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Replacement of Fish Meal with Crustacean Meals in Diets for Long-Snouted Seahorse, *Hippocampus guttulatus*: Digestibility and Growth Performance

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Abstract: This study investigated the effect of partially replacing fish meal with krill and copepod meals in inert diets co-fed with shrimp on the growth and nutrient digestibility of long-snout seahorses (*Hippocampus guttulatus*). A control diet (Diet 1) using raw starch and four experimental diets with similar protein (≈44.8%) and energy (≈15.1 MJ/kg) levels were tested. Diet 2 used fish meal as the sole protein source, while in Diets 3–5, krill and copepod meals replaced 44% of the fish meal. Seahorses fed shrimp + Diets 2–5 showed significantly higher growth rates ($p < 0.05$) than those fed shrimp + Diet 1, though there were no significant growth differences among Diets 2–5. Digestibility of dry matter (46.1% to 72.2%), lipids (73.3% to 85.5%), crude protein (89.8% to 95.8%), energy (82% to 92.2%), and phosphorus (28.7% to 64.4%) varied with diet, being consistently lower in seahorses fed shrimp + Diet 1. As an agastric species, *H. guttulatus* did not exhibit impaired digestibility for any of the tested nutrients, minerals, or energy. This study suggests that crustacean meals can effectively substitute fish meal in inert diets for this species, contributing to the sustainability and optimization of captive seahorse husbandry practices.

Keywords: seahorse; *Hippocampus guttulatus*; digestibility; fish meal; crustacean meals

Key Contribution: This study demonstrates that seahorse species have a digestive capacity comparable to other fish species, with a high nutrient digestibility rate despite their agastric physiology. When formulating inert diets for seahorses, fish meal can be successfully replaced by crustacean meals without compromising nutrient digestibility or growth performance.

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1. Introduction

In marine aquaria, seahorses (*Hippocampus* spp.) are highly sought after due to their distinctive morphology and unique mating behaviors [1–3]. However, the escalating demand for live and dried seahorses for use in Traditional Chinese Medicine (TCM) across several Asian countries [4,5] has led to a decline in wild populations and their overexploitation [5]. In response, seahorse aquaculture emerges as the potential to meet the demand for the marine ornamental aquarium trade [6] while simultaneously alleviating pressure on wild populations. According to Koldewey and Martin-Smith (2010) [7], the sale of live seahorses dominates the market for aquaculture-sourced seahorses. Nonetheless, seahorses remain as candidate species for aquaculture due to lingering uncertainties surrounding their nutritional requirements, with feeds representing a crucial bottleneck in their cultivation. Seahorses are agastric teleosts (lack a stomach) [8], and in such species, digestion primarily occurs in the intestine [9]. Seahorses whose offspring undergo direct development in the

male's brood pouch hatch with a fully developed digestive tract [10,11], enabling exogenous feeding within a few hours after release from the male's pouch [11,12]. In aquaculture, high seahorse mortality rates at early life stages have been linked to nutritional factors, diet digestibility [11,13,14], and low digestive efficiency and nutrient absorption due to the incomplete functionality of the digestive tract at those stages [13,15].

Regardless of their life stage, seahorses have a requirement to be fed natural diets, whether live or frozen, driven by their imprinted feeding behavior, which compels them to accept and consume only feed items that resemble recognizable prey shapes [14]. Although adult seahorses can be fed natural frozen diets [14,16,17], these can be enriched to enhance and meet the seahorse's nutritional requirements [14]. In the wild, regardless of the species' geographic distribution, seahorses primarily feed on available crustacean species [18–20], mostly amphipods, mysids, and small Caridae shrimp, a dietary specificity that can be replicated using crustacean meals.

One of the first critical steps in developing diets for candidate aquaculture species is determining the digestibility coefficients (ADC) for various potential feed ingredients [21,22]. Assessing ingredient digestibility is essential for understanding the nutritional value of a feed ingredient and formulating research or commercial diets based on digestible nutrients [23]. Historically, fish meal has been the primary dietary ingredient due to its undeniable qualities, including high protein content, a well-balanced amino acid profile, high digestibility and palatability, and the absence of anti-nutritional factors [24,25]. However, as the global supply of fish meal made from wild-captured forage fish is at risk, with limited or no prospects for increased production in the future [26,27], the industry is increasingly shifting toward reducing or strategically segmenting the use of fish meal according to production stages (e.g., broodstock, larval, starter, and grower feeds) [28]. In the contemporary pursuit of more sustainable and cost-effective food sources, numerous studies have focused on identifying alternatives to fish meal. Progress has been made in replacing fish meal with plant protein ingredients such as soybeans, lupins, peas, and canola [24,29]. However, plant-based meals may have detrimental effects on growth performance [30–33], feed efficiency [31,34], nutrient digestibility [35,36], digestive enzyme activities [35,37], and overall health [24,33]. This is primarily because the use of plant ingredients for carnivorous species may be inadequate due to their high carbohydrate content, unbalanced amino acid profiles, and the presence of anti-nutritional factors [38], such as protease/trypsin inhibitors, saponins, and mycotoxins.

Unlike fish meal, untapped marine resources with substantial biomass can be found at lower trophic levels. This suggests that sustainable sources of crustacean meals can be obtained, with Antarctic krill (*Euphausia superba*) emerging as a promising candidate [39–42]. Current estimates indicate a standing biomass of 379 million metric tons [43], with just over 300,000 metric tons harvested in the Atlantic Sector (Area 48) in 2018 [44], and the annual catch quota is limited to 1% of the total estimated biomass in specific designated areas [45]. This conservative catch quota and the trends in biomass explain why krill stocks are considered among the best-managed and underutilized marine resources to date and are currently regarded as a sustainable resource. Krill meal offers high protein content, favorable amino acid and fatty acid profiles, and enhanced palatability properties [42]. In previous studies, tested krill meal proved to be an effective and beneficial alternative to traditional fish meal, enhancing growth performance and health indicators of several fish species (e.g., Asian swamp eel (*Monopterus albus*) [46,47], Red-White Koi carp (*Cyprinus carpio* var. koi) [48], salmon (*Salmo salar*) [41], rainbow trout (*Oncorhynchus mykiss*) [49], yellow croaker (*Larimichthys crocea*) [50], yellowhead catfish (*Pelteobagrus fulvidraco*) [51], tongue sole (*Cynoglossus semilaevis*) [52], and European seabass (*Dicentrarchus labrax*) [53]).

Thus, this study aimed to assess the efficacy of crustacean proteins as a replacement for fish meal in practical co-feed diets for the long-snout seahorse, *Hippocampus guttulatus*.

2. Materials and Methods

Ninety six-month-old F3 generation *H. guttulatus* individuals were selected from a captive stock. Initially, the fish were manually sorted to determine their sex. Only males showing no visible signs of pregnancy were included to prevent initial wet weight bias. The experimental tanks were set up within the same rearing system where seahorses had previously been maintained. All experimental conditions, including environmental parameters such as temperature, water quality, and lighting, were kept identical to those in the original system. The only variable that differed was the tank size. The seahorses were acclimated to the tank for one week, during which they were fed frozen Atlantic ditch shrimp (*Palaemonetes varians*).

The digestibility trial followed a completely randomized design, with three replicate tanks assigned to each of the five dietary treatments. Each tank was a 110 L fiberglass square-bottom unit assembled in a flow-through system. Fifteen tanks were used in total, and each tank housed six animals (three males and three females) for a total of $n = 18$ fish for each dietary treatment. The tanks had a constant water flow of 110 L/h and moderate aeration. Seawater, filtered through continuous sand and biological filtration, entered the tanks through black polystyrene tubing placed at the surface level in the corner of the tank. The water outflow structure, located in the center of the tanks, consisted of a PVC tube structure covered with a mesh screen (500 µm diameter) at the water surface. Three nylon nautical rope holdfasts (1 cm ø) were placed inside each tank.

The temperature, dissolved oxygen, salinity, and pH of the water were maintained at 20.5 ± 0.3 °C, 8.4 ± 0.2 mg L^{-1}, 36.2 ± 0.1 ppt, and 7.8 ± 0.1, respectively. The tanks were illuminated from above using two 36 W fluorescent tubes, providing an intensity of 547.5 ± 20.5 lux at the water surface. The photoperiod was controlled by a timer set to a 14-h light and 10-h dark cycle (600–2000 h). Water quality parameters, including ammonia, nitrates, and nitrites, were measured twice a week and remained stable throughout the experiment. Ammonia levels were consistently below detectable levels, nitrate levels were below 0.3 mg/L, and nitrite levels were below 1.25 mg/L. The experiment was conducted over a period of 90 days.

2.1. Experimental Diet Preparation

One control diet (Diet 1) consisting of raw starch without any protein or lipid sources was prepared to serve as the baseline diet for later analysis of apparent digestibility coefficients (ADC) for the non-supplemented shrimp diet (Table 1). Four additional isoproteic (≈44.8% crude protein [CP]) and isoenergetic (≈15.1 MJ digestible energy [DE]/kg) experimental inert diets were formulated using different animal proteins, including fish meal, krill meal, and copepod meal (Cyclop-eeze®; Argent Chemical Laboratories, Redmond, WA, USA). In Diet 2, fish meal was included as the sole animal protein source. In Diets 3 to 5, krill and copepod meals were added at varying inclusion rates to partially replace fish meal, resulting in a 44.4% replacement (Table 1). All other ingredients remained consistent across the four diets (Table 1).

To evaluate the ADC, 10 g/kg of chromic oxide (Cr_2O_3; Merck KGaA, Darmstadt, Germany) was added to each diet as an inert marker. Once the ingredients were mixed, the diets were steam-pelleted using a laboratory pellet mill (California Pellet Mill, San Francisco, CA, USA), dried overnight under forced air at 35 °C, and stored at 4 °C until use.

Table 1. Ingredients and proximate composition of the experimental diets (g/100 g dry diet).

Ingredient (g/100 g)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Fish meal, herring	0	45	25	25	25
Krill meal	0	0	20	0	10
Cyclop-eeze	0	0	0	20	10
Soy protein concentrate	0	10	10	10	10
Starch, raw	95	0	0	0	0
Wheat flour	0	23.5	24	26	25
Wheat gluten	0	10	10	10	10
Krill oil	0	6.5	6	4	5
Vitamin premix[1]	1	1	1	1	1
Mineral premix[2]	1	1	1	1	1
$CaHPO_4$	2	2	2	2	2
Marker (Cr_2O_3)	1	1	1	1	1
Carophyl pink	0.1	0.1	0.1	0.1	0.1
Analyzed composition (g/100 g)					
Dry matter	90.5	91.1	91.1	90.9	91.0
Crude Protein	0	46.7	45.1	43.3	44.2
Lipid	0	11.8	12.3	14.3	13.3
Ash	2.5	8.7	8.6	6.9	7.7
Gross energy (MJ/kg)	16.7	16.0	14.9	13.5	14.2

2.2. Shrimp Culture

Supplemented shrimps were prepared following the method outlined by [14]. In brief, Atlantic ditch shrimp (*P. varians*) were captured in a single fishing event and sorted to an average size of 18.4 ± 1.8 mm (total length from the tip of the rostrum to the end of the telson). These shrimps were then distributed among five fiberglass tanks, with one tank assigned to each diet. The tanks were set up under the same conditions as described earlier.

The shrimps were starved for 24 h to ensure empty stomachs and then fed with each of the five diets (Diets 1 to 5) until they reached satiation. The feeding duration was approximately 15 min, during which the shrimps consumed their fill and their stomachs became full. Following this feeding period, the shrimps were collected, immediately frozen, and stored at a temperature of −18 °C until further use. Prior to freezing, a sample weighing 100 g (wet weight) from each dietary treatment was preserved for future proximate analysis (Table 2).

Table 2. Proximate composition (dry matter basis) of the tested diets (shrimp + each of the inert diets).

Diets	Shrimp + D1	Shrimp + D2	Shrimp + D3	Shrimp + D4	Shrimp + D5
% Dry Matter	96.8	96.9	95.7	93.4	97.2
Ash %	20.0	20.4	20.3	20.5	20.1
Lipid %	5.4	5.6	5.7	5.5	5.7
Protein %	69.0	73.2	72.4	74.2	72.5
Phosphorus %	1.1	1.2	1.2	1.2	1.2
Gross Energy (kJ/g)	17.9	18.6	18.8	19.2	18.5

2.3. Digestibility and Growth Trial

The seahorses were fed the experimental diets once a day (~9:00 h) at approximately 6% body weight day^{-1}, ensuring a slight excess of feed. The daily shrimp ration was thawed in seawater before use. Once defrosted, excess water was drained from the diets, and they were gently dried, weighed, and fed to the seahorses. After a period of 2 h, any remaining food was collected and subtracted from the initial amount offered. Seahorses produce fecal pellets that remain relatively intact, allowing for collection with minimal loss of dry matter. Fecal samples were collected in the morning before feeding using a siphoning

method. The fecal collections from each tank were combined daily and stored frozen at −18 °C until a sufficient quantity was obtained for chemical analyses. The apparent digestibility coefficients (ADC) of dry matter, energy, and protein were determined using the following formulas:

$$\text{ADC of dry matter (\%)} = (100 - (\text{dietary } Cr_2O_3/\text{fecal } Cr_2O_3) \times 100) \quad (1)$$

$$\text{ADC of nutrients or energy (\%)} = 100 \times [1 - (Cr_2O_3 \text{ in diet}/\ Cr_2O_3 \text{ in feces}) \times (\text{Y in feces}/\text{Y in diet})]. \quad (2)$$

where Y is the nutrient or energy content.

Fish sampling was conducted every 2 weeks to record weight and length measurements, as well as to adjust the daily feed ration accordingly. After each sampling event, the daily wet weight of each ration was modified based on the average wet weight increase observed in each treatment group. This adjustment ensured that appropriate feed rations were maintained throughout the study period (i.e., "2-week ration adjustment"). To minimize stress during the sampling process, seahorses were measured differently than the three-length measurements proposed by [54] for the purpose of obtaining the total length. Instead, seahorses were measured by summing the head length (from the tip of the snout to the midpoint of the cleithral ring) and the body height (from the same point on the cleithral ring to the tip of the outstretched tail). This measurement approach aimed to minimize stress on the seahorses during sampling. Data were used to calculate: (1) weight gain (WG, g/fish) = $(W_f - W_i)/W_i$, where W_f is the final seahorse wet weight and W_i is the initial wet weight; (2) the growth rate of the fish was calculated using the thermal-unit growth coefficient (TGC) = $([{W_f}^{1/3} - {W_i}^{1/3}]/\Sigma[T \times D]) \times 1000$, where W_f is the final seahorse wet weight and W_i is the initial wet weight, T is the water temperature (C), and D is the number of days; (3) food conversion rate (FCR) = estimated individual feed consumption (dry weight)/increase in the individual wet weight; and (4) condition factor (CF) = (wet weight (g)/height^3 (cm)) × 100.

2.4. Chemical Analyses

Diets and feces were analyzed following the procedures outlined in [55]. In brief, the dry matter was analyzed by drying samples at 105 °C to a constant weight; ash by incineration of samples at 550 °C for 5 h; crude protein (N × 6.25) by the Kjeldahl method using a Leco® nitrogen analyzer (Leco Corporation, St. Joseph, MO, USA); crude lipid by extraction with methyl-ether (ANKOM® XT10 Extractor); and crude fiber by acid and basic digestion (Fibertec® System M., 1020 Hot Extractor, Tecator, Höganäs, Sweden). The chromic oxide in diets and feces was quantified according to the protocol of [56]. Feeds and feces were incinerated at 650 °C for 16 h, followed by 10 min of acid digestion in boiling HCl (1 N) and a second incineration at 650 °C for 16 h. All analyses were performed in triplicate.

2.5. Statistical Analyses

After confirming normality and homoscedasticity, variance analyses were performed. Data are presented as mean ± standard deviation (mean ± s.d.). Growth parameters, including length, wet weight, CF, TGC, and FCR, were analyzed using nested ANOVA, followed by Neuman–Keul's (NK) multiple comparison test ($p < 0.05$). For digestibility analysis, the mean values and standard deviations of the five compound diets were calculated, and differences between diets were assessed using ANOVA again followed by the Neuman–Keuls (NK) multiple comparison test ($p < 0.05$). All statistical analyses were performed using the GraphPad Prism® (version 6.00 for Windows; GraphPad Software, San Diego, CA, USA) software package.

3. Results

The data on final length, final weight, WG, TGC, CF, FCR, and survival are presented in Table 3. The growth rate was significantly higher ($p < 0.05$) in seahorses fed shrimp + Diets 2 to 5 compared to those fed shrimp + Diet 1. However, there were no significant differences ($p > 0.05$) in the growth rate between seahorses fed each of the four shrimp-supplemented diets (Table 3).

Table 3. Growth performance of adult *H. guttulatus* fed different diets (shrimp + each of the inert diets) at the end of growth trial (mean ± s.d., $n = 3$). Different superscripts in the same row denote statistically significant differences ($p < 0.05$).

	Shrimp + D1	Shrimp + D2	Shrimp + D3	Shrimp + D4	Shrimp + D5
Initial length (cm)	15.9 ± 0.3 ^{a}	15.7 ± 0.3 ^{a}	15.7 ± 0.2 ^{a}	15.8 ± 0.2 ^{a}	15.9 ± 0.3 ^{a}
Final length (cm)	17.4 ± 0.5 ^{a}	17.5 ± 0.4 ^{a}	17.7 ± 0.7 ^{a}	17.5 ± 0.7 ^{a}	17.7 ± 0.6 ^{a}
Initial weight (g)	9.2 ± 0.6 ^{a}	9.2 ± 0.5 ^{a}	9.3 ± 0.4 ^{a}	9.3 ± 0.5 ^{a}	9.2 ± 0.7 ^{a}
Final weight (g)	13.2 ± 1.1 ^{b}	14.9 ± 1.3 ^{a}	15.3 ± 1.4 ^{a}	15.4 ± 1.6 ^{a}	15.2 ± 1.5 ^{a}
WG (g/fish)	0.43 ± 0.21 ^{b}	0.62 ± 0.2 ^{a}	0.65 ± 0.18 ^{a}	0.66 ± 0.24 ^{a}	0.65 ± 0.27 ^{a}
TGC	0.07 ± 0.02 ^{b}	0.10 ± 0.01 ^{a}	0.11 ± 0.01 ^{a}	0.11 ± 0.02 ^{a}	0.11 ± 0.02 ^{a}
Initial CF	0.23 ± 0.02 ^{a}	0.24 ± 0.02 ^{a}	0.24 ± 0.03 ^{a}	0.24 ± 0.02 ^{a}	0.23 ± 0.03 ^{a}
Final CF	0.25 ± 0.05 ^{a}	0.28 ± 0.04 ^{a}	0.28 ± 0.06 ^{a}	0.29 ± 0.05 ^{a}	0.27 ± 0.06 ^{a}
FCR	3.8 ± 0.5 ^{b}	2.7 ± 0.3 ^{a}	2.6 ± 0.5 ^{a}	2.5 ± 0.5 ^{a}	2.6 ± 0.4 ^{a}
% survival	100	100	100	100	100

Similarly, the WG, TGC, and FCR values were significantly higher ($p < 0.05$) in seahorses fed shrimp with supplemented diets. At the end of the experiment, there were no significant differences ($p > 0.05$) in CF among the animals fed each of the tested diets.

Table 4 presents the apparent digestibility coefficients of the tested diets and the derived coefficients from the test feed ingredients for long-snout seahorses. The apparent digestibility of dry matter (46.1–72.2%), lipids (73.3–85.5%), crude protein (89.8–95.8%), energy (82–92.2%), and phosphorus (28.7–64.4%) was influenced by the tested ingredients ($p < 0.05$). Dry matter and nutrient digestibility were consistently lower ($p < 0.05$) for shrimp + Diet 1 compared to all other tested diets.

Table 4. Apparent digestibility of nutrients, energy, and phosphorus in the test ingredients for *H. guttulatus*. Different superscripts in the same row denote statistically significant differences ($p < 0.05$).

	Shrimp + D1	Shrimp + D2	Shrimp + D3	Shrimp + D4	Shrimp + D5
Dry Matter	46.1 ± 0.7 ^{b}	72.1 ± 1.1 ^{a}	72.3 ± 2.6 ^{a}	71.5 ± 2.6 ^{a}	72.2 ± 0.5 ^{a}
Lipid	73.3 ± 0 ^{b}	85.5 ± 0.7 ^{a}	85 ± 2.9 ^{a}	83.9 ± 1.1 ^{a}	83.9 ± 1.6 ^{a}
Protein	89.8 ± 0.3 ^{b}	95.8 ± 0.2 ^{a}	95.2 ± 0.5 ^{a}	95.6 ± 0.4 ^{a}	95.3 ± 0.1 ^{a}
Energy	82 ± 0.5 ^{b}	92.2 ± 0.4 ^{a}	90.5 ± 0.6 ^{a}	91.5 ± 0.5 ^{a}	90.6 ± 0.3 ^{a}
Phosphorus	28.7 ± 0.1 ^{b}	64.4 ± 1.7 ^{a}	59.1 ± 2.7 ^{a}	63 ± 3.2 ^{a}	61.6 ± 2 ^{a}

Crude protein digestibility was generally high and consistent across the four shrimp + supplemented diets, regardless of the protein source (fish, krill, or copepod meal). Gross energy digestibility followed a similar trend to protein, with similarly high values. However, phosphorus digestibility was the lowest among the nutrients tested, with particularly poor digestibility in shrimp + Diet 1 (28.7 ± 0.1) compared to shrimp + D2 (64.4 ± 1.7).

4. Discussion

Digestibility studies play a critical role in understanding nutrient utilization and facilitate precise formulation of diets for specific fish species [57]. Seahorses remain promising candidates for aquaculture despite their dependence on natural prey. Consequently, there is limited knowledge concerning seahorse nutrient requirements, retention of major nutrients,

and energy utilization. Providing a mixed prey diet to closely resemble their natural diet is challenging, if not impossible. Hence, natural prey supplementation becomes essential in their production, and as carnivorous species, dietary supplementation should aim to closely mimic their natural diet. In carnivorous fish diets, fish meal (FM) is often used as a main ingredient, as different fish meals, which provide high levels of essential amino acids, are typically well digested and contain few anti-nutritional factors [58]. However, the extensive use of FM in aquafeed production exerts significant pressure on global fish stocks. Consequently, reducing its usage is imperative to bolster the sustainability of aquaculture feeds, especially when formulating diets for candidate species like seahorses.

Various animal protein ingredients have been tested as partial or complete alternatives to FM in diets for different fish species, with varying success [59]. Replacement of FM with alternative protein sources has proven effective in supporting or even enhancing growth performance in numerous fish species, including European seabass (*Dicentrarchus labrax*) [29,53,60], largemouth bass (*Micropterus salmoides*) [61,62], white snook (*Centropomus viridis*) [63], rainbow trout (*Oncorhynchus mykiss*) [64,65], California yellowtail (*Seriola dorsalis*) [66], red tilapia (*Oreochromis niloticus* × *O. mossambicus*) [67], Atlantic salmon (*Salmo salar*) [68], gilthead sea bream (*Sparus aurata*) [69], and olive flounder (*Pleuronectes platessa*) [70]. In the present study, FM was partially replaced with crustacean meals without compromising the growth performance or digestibility of the supplementary diets. Concordantly, seahorses fed shrimp with diet supplementation exhibited significantly higher growth rates ($p < 0.05$) compared to those fed non-supplemented shrimp diets. Notably, all four supplemented shrimp diets significantly improved growth performance ($p < 0.05$) compared to the non-supplemented shrimp diet, with no significant differences ($p > 0.05$) observed among the supplemented diets. This suggests that using alternative protein sources is a viable option, allowing for an effective reduction in the amount of fish meal (FM) used. These findings provide valuable insights, supporting the conclusion that more sustainable crustacean meals can be a feasible alternative to reduce or even replace FM in seahorse diets. These results are consistent with those of [14], which reported increased growth and positive effects on reproductive rate and brood quality in *H. guttulatus* when fed a similarly supplemented diet. Additionally, the use of crustacean meals in seahorse diets is fully justified, as it mimics the natural crustacean-based diet of these species. The results of the present study align with other research focused on replacing FM with krill meal. In various experiments, numerous benefits have been observed across different species. For instance, [46,47] reported that the inclusion of krill meal in the diet of Asian swamp eel (*Monopterus albus*) significantly improved growth performance and fecundity, boosted immunity, enhanced muscle textural quality, and positively influenced the expression of myogenic regulatory factors. The study cited in [48] demonstrated that replacing FM with krill meal in the diet of Red-White Koi carp (*Cyprinus carpio* var. koi) led to better growth performance, enhanced body coloration, and improvements in serum biochemical indexes. Similarly, [41] observed improved growth performance in salmon (*Salmo salar*) when FM was partially replaced with krill meal.

Krill meal has also shown positive effects on muscle quality and overall health. The study cited in [49] found improved growth performance and muscle quality in rainbow trout (*Oncorhynchus mykiss*), while [50] noted improvements in growth performance, intestinal morphology, body composition, and organoleptic quality in large yellow croaker (*Larimichthys crocea*). Furthermore, [51] observed enhanced growth performance, immune responses, and muscle quality in yellowhead catfish (*Pelteobagrus fulvidraco*), and [52] reported improved reproductive performance in tongue sole (*Cynoglossus semilaevis*). Finally, [53] documented improvements in growth performance and feed utilization in European seabass (*D. labrax*), further reinforcing the efficacy of krill meal as a high-quality replacement for FM in various fish species. These studies, along with the present research, collectively demonstrate that krill meal can serve as an effective and beneficial alternative to traditional fish meal, enhancing growth performance, health indicators, and overall product quality in a wide range of aquaculture species.

The chromic oxide method [71] has been extensively utilized to assess the apparent digestibility of fish feeds. In the present study, the concentration of chromium oxide (in g/kg of diet) in the inert diets was intentionally higher than typically observed in most studies. This adjustment was made to account for the natural diet portion and ensure an adequate level of chromium oxide in the overall diet provided. This enabled accurate quantification of nutrients in both the diets and feces. This approach was successfully validated, as the quantity of chromic oxide employed allowed for the calculation of apparent digestibility coefficients (ADC) for the tested feed ingredients.

Protein digestibility ranged between 89.8% and 95.8%, with the non-supplemented shrimp diet displaying the lowest digestibility. This suggests that the long-snout seahorse possesses a notable capacity to digest protein despite its agastric physiology. In agastric fish species, an intestinal bulb or expansion in the anterior intestine, which serves as a food storage area [72], is present, functioning like a stomach. In the absence of pyloric caeca, agastric fish may have relatively longer intestines measuring several times the length of the animal's body, which compensates for the lack of a stomach and increases the overall surface area of the intestine [73]. Therefore, these aforementioned agastric physiological characteristics do not impair protein digestibility. In fact, it was even higher than that observed in other species (e.g., *M. salmoides* by [74] and *Morone saxatilis* by [75], and it was similar to *O. mykiss* [64] and *Bidyanus bidyanus* [76] when testing FM digestibility). When evaluating protein digestibility in crustacean meals, the increased chitin content in these diets should also be considered. Chitin is a nitrogen-containing polysaccharide composed of proteins and carbohydrates, and is primarily found in the exoskeletons of invertebrates. It consists of calcium oxide and protein units [77]. The inclusion of chitin-containing ingredients in diets has been reported to benefit fish health. However, its impact on growth performance and nutrient digestibility remains controversial, with mixed results in the literature [78]. Although chitin was not individually analyzed in this study, its levels were naturally elevated with the addition of crustacean meals. The enhanced growth performance observed in fish fed shrimp and Diets 2–5 was consistent with the higher chitin levels in these diets.

However, the apparent digestibility of dry matter (DM) and phosphorus was generally lower, particularly for the control shrimp diet without dietary supplementation. Phosphorus is a crucial mineral required for normal growth, reproduction, and overall health in fish [79]. It serves as a major component of the skeleton, nucleic acids, and cell membranes, and plays a direct role in all energy-producing cellular reactions [80]. While fish can absorb phosphorus from water, its concentration is typically low in both fresh water and seawater, making food the primary source for fish [81].

The apparent digestibility coefficient (ADC) of phosphorus (P) from fish meal has been reported to range from 17% to 81% for rainbow trout (*Oncorhynchus mykiss*) [82–85], from 0% to 61% for common carp (*Cyprinus carpio*) [82,86,87], from 27% to 65% for tilapia (*Oreochromis niloticus*) [88–91], 58% for Senegalese sole (*Solea senegalensis*) [92], and 33% for grass carp (*Ctenopharyngodon idella*) [88]. In the present study, the ADC of P ranged from 28.7 $\pm$ 0.1% to 64.4 $\pm$ 1.7%, falling within the reported range for these species. Although the percentage of P included in each of the tested diets was quite similar, the ADC of P increased in fish fed supplemented diets (59.1 $\pm$ 2.7% to 64.4 $\pm$ 1.7%) compared to those fed the non-supplemented shrimp base diet (28.7 $\pm$ 0.1%).

While feed ingredients inherently contain P, its digestibility varies significantly [93]. Inorganic sources of P, such as bones or crustacean exoskeletons, are solubilized in the stomach by gastric acid. The solubilized inorganic phosphorus is then absorbed in the intestine, primarily in the duodenum and pyloric caeca [93]. As a result, agastric fish are unable to utilize insoluble bone P or hydroxyapatite in their feed [93]. Additionally, while crustaceans, like most mineralized invertebrates, use calcium carbonate for skeletal reinforcement, they also incorporate calcium phosphate at specific exoskeleton sites [94]. Consequently, agastric species, such as seahorses, have a negligible capacity to utilize the inorganic P fraction present in crustacean meals. In contrast, organic sources of P in feeds

are broken down enzymatically in the intestine, enabling their digestibility by agastric species [93]. Therefore, P digestibility appears to depend on its source and origin, with higher digestibility observed when the tested ingredients (fish, krill, and copepod meals) were included in the diet.

In conclusion, the results of this study demonstrate that supplementing a shrimp-based diet with inert diets that enhance the nutritional profile not only improves the growth performance of the species, but also shows that, despite being an agastric species, this physiological trait does not limit its ability to digest key nutrients and energy. Thus, their apparent digestibility of the tested diets was proportional to the nutritional quality of the diets themselves. However, this co-feeding technique is only applicable to medium- to large-sized *Hippocampus* species, which are capable of consuming larger prey, and further research is necessary to adapt it to smaller seahorse species.

These results support the beneficial use of crustacean and krill meals in seahorse inert diets, and further experiments are needed to evaluate the complete replacement of FM with krill meal. As a sustainable protein source with potential for increased use and proven efficacy in successfully replacing FM in diets for both fish and crustacean species, krill meal's inclusion in aquaculture diets can enhance the sustainability of aquafeeds, thereby improving the overall sustainability of the aquaculture industry.

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