Enzymological, Histological and Serum Biomarkers Responses of Snubnose Pompano on Complete Replacement of Fishmeal Using Cottonseed Meal Supplemented With Lysine and Methionine in the Diet

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Abstract

In a feeding experiment, cottonseed meal (CSM) was used to replace fishmeal (FM) in the diet of snubnose pompano supplemented with lysine and methionine to assess the growth, nutritive profile, hematological, histological and stress biomarkers response. Experimental fishes were randomly stocked in five treatments each with triplicates. Five isonitrogenous and isolipidic diets with graded level of CSM (0, 8.7, 17.4, 26.0 and 34.7%) as replacement for FM protein (0, 25, 50, 75, and 100%) were formulated and fed to respective treatments. Comparison between various parameters among the treatments was made using orthogonal polynomial contrasts to indicate the statistical significance. Higher alkaline phosphatase, acid phosphatase, lactate dehydrogenase, malate dehydrogenase, aspartate and alanine aminotransferase activities were observed in 0CSM group and followed by 100CSM group as higher inclusion level of CSM with higher free gossypol content did not affect the metabolic enzyme activities. The maximum muscular free gossypol accretion of 1.28 mg kg^{-1} (on wet basis) was recorded in 100CSM group which was very well below the critical limit set by FDA. As a conclusion, fishmeal can be completely replaced using cottonseed meal in the diet of pompano without adverse effect on growth, metabolism and general health.

Introduction

As the global fish production increases, fish feed production is also increasing tremendously. Even though aquafeed production increases, cost effective feed is not available in the market, especially for carnivorous marine fishes. The reason behind such situation is higher inclusion level of fishmeal (FM) and fish oil in the diet of carnivorous marine fishes. Thus, fishmeal became one of the most indispensable components in marine fish feeds and its requirement is increasing exponentially. But, fishmeal production is almost static around 7 mmt (Hardy and Tacon 2002) in which 73% is used by aquaculture industry alone (Shepherd 2012). However, unfortunately there is no scope to escalate the FM production in future and the FM price is increasing steadily. Therefore, utilization of FM in marine carnivorous fish feeds has started declining over the years. The increased demand of FM coupled with rise in prices led to the search of alternative protein sources suitable for the replacement of FM to the maximum extent possible. Thus, the main challenges ahead for the aquafeed industry are to reduce the inclusion rate of FM in feeds and to source out economically viable and environment friendly alternative ingredient sources (Agbo 2008). In this context, fishmeal replacement is tried with several plant source protein ingredients both commercial and non-conventional sources as they are widely available, renewable and reasonably priced. Among the different plant source proteins, cottonseed meal (CSM) is one of the most promising alternatives to marine source ingredients due to its high protein content (42-45%), palatability, wide availability at reasonably low price (Li and Robinson, 2006). The CSM is an unconventional feed ingredient obtained as a residue from cottonseed oil extraction process which generally priced lower than groundnut oilcake and soybean meal, would be beneficial in reducing feed costs for commercial fish farming (Luo et al. 2006).

Like other ingredients of plant origin, CSM is also contains some anti-nutritional factors including free gossypol (FG), a yellow coloured polyphenolic aldehyde that is the most harmful to monogastric animals including fish species (Cheng and Hardy 2002). The major limitation associated with the use of CSM is the FG toxicity which could decrease the digestibility of lysine when fish ingested diets with high concentration of gossypol (Liu et al. 2009) and also causes anorexia, anemia, pathological changes in liver and kidney, and abnormal gametogenesis (Yue and Zhou 2008; Yan et al. 2014). The CSM was examined for many commercial important fish species such as rainbow trout (Rinchard et al. 2003a; Luo et al. 2006), silver crucian carp (Cai et al. 2011), black sea bream (Sun et al. 2015), Ussuri catfish (Bu et al. 2017) and black sea bass (Anderson et al. 2016) to determine the suitable proportions of inclusions as alternatives to fishmeal by assessing its impacts on physiological and biochemical responses. Liu et al. (2016) suggested that the adverse effect of increasing CSM in the diet on growth performance is species-specific and related to the age of the fish (Rinchard et al., 2003a) and dietary free gossypol content of the diet (Wang et al. 2014).

Among the different species of mariculture interest, snubnose pompano is one of the commercially important species because of its fast growth, relatively lower protein requirement, tasty white meat and tolerance to wide range of salinity and

temperature (Kalidas et al. 2012; Prabu et al. 2020). The farming of snubnose pompano successfully established in several Asian-Pacific countries such as Taiwan, Indonesia, China and India and being carried out in coastal ponds and floating open sea cages (Kalidas et al. 2020). Therefore, the present study is aimed to replace FM using CSM to evaluate the effect on physiological, hematological, biochemical and histological response in the juveniles of snubnose pompano.

Material And Methods

Cottonseed meal

Extruded cottonseed meal (CSM) was procured from Growel Feeds Pvt. Ltd., Andhra Pradesh, India. The CSM was milled using a kitchen mixer grinder to reduce the particle size about 100 µm for feed preparation.

Experimental fishes

Juveniles of snubnose pompano, *T. blochii* (Lacepede, 1801) were procured from Mandapam Regional Centre of ICAR-CMFRI, Tamil Nadu to Tuticorin Research Centre of ICAR-CMFRI, Thoothukudi in oxygen filled polyethylene bags. On arrival, the pack of fishes were acclimated to the prevailing water temperature of the wet lab and followed by quarantine measures were carried out. The fishes were stocked in seawater of 25 g kg^{1}salinity. The salinity of rearing medium were gradually reduced to 15 g kg^{1} in 15 days duration and maintained at 15 g kg^{1} for further research.

Setting up of experimental units

The experiment was conducted in indigenous Re-circulatory Aquaculture System (i-RAS) unit designed for fish nutrition experiment (Prabu et al. 2017). After acclimation, the fishes with the average weight of 12.5±0.5 g were stocked by following completely randomized design (CRD) in which fishes were subjected to five number of treatments each with triplicates. In each replicate 12 fishes were stocked. The treatments were named according to the level of replacement of FM protein with CSM in the diet as 0CSM (0% FM protein replaced with CSM), 25CSM, 50CSM, 75CSM and 100CSM (100% FM protein replaced with CSM).

Experimental feed

There were five experimental diets formulated with graded levels of CSM to replace FM in the isonitrogenous (40% CP) and isolipidic (6% CF) diets. Feed ingredients (Table 1) were weighed accurately, mixed thoroughly, blended with required quantity of water and cooked; after which passed through laboratory scale motorized pelletizer using 4 mm diameter die. Wet feed strings were further dried, crushed and sieved to collect 2 and 3 mm slow-sinking crumble feeds (Prabu et al. 2020).

Table 1

Feed for cottonseed meal experiment (g kg^{-1})

Ingredients	0CSM	25CSM	50CSM	75CSM	100CSM
Cottonseed meal[1]	**0**	**87**	**174**	**260**	**347**
Fishmeal[2]	**240**	**180**	**120**	**60**	**0**
Soy bean meal[3]	140	150	160	169	176
Squid meal[4]	40	40	40	40	40
Groundnut oil cake [5]	126	129	127.5	129	129
Meat&bone meal [6]	50	50	50	50	50
De-oiled rice bran[7]	73	42.5	15	0	0
Shrimp meal[8]	50	50	50	50	50
Wheat powder[9]	200	190	180	155	117.5
Fish oil[10]	15	15	15	15	15
Sunflower oil[11]	10	10	10	10	10
Vitamin[12,†]	15	15	15	15	15
Mineral[13‡]	15	15	15	15	15
Sodium meta bisulphate[14]	1	1	1	1	1
Methionine[15]	**3.5**	**3.5**	**4**	**4.5**	**5**
Lysine[16]	**3**	**3.5**	**5**	**6**	**7**
Vitamin C[17]	2.5	2.5	2.5	2.5	2.5
Lecithin[18]	5	5	5	5	5
Dicalcium phosphate [19]	5	5	5	5	5

1Gima cotton seed Bio–technology, Wardha, Maharastra; 2Raj Fishmeal and Oil Co., Malpe, Mangalore; 3SakthiSoya, Coimbatore; 4King Fish Products Pvt Ltd., Veraval; 5,7,9&11 Locally procured; 6Arogya Bio Proteins, Vellore; 8KhajaMohideen, Wall Tax Road, Chennai; 10Crude sardine oil from Kiriyathan Trading Co., Narakkal, Kerala; 12Supplevite – M from Sarabhai Zydus Animal Health Pvt. Ltd, Vadodara; 13Agrimin from Vibrac Healthcare India, Pvt. Ltd., Mumbai; 14,18,19&24 Hi–Media, Mumbai; 15Evonik Degussa, Germany; 16BestAmino, CJ Corporation, South Korea; 17Stay – C from DSM Nutritional Technologies, Mumbai; 20&21 Locally collected; 22Collected from Arunachal Pradesh; 23Pega Bind, Bentoli, USA

SWM, Seaweed meal (*Sargassum wightii*); NFM, Neem flower meal (*Azadirachta indica*); FWM, Fishwort meal (*Houttuynia cordata*).

† Composition of vitamin mix (unit kg^{-1}): Vitamin A, 500,000 IU; Vitamin D3, 250,000 IU; Vitamin E, 2000 mg; Vitamin K, 1,000 mg; Thiamin, 100 mg; Riboflavin, 2,000 mg; Pyridoxine,500 mg; Cyanacobalamin, 400 mg; Calcium Pantothenate, 2,500 mg; Niacin, 4000 mg; Biotin,4000 mg; Folic acid 200 mg.

‡ Composition of mineral mix (unit kg^{-1}): Manganese oxide, 500 mg; Potassium iodide, 500mg; Ferrous sulphate, 10 g; Zinc oxide, 1000 mg; Copper sulphate, 250 mg; Cobalt carbonate, 2mg; Sodium selenite, 10 mg; Chromium chloride, 100 mg; Calcium lactate, 250 g; Calcium phosphate (monobasic), 350 g; Magnesium sulphate, 50 g.

Ingredients	0CSM	25CSM	50CSM	75CSM	100CSM
Immunostimulants	5	5	5	5	5
SWM[20]+NFM[21] +FWM[22]					
Binder[23]	0	0	0	2	4
Butylated hydroxyl toluene[24]	1	1	1	1	1

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Fish rearing

Fishes were fed to apparent satiation twice daily at 10.00 and 16.00 h during the 10-week feeding trial and feed intake by the experimental fishes was recorded. Round the clock filtered water circulation was made through iRAS with 15 g kg^{-1} saline water and the flow-rate of water in each tank was kept at 5 L min^{-1}. Faecal matter generated in the experimental tanks was siphoned out daily and the released water was replenished with 15 g kg^{-1} saline water. The water quality parameters such as dissolved oxygen, pH, temperature, ammonia, nitrite and nitrate level were kept optimal during the experimental period.

Growth assessment and feed utilization

At the end of the rearing period, the body weight of experimental fish was measured to assess the growth response and feed utilization. The fish were starved overnight before taking the body weight and to avoid the fecal matter at the time of sampling. Experimental fishes were weighed accurately with a precision of 0.1 g using electronic balance at the sampling

site. The growth parameters weight gain percentage (WG%), specific growth rate (SGR), average daily growth (ADG), geometric mean body weight (W_G), growth rate on metabolic body weight (GR_{MBW}), thermal growth coefficient (TGC) and feed utilization parameters such as absolute feed intake (FI_{ABS}), feed intake of fish on % of body weight (FI_{PCT}), feed intake per metabolic body weight (FI_{MBW}), feed conversion ratio (FCR), protein efficiency ratio (PER), feed efficiency (FE) and protein productive value (PPV) were calculated as described by Prabu et al. (2020).

Sampling, blood collection and homogenate preparation

Two fish from each replicate were anesthetized with MS222 at 100 mg L^{-1} water. The fishes were euthanized and liver, gill and intestine tissue samples were removed carefully. Tissue samples were homogenized with 0.25 M chilled sucrose solution for 10% tissue homogenate using tissue homogenizer (IKA® India Pvt. Ltd., Bengaluru, India), centrifuged by refrigerated centrifuge (Remi, India), supernatant collected and stored at -20°C freezer (Blue Star Ltd., India) until used (Tok et al. 2017) for the assay of digestive and metabolic enzymes. From all treatments three more fishes were euthanized for the collection of blood and serum from the caudal vein as described by Prabu et al. (2016). Subsequently, the fishes were sliced into pieces and oven dried at 105°C for 6 h for determination of whole body proximate composition and nutrition profiling.

Free gossypol estimation

The free gossypol content of CSM, experimental diets and muscle samples of experimental fishes were assessed through HPLC (Waters, USA) by the method of Karishma et al. (2016).

Proximate composition

The proximate composition of experimental feeds and whole body fish samples were carried out (AOAC 1995). The crude protein content (nitrogen × 6.25) was determined by the Kjeldahl method after acid digestion using a Kjeldahl System (FOSS Kjeltec 2300). Crude lipid content was estimated using a Soxhlet System (FOSS Soxtec 2043). Total ash content was determined by incinerating the feeds and tissue samples in a muffle furnace (Kemi Lab Equipment, Kerala, India) at 600 ºC for 4 h and moisture content was estimated using hot air oven.

Essential amino acid profile of feed and tissue samples

The amino acid profile of experimental feeds and whole-body fish tissue samples were estimated using reverse-phase high-performance liquid chromatography (HPLC) (Waters, USA). The feed and tissue samples were hydrolyzed using 6 M HCl at 110 ºC for 24 h. Further, the hydrolysates of samples were loaded to a C-18 column and amino acids were separated via reverse phase HPLC. Essential amino acids were quantified with a photodiode array detector following post-column derivatization with ninhydrin (Smith 1997).

Hematological parameters

The estimation of haemoglobin level was done by cyanmethemoglobin method (Larsen and Snieszko, 1961) using Drabkin's fluid (Himedia, India). Red blood corpuscles (RBC) and white blood corpuscles (WBC) were counted in a Neubauer's haemocytometer (Rohem, India) using RBC and WBC diluting fluids (Himedia, India), respectively. The packed cell volume (PCV) or Heamatocrit (Hct %) was determined by the Wintrobe and Westergreen method as described by Blaxhall and Daisley (1973). The mean cell volume (MCV) and the mean cell hemoglobin concentration (MCHC) were calculated from the Hct, Hb and RBC by standard formulas (Blaxhall and Daisley 1973).

Serum biochemical markers

Serum biochemical parameters were assessed by kits manufactured by Coral Clinical Systems, Goa, India. Serum total protein was determined by biuret method (Reinhold 1953) using the protein estimation kit. Albumin was estimated by Bromocresol green binding method (Doumas et al. 1971) using kit whereas globulin level was determined by subtracting the values of serum total protein with serum albumin. Serum triglyceride was determined by glycerol phosphate oxidase/peroxidase method (Fossati and Prencipe 1982) using triglyceride kit. Serum cholesterol was estimated by

cholesterol oxidase/peroxidase method (Meiattini et al. 1978) using cholesterol kit. Phospholipid content was calculated from cholesterol content empirically by the method of Covaci et al. (2006). Serum total lipid content was calculated by the summation of serum triglycerides, phospholipids and cholesterol contents. Serum very low density lipoprotein (VLDL) level was calculated as per the methods of Friedewald et al. (1972).

Digestive and metabolic enzymology

Intestinal α-amylase activity was estimated by dinitro-salicylic acid method as described by Rick and Stegbauer (1974). As described by Drapeau, (1976) intestinal protease activity of samples from experimental fishes was determined by the casein digestion method. The intestinal lipase activity was determined by the *p*-nitrophenyl palmitate (*p*NPP) hydrolysis method (Katsivela et al. 1995). Aspartate aminotransferase (AST) activity was assayed in liver and muscle tissue homogenates as described by Wootton (1964) and alanine aminotransferase (ALT) activity was assayed similarly except the substrate 0.2 M DL- alanine used instead of aspartic acid. Alkaline phosphatase (ALP) activity of liver and intestine samples was determined by the method of Garen and Levinthal (1960). Whereas acid phosphatase (ACP) activity was estimated by the same method of ALP, except acetate buffer (0.2M, pH 5) was used in lieu of bicarbonate buffer. Lactate dehydrogenase (LDH) activity from muscle and liver samples of experimental fishes was assayed by the method of Wroblewski and La Due (1955). Malate dehydrogenase (MDH) activity from liver and muscle samples of experimental fishes was assayed by the method of Ochoa (1955).

Histology

Liver samples of experimental fishes from respective treatments for histological observations were fixed in Bouin's Fluid. After the fixation, the liver samples were dehydrated in ethanol series, embedded in paraffin, sectioned at 5µm, mounted on albumin coated glass slides and stained with hematoxylin and eosin (Humason 1979). The mounted liver sections were examined using the Euromex Oxion microscope with the imaging software Image focus 4.

Statistical analysis

The data were statistically analyzed by statistical package, SPSS version 20 (SPSS Inc., Chicago, IL, USA). Comparison among different treatments was made for each responses using linear model through one-way ANOVA and further checked for the presence of quadratic effects using orthogonal polynomial contrasts. Statistical significance was determined at 5% ($p \leq 0.05$) probability levels. The second degree polynomial regression analysis was carried out to determine the optimum level of inclusion of CSM in the diet by exploring the relationship between various responses and CSM level in the diet. The model form used as follows:

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

Where x_u represents the CSM levels, β_i (i =0, 1, 2) represents parameters of the model which were estimated using the Ordinary Least Square (OLS) method and e_u is the random error and the model fitness was assessed using R^2. Optimum CSM was estimated by taking partial differential of the fitted model as follows:

$$\frac{?f\left(x_u\right)}{?x_u} = 0 \text{andthemaximum(orminimum)isdecidedwhen} \frac{?^2 f\left(x_u\right)}{?x_u^2}$$

Results

Growth response and feed utilization

The growth performance of snubnose pompano fed with graded levels of CSM to replace protein content of FM in the diet showed significant difference (linear, quadratic, cubic and quartic, $P<0.05$) except for initial weight (Table 2). Higher WG%,

SGR, ADG, TGC, geometric mean body weight and growth rate on metabolic body weight were observed in 0CSM group followed by 100CSM. Absolute feed intake (FI_{ABS}) showed a significant difference (all order, $P<0.05$) and feed intake on % of body weight (FI_{PCT}), feed intake per metabolic body weight (FI_{MBW}) and PPV showed significant difference ($P<0.05$) at linear, quadratic and cubic order of polynomial contrast. The FCR, PER and FE showed quadratic, cubic and quartic order of significant difference ($P<0.05$). Lower FI_{PCT} and FI_{MBW} were observed in 25CSM group, followed by 50CSM and 100CSM group. Among the treatments, better FCR was observed in 100CSM group and better PER and PPV were witnessed in 0CSM group.

Table 2
Growth performance and feed intake of snubnose pompano fed with graded level of CSM

Parameters	0CSM	25CSM	50CSM	75CSM	100CSM	SEM	LOP	SOP	COP	QOP
Growth performance										
Initial weight	12.69	12.75	12.73	12.62	12.61	0.02	0.033	0.132	0.159	0.491
Final weight	61.65	56.71	57.90	57.27	59.06	0.47	0.001	0.001	0.001	0.001
Weight gain %	385.68	344.81	354.72	353.78	368.30	3.87	0.001	0.001	0.001	0.001
Specific growth rate (%)	2.25	2.13	2.16	2.16	2.20	0.01	0.002	0.001	0.001	0.001
ADG (g day^{-1})	0.70	0.63	0.65	0.64	0.66	0.006	0.001	0.001	0.001	0.001
W_G (g)	27.97	26.89	27.15	26.88	27.29	0.11	0.001	0.001	0.033	0.002
GR_{MBW} (g $kg^{-0.8}$ day^{-1})	31.25	29.19	29.70	29.66	30.39	0.19	0.001	0.001	0.001	0.001
Thermal growth coefficient	0.80	0.74	0.75	0.75	0.77	0.02	0.001	0.001	0.001	0.001
Feed utilization										
FI_{ABS} (g DM $fish^{-1}$ day^{-1})	1.03	0.96	0.97	0.99	0.99	0.006	0.001	0.001	0.001	0.038
FI_{PCT} (% day^{-1})	3.70	3.57	3.59	3.67	3.61	0.014	0.009	0.001	0.001	0.063
FI_{MBW} (g DM $kg^{-0.8}$ day^{-1})	46.28	44.63	44.85	45.91	45.17	0.17	0.009	0.001	0.001	0.063
Feed conversion ratio	1.27	1.28	1.27	1.30	1.26	0.003	0.955	0.001	0.001	0.001
Protein efficiency ratio	1.56	1.52	1.53	1.50	1.56	0.006	0.124	0.001	0.020	0.001
Feed efficiency	0.62	0.61	0.61	0.60	0.62	0.002	0.124	0.001	0.020	0.001
Protein productive value (%)	34.08	33.36	32.82	32.3	33.67	0.18	0.019	0.001	0.029	0.281
ADG, Average daily growth; W_G, Geometric mean body weight; GR_{MBW}, Growth rate on metabolic body weight; FI_{ABS}, Absolute feed intake; FI_{PCT}, Feed intake of fish on % of body weight; FI_{MBW}, Feed intake per metabolic body weight.										
Data expressed as Arithmetic mean with three replications.										

Proximate composition of experimental diets and fishes

The total ash and fibre content of experimental diets showed significant difference (linear, quadratic order, P<0.05). The calcium, phosphorous and free gossypol content showed linear order of polynomial significant difference (P<0.05). Total ash, calcium and phosphorous content exhibited a decreasing trend as the level of inclusion of CSM increased where as free gossypol (FG) and fibre content showed an increasing trend as the level of inclusion of CSM increased in the diet (Table 3). The FG content of CSM used in this experiment was 965 mg kg^{-1}.

Table 3
Proximate composition of experimental feed and whole body of experimental fish

Parameters	0CSM	25CSM	50CSM	75CSM	100CSM	SEM	LOP	SOP	COP	QOP
Feed proximate composition (%)										
Moisture	7.61	7.14	7.37	7.06	7.36	0.07	0.155	0.050	0.830	0.039
Crude protein	40.16	40.18	40.32	40.15	40.17	0.02	0.945	0.095	0.586	0.037
Crude fat	6.16	06.13	6.17	6.18	6.19	0.02	0.410	0.431	0.555	0.775
Total ash	10.67	10.48	10.33	10.26	10.14	0.05	0.001	0.019	0.156	0.400
Crude fibre	3.11	3.13	3.21	3.29	3.48	0.04	0.001	0.023	0.612	0.643
Nitrogen free extract	32.28	32.93	32.63	33.05	32.65	0.09	0.063	0.019	0.772	0.016
GE (kcal g^{-1})	4.04	4.07	4.06	4.08	4.07	0.003	0.007	0.003	0.841	0.031
Calcium	2.48	2.20	1.92	1.66	1.37	0.11	0.001	0.978	0.699	0.961
Phosphorous	1.54	1.44	1.32	1.23	1.13	0.04	0.001	0.956	0.931	0.713
Free gossypol (mg kg^{-1})	0.00	85.86	177.15	252.63	343.62	5.96	0.001	0.411	0.267	0.152
Whole body proximate composition on DM basis (%)										
Crude protein	65.01	65.19	65.23	65.21	65.23	0.05	0.207	0.352	0.633	0.984
Crude fat	21.07	21.27	21.39	21.20	21.34	0.05	0.197	0.318	0.250	0.399
Total ash	13.48	13.05	12.90	13.06	12.92	0.07	0.019	0.092	0.167	0.550
Crude fibre	0.19	0.24	0.25	0.30	0.30	0.01	0.001	0.298	0.860	0.241
Nitrogen free extract	0.26	0.24	0.23	0.21	0.22	0.02	0.458	0.894	0.896	0.905
GE (kcal g^{-1})	5.42	5.45	5.46	5.43	5.45	0.005	0.086	0.163	0.179	0.409

GE, Gross energy; DM, Dry matter. Data expressed as mean ±SE

The whole-body total ash and fibre content showed linear order of significance (P<0.05). Higher level of whole-body crude protein and lipid contents were observed in 0CSM and 100CSM groups, respectively. Higher total ash content of experimental fishes was observed in 0CSM and lower value was registered in 50CSM group (Table 3). The whole-body calcium content showed significant difference (linear order polynomial, P<0.05) and the level was showing a decreasing trend as the level of inclusion of CSM increased in the diet (Fig. 1A). The phosphorous content was also showing a similar trend (Fig. 1B).

Tissue level free gossypol content

The FG content of muscle samples showed significant difference (linear, quadratic, P<0.05) (Fig. 2A). The higher FG content of 1.28 mg kg^{-1} on as is basis was observed in 100CSM group. The level of FG contents of CSM based diet fed fishes were

very well below the limit set by FDA (450 mg kg^{-1}). The relationship between muscular FG accretion and dietary FG content showing a linear equation of y = 0.0115x + 0.1712;

R^2 = 0.9892 (Fig. 2B).

Essential amino acid profile of experimental feed and tissue samples

Essential amino acid composition of experimental feeds and whole body tissue samples of experimental fishes were showed significant difference ($P<0.05$) at a different order of polynomial contrasts among the feeds (Table 4). The level of arginine and cystine were found higher as the level of CSM increased in the diet. There was a slight decrease in the lysine content of the experimental diets among the treatments as the level of inclusion of CSM increased in the diets; however, the levels were almost on par with the lysine requirement of snubnose pompano. Rest of the essential amino acid values showing a decreasing trend as the level of FM decreased in the experimental diets.

Table 4
Essential amino acid profile of experimental feeds and fished fed with graded level of CSM

Amino acids	0CSM	25CSM	50CSM	75CSM	100CSM	SEM	LOP	SOP	COP	QOP
Experimental feed (g kg^{-1} feed)										
Arginine	20.30	21.77	22.08	21.41	20.91	0.18	0.085	0.001	0.012	0.435
Histidine	10.15	9.70	8.80	8.19	7.66	0.25	0.001	0.770	0.165	0.373
Isoleucine	13.39	12.75	12.41	12.07	11.80	0.15	0.001	0.151	0.544	0.735
Leucine	33.06	32.48	32.07	31.73	31.44	0.16	0.001	0.120	0.731	0.952
Lysine	24.46	24.20	24.15	23.96	23.81	0.06	0.001	0.710	0.288	0.201
Methionine	11.66	11.71	11.71	11.68	11.69	0.01	0.398	0.049	0.024	0.845
Phenylalanine	17.93	16.90	16.40	15.87	15.51	0.23	0.001	0.008	0.301	0.353
Therionine	14.96	14.41	14.06	13.56	13.22	0.17	0.001	0.517	0.910	0.510
Valine	15.04	14.70	14.20	14.07	13.94	0.11	0.001	0.017	0.514	0.181
Cystine	6.11	6.65	7.22	8.08	8.61	0.24	0.001	0.222	0.053	0.094
EAA	165.81	163.88	161.70	159.62	157.78	0.90	0.001	0.952	0.906	0.987
Whole body samples of experimental fish (g kg^{-1} body mass on as is basis)										
Arginine	12.82	13.85	14.10	13.44	13.21	0.12	0.015	0.001	0.001	0.001
Histidine	8.05	7.79	8.07	8.17	7.93	0.04	0.127	0.190	0.001	0.022
Isoleucine	10.43	10.29	10.02	9.76	9.47	0.10	0.001	0.238	0.607	0.743
Leucine	17.87	17.75	16.68	16.50	16.87	0.15	0.001	0.001	0.001	0.001
Lysine	20.69	20.36	19.91	20.13	19.77	0.11	0.004	0.400	0.440	0.202
Methionine	7.10	6.95	6.88	6.80	6.93	0.03	0.005	0.004	0.334	0.412
Phenylalanine	8.32	7.97	7.77	7.92	7.73	0.06	0.001	0.035	0.058	0.160
Therionine	7.54	7.65	7.53	7.16	6.99	0.09	0.001	0.016	0.104	0.435
Valine	10.39	10.36	10.13	10.01	10.16	0.04	0.001	0.004	0.001	0.618
Cystine	4.54	4.75	4.43	4.64	4.84	0.04	0.024	0.033	0.018	0.008
EAA	107.76	107.30	105.29	104.53	104.04	0.37	0.001	0.350	0.107	0.196

Data expressed as mean ±SEM; EAA, Essential amino acids.

The limiting amino acids lysine and methionine levels in whole body tissue samples of experimental fishes were slightly lower in the CSM based diets fed fishes than the control diet fed fishes (0CSM). Arginine value was found increasing until 50CSM group after which a slight reduction was noticed in 75CSM and 100CSM groups and rest of the EAAs were showing a fluctuating trend among the treatments with maximum values at 0CSM group.

Hematological response

Among the complete blood count indices, RBC count showed linear order of polynomial (LOP), WBC and PCV showed LOP and SOP and MCH showed LOP and COP significant difference ($P<0.05$). Rest of the indices such as MCV, MCHC and hemoglobin (Hb) level of experimental fishes from differential treatments does not show significant difference ($P>0.05$)

(Table 5). The RBC, WBC, Hb and PCV were found higher in 0CSM group. Higher MCH and MCV values were recorded in 75CSM group and MCHC value was found higher in 100CSM group.

Table 5
Hematological response of experimental fish fed with graded level of CSM in place of FM

Parameter	0CSM	25CSM	50CSM	75CSM	100CSM	SEM	LOP	SOP	COP	QOP
Hb (g dL^{-1})	9.25	8.43	8.56	8.56	8.75	0.14	0.374	0.115	0.431	0.591
RBC (10^6 mm^{-3})	3.24	3.19	2.92	2.81	2.94	0.05	0.004	0.128	0.104	0.686
MCH (pg)	28.64	26.40	29.30	30.52	29.77	0.47	0.025	0.657	0.015	0.330
MCHC (%)	27.41	26.14	27.44	27.56	28.54	0.45	0.298	0.421	0.624	0.529
MCV (fL)	104.89	101.27	107.02	110.71	104.43	1.53	0.449	0.577	0.104	0.905
WBC (10^3 mm^{-3})	6.12	5.44	4.78	4.12	4.48	0.20	0.001	0.003	0.055	0.412
PCV (%)	33.80	32.26	31.20	31.07	30.67	0.32	0.001	0.013	0.430	0.496

Hb, Hemoglobin; RBC, Red blood corpuscles; WBC, White blood corpuscles; MCH,Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin; MCV, Mean corpuscular volume; PCV, Packed cell volume. Data expressed as Mean.

Biochemical response

The serum biochemical parameters such as total protein, albumin, globulin and A:G ratio does not showed significant difference (P>0.05) (Table 6). However, higher protein and albumin content was registered in 0CSM group followed by 100CSM group. The globulin content was found higher in 100CSM group followed by 0CSM group. Serum triglycerides, total lipids and VLDL contents showed a significant difference (LOP; SOP; P<0.05) and cholesterol, and phospholipids contents showed significant difference (P<0.05) at LOP (Table 6). The level of triglycerides, cholesterol, phospholipid, VLDL and total lipid contents were showing a decreasing trend as the level of inclusion of FM was decreased by replacing with CSM.

Table 6
Serum biochemical parameters of experimental fishes fed with varying levels of CSM in the diet

Parameters	0CSM	25CSM	50CSM	75CSM	100CSM	SEM	LOP	SOP	COP	QOP
Protein profile										
Total protein (g dL^{-1})	4.88	4.72	4.75	4.69	4.83	0.04	0.641	0.167	0.945	0.453
Albumin (g dL^{-1})	1.97	1.83	1.85	1.81	1.87	0.03	0.252	0.188	0.754	0. 460
Globulin (g dL^{-1})	2.91	2.89	2.90	2.87	2.96	0.02	0.546	0.308	0.594	0.610
A:G ratio	0.68	0.63	0.64	0.63	0.63	0.008	0.126	0.354	0.521	0.595
Lipid profile (mg dL^{-1})										
Triglyceride	122.62	116.07	113.39	112.80	115.71	1.03	0.001	0.002	0.123	0.689
Cholesterol	150.81	147.57	146.15	144.47	143.56	0.85	0.002	0.402	0.813	0.788
Phospholipids	200.09	197.73	196.69	195.46	194.80	0.62	0.002	0.402	0.813	0.788
Total lipid	473.52	461.37	456.62	453.62	451.16	2.30	0.001	0.035	0.388	0.919
VLDL	24.52	23.21	22.67	22.73	22.55	0.20	0.001	0.002	0.123	0.689

VLDL, Very low density lipoprotein. Data expressed as Mean.

Digestive enzymology

The intestinal α-amylase and protease activities showed significant difference (linear, quadratic, quartic, $P<0.05$) and lipase showed significant difference (linear, $P<0.05$) (Fig. 3A, B). The α-amylase activity was higher in 50CSM treatment and lower in 0CSM group. The maximum protease and intestinal lipase activity were noticed in 0CSM treatment.

Metabolic enzyme activities

The hepatic ACP, ALP and intestinal ALP activities showed significant difference (LOP, SOP, $P<0.05$) and intestinal ACP showed significant difference (SOP, $P<0.05$) (Table 7). Higher hepatic and intestinal ACP and ALP activities were observed in 0CSM group and lower activities were registered in 75CSM group. The hepatic AST showed significant difference (LOP, SOP, $P<0.05$) and muscular AST, ALT and hepatic ALT activities showed significant difference (SOP, $P<0.05$). Higher AST and ALT activities were observed in 0CSM group followed by 100CSM group. The muscular LDH and MDH activities showed significant difference (LOP, SOP, $P<0.05$) and hepatic MDH showed significant difference (SOP, $P<0.05$). Better LDH and MDH activities were observed in 0CSM group followed by 100CSM group.

Table 7
Metabolic enzyme activities of experimental fishes fed with varying levels of CSM in the diet

Parameter	Tissue	0CSM	25CSM	50CSM	75CSM	100CSM	SEM	LOP	SOP	COP	QOP
ACP	Liver	70.04	62.42	59.42	54.23	59.89	1.72	0.008	0.038	0.486	0.408
	Intestine	31.85	28.68	27.96	27.54	29.19	0.57	0.091	0.035	0.916	0.674
ALP	Liver	108.83	99.29	95.81	88.05	94.65	1.99	0.001	0.006	0.252	0.139
	Intestine	54.31	44.62	40.18	37.90	43.74	1.59	0.001	0.001	0.521	0.449
AST	Liver	31.85	27.81	25.04	26.61	27.32	0.75	0.020	0.011	0.579	0.421
	Muscle	17.62	16.51	15.97	15.34	17.02	0.25	0.068	0.002	0.168	0.341
ALT	Liver	24.63	20.80	21.23	22.34	22.13	0.45	0.143	0.021	0.041	0.883
	Muscle	11.83	10.62	9.83	10.20	11.07	0.29	0.341	0.047	0.889	0.997
LDH	Liver	0.99	0.83	0.85	0.90	0.89	0.02	0.431	0.111	0.191	0.893
	Muscle	1.40	1.24	1.14	1.19	1.28	0.03	0.022	0.001	0.779	0.546
MDH	Liver	2.78	2.61	2.46	2.53	2.70	0.04	0.291	0.003	0.677	0.577
	Muscle	3.78	3.61	3.51	3.35	3.74	0.04	0.013	0.001	0.002	0.039

ALT, Alaninae aminotransferase; AST, Aspartate aminotransferase; LDH, Lactate dehydrogenase; MDH, Malate dehydrogenase, ACP, Acid phosphatase; ALP, Alkaline phosphatase.

LDH and MDH activity was expressed as Unitmg protein^{-1} min^{-1} where 1 unit was equal to A0.01 OD min^{-1}. AST and ALT activity was expressed as nanomoles oxaloacetate formed mg protein^{-1} min^{-1} and nanomoles pyruvate formed mg protein^{-1} min^{-1} respectively. ACP sand ALP activity was expressed as nanomoles p-nitrophenol released min^{-1} mg protein^{-1}. Data expressed as Mean.

Histology of liver

Liver sections of snubnose pompano from various treatments indicated no much variation among the treatments (Fig. 4). Liver section of snubnose pompano from 25CSM group showing the hepatocytes with mild intra cytoplasmic vacuolation and also in 50CSM group very few mild vacuolated hepatocytes, hepatocytes with few pyknotic nucleus and very few hepatocytes with hypertrophy were present. The hepatocytes of liver section of 100CSM group were quiet normal with no abnormalities and were comparable with 0CSM.

Optimize the CSM inclusion

The hematological parameter (MCV), biochemical parameters (triglycerides and total lipid) and enzymological parameter (α amylase) were subjected to second degree polynomial regression analysis to determine the optimum level of inclusion of CSM in the diet of snubnose pompano. The relationship between these parameters and dietary CSM levels revealed that CSM can be included in the range of 27.8-34.3% of diet (Fig. 5).

Discussion

The growth performance of snubnose pompano in the present study, in terms of WG%, SGR, ADG and TGC were found higher in control group followed by 100CSM containing 343 mg FG kg $^{-1}$ diet, which indicated that it can tolerate the FG than most of fishes and thus growth performance was not affected. This is in agreement with Rinchard et al. (2003a) found that complete replacement of FM using CSM does not influence the growth performance in terms of WG% and SGR% in male rainbow trout.

The major limitation associated with the use of CSM is the free gossypol toxicity which could decrease the digestibility of lysine and rendering it less bioavailable when fish ingested diets with high concentration of gossypol (Liu et al. 2009). According to Rinchard et al. (2003b) growth depression in fishes fed with CSM based diets are usually linked with the reduction in the bioavailable lysine contents of the diets. In the present study, the maximum inclusion level of CSM was 34.7% of diet which did not show any negative effects on growth due to supplementation of adequate level of lysine in the diet and availability of more quantity of arginine in the CSM replaced diets when compared with 0CSM diet. This is because the fact that FG present in CSM reacts with the free epsilon-amino groups from lysine and arginine to form bound gossypol which affects the digestibility of these amino acids (Fernandez et al. 1995). In the same way, higher inclusion rate of 24.5% of CSM was reported for on-growing grass carp (Yan et al. 2014) and 36.6% for sub-adult stage of grass carp (Liu et al., 2016) without adverse effects on growth. As the FM replaced diets in the present study were supplemented with lysine and methionine on par with the control diet and availability of higher arginine content as the level of CSM increased in the diet, the growth performance of pompano fed with CSM was not depressed.

Haematological status of fish is a useful indicator for monitoring fish health and physiological responses to any stressors (Ahmed et al. 2020). Free gossypol found in the CSM readily binds with dietary iron to form a gossypol-iron complex, which inhibits its absorption that leads to iron deficiency and affects erythropoiesis (Gadelha et al. 2014). The FG may also cause a reduction in Hct and Hb of fish (Mbahinzireki et al. 2001) and leukocyte counts which affect the immune-competence of the animal (Braga et al. 2012). The hemoglobin, RBC, WBC and hematocrit values in the present study were not significantly influenced by the complete replacement of FM using CSM in snubnose pompano. This result is in line with Rinchard et al. (2003a) who reported similar response in terms of RBC and hematocrit in male rainbow trout fed with graded levels of CSM in the diet. Similarly, Dabrowski et al. (2000) fed adult rainbow trout with the diets containing up to 990 mg FG/kg through dietary CSM for 131 days had lower hematocrit and hemoglobin than fish fed 495 mg/kg or less concentration. The deleterious effects of CSM on hematological response of fishes are species-specific (Liu et al. 2016) and fish can endure relatively higher levels of FG than other monogastric animals (Cheng and Hardy 2002). In the present study, the levels of FG content was lower and also the diets were adequately supplemented with lysine which may bound with FG and effectively minimized the formation of gossypol-iron complex and hence, did not affected the RBC and hematocrit values seriously.

According to Summerfelt and Penne (2007) the nutrient accretion in different fish species is primarily dependent on the level of dietary nutrient composition and other associated biological factors. In the current study, whole-body proximate composition of experimental fishes in terms of total ash, calcium and phosphorous content was influenced by the reduction in the level of inclusion of FM content of the diet and thus the lower values were observed in FM free diet fed fishes (100CSM). This is in agreement with Garcia-Abiado et al. (2004) and Hassaan et al. (2019) reported the reduction of total ash content in tilapia as the level of inclusion of CSM in the diet increased by reducing FM content of the diet. Calcium (Ca) and phosphorous (P) concentration of fish vertebra, scales and whole body tissue have been commonly used as an insightful indicator of dietary Ca or P status in fish nutritional research (Zhang et al. 2006; Hossain and Yoshimatsu 2014). In corroboration with the present result, Mbahinzireki et al. (2001) reported that dietary CSM with varying level of FG on mineral composition especially calcium and phosphorous content of whole body of tilapia found a decreasing trend as the CSM inclusion increased. Similarly, Hassaan et al. (2019) reported the plasma calcium and phosphorous content of comparable response in tilapia. In the present study, other proximate parameters such as crude protein, lipid and NFE were not affected by CSM inclusion in the diet. The results of the current study corroborates the results of Ahmed et al. (2018) and Zhou et al. (2017) in juvenile blunt snout bream fed with complete plant based diet and cottonseed meal, respectively.

The essential amino acid profile of the experimental diets indicating that the levels of limiting amino acids, lysine and methionine were on par with their requirements of lysine (2.4% of diet) and methionine (1.16-1.18% of diet) of snubnose pompano (Ebeneezar et al. 2019; Ebeneezar et al. 2020). In the present study, the experimental diets were supplemented with external sources of lysine and methionine to maintain their levels equally in all the FM replaced diets on par with the requirements of snubnose pompano. The lysine content was slightly reduced to the level of requirements in the higher inclusion of CSM groups (75CSM and 100CSM) which may be due to the reaction of FG with lysine and formation of bound gossypol. This is in accord with the results of Liu et al. (2020) in the experimental diets prepared with graded level of

inclusion of low-gossypol CSM in the diet of silver sillago, *Sillago sihama.* The EAA profile of whole body samples of experimental fishes showing that lysine, methionine and arginine contents were comparatively lower in CSM incorporated diets than the than 0CSM groups. This result is comparable with Choi et al. (2020) in sub-adult olive flounder, *Paralichthys olivaceus* fed the diet containing FM replaced with either animal proteins or animal and plant proteins revealed that most of the amino acids were not significantly changed among the treatments.

The levels of energetic metabolites such as triglycerides and cholesterol of fish are considered as important indices reflecting the nutritional and physiological status of fish (Xu et al. 2013) and body fat metabolism (Shen et al. 2020). Gossypol content of dietary CSM reduces the cholesterol in the body which may be attributed through reduced intestinal absorption of dietary cholesterol and may reduce the synthesis of LDL in the liver (Wang et al., 2009). In the present study, serum cholesterol, total lipid, LDL and VLDL contents of experimental fishes showing a declining trend as the level of inclusion of CSM increased in the diet which may be due to FG contents of the diets that reduced the intestinal absorption. This is in agreement with Liu et al. (2016) reported similarly lower level of cholesterol and LDL content in the pre-adults of grass carp fed with varying levels of CSM. Correspondingly, Choi et al. (2020) reported that FM replaced with either animal proteins or animal and plant proteins in sub-adult olive flounder effectively reduced the levels of cholesterol when compared with FM based diet.

Serum protein profile in terms of higher albumin and globulin is a valuable indicator of better health status of fish through enhanced innate immune system function (Liang et al. 2018). In the present study, the serum protein profile did not show significant changes among the treatments which indicate that CSM content of experimental diets did not influenced the health status of snubnose pompano. This is in agreement with Shen et al. (2020) found no significant difference in total protein content in golden pompano fed with the diet containing varying levels of FM substitution with cottonseed protein concentrate. Similarly, Ahmed et al. (2018) reported that complete replacement of FM with plant proteins including CSM) and supplemented EAA in juvenile of *M. amblycephala* did not affect plasma total protein, albumin and triglyceride which in turn did not affect the immune response.

Digestive enzyme activities depend on the age, feeding habits and nutritive profile of the ingredients used for feed formulation (Deguara et al. 2003). In the present study, the protease activity was decreased as the inclusion level of CSM increased in the diet of snubnose pompano. This is in agreement with Liu et al. (2016) who reported likewise decreasing trend of protein digesting enzyme, chymotrypsin activity in grass carp fed with increasing level of CSM. In the present study, the amylase activity was increasing with increased inclusion of CSM in the diet. This is true in silver sillago that showed an increasing amylase activity as the level of inclusion of CSM was increased in the diet (Liu et al. 2020).

Activities of the metabolic enzymes such as AST, ALT and ALP are the indicators of the metabolic processes and function of liver (Prabu et al. 2021). In the current study, the activities of AST, ALT and ALP in the FM replaced with CSM groups were found slightly lower than the fishes fed with control diet without cottonseed meal (0CSM). This is agreement with Bu et al. (2017) who reported similarly lower AST and ALT enzyme activities in juvenile Ussuri catfish fed with FM replaced using CSM in the diet. Wang et al. (2014) reported that the AST and ALP activities showing a fluctuating trend among the treatments and suggested that asynchronous effect of dietary CSM on their activities. Further, Cai et al. (2011) reported that dietary CSM up to 400 g kg^{-1} diet did not show any difference in plasma levels of ALT and AST of crucian carp. In the present study, the LDH and MDH activities were showing a slightly decreasing trend as the level of inclusion of CSM increased in the diet. This result is in line with Rinchard et al. (2003a) who reported similar trend of LDH activity as the level of CSM increased in the diet of male rainbow trout.

Dietary FG accumulates higher amount in the liver and kidneys of animals (Wang et al. 2014) and in the muscle tissue the accumulation was very meagre that depends on the levels of dietary FG content and the nutritive profile of diet (Li and Robinson 2006). In the present study, the FG content of muscle tissues of snubnose pompano was at the maximum of 1.28 mg kg^{-1} body mass on as is basis in 100CSM which was insignificant while comparing the permissible limit of FG (450 mg kg^{-1}) in any cotton seed related products (FDA 1974). This result is in accord with the results of Lee et al. (2006) who fed the rainbow trout with 58.8% dietary CSM for 3-years and concentration of FG in the muscle tissue was lower than 1 mg kg^{-1} of

wet tissue sample. Similarly, Wang et al. (2014) reported that juvenile common carp fed with 54% CSM corresponding to 647 mg kg^{-1} FG for 8 weeks showed the FG concentration in the muscle tissue was 6.07 mg kg^{-1} on DM basis. Based on these results, it is evident that it is very safe to consume the fish fed with CSM based diets as the accretion was insignificant. Further, the half-lives ($t_{1/2}$) of (+) and (−) FG in rats was estimated at 25.26 and 10.53 h, respectively (Chen et al. 1987) which indicated that the accumulated FG may be completely depleted from the fish tissues once the animals fed with CSM free diet for a short stint.

The alterations in liver morphological structure and cytology significantly affect the normal liver function as liver is an excellent indicator of water quality, nutritional and physiological status of fishes (Xu et al. 2013). In the present study, the liver sections of experimental fishes did not show any serious gross structural changes even at higher FG content of 100CSM group except for the mild vacuolation of hepatocytes, few hypertrophic hepatocytes and few hepatocytes observed with pyknotic nucleus in 50CSM group. The present observation is in accord with the histological observations of Wang et al. (2014) who noticed that slight decrease in the volume of the hepatocytes with increased numbers in the juveniles of common carp.

Conclusion

Snubnose pompano can effectively utilize the diet devoid of FM which was evident from various growth and feed utilization parameters, nutritive profile and histological observation in the 100CSM group. Further, the second degree polynomial regression analysis of hematological, biochemical and enzymological parameters revealed that the dietary inclusion of 27.8-34.3% of CSM was optimum for better growth and health of snubnose pompano. The use of CSM in marine fish diets largely depends on the tolerance level of fish to free gossypol and also the requirements of lysine and methionine of the fish. In the current study, the fish effectively tolerated to the concentration of 345 mg free gossypol kg^{-1} diet without any significant negative effects. This indicates that snubnose pompano can tolerate at least 350 mg free gossypol kg^{-1} and thus, it is most warranted to determine the maximum tolerable level of free gossypol content in snubnose pompano. This study reveals that fishmeal can be completely replaced with cottonseed meal without any negative impacts on growth and general health of snubnose pompano.

Declarations

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Conflicts of interest/ Competing interests

All authors of this manuscript are declared that no conflict of interest present in this manuscript.

Availability of data and material (data transparency): The data made available upon reasonable request

Consent to participate/ Ethical statement:

The use of juveniles of snubnose pompano for this experiment was carried out according to the guidelines of animal ethical procedures of ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI), Kochi, by carefully considering the welfare of the fishes.

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All authors agreed with the content and that all gave explicit consent to submit this manuscript in Fish Physiology and Biochemistry.

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Author contribution

Dhanasekaran Linga Prabu: Conceptualization; Investigation; Methodology; writing- original draft

Pananghat Vijayagopal: Funding acquisition; Supervision; Writing - review & editing

Sanal Ebeneezar: Validation; Visualization; Writing - review & editing

Chellappa Kalidas: Methodology; Visualization.

Palsamy Rameshkumar: Formal analysis; Data curation; Validation.

Eldho Varghese: Data curation; Statistical analysis.

Bose Ramar Muniswaran: Formal analysis; Software.

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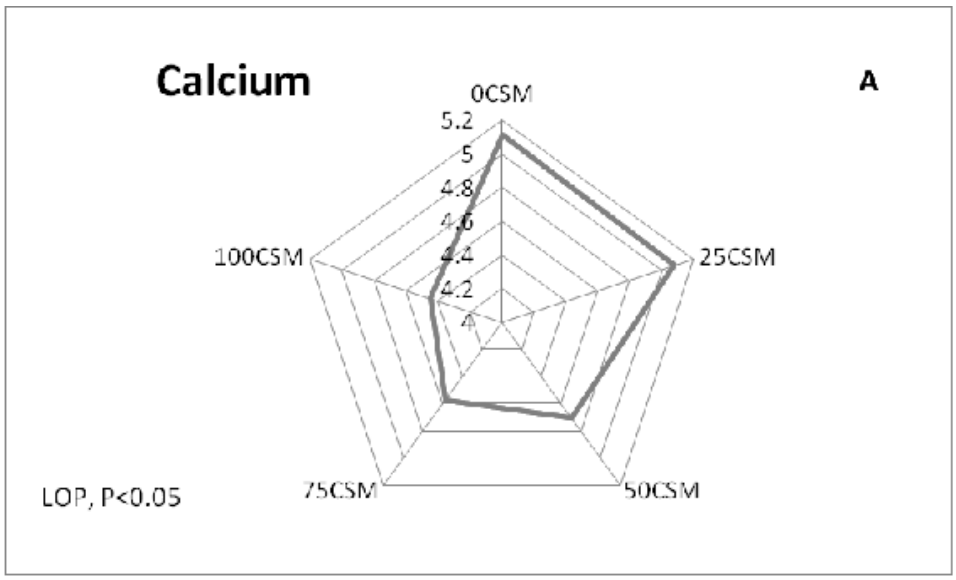
Figures



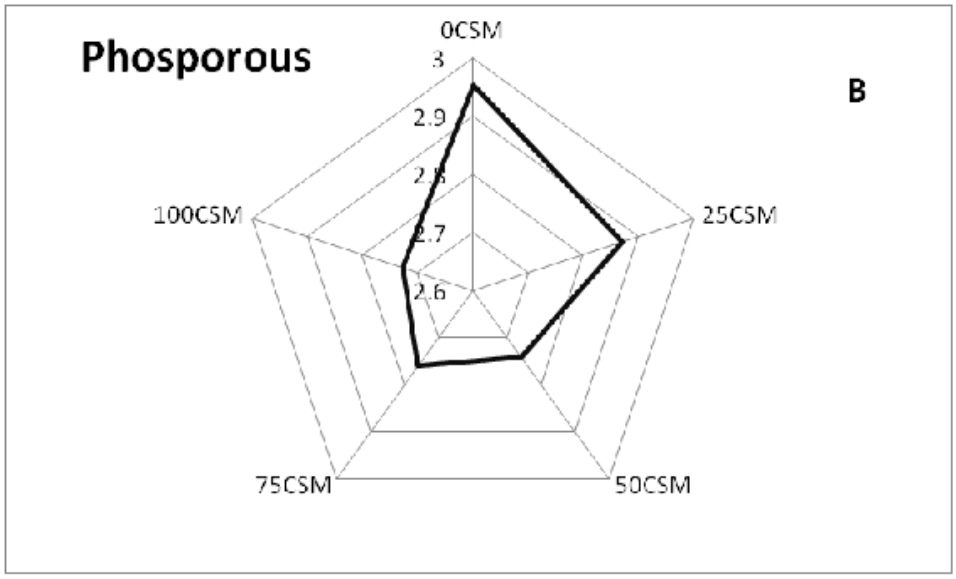
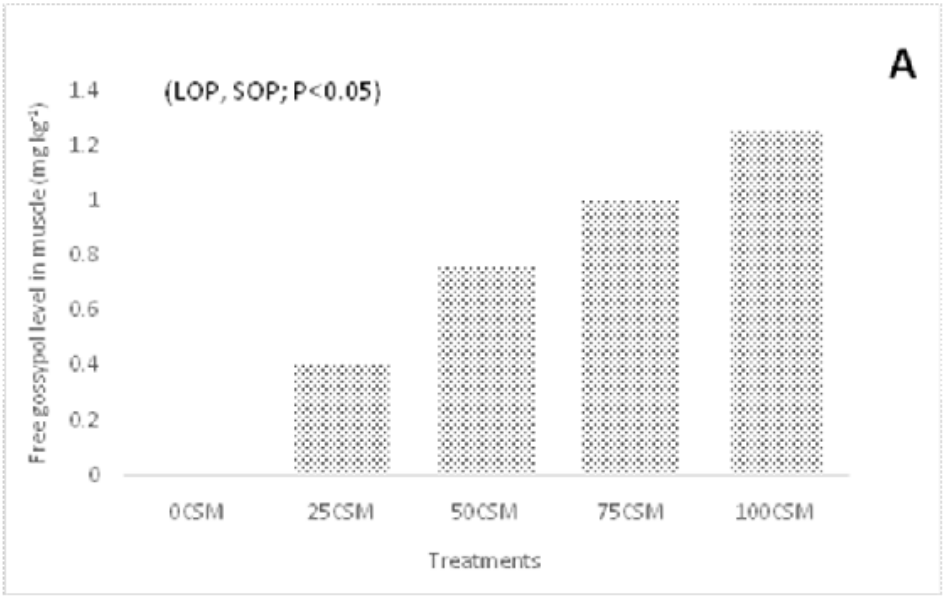


Figure 1

Whole body calcium (A) and phosphorous (B) content of experimental fishes fed with varying levels of CSM incorporated diet The result is expressed as percentage (%). Data expressed as Arithmetic Mean of three replications.



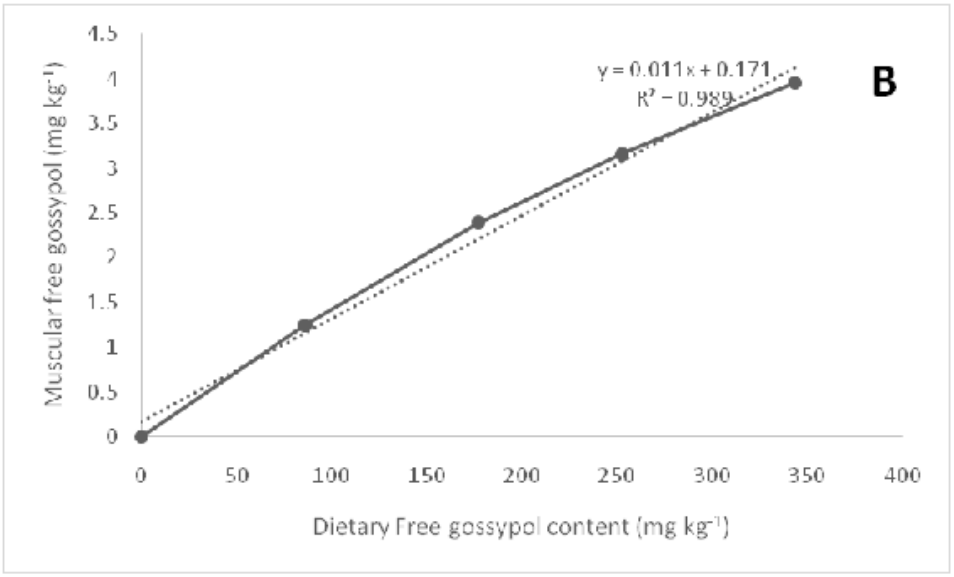
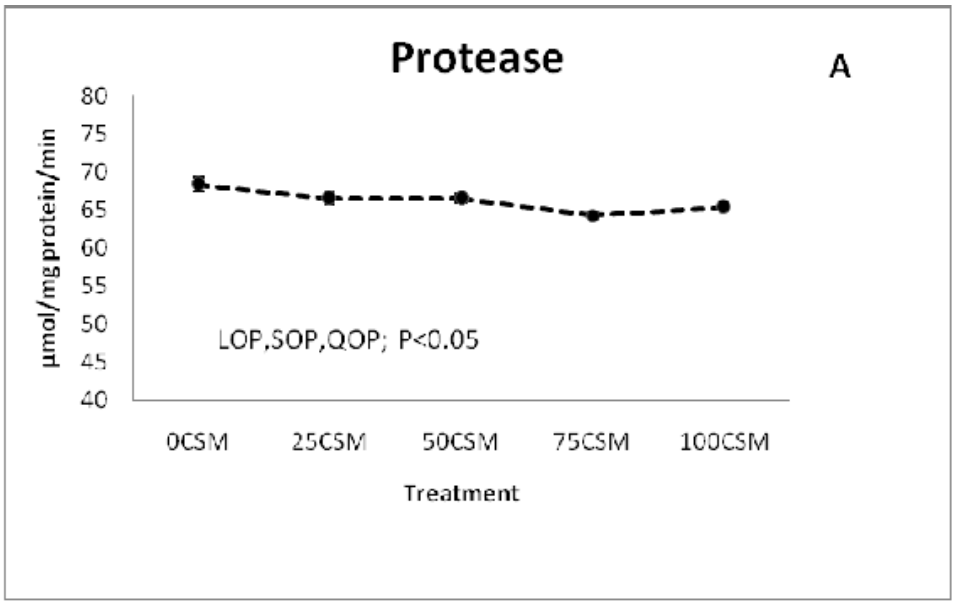


Figure 2

Free gossypol content in the muscle tissues of experimental fishes A. Free gossypol content in the muscle tissues of experimental fishes on as is basis. B. The relationship between dietary FG content and muscular FG accretion (DM basis) in the experimental fishes fed with varying levels of CSM incorporated diet. Data expressed as Arithmetic Mean of three replications.



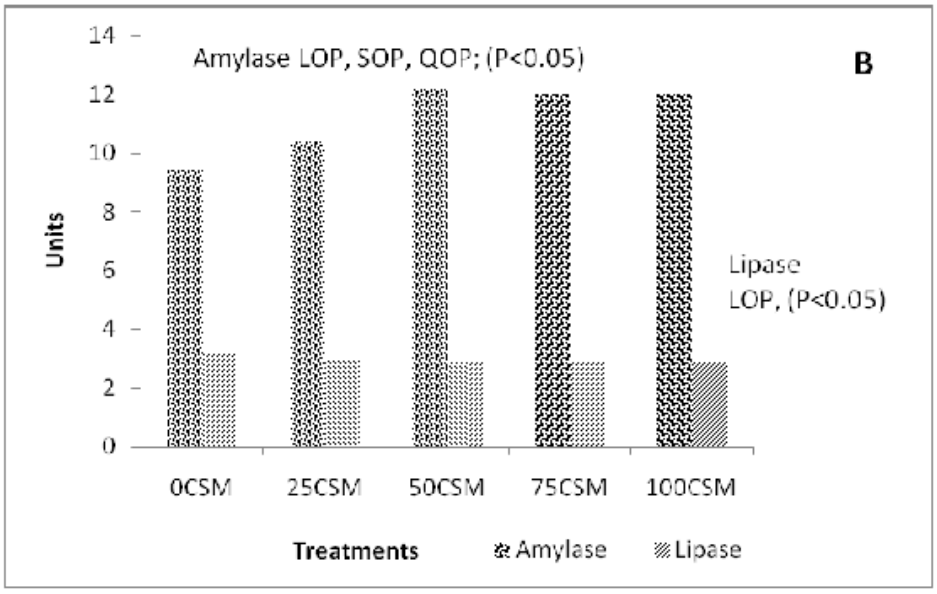


Figure 3

Digestive enzyme activities of experimental fish fed with FM replaced with CSM in the diets A. Intestinal protease activity of juvenile snubnose pompano; B. Intestinal α-amylase and lipase activities of juvenile snubnose pompano. Data expressed as Arithmetic Mean of three replications. Amylase activity: nanomoles of maltose released min-1 mg protein-1; Lipase activity: nanomoles of pNP liberated min-1 mg protein-1; Protease activity: nanomoles of tyrosine released/min/mg protein.

Figure 4

Liver histology of experimental fishes fed with graded level of CSM to replace fishmeal in the diet (stained with H&E; x20) A. Liver section of snubnose pompano from 0CSM group showing the hepatocytes with normal nucleus. The erythrocytes found in the blood vessels are normal; B. Liver section of snubnose pompano from 25CSM group showing the hepatocytes with mild intra cytoplasmic vacuolation (red arrow); C. Liver section of snubnose pompano of 50CSM group showing the hepatocytes with few pyknotic nucleus (yellow arrow) and very few hepatocytes with hypertrophy (black arrow) are also present. The erythrocytes found in the blood vessels are normal; D. Liver section of snubnose pompano of 75CSM group

showing normal hepatocytes with normal nucleus; E. The hepatocytes of liver section of 100CSM group are quiet normal and no abnormalities are seen.

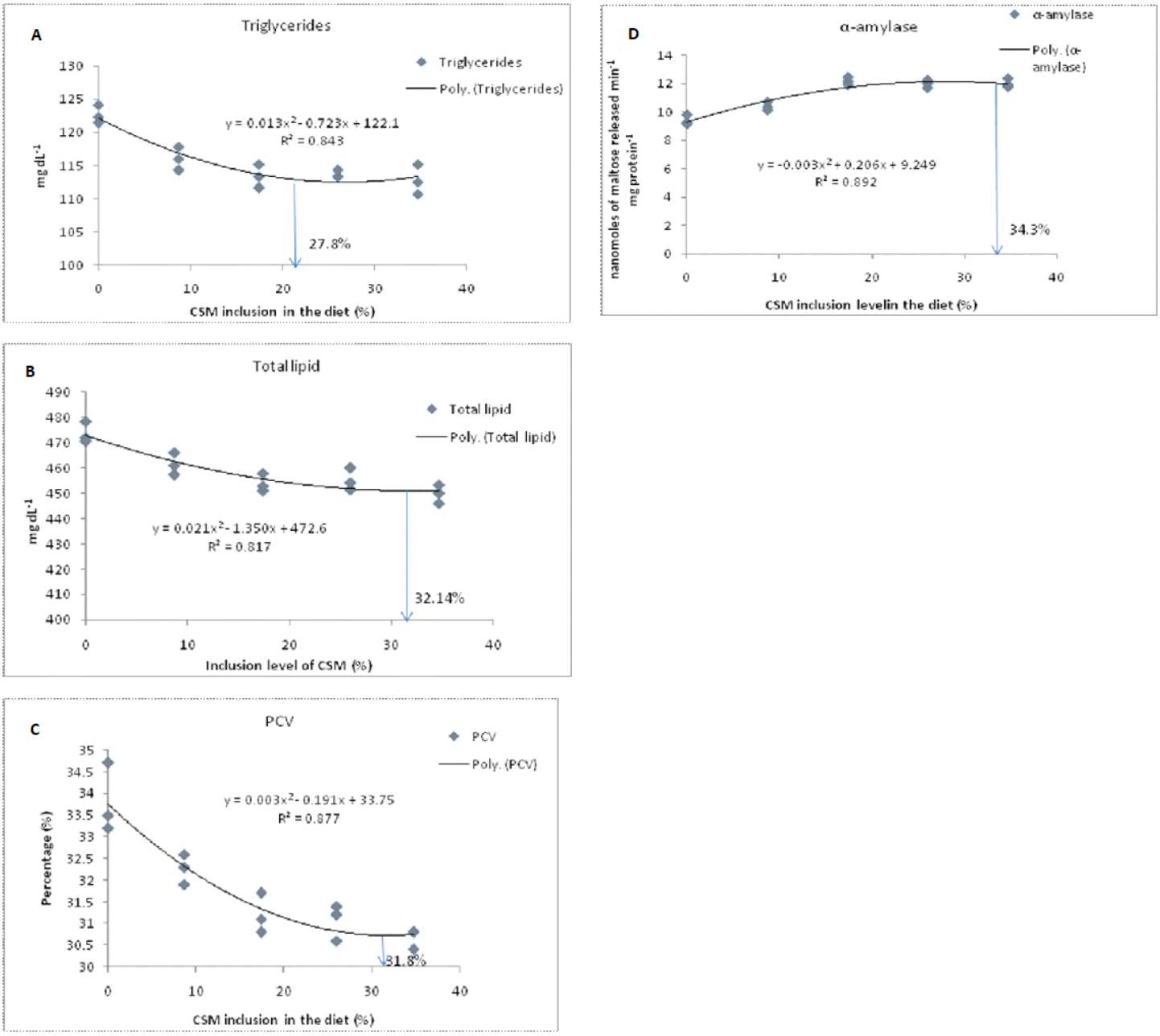


Figure 5

Second degree polynomial regression analysis of MCV, PCV, triglycerides, total lipids, amylase and alkaline phosphatase in snubnose pompano fed with varying levels of dietary CSM. Data expressed as Arithmetic Mean of three replications.