sources into the developing gamete is essential for the reproductive process and embryonic development of fish (Fei et al., 2020). PLs-supplemented diets for *P. nasutus* have not been reported in published literature, though it has the potential to promote the elevation of male sex steroid hormones which are important for the promotion of gametogenesis as reported in other fish species. This approach could be a useful tool for broodstock conditioning of *P. nasutus* for effective seed production in captive facilities. Research on the utilization of PLs in the diet of finfish broodstocks to enhance their reproductive performance is limited. Further studies are recommended on other sources of PLs such as egg yolk lecithin, and krill oil for finfish broodstock including *P. nasutus* to ascertain the roles of PLs on sex steroid hormones and general reproductive parameters. While this study did not comprehensively investigate the reproductive performance of male *P. nasutus*, including sperm quality and the hatchability of fertilized eggs, the observed, increase in GSI, and elevated 11-KT levels suggest positive effects of phospholipids on improving reproductive ability of male *P. nasutus* broodstock.

In conclusion, this study confirms the beneficial role of phospholipids in promoting significant positive effects on haematological and blood biochemistry indices, sex steroid hormones, and biochemical and fatty acid composition of *P. nasutus* male broodstock. Although there was no significant increase in the growth of the broodstock, the results suggest that incorporating phospholipids into the diet could be a valuable strategy for optimizing the nutrition of these fish and enhancing the aquaculture production of this species.

Ethical Statement

The study obtained ethical approval for the utilization of animals in research from the Institutional Animal Care and Use Committee (IACUC) at the Research Management and Innovation Centre, University Malaysia Terengganu.

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Author Contribution

Donald Torsabo and Nurizzati Idris: Data curation, Writing- Original draft preparation. Benedict Terkula Iber, Ataguba Gabriel Arome: Conceptualization, Methodology, Software. Ivan Koh Chong Chu, Muhammad Yazed Abduh: Visualization, Investigation. Caterina Faggio, Noordiyana Mat Noordin, Ambok Bolong Abol-Munafi: Supervision. Mohamad Nor Azra: Software, Validation. Federica Impellitteri: Writing-Reviewing and Editing.

Conflict of Interest

The authors affirm that they have no conflicts of interest to disclose.

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