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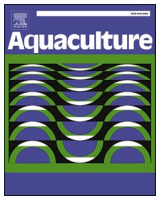

Effects of digestible protein and energy on growth, amino acid requirements and protein utilisation in juvenile Malabar snapper (*Lutjanus malabaricus*): A four-protein by three-energy factorial design study

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ABSTRACT

The Malabar snapper (*Lutjanus malabaricus*) is a commercially valuable marine fish widely farmed in the Indo-Pacific region. To address the lack of nutritional information for this species, a factorial design with 12 diets comprising four protein levels (P540, P480, P420 and P360) and three energy levels (Low, Mid and High) was used to evaluate the effects of digestible protein (DP) and digestible energy (DE) on growth, protein retention and utilisation efficiency in juvenile Malabar snapper with an initial size of 39.0 ± 0.34 g. Fish were fed to apparent satiation for 56 days and gained an average body weight ranging from 13.6 g to 34.1 g. Optimal growth of up to 73.1 g was achieved with a dietary DP level of 479.1 g kg^{-1} at a DP/DE ratio of 25.78 g MJ^{-1}. However, the optimal protein deposition could not be estimated from the range of DP levels investigated. This indicated that a higher dietary DP, above 518 g kg^{-1}, is required for optimal protein deposition. An indirect approach was used to estimate dietary amino acid (AA) requirements in conjunction with the estimation of protein requirements. This indirect method is useful for suggesting EAA levels to be included in an actual dose-response trial. High carbohydrate, low protein diets resulted in reduced feed intake, growth performance, protein retention and utilisation. Growth was optimised with digestible carbohydrates close to the minimum level tested, at 185.8 g kg^{-1}, indicating that either high carbohydrate inclusion limits growth or protein promotes growth. Increased dietary digestible fat led to greater whole-body fat deposition. Still, it did not improve growth rate or protein retention efficiency, indicating no apparent protein-sparing effect from non-protein energy sources. These results highlight the Malabar snapper's high digestible protein requirement and its lack of growth or efficiency benefits from dietary carbohydrates or lipids. The practical implications of these findings are significant, as they provide essential baseline knowledge for formulating appropriate feeds for this species, thereby enhancing the efficiency and sustainability of Malabar snapper aquaculture.

1. Introduction

Malabar snapper, *Lutjanus malabaricus* (Bloch and Schneider, 1801), is an important commercial and recreational fish of the tropical and subtropical Indo-Pacific region (Allen, 1985; Blaber et al., 2005; Takahashi et al., 2023). The high market value of tropical snappers (*Lutjanus* sp.) has led to the development of aquaculture in Taiwan, Malaysia, Indonesia and Singapore (FAO, 2024). Utilising a species-specific diet can improve feed conversion ratios and mitigate dietary-related impacts on the environment (Hua et al., 2019; Glencross et al., 2023). However, the nutritional requirements of Malabar snapper are currently unknown, and commercial farming typically relies on generic marine feeds or those formulated for other fish species. The compromises inherent in using generic feeds are highlighted by the limited nutritional requirements proposed for *Lutjanus* species. The recommended dietary protein levels for mangrove snapper (*L. argentimaculatus*) (Catacutan et al., 2001; Abbas and Siddiqui, 2013), spotted rose snapper (*L. guttatus*) (Parra et al., 2010), yellow snapper (*L. argentiventris*) and red snapper (*L. campechanus*) (Miller et al., 2005) varied between 32 % and 55 %, while the suggested dietary fat content for mutton snapper (*L. analis*) and red snapper is 6 % and 10 %, respectively (Watanabe et al., 2001; Miller et al., 2005). Furthermore, the optimum dietary protein-to-energy

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ratio ranges from 20.1 to 23.3 g MJ^{-1} for mangrove snapper (Catacutan et al., 2001; Abbas et al., 2011) and 27.5 to 29.5 g MJ^{-1} for mutton snapper (Watanabe et al., 2001). While these guidelines assist in diet formulation, the specific nutritional requirements of Malabar snapper must be determined to develop an optimised diet for this species.

Protein, fats, and carbohydrates are major constituents in feed that provide fish with the energy required for growth and metabolism. Proteins are the most expensive macronutrient, however their insufficiency can restrict growth. Feed formulation for high-value aquaculture products aims to optimise growth while minimising costs, necessitating an understanding of the optimum dietary protein inclusion levels (NRC, 2011). Understanding the appropriate dietary energy level is also essential, as it influences protein requirements, feed intake, and conversion efficiency. A diet low in energy from fats and carbohydrates may cause fish to use dietary protein for metabolism rather than for somatic growth, increasing protein demand and potentially reducing growth rates. Conversely, excessive dietary energy may cause fish to cease feeding before attaining the required protein level for optimal growth, affecting their protein utilisation (Bureau et al., 2003). Adequate intake of non-protein energy sources may reduce protein demand for energy, a phenomenon known as the "protein-sparing" effect (Shiau and Peng, 1993; Thirunavukkarasar et al., 2022). This effect has been demonstrated in fish through the use of carbohydrates (Tekinay and Davies, 2001; Mohanta et al., 2007; Orire and Sadiku, 2011), and lipids (Nankervis et al., 2000; Welengane et al., 2019; Thirunavukkarasar et al., 2022). However, the effectiveness of non-protein energy sources in sparing protein can vary among species, as seen with juvenile cobia (*Rachycentron canadum*) (Chou et al., 2001; Craig et al., 2006), gilthead sea bream (*Sparus aurata*) (Santinha et al., 1996; Santinha, and Corraze, and Gomes., 1999), snubnose pompano (*Trachinotus blochii*) (Prabu et al., 2020) and juvenile spotted rose snapper (Benitez-Hernández et al., 2022). The capacity of fish to utilise carbohydrates as an energy source depends on species (Maas et al., 2020), and excessive lipids can impair growth (Fan et al., 2021).

Understanding protein requirements in fish is intricate. Rather than having a direct requirement for protein, it is the quantity and balance of essential amino acids (EAA), as well as the total quantity of amino acids, that contribute to growth (Wilson, 1986). While crude protein is a crucial consideration in diet formulation, the quality of the protein or its amino acid profile is often overlooked. Moreover, protein bioavailability must be considered, as poor digestibility can lead to higher protein requirements (Hussain et al., 2024).

Early estimations of EAA requirements were based on the amino acid profile of the whole body (Akiyama et al., 1997). Nowadays, the conventional methods for determining EAA requirements involve growth trials using body weight gain or thermal growth coefficient (TGC) as measured responses, expressed as g/16 g N or % CP (Cowey, 1995; Xing et al., 2024). These studies utilise a basal diet deficient in the target EAA, with other nutrient levels kept constant or in excess. The target EAA is then supplemented at graded levels, creating a dose-response experiment (Wang et al., 2016; Candebat et al., 2023). While EAA requirements based on the whole-body amino acid profile have their merits, dose-response studies are still considered the standard approach for estimating specific EAA requirements. However, many trials are needed to evaluate EAAs individually. Furthermore, trials may often have to be repeated because the initial range of EAA levels selected did not yield a break-point requirement estimation (Poppi et al., 2017). Therefore, we propose a novel rudimentary (indirect) approach to estimate crude amino acid requirements based on graded dietary protein levels and TGC to provide a preliminary snapshot of the EAA requirement. This approach is beneficial for species with limited nutritional information. It should be complemented by evaluating optimal digestible protein (DP) levels and digestible protein-to-digestible energy (DP/DE) ratios for growth. Given the lack of nutritional requirements for Malabar snapper, this study aims to use a four-protein by three-energy level factorial diet design to (1) evaluate the optimal DP levels and DP/DE ratio for growth, (2) provide a crude estimation of dietary EAA requirements, and (3) investigate the effects of varying dietary energy levels on protein utilisation.

2. Materials and methods

2.1. Experimental diets

A total of 12 practical test diets were formulated based on a four-by-three factorial design to have four levels of dietary protein: P540, P480, P420 and P360 (g kg^{-1}, DM basis) and three dietary energy levels: Low, Mid, and High having 21, 22.3 and 23.6 (MJ kg^{-1}, DM basis), respectively (Table 1). Macro ingredients such as fishmeal, wheat flour, wheat gluten, soybean meal, corn starch, and fish oil were included proportionally to produce a wide DP/DE ratio, ranging from 15.1 to 27.2 g MJ^{-1} (Table 1). The diets were manufactured in an R&D feed mill facility at the Marine Aquaculture Centre (MAC), Singapore Food Agency (SFA). Dry ingredients supplied by MAC were milled and sieved below 500 μm before use. Dry powdered ingredients were mixed homogeneously for 30 min using a 100 L horizontal paddle mixer (KSE-PM100, Kong Shiang Engineering Pte. Ltd.) before extrusion. All the test diets were produced using a co-rotational twin-screw extruder (Evolum 25, Clextral) with a 4 mm die. The twin-screw extruder has a length-to-diameter ratio L/D of 24:1 and consists of six modules of 100 mm, each equipped with temperature control. The highest temperature setting at the end of the barrel was set at 120 °C to create semi-floating diets. Extruded pellets were dried below 10 % moisture using a fluidised dryer at 70 °C for at least 40 min. Dried pellets were de-dusted before fish oil was vacuum coated at 200 mbar of absolute pressure. The test diets were cooled to room temperature and stored at four °C before use.

2.2. Fish and growth trial

Juvenile Malabar snapper was obtained from the MAC hatchery, and the experiment was conducted in MAC's shared experimental tank system, which comprised 36 replicated 200 L fibreglass tanks. Each tank in the system had a faecal sedimentation column to collect faecal matter for the nutrient apparent digestibility coefficient (ADC) calculation. Fish were acclimatised to the system for one week and fed using a commercial diet (M502, Uni-president, Vietnam) to apparent satiation before the start of the trial. At Day 0, fish were graded by size, and 30 fish were bulk-weighed and allocated to each of the 36 experimental tanks, with an average initial body weight (IBW) of 38.96 ± 0.34 g. The experiment was conducted for 56 days (8 weeks) under flow-through seawater conditions with a water exchange rate of 150 % per hour. Incoming seawater was sand-filtered and UV-treated before entering the tank system. The daily water parameters were monitored throughout the trial using Aqua Troll 500 (In-Situ, Colorado). The average water temperature, dissolved oxygen (DO), salinity, and pH were 29.22 ± 0.51 °C, 6.08 ± 0.33 mg/L, 33.59 ± 1.12, 8.53 ± 0.30 respectively. Ammonia, nitrite, and nitrate concentrations were 0 mg/L (API® saltwater master test kit) throughout the trial. A photoperiod of 12 h of light and dark was maintained throughout the experiment.

The diets were hand-fed to apparent satiation twice daily. Each feeding session was completed within an hour. Apparent satiation was determined when uneaten feeds were observed at the bottom of the tank. Any uneaten feed pellets at the end of each feeding session were removed from the tank, and the dry weight of the pellets was deducted from the total feed offered to calculate total feed intake (TFI). Each treatment group was conducted in triplicate ($n = 3$) and fed with one of the 12 diets. Tank configurations were randomised using the sample function in *R* (R Core Team, 2022).

2.3. Fish sample collection and processing for whole body composition

At the start of the trial, 50 fish from the initial population were

Table 1
Diet formulation and measured composition of test diets (g kg^{-1} or MJ kg^{-1}, DM).

Energy levels (MJ kg^{-1}, DM)	Low (21)				Mid (22.3)				High (23.6)			
Protein levels (g kg^{-1}, DM)	P540	P480	P420	P360	P540	P480	P420	P360	P540	P480	P420	P360
Diet formulation (g kg^{-1})												
Fish meal[a]	350	300	250	200	350	300	250	200	350	300	250	200
Wheat Flour[b]	10	95	180	265	10	95	180	265	10	95	180	265
Wheat gluten[c]	130	90	50	10	130	90	50	10	130	90	50	10
Soybean meal[d]	180	170	160	150	180	170	160	150	180	170	160	150
Soybean concentrate[e]	50	50	50	50	50	50	50	50	50	50	50	50
Corn Gluten Meal[f]	60	60	60	60	60	60	60	60	60	60	60	60
Corn Starch[g]	170	170	170	170	115	115	115	115	50	50	50	50
Sardine Oil[h]	20	35	50	65	75	90	105	120	140	155	170	185
MDCP	9	9	9	9	9	9	9	9	9	9	9	9
Betaine HCL[i]	2	2	2	2	2	2	2	2	2	2	2	2
Vitamin & Mineral[§]	15	15	15	15	15	15	15	15	15	15	15	15
Choline chloride[j]	1	1	1	1	1	1	1	1	1	1	1	1
Vitamin C, Stay C-35[k]	1	1	1	1	1	1	1	1	1	1	1	1
Mold inhibitor[l]	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Antioxidant[m]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Yttrium Oxide[n]	1	1	1	1	1	1	1	1	1	1	1	1
Analysed nutrient and energy composition (g kg^{-1} or MJ kg^{-1}; DM)												
Moisture (g kg^{-1}, as is)	76.7	57.8	71.7	72.9	69.4	73.3	70.9	74.0	59.1	44.6	49.3	48.3
CP (g kg^{-1})	553	487	423	359	538	489	421	355	535	468	411	348
CF (g kg^{-1})	78	91	104	118	145	151	153	167	218	220	237	236
Fibre (g kg^{-1})	14	13	6	19	7	7	11	7	13	10	9	12
Carb[†] (g kg^{-1})	281	342	401	454	222	275	354	408	158	234	283	354
Ash (g kg^{-1})	88	79	72	69	95	86	73	69	89	78	69	62
GE[‡] (MJ kg^{-1})	21.0	21.0	21.0	20.9	22.2	22.2	22.1	22.0	23.9	23.7	23.9	23.6
Digestible nutrients and energy content (g kg^{-1} or MJ kg^{-1}; DM)												
DP (g kg^{-1})	518	447	378	321	512	453	378	315	500	435	374	303
DF (g kg^{-1})	72	87	97	110	139	143	138	151	206	206	220	204
DC[†] (g kg^{-1})	230	282	334	399	187	227	294	352	100	174	220	284
DE[‡] (MJ kg^{-1})	19.0	18.8	18.5	18.8	20.8	20.2	19.4	19.5	21.7	21.4	21.3	20.1
DP/DE[‡] (g MJ^{-1})	27.2	23.7	20.4	17.1	24.6	22.4	19.5	16.2	23.1	20.3	17.6	15.1

CP: crude protein; CF: crude fat; Carb: carbohydrate; GE: gross energy; DP: digestible protein; DF: digestible fat; DC: digestible carbohydrate; DE: digestible energy.
[†] Dietary carbohydrate calculated as: 100 % - (CP% + Fat% + Ash% + Moisture%).
[‡] Gross energy is calculated from energetic values of 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for fat and 17.2 kJ g^{-1} for carbohydrate.
[§] Provide per kg of diet: Vitamin A, 2000 MIU; Vitamin D3, 400 MIU; Vitamin E, 20 g; Vitamin B2, 5 g; Vitamin K3, 2 g; Nicotinic Acid, 15 g; Calcium Pantothenate, 10 g; Folic acid, 0.5 g; Vitamin B1, 2 g; Vitamin B6, 2 g; Vitamin B12, 10 mg; Iron, 100 g; Copper 10 g; Manganese, 70 g; Zinc, 80 g; Cobalt, 300 mg; Iodine, 1000 mg.
[a] FF Classic, FF SKAGEN A/S, Denmark; [b] Little Shephard Brand, Prima Ltd., Singapore; [c] Vital wheat gluten, Malindra group, USA; [d] Hipro soybean meal, Argentina; [e] X-soy 200, CJ selecta, Brazil; [f] Cargill, USA; [g] Daesang corporation, Korea; [h] Sardine fish oil, Indonesia; [i] Feed grade (≥ 98), CAS:590–46-5, Xi'an Bactrian Camel Biotech Co., Ltd., China; [j] 60 % corn cob, Shandong Jujia Biotech Co., Ltd., China; [k] L-ascorbate-2-polyphosphate, Rovimix Stay-C® 35, DSM, Netherlands; [l] Funginat, Norel animal nutrition, Singapore; [m] Butylated hydroxytoluene (BHT), 2 %; Butylated hydroxyanisole (BHA), 0.5 %; Ethoxyquin, 3.2 %; Haltox, Zargo, Singapore; [n] Yttrium(III) oxide, 99.9 %, CAS: 1314-36-9, Thermo Fisher Scientific, Singapore.

euthanised by an overdose of Aqui-S® (AQUI-S New Zealand Ltd., New Zealand) to analyse the initial whole-body composition (WBC). On Day 57, all the fish from each tank were euthanised to determine the final body weight (FBW) and the final WBC. Fish samples were kept at −20 °C before processing. The WBC processing for chemical analysis followed the methods reported by Bureau et al. (2006) with minor modifications. Whole fish samples from each tank were thawed at four degrees overnight and autoclaved at 105 °C for 40 min or until the samples were fully cooked. Samples were cooked to allow complete homogenisation of the whole fish, including scales and bones, by blending in a blender (HR3656/01, Philips) in 500–800 g sub-batches. All the blended sub-batches were mixed homogeneously using a Kitchen Aid stand mixer for 10 min. Blended fish samples were freeze-dried, ground using a coffee grinder, and stored at −20 °C until chemical analysis.

2.4. Faecal sample collection and processing for estimating digestibility

The fish in the trial were given a week to acclimatise to their respective diets before daily faecal samples were collected starting from Day 8. Faecal samples from each tank were collected using individual faecal settling columns according to Cho et al. (1982), with some modifications. One hour after the last feeding, all tanks and settling columns were cleaned daily to minimise and prevent contamination from uneaten feeds in the faecal samples. Overnight faecal samples were collected the next day at 9:00, before the first feeding. A modified 350 mL syringe with an enlarged opening was used to prevent any breakage of faecal materials and to transfer the faecal samples into a 250 ml conical centrifuge bottle. The faecal samples were centrifuged using a refrigerated centrifuge (Centrifuge 5920 R, Eppendorf) at 3000 RCF for 15 min at four °C. Daily wet faecal residuals were pooled individually according to each tank and stored at −20 °C before freeze-drying. The freeze-dried faecal samples were sieved using a 0.3 mm screen mesh to remove contaminating fish scales and stored at −20 °C before chemical analysis.

2.5. Chemical analyses

Analyses of crude protein (CP) and crude fat (CF) in ingredients, diets, WBC and faecal samples were done by an ISO-accredited analytical service laboratory, Eurofins Food Testing Singapore Pte Ltd., Singapore (Eurofins). CP (N% X 6.25; Dumas) was analysed following AOAC 968.06 and 992.15 with modifications made by Eurofins. CF in samples was analysed by hydrochloric acid hydrolysis and extracted using ether and hexane following AOAC 922.06 and 954.02 with internal modification made by Eurofins. The amino acid profiles of the diets were analysed by Eurofins using HPLC after hydrochloric acid

Table 2
Growth performance of juvenile Malabar snapper[§] after 56 days.

	Dietary energy levels[†]	Dietary protein levels				Main effects of energy levels	Two-way ANOVA *P*-value		Contrast *P*-value	
		P540	P480	P420	P360		Energy	P X E	Linear	Quadratic
BWG (g)	Low	33.5 ± 1.82	29.1 ± 3.40	21.8 ± 2.61	12.6 ± 1.57	24.3 ± 8.52	0.874	0.918	<0.001	0.693
	Mid	35.6 ± 8.09	27.6 ± 1.68	21.4 ± 4.20	15.1 ± 0.59[†]	25.8 ± 8.67				
	High	33.3 ± 3.70	29.9 ± 1.38	20.9 ± 4.30	13.0 ± 2.02	24.2 ± 8.69				
TGC	Low	0.5 ± 0.02	0.4 ± 0.04	0.3 ± 0.04	0.2 ± 0.02	0.36 ± 0.11	0.831	0.936	<0.001	0.547
	Mid	0.5 ± 0.09	0.4 ± 0.02	0.3 ± 0.06	0.2 ± 0.01[†]	0.38 ± 0.11				
	High	0.5 ± 0.05	0.4 ± 0.02	0.3 ± 0.06	0.2 ± 0.03	0.36 ± 0.11				
SGR (% day^{-1})	Low	1.1 ± 0.05	1.0 ± 0.09	0.8 ± 0.08	0.5 ± 0.05	0.85 ± 0.25	0.807	0.888	<0.001	0.519
	Mid	1.2 ± 0.19	1.0 ± 0.05	0.8 ± 0.13	0.6 ± 0.02[†]	0.90 ± 0.24				
	High	1.1 ± 0.10	1.0 ± 0.04	0.8 ± 0.12	0.5 ± 0.07	0.84 ± 0.25				
TFI (g)	Low	46.5 ± 3.00	47.8 ± 3.55	45.0 ± 1.68	39.7 ± 3.09	44.7 ± 4.05^{x}	<0.001	0.097	<0.001	0.007
	Mid	48.6 ± 4.68	43.2 ± 1.68	43.2 ± 2.82	39.6 ± 1.66[†]	44.0 ± 4.17^{x}				
	High	45.1 ± 3.01	42.7 ± 0.9	35.2 ± 2.49	32.0 ± 2.64	38.7 ± 5.94^{y}				
FCE (%)	Low	72.1 ± 3.01	60.9 ± 3.33	48.4 ± 4.18	31.8 ± 1.57	53.3 ± 15.88^{x}	0.004	0.613	0.084	0.354
	Mid	72.6 ± 10.16	63.9 ± 2.7	49.4 ± 6.53	38.3 ± 0.14[†]	57.7 ± 14.32				
	High	73.7 ± 3.32	69.9 ± 2.05	58.9 ± 7.93	40.4 ± 2.94	60.7 ± 14.08^{y}				
SR (%)	Low	94.4 ± 1.92	95.6 ± 1.92	94.4 ± 5.09	92.2 ± 7.70	94.2 ± 4.29	0.051	0.575	0.439	0.215
	Mid	100.0 ± 0.00	98.9 ± 1.92	100.0 ± 0.00	95.0 ± 7.07[†]	98.8 ± 3.08				
	High	95.6 ± 5.09	97.8 ± 1.92	93.3 ± 3.33	97.8 ± 3.85	96.1 ± 3.72				
Main effects of protein levels							Protein			
BWG (g)		34.1 ± 4.67^{a}	28.9 ± 2.25^{b}	21.4 ± 3.30^{c}	13.4 ± 1.76^{d}		<0.001			
TGC		0.48 ± 0.05^{a}	0.42 ± 0.03^{b}	0.33 ± 0.04^{6}	0.21 ± 0.03^{d}		<0.001			
SGR (% day^{-1})		1.12 ± 0.11^{a}	0.99 ± 0.06^{b}	0.78 ± 0.10^{c}	0.53 ± 0.06^{d}		<0.001			
TFI (g)		46.7 ± 3.51^{a}	44.6 ± 3.14^{ab}	41.1 ± 4.95^{b}	36.8 ± 4.58^{c}		<0.001			
FCE (%)		72.8 ± 5.60^{a}	64.9 ± 4.66^{b}	52.2 ± 7.48^{c}	36.6 ± 4.50^{d}		<0.001			

Values are mean ± SD (n = 3).
abcd For parameters with a significant effect on protein levels, values within a row lacking a common superscript differ (P < 0.05).
P: Protein levels; E: Energy levels; IBW: Initial body weight; FBW: Final body weight; BWG: Body weight gain; TGC: Thermal-unit coefficient; SGR: Specific growth rate; FCE: Feed conversion efficiency; SR: Survival rate.
[†] Mean values of treatment groups (n = 2).
[‡] Energy levels Low, Mid and High are 21.0, 22.3 and 23.6 MJ kg^{-1} in DM basis, respectively.
[§] Initial body weight (g) = 38.96 (SD 0.34).

hydrolysis with phenol added for 24 h at approximately 110 °C, following methodology from Henderson and Brooks (2010), Henderson et al. (2000), Barkholt and Jensen (1989) and Schuster (1988) (Supplementary Table 1). Moisture content and ash of test ingredients, diets, WBC slurry and WBC freeze-dried powdered, and faecal samples were determined in triplicates per sample following AOAC 930.15 and 942.05, respectively. Pacific Lab Services determined fibre content in diets following AOAC 978.10 and determined yttrium contents in diets and faecal samples using Pacific Lab Method 4.3, ICP-OES. Carbohydrate was determined by nutrient difference as follows: Carbohydrate = 100 % – (% moisture + % protein + % fat + % ash) (Rahman et al., 2016). Gross energy (GE) was calculated using the mean energetic values of protein, fat and carbohydrate of 23.6, 39.5 and 17.2 kJ g^{-1}, respectively, according to Blaxter (1989).

2.6. Growth performance, digestibility and nutrient balance calculations

Growth performance parameters such as body weight gain (BW_{gain}), thermal growth coefficient, specific growth rates, feed conversion efficiency (FCE), and survival in Table 2 were calculated using the following equations:

$$BW_{gain} = BW_f - BW_i$$

$$TGC = \left[\left(BW_f^{\left(\frac{1}{3}\right)} - BW_i^{\left(\frac{1}{3}\right)} \right) \div (T \times d) \right] \times 1000$$

$$SGR = \left(ln BW_f - ln BW_i \right) \div d \times 100$$

$$FCE = BW_{gain} \div TFI \times 100$$

where BW_f and BW_i are the final and initial body weights (g fish^{-1}), T is the average temperature of the trial (°C), d is the number of days, TFI is the total feed intake (g fish^{-1}), and CP_{Diet} represents the crude protein content of the diet (g kg^{-1}).

The apparent digestibility coefficient (ADC) for proximate composition and energy in diets was calculated following Cho et al. (1982):

$$ADC = [1 - (N_{Faecal} \div N_{Diet} \times Y_{Diet} \div Y_{Faecal})] \times 100$$

where N_{Diet} and N_{Faecal} represent the percentages of nutrients or kJ g^{-1} GE of the diet and faecal matter, respectively. Y_{Diet} and Y_{Faecal} represent the percentages of Yttrium in diet and faecal samples, respectively.

The digestible nutrient content of the diet was calculated by multiplying the nutrient composition with the ADC of each respective nutrient in each diet. Digestible protein intake (DPI, g kg$^{-0.7}$ day^{-1}), protein deposition (P_{gain}, g kg$^{-0.7}$ day^{-1}) and protein retention efficiency (PRE, %) in Table 3 were calculated using the following equations:

Table 3
Protein balance of juvenile Malabar red snapper fed with experimental diets for 56 days.

	Energy levels‡	Protein levels				Main effects of energy levels	Two-way ANOVA *P*-value		Contrast *P*-value	
		P540	P480	P420	P360		Energy	P X E	Linear	Quadratic
DPI	Low	3.10 ± 0.17	2.86 ± 0.17	2.35 ± 0.05	1.86 ± 0.11	2.55 ± 0.51^{x}	<0.001	0.092	<0.001	0.005
(g kg$^{-0.7}$ day^{-1})	Mid	3.20 ± 0.19	2.60 ± 0.07	2.25 ± 0.10	1.80 ± 0.07†	2.52 ± 0.53^{x}				
	High	2.94 ± 0.13	2.51 ± 0.04	1.86 ± 0.09	1.44 ± 0.10	2.19 ± 0.61^{y}				
P_{gain}	Low	0.91 ± 0.06	0.82 ± 0.1	0.61 ± 0.07	0.38 ± 0.04	0.68 ± 0.22^{x}	0.034	0.711	<0.001	0.542
(g kg$^{-0.7}$ day^{-1})	Mid	0.91 ± 0.16	0.7 ± 0.01	0.61 ± 0.04	0.43 ± 0.05†	0.68 ± 0.19^{x}				
	High	0.84 ± 0.06	0.7 ± 0.06	0.52 ± 0.09	0.32 ± 0.03	0.60 ± 0.21^{y}				
PRE	Low	29.3 ± 1.27	28.5 ± 2.21	25.8 ± 2.65	20.5 ± 0.82	26.0 ± 3.94	0.771	0.544	0.148	0.655
(%)	Mid	28.3 ± 3.46	26.9 ± 0.99	26.9 ± 1.10	23.7 ± 1.86†	26.7 ± 2.41				
	High	28.5 ± 1.19	28.0 ± 1.90	27.9 ± 3.62	22.0 ± 1.48	26.6 ± 3.39				
Main effects of protein levels							Protein			
DPI (g kg$^{-0.7}$ day^{-1})		3.08 ± 0.18^{a}	2.66 ± 0.18^{b}	2.16 ± 0.24^{c}	1.69 ± 0.22^{d}		<0.001			
P_{gain} (g kg$^{-0.7}$ day^{-1})		0.89 ± 0.10^{a}	0.74 ± 0.08^{b}	0.58 ± 0.08^{c}	0.37 ± 0.06^{d}		<0.001			
PRE (%)		28.7 ± 1.99^{a}	27.8 ± 1.7^{a}	26.9 ± 2.48^{a}	21.9 ± 1.76^{b}		<0.001			

Values are mean ± SD ($n = 3$).
abcd For parameters with a significant effect on protein levels, values within a row lacking a common superscript differ ($P < 0.05$).
xy For parameters with a significant effect on energy levels, values within a column lacking a common letter superscript differ ($P < 0.05$).
P: Protein levels; E: Energy levels; DPI: Digestible protein intake; P_{gain}: Protein deposition; PRE: Protein retention efficiency.
† Mean values of treatment groups ($n = 2$).
‡ Energy levels Low, Mid and High are 21.0, 22.3 and 23.6 MJ kg^{-1} in DM, respectively.

$$DPI = TFI \times (CP_{Diet} \div 1000) \div MBW_G \div d \times CP_{ADC}$$

$$P_{gain} = \left[(BW_f \times CP_{WBCf} \div 1000) - (BW_i \times CP_{WBCi} \div 1000)\right] \div MBW_G \div d$$

$$PRE = P_{gain} \div DPI \times 100$$

where BW_f and BW_i are the final and initial body weights (g fish^{-1}), TFI is the total feed intake (g fish^{-1}), CP_{Diet} and CP_{WBC} represent either the crude protein content of the diet or WBC, respectively (g kg^{-1}), *f* and *i* represent the final or initial samples, and d is the number of days. The geometric mean body weight (BW_G; in g) is calculated as $\sqrt{IBW \times FBW}$, from which the geomean metabolic body weight (MBW_G; in kg$^{0.7}$) is calculated as $(BW_G/1000)^{0.7}$ using the metabolic exponent of 0.7 to describe the relationship between protein rate and body mass (Lupatsch et al., 1998; Raposo et al., 2024).

2.7. Statistical analyses and broken line regression

Data were analysed using IBM SPSS Statistics version 27. Data are screened for outliers using box and whisker plots. Data with at least 1.5 interquartile ranges below the first quartile or at least 1.5 interquartile ranges above the third quartile are identified as potential outliers and are removed from subsequent analyses. In this study, one potential outlier was identified from treatment group P360Mid (tank G9) and removed from subsequent analyses (Supplementary Fig. 1).

Residuals were assessed for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. Parameters that conformed to the assumptions of normality and homogeneity of variance were analysed with two-way ANOVA. Post-hoc multiple comparisons between treatment groups were conducted using Tukey's HSD test to identify differences in the main effects mean values. The associations between the parameters in Fig. 1 were performed using Pearson correlation (R), the corrplot package in the *R* program (Wei and Simko, 2021). Statistical differences were considered significant when *P* values were < 0.05.

The linearity effects of diets were examined using polynomial orthogonal contrast (Steel et al., 1997). Analyses of broken-line (BL) regression to estimate nutrient requirements of DP (g kg^{-1}), DP/DE (g MJ^{-1}), amino acids (g 100 g^{-1} of diet) and acceptable carbohydrate levels (g kg^{-1}) in DM were performed using easyreg package version 4.0 (Arnhold, 2018) in the *R* program (Kaps and Lamberson, 2009; R Core Team, 2022). BL regression models of either linear-plateau (L-P) or linear-linear (L-L) were used based on the highest R-squared (R^2) coefficient of determination. The requirement is the *x* value of the point common to both lines (Pesti et al., 2009).

For L-P,

$y = \mu + b \times x + e_i$ if $x \leq$ requirement, and.

$$y = \text{maximum (or minimum)}, \text{if } x \geq \text{requirement} \quad (1)$$

For L-L,

$$y = \mu + b \times x + e_i \text{ if } x \leq \text{requirement and if } x \geq \text{requirement} \quad (2)$$

where *y* is the dependent response, either TGC or P_{gain}; *x* is the fixed factor; μ is the coefficient for constant; *b* is the coefficient of *x*; e_i is the standard error, and $i = n$, with $n = 3$ for each diet (except diet P360Mid, $n = 2$).

To express amino acid requirements in (% CP), the estimated CP requirement (g kg^{-1}) based on TGC was calculated by dividing the estimated DP requirement of 479.1 g kg^{-1} by the average protein ADC of P540. The estimated EAA requirements were expressed as (% CP) by dividing by the estimated CP required for optimal growth.

The efficiency of protein utilisation for protein gain was estimated from the slope of the regression of protein deposition on DPI. The pairwise comparisons of the regression slopes between the different energy diets were tested for significance using a general linear model with protein deposition as the dependent parameter, DPI as a covariate and energy diets as a fixed factor. When the interaction effect between energy diets and DPI was significant ($P < 0.05$), the slopes differed between the two energy diets.

3. Results

3.1. Effects of diets on growth performance and protein retention

Juvenile Malabar snapper grew well over the 56 trial days, with a survival rate higher than 92 % across all the treatment groups (Table 2). The fastest-growing group (P540Mid) gained 91.4 % of its initial body

weight, and the average body weight gain (BWG) for all treatment groups was 24.7 g. Based on two-way ANOVA analysis, growth performance parameters such as thermal growth coefficient (TGC), specific growth rate (SGR), and BWG were only influenced by dietary protein levels but not by dietary energy levels. Juvenile Malabar snapper grew the largest when fed diets containing the highest dietary protein level of 540 g kg^{-1}, regardless of the dietary energy content, and all lower dietary protein levels resulted in significantly reduced growth ($P < 0.05$). The effects of higher dietary protein leading to better performance values were similarly observed in total feed intake (TFI) and feed conversion efficiency (FCE) (Table 2), as well as in digestible protein intake (DPI), protein deposition and protein retention efficiency (PRE)

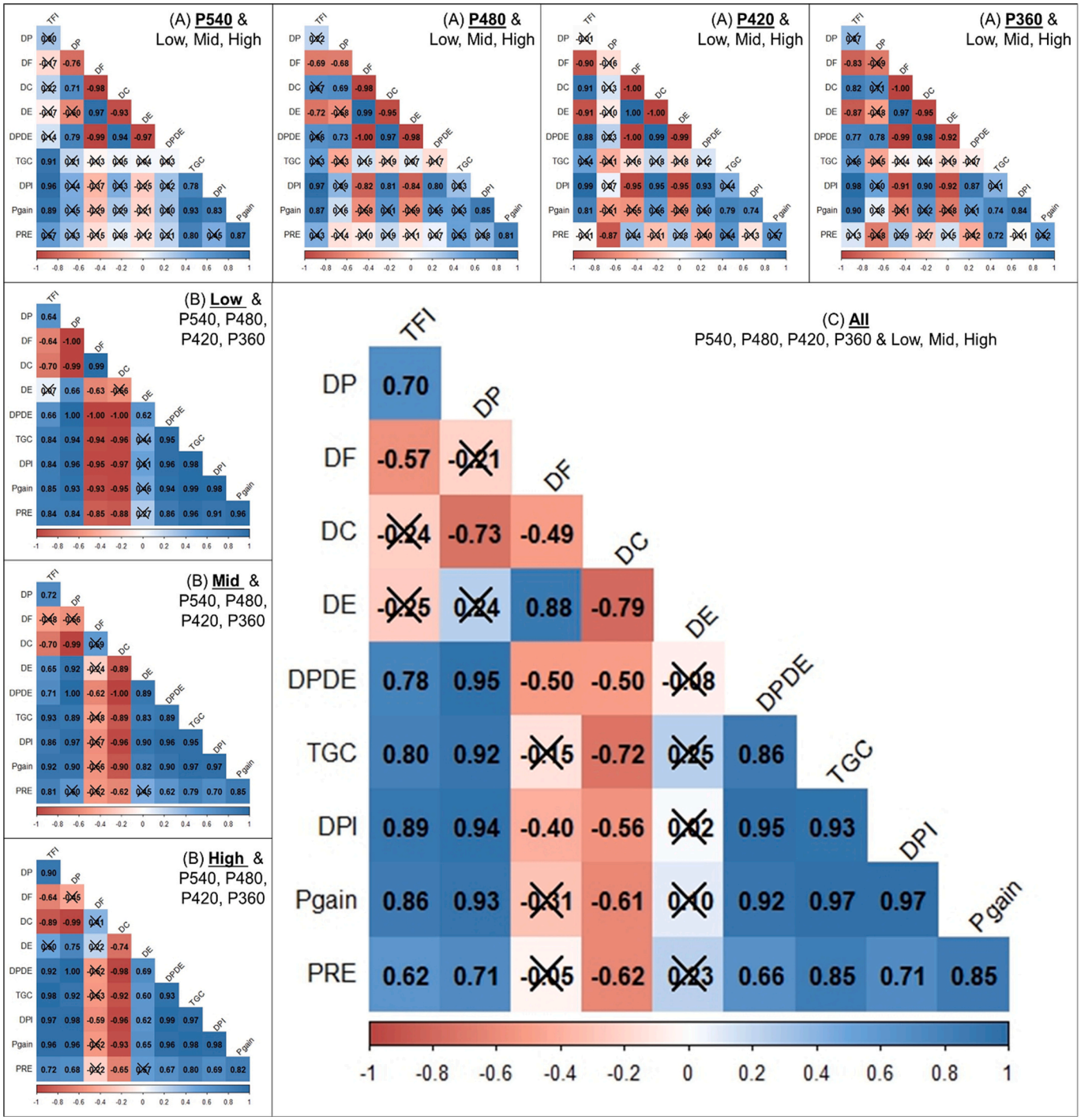

Fig. 1. Associations between digestible nutrients (e.g., DP, DF, DC, DE and DP/DE), growth parameters (TGC) and protein balance (DPI, P_{gain} and PRE) within (A) a constant protein level with different dietary energy levels, within (B) a constant energy level with different dietary protein levels, and (C) all dietary protein and energy levels. The correlation coefficient 'R' values were shown in the text within the colour box. The strength of R was illustrated by the colour tone in which the red represented negative correlations (−1), and the blue represented positive correlations (1). R values lacking statistically significant correlation coefficients were marked with a cross ($P > 0.05$). DP: digestible protein; DF: digestible fat; DC: digestible carbohydrate; DE: digestible energy; DP/DE: digestible protein to digestible energy ratio; DPI: digestible protein intake; Pgain: Protein deposition; PRE: protein retention efficiency; P: Protein levels of 540, 480, 420, 360 g kg^{-1} in DM; Energy levels Low, Mid and High are 21.0, 22.3 and 23.6 MJ kg^{-1} in DM, respectively; All: inclusive of all twelve diets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Table 3). To the contrary, higher dietary energy levels significantly reduced TFI, DPI and protein deposition but improved FCE (Tables 2 and 3, $P < 0.05$). However, the effects of dietary protein and energy were independent, and no interactions between them were observed. The correlation coefficient (R) between TFI, digestible dietary nutrients, TGC, PER, DPI, protein deposition, and PRE can be visualised in Fig. 1. Correlations that were not significant were marked with a cross ($P > 0.05$). Based on significant associations from all 12 diets, TFI positively correlated with all parameters except digestible carbohydrate (DC) and DE, and negatively correlated with digestible fat (DF). Focusing on TGC, protein deposition and PRE, strong positive correlations were observed between them and TFI, DP, DP/DE and DPI. Among the digestible nutrients, DC negatively correlated with TGC, DPI, protein deposition and PRE. The effects of DC on growth parameters and protein balance differed from those of DP, DF, and DE, such that DP positively influenced them, while DF and DE had no significant associations (Fig. 1C). Based on differences in dietary protein levels at fixed dietary energy levels (either Low, Mid or High), DE was not associated with TGC, DPI, protein deposition and PRE in Low-energy diets. Instead, DF and DC negatively impacted growth and protein retention in Low. Based on Mid and High energy diets, DC still contrasted growth and protein retention; however, DF had no significant association, and DE was positively associated with growth and protein retention (Fig. 1B). At fixed dietary protein levels (either P540, P480, P420, P360) but varying energy diets, DE did not affect TGC, protein deposition and PRE (Fig. 1A, $P > 0.05$).

Polynomial contrast examination of the effect of diets on growth performance and protein balance indicated that most of the parameters had a linear relationship with diets ($P < 0.05$) except for TFI and DPI, where the data also had a quadratic relationship ($P < 0.05$). On the other hand, no significant contrast was observed for FCE, survival, and PRE, and those parameters were not used for estimating nutrient requirements.

3.2. Effect of diets on whole-body nutrient composition and apparent digestibility

Data on the proximate nutrient and energy content of juvenile Malabar red snapper whole-body composition (WBC) are presented in Supplementary Table 2. The initial moisture, protein, fat, ash, and gross energy contents were 666 g kg^{-1}, 167 g kg^{-1}, 109 g kg^{-1}, 57 g kg^{-1} and 8.3 MJ kg^{-1}, respectively. After the 56-day trial, the final WBC protein content ranged from 165 g kg^{-1} to 180 g kg^{-1}. Higher dietary protein levels increased WBC protein content but decreased WBC fat and ash content ($P < 0.05$). In contrast, higher dietary energy levels decreased WBC moisture, protein and ash content but increased WBC fat and energy content. Significant interactions between protein and energy were observed in WBC fat and gross energy content ($P < 0.05$). The influence of dietary energy on WBC fat content was likely because of the dietary fat and not carbohydrate contents in the diets, since a strong correlation could be observed between WBC fat and dietary fat contents ($R^2 = 0.89$) but not dietary carbohydrates ($R^2 = 0.18$). The changes in WBC fat content were likely balanced by WBC moisture content, for which body fat content was observed to be negatively correlated with moisture content in WBC ($R^2 = -0.85$).

Dietary protein, energy levels, and their interaction significantly affected the apparent digestibility coefficient (ADC) of dietary macronutrients and energy (Supplementary Table 3). Regarding the effect of dietary protein, the ADC of protein, dry matter (DM), and gross energy (GE) were reduced significantly as the protein level in the diet decreased. The ADC of fat was similar between P540 and P480, but the ADC of fat was significantly lower in P420 and even lower in P360. On the contrary, ADC of carbohydrates increased with decreasing dietary protein content. Regarding the effect of dietary energy, the highest ADC of protein, DM, GE, fat and carbohydrate were observed for Mid-energy diets, followed by Low diets, and the lowest ADC values were in High diets. However, the Low-energy and mid-energy diets ADC values for fat and carbohydrates were not significantly different ($P > 0.05$).

3.3. Digestible nutrient and dietary amino acid requirement

Broken-line regression analyses with either L-P or L-L models were used to estimate the digestible nutrient requirements for optimal growth based on TGC and protein deposition (Table 4). The required DP and DP/DE ratio for optimal growth performance was estimated to be 479.1 g kg^{-1} and 25.78 g MJ^{-1}, respectively. Separately, the DP/DE ratio requirements for maximum protein deposition were estimated to be 25.32 g MJ^{-1} (Fig. 2). The requirement of DP for optimal protein deposition could not be estimated as the relationship between DP and protein deposition was linear, and a breakpoint was not observed before the maximum DP level. Similar nutrient estimations for digestible fat and DE with TGC had been conducted; however, the correlations were poor ($R^2 < 0.15$, data not shown) because the experiment had only three energy levels. The recommended digestible carbohydrate levels in diets were less than 185.8 g kg^{-1}, and inclusions above the recommended level have resulted in a decline in TGC and protein deposition (Table 4).

Indirect estimation of dietary amino acid requirements was also performed in this study (Table 4). Dietary amino acids could be estimated with the design of this experiment, as dietary protein contents were formulated using macro ingredients added proportionally. This resulted in the diets having four graded dietary amino acid levels corresponding to the four dietary protein levels at each of the three dietary fat levels. All 17 amino acid (except tryptophan and combining aspartic acid with asparagine and glutamic acid with glutamine) requirements were estimated (Table 4). Among the EAAs, the dietary lysine and methionine requirements (g kg^{-1} diet, DM) for optimal growth performance based on TGC were estimated to be 29.7 g kg^{-1} and 11.6 g kg^{-1}, respectively.

The estimated CP requirement of 508.9 g kg^{-1} was calculated by dividing the estimated DP requirement of 479.1 g kg^{-1} by the average P540 protein ADC of 94.14 %. The estimated EAA requirements were expressed as (% CP) by dividing by the estimated CP requirement required for optimal growth (Fig. 4). Juvenile Malabar snapper's EAA requirements are highly correlated, $R = 0.89$, and are higher than the average EAA requirement of fish, except for the total sulphur amino acids (TSAA, Met + Cys) and tryptophan, which were not analysed. The average EAA fish requirement was obtained from Xing et al. (2024).

3.4. Effects of diets on digestible protein utilisation efficiency for growth

The utilisation efficiency of digestible protein for protein deposition can be estimated through the relationship between protein deposition and DPI, as visualised in Fig. 3. The following regression equations of digestible protein utilisation efficiency were obtained for Low, Mid and High energy diets, respectively.

$$\text{Low}; P_{gain} = -0.404\ (\text{SE}\ 0.062) + 0.426\ (\text{SE}\ 0.024)\ \text{DPI}, R^2 = 0.970 \quad (3)$$

$$\text{Mid}; P_{gain} = -0.200\ (\text{SE}\ 0.079) + 0.349\ (\text{SE}\ 0.031)\ \text{DPI}, R^2 = 0.935 \quad (4)$$

$$\text{High}; P_{gain} = -0.150\ (\text{SE}\ 0.052) + 0.341\ (\text{SE}\ 0.023)\ \text{DPI}, R^2 = 0.957 \quad (5)$$

Based on the coefficient value of digestible protein intake (i.e., the regression slope), the protein utilisation efficiencies for protein deposition decreased with higher dietary energy intake. Although no significant differences were observed between Low-energy and Mid-energy diets, Eq. (3) and (4), and between Mid-energy and High-energy diets, Eq. (4) and (5) ($P > 0.05$), the protein utilisation efficiencies were significantly higher in Low-energy compared to High-energy diets by 24.9 % ($P < 0.05$).

Table 4
Regression equations and estimated requirement values for digestible protein (DP), digestible carbohydrate, DP/DE ratio, and dietary amino acid composition as a function of TGC or protein gain.

Dependent Variable	Fixed Factor	Model	Equations		R^2	Estimated Requirement
			when, x < Req	When, x ≥ Req		
Nutrient and energy (DM)						
DP (g kg^{-1})	TGC	L-P	$y = -0.27 + 0.002\times$	$y = 0.48$	0.87	479.1
DP (g kg^{-1})	P_{gain}	Linear	$y = -0.56 + 0.003\times$	N.A.	0.87	N.A.
DC[†] (g kg^{-1})	TGC	L-P	$y = 0.50\times$	$y = 0.71\times$	0.56	185.8
DC[†] (g kg^{-1})	P_{gain}	L-P	$y = 0.88\times$	$y = 1.23\times$	0.41	185.8
DP/DE[‡] (g MJ^{-1})	TGC	L-P	$y = -0.21 + 0.03\times$	$y = 0.51$	0.79	25.78
DP/DE[‡] (g MJ^{-1})	P_{gain}	L-P	$y = -0.52 + 0.06\times$	$y = 0.91$	0.88	25.32
Essential and semi-essential amino acids (g 100 g^{-1} of diet, DM)						
Arginine	TGC	L-P	$y = -0.50 + 0.35\times$	$y = 0.45$	0.73	2.68
Cysteine	TGC	L-L	$y = -0.34 + 1.30\times$	$y = 0.22 + 0.35\times$	0.49	0.58
Histidine	TGC	L-P	$y = -0.33 + 0.65\times$	$y = 0.45$	0.78	1.20
Isoleucine	TGC	L-L	$y = -0.35 + 0.36\times$	$y = 0.78–0.13\times$	0.71	2.32
Leucine	TGC	L-P	$y = -0.58 + 0.25\times$	$y = 0.45$	0.72	4.07
Lysine	TGC	L-P	$y = -0.20 + 0.22\times$	$y = 0.45$	0.75	2.97
Methionine	TGC	L-P	$y = -0.51 + 0.85\times$	$y = 0.46$	0.76	1.16
Phenylalanine	TGC	L-P	$y = -0.50 + 0.45\times$	$y = 0.45$	0.74	2.31
Threonine	TGC	L-L	$y = -0.53 + 0.52\times$	$y = 0.32 + 0.06\times$	0.75	1.86
Tyrosine	TGC	L-L	$y = -0.54 + 0.57\times$	$y = 0.20 + 0.13\times$	0.78	1.69
Valine	TGC	L-L	$y = -0.45 + 0.38\times$	$y = 0.23 + 0.09\times$	0.70	2.31
Non-essential amino acids (g 100 g^{-1} of diet, DM)						
Alanine	TGC	L-L	$y = -0.58 + 0.39\times$	$y = 0.34 + 0.04\times$	0.75	2.63
Aspartic Acid	TGC	L-P	$y = -0.37 + 0.19\times$	$y = 0.45$	0.77	4.29
Glutamic Acid	TGC	L-L	$y = -0.39 + 0.09\times$	$y = 0.24 + 0.02\times$	0.79	9.14
Glycine	TGC	L-P	$y = -0.26 + 0.27\times$	$y = 0.45$	0.76	2.47
Proline	TGC	L-L	$y = -0.28 + 0.22\times$	$y = 0.53–0.02\times$	0.75	3.39
Serine	TGC	L-P	$y = -0.53 + 0.47\times$	$y = 0.46$	0.8	2.13

DP: digestible protein; DC: digestible carbohydrate; DP/DE: digestible protein/digestible energy; N.A.: not available.
[†] Dietary carbohydrate calculated as: 100 % - (CP% + Fat% + Ash% + Moisture%).
[‡] Gross energy is calculated from energetic values of 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for fat and 17.2 kJ g^{-1} for carbohydrate.

4. Discussion

4.1. Juvenile Malabar snapper requires high levels of digestible protein for optimal growth

Fish dietary protein requirements have been extensively reviewed by Oliva-Teles et al. (2020). From that review that compiled data from over 300 studies, the dietary protein requirements across 150 fish species ranged from 24 % to 62 %, averaging 42 %. In this study, the highest growth and protein deposition in juvenile Malabar snapper were achieved with P540 diets containing an average of 54.2 % protein on DM basis, indicating that their protein requirement is towards the upper end of the expected range. However, since P540 diets were the highest protein level tested, further studies investigating higher dietary protein levels beyond 54.2 % are needed to validate the optimal dietary protein requirement for growth. Compared to other snapper species, the Malabar snapper's requirement is higher than red snapper (Miller et al., 2005), mangrove snapper (Catacutan et al., 2001; Abbas and Siddiqui, 2013), mutton snapper (Watanabe et al., 2001) and spotted rose snapper (Parra et al., 2010), and is closer to the 55 % protein requirement of yellow snapper (Maldonado-García et al., 2012). These findings indicated that Malabar snapper requires a high-protein diet for optimal growth, and the estimated requirement levels were within the expected range for snappers.

Nowadays, aquaculture diets are commonly formulated based on digestible nutrients and energy needs (Cho and Kaushik, 1990). A good-quality feed is highly dependent on its ingredient characteristics and nutrient bioavailability (Glencross, 2020), and diets composed of poorly digestible ingredients can lead to higher requirements (Hussain et al., 2024). Therefore, reporting nutrient requirements in their digestible form is critical, yet very few studies have done so. The significant findings that dietary protein and energy contents influenced the dietary ADC nutrient values highlight the importance of evaluating nutrient requirements in their digestible form (Supplementary Table 3). Based on the factorial design of this study, the estimated DP requirement of 479.1 g kg^{-1} at a DP/DE ratio of 25.78 g MJ^{-1} was optimal for growth in juvenile Malabar snapper from 39 g to 73 g. These requirements were much higher than the recommended DP and DP/DE ratio for juvenile mangrove snapper at 384 g kg^{-1} and 22.5 g MJ^{-1}, respectively (Abbas and Siddiqui, 2013). To our knowledge, digestible nutrient requirements have only been evaluated in mangrove snapper but not for other snappers. In comparison with other carnivorous species, the estimated DP requirement of juvenile Malabar snapper between body weight of 39 g and 73 g was closer to 470 g kg^{-1} for juvenile orange-spotted grouper (*Epinephelus coioides*) at an initial size of 10 g (Yan et al., 2021), but higher than 421 g kg^{-1} for juvenile barramundi (*Lates calcarifer*) at an initial weight of 76 g (Williams and Barlow, 1999) and 439 g kg^{-1} for juvenile gilthead sea bream (*Sparus aurata*) at an initial size of 10 g (Santinha et al., 1996). Comparison of optimal nutrient requirements across species is often challenging since the utilisation and requirements can be dependent on a multitude of factors such as culture conditions (Kieffer et al., 1998; Langi et al., 2024), the developmental size of fish (Glencross and Bermudes, 2012), and the type and quality of the ingredients used (Glencross et al., 2007; Huangfu et al., 2024). Although the juvenile Malabar snapper in this study grew well with over 90 % survival rate for 56 days, the SGR and TGC of the P540 diets seemed slow at 1.12 % day^{-1} and 0.48, respectively. Direct growth rate comparison within and between species is difficult since smaller fish have faster relative growth rates than bigger fish. The SGR of juvenile Malabar snapper of P540 diets that grew from 39.0 g to 73.1 g in this study were similar to juvenile spotted rose snapper cultured in floating net cages for a year, which grew from 14.2 g to 437.0 g at 0.95 % day^{-1} (Hernández et al., 2016), and in tank conditions for 70 days, growing from 77.4 g to 165.6 g at 1.09 % day^{-1} (Osuna-Salazar et al., 2023). However, there are growth discrepancy within the same species. Faster SGRs of 1.56 % day^{-1} and 1.94 % day^{-1} have also been reported for juvenile spotted rose snapper, which has grown from 19.4 g to 57.8 g and from 31.3 g to 120.3 g, respectively, after a 70-day trial (Hernández et al., 2021;

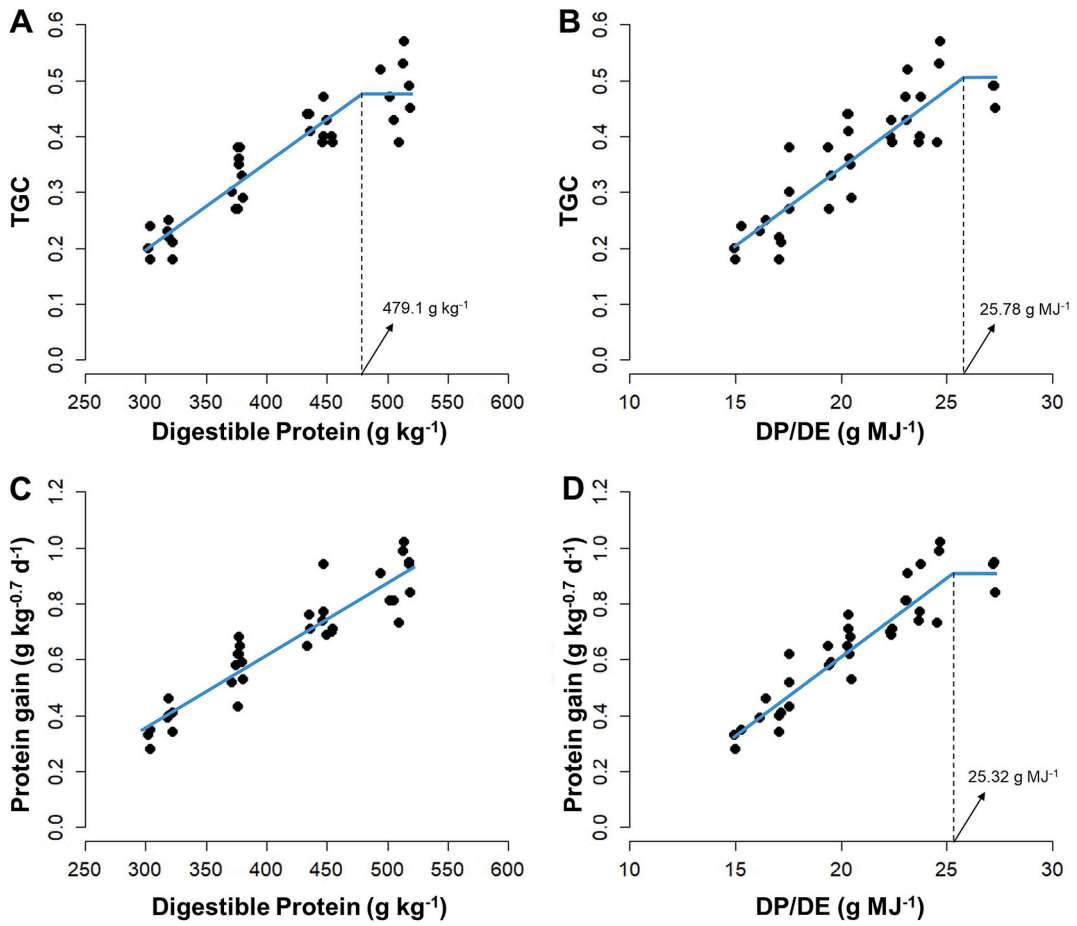

Fig. 2. Linear regression using broken-line analysis was conducted to estimate the optimal digestible protein (DP, g kg^{-1}, DM) or digestible protein-to-digestible energy ratio (DP/DE, g MJ^{-1}, DM) for thermal growth coefficient (TGC) or protein gain (g $kg^{-0.7}$ d^{-1}). (A) Relationship between DP and TGC. (B) Relationship between DP/DE and TGC. (C) Relationship between DP and protein gain. (D) Relationship between DP/DE and protein gain. Gross energy is calculated from energetic values of 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for fat and 17.2 kJ g^{-1} for carbohydrate. DM: Dry matter.

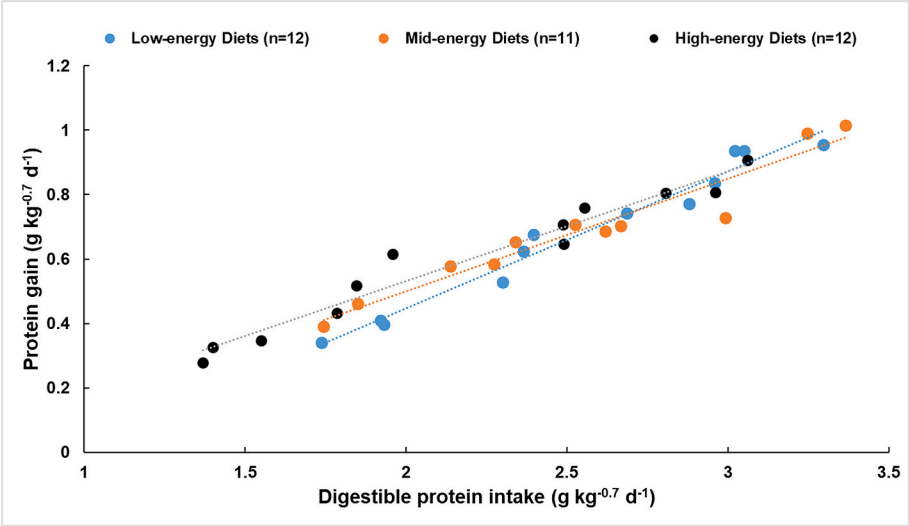

Fig. 3. Relationship between protein gain (P_{gain}, g $kg^{-0.7}$ d^{-1}) as a function of digestible protein intake (DPI, g $kg^{-0.7}$ d^{-1}) of L. *malabaricus* fed either Low, Mid or High energy diets for 56 days. Low energy diets (P_{gain} = −0.404 (SE 0.062) + 0.426 (SE 0.024) DPI, R^2 = 0.970), Mid energy diets (P_{gain} = −0.200 (SE 0.079) + 0.349 (SE 0.031) DPI, R^2 = 0.935) and High energy diets (P_{gain} = −0.150 (SE 0.052) + 0.341 (SE 0.023) DPI, R^2 = 0.957). The regression slope between High-energy and Low-energy diets and between Mid-energy and Low-energy diets differed significantly ($P < 0.05$).

Benitez-Hernández et al., 2022). The relatively slower growth rate in Malabar snapper compared to spotted rose snapper may be limited by its genetic potential as the batch of fish has not undergone selection for fast growth nor has its culture condition been optimised (Ibarra-Castro et al., 2020). The feeding frequence of twice a day in this study might also be suboptimal to maximise the growth potential of this fish. Nonetheless, juvenile Malabar snapper at 39 g in the current culture conditions and diet formulation seemed to require higher digestible protein levels in its diet than other juvenile carnivorous fish with body weight less than 100 g.

4.2. Indirect estimation of amino acid requirements based on dietary protein requirement

It is commonly recognised that fish require a balanced composition of essential amino acids (including cysteine and tyrosine) rather than crude protein in their diet (Wilson, 1986). Over the years, several reviews on EAA requirements have been conducted on fish (Cowey, 1995; NRC, 2011; Guillaume et al., 2017; Xing et al., 2024), and there are considerable variations in the recommended EAAs within- and between species (summarised by Xing et al. (2024)). In summary, variability in data might be attributed to the experimental design, including the quality and composition of diets, the levels and range of EAA contents evaluated, culture condition, size of fish, trial duration, statistical tools and models used for data analysis, and estimating requirements (Xing et al., 2024).

The standard and conventional approach to evaluating target EAA requirement is through multiple dose-response trials by using a range of target EAA concentrations (deficient to excess) commonly achieved by adding crystalline amino acids to diets that are either purified or practically formulated (Xing et al., 2024). Although such iterative approaches can yield reliable results, ample time and resources would be needed to study the requirements of every individual EAA. For example, in barramundi studies that focused on individual amino acid requirements, only methionine, lysine, arginine and tryptophan were estimated out of the ten essential amino acids (Murillo et al., 2001; Coloso et al., 2004; Poppi et al., 2017). Furthermore, crystalline EAA is commonly supplemented to meet target values, so it is vital to verify that crystalline amino acids are utilised efficiently since the addition of target EAA in excess can also disrupt the balance of amino acid composition (EAA to non-EAA ratio), affecting growth and protein utilisation (Peres and Oliva-Teles, 2006, 2017). Hence, this study proposed an indirect method for evaluating dietary amino acid requirements in conjunction with dietary protein requirements. The practical application of the indirect method is demonstrated in this study by comparing the results with known fish EAA requirements. A recent meta-analysis reviewed by Xing et al. (2024) provided re-estimations of the 10 EAA requirements (expressed as % CP) on fish based on 320 studies. Malabar snapper's estimated dietary EAA requirements were higher for every EAA except for the TSAA, which correlated positively with the compiled fish requirements (Fig. 4). This demonstrated the robustness of the indirect method and supported the high CP requirement estimated for Malabar snapper, which is higher than the average fish (as discussed in section 4.1). The Malabar snapper's 29.7 g kg^{-1} lysine requirement is much higher than the 20.6 g kg^{-1} lysine requirement of barramundi (Murillo et al., 2001) and 21.6 g kg^{-1} lysine requirements of hybrid grouper (*E. fucoguttatus* X *E. lanceolatus*) (Li et al., 2019). On the contrary, the 11.6 g kg^{-1} methionine and 17.4 g kg^{-1} TSAA requirement in this study is within and closer to the lower limit of 10.5 to 13.6 g kg^{-1} methionine and 17.1 to 20.2 g kg^{-1} TSAA requirement for barramundi (Poppi et al., 2017). However, it is lower than the 14.5 g kg^{-1} methionine and 21.4 g kg^{-1} TSAA requirement for hybrid grouper (Li et al., 2020) and 15.8 g kg^{-1} methionine and 20.3 g kg^{-1} TSAA requirement for giant grouper (*E. lanceolatus*) (Candebat et al., 2023). These results indicated that Malabar snapper requires much higher levels of dietary lysine and lower levels of methionine and TSAA than other carnivorous fish such as barramundi and groupers. The indirect approach has estimated the total AA requirements faster and more efficiently than the iterative methods at the expense of accuracy. It is also useful in providing suggestions for the amino acid levels to be included in specific EAA dose-response trials.

4.3. Effects of non-protein dietary energy on feed intake and carbohydrate tolerance

Like other terrestrial animals, fish consume food to satisfy their energy needs, and they may stop eating when their energy requirements are met (Cho and Kaushik, 1990; Bureau et al., 2003). This phenomenon was observed in this study, in which Malabar snapper fed with High- and Mid-energy diets had lower feed intake than those on Low-energy diets, regardless of dietary protein content ($P < 0.05$). In contrast, feed intake amounts were lowered significantly in fish that consumed diets with less DP and more DC ($P < 0.05$), and the DC acceptable levels were estimated to be 185.8 g kg^{-1} in the diets. The DC contents in this study ranged from 100 to 399 g kg^{-1} and are inversely proportional to DP contents. From Fig. 1, the digestible carbohydrate levels compared to isoenergetic diets negatively correlated with feed and protein intake, which explains the reduction in growth performance and protein retention. However, an important caveat should be noted when discussing the potential adverse effects of DC in this study since the observed reduction in feed intake amount and growth could also be equally explained by the reduced levels of DP in the diet. Nonetheless, DC levels above 185.8 g kg^{-1} in the diets might affect growth performance in juvenile Malabar snapper and further studies are required to validate this acceptance level.

Digestibility and utilisation of carbohydrates are highly variable in fish, especially carnivorous fish, and influenced by numerous factors, including origin or type, dietary inclusion level, changes in farming conditions (i.e., salinity), and technological processes of the starch or feed (Krogdahl et al., 2004; Hua and Bureau, 2009; Kamalam et al., 2017). In this study, the experimental diets were extruded, and the apparent digestibility of dietary carbohydrates ranged from 63.5 % in P540High to 87.8 % in P360Low. This indicated that juvenile Malabar snapper could digest carbohydrates, which is similar to other carnivores such as barramundi (Booth and Pirozzi, 2021), brown-marbled grouper (*E. fuscoguttatus*) (Mamauag et al., 2019) and rainbow trout (*Oncorhynchus mykiss*) (Krogdahl et al., 2005a, 2005b). Although carbohydrates (i.e., starch) can be digested, fish such as barramundi have reported limited ability to utilise starch for growth (Nankervis et al., 2000; Palma et al., 2020), and higher digestible starch contents in the diets were associated with an increased feed conversion ratio (Glencross et al., 2014) and reduced protein utilisation efficiency (Glencross et al., 2017). A recent study from our lab evaluated the energy utilisation efficiencies of digestible nutrients and observed that Malabar snapper is similarly unable or has a limited capacity to metabolise carbohydrates efficiently for growth (Ngoh et al., 2024). However, a separate study on spotted rose snapper demonstrated that gelatinising or pre-cooking the starch could increase starch inclusion in diets and improve carbohydrate utilisation without affecting feed intake (Benitez-Hernández et al., 2022). Therefore, the low digestible carbohydrate acceptance level might apply only to the extrusion processing conditions used in this study. Additional studies are also warranted to determine the acceptable dietary carbohydrate inclusion level after the utilisation efficiency of digestible carbohydrates in Malabar snapper has been optimised.

Carbohydrate contents in diet and faecal material are typically calculated by nutrient difference because of the difficulties in analysing non-starch polysaccharides (Saunders and Hautala, 1979). Calculated carbohydrate values can lead to erroneous results due to experimental errors arising from the analytical tests of other macronutrients. Therefore, the results on carbohydrates (i.e., carbohydrate content, digestibility and acceptable carbohydrate level) should be interpreted with discretion (Krogdahl et al., 2005a, 2005b). Moreover, the gross energy values in this study were not determined by a bomb calorimeter but calculated using the energy values of protein, fat and carbohydrate

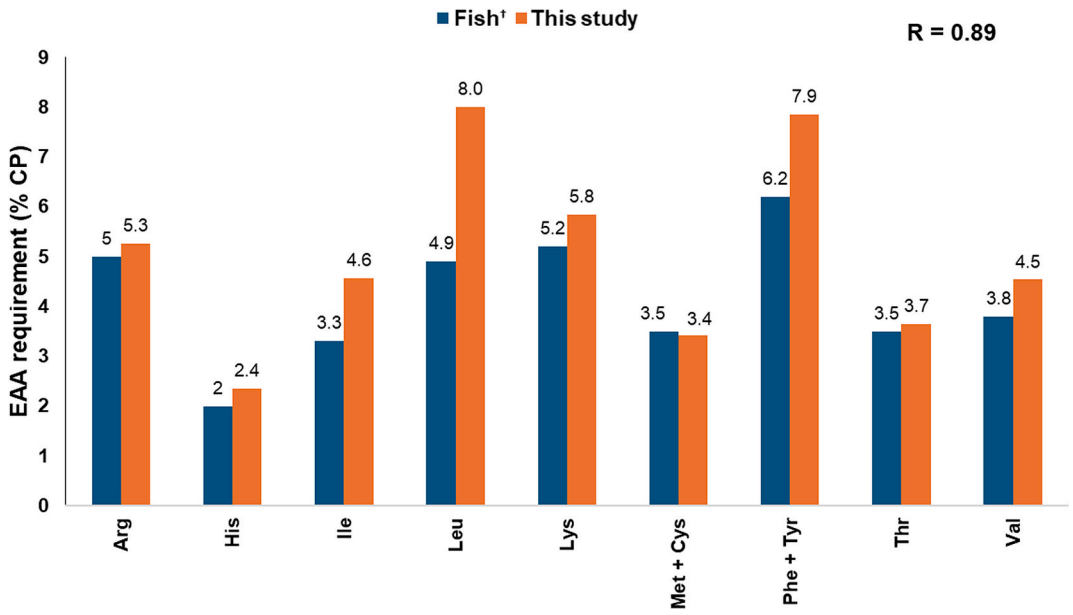

Fig. 4. Essential amino acid requirements (% CP) of average and Juvenile Malabar snapper. †Data were adapted from Xing et al. (2024).CP: Crude protein; EAA: Essential amino acid; Arg: arginine; His: histidine; Ile: isoleucine; Leu: leucine; Lys: lysine; Met + Cys: methionine + cysteine; Phe + Tyr: phenylalanine + tyrosine; Thr: threonine; Val: valine.

(Blaxter, 1989). The data associated with energy, such as the estimated requirements of DP/DE for optimal growth and protein deposition arising from this study, must also be interpreted with care. The carbohydrate and energy values should be used based on the relative association and trend of the data rather than actual values, and further studies using actual analytical values of carbohydrate and energy (i.e., via a bomb calorimeter) should be conducted to validate the results of this study.

4.4. *No noticeable protein-sparing effect below optimal digestible protein requirement in Malabar snapper*

Previous studies have reported that adequate dietary non-protein energy intake could spare dietary protein as an energy source in a variety of aquaculture species, such as barramundi (Nankervis et al., 2000), Atlantic salmon (*Salmo salar*) (Grisdale-Helland and Helland, 1997), Atlantic cod (*Gadus morhua*) (Morais et al., 2001), brown-marbled grouper (Shapawi et al., 2014), and rainbow trout (Segler et al., 2023). However, excessive dietary fat has also been reported to reduce feed intake, increase body fat deposition (Lin and Shiau, 2003; Shapawi et al., 2014; Yong et al., 2015), and even negatively affect growth performance for some species, such as greasy grouper (*E. malabaricus*) (Lin and Shiau, 2003) and common carp (*Cyprinus carpio*) (Fan et al., 2021). In our study, digestible fat contents in the diets only negatively correlated with feed intake and DPI when dietary protein levels were below the recommended level and when dietary energy content was low (Fig. 1). Dietary fat content was observed to be the primary reason for fat accumulation in the body as WBC fat correlated strongly with dietary fat ($R^2 = 0.89$), but not dietary carbohydrate ($R^2 = 0.18$). Nevertheless, since the body moisture content is often proportional to body fat content (Zhang et al., 2014; Ali et al., 2020), the increment in WBC fat in this study did not contribute to somatic growth and no apparent protein-sparing effects from dietary fats were observed.

The presence of protein-sparing effects from non-protein energy sources could be determined by comparing a series of isonitrogenous diets with varying digestible energy levels, where protein efficiency ratio (PER) or PRE are used as indicators. When the protein-sparing effect is present, fish would exhibit higher PRE values after consuming diets with higher energy content (i.e., lower DP/DE ratio) and the dietary protein consumed would be 'spared' from being converted into fuel for energy and instead used for protein synthesis. After consuming diets with reduced DP/DE, higher PER and PRE values have been reported for snappers (Abbas et al., 2011; Abbas and Siddiqui, 2013) and hybrid grouper (Jiang et al., 2016). However, the diets investigated in those studies differed in dietary protein content, and concluding the presence of protein-sparing activity would be challenging since inadequate or excessive protein intake levels might also affect PRE. This is because when fish are fed protein levels below optimum requirements, more feed may be consumed (before the energy requirement is met) to satisfy the protein level required for growth and metabolism, which lowers PRE. At excess and optimum protein levels, protein gain would be maximised and remain constant, such that any excess feed or protein intake would also reduce PRE. The present study investigated a range of DP/DE ratios from 15.1 to 27.2 g MJ^{-1} at four protein and three energy levels. The isonitrogenous diets containing different energy levels did not influence the PRE values. On the other hand, TFI, DPI, and DP/DE ratios correlated positively with PRE in isoenergetic diets containing different protein levels, in which higher protein intake increased PRE values (Fig. 1). Furthermore, based on the coefficient value of DPI derived from linear regression analyses, the utilisation efficiency of digestible protein for protein deposition decreased significantly when High-energy diets were consumed instead of Low-energy diets (Fig. 3). These results collectively indicated an absence of protein-sparing action from non-protein energy sources in juvenile Malabar snapper for the current range of dietary protein levels investigated, and increasing energy contribution from lipids or carbohydrates can reduce protein utilisation efficiency. The estimated DP/DE ratio requirement based on protein gain was 25.32 g MJ^{-1}. However, a linear relationship was observed between protein deposition and digestible protein level, such that the highest protein deposition was observed with the highest DP level. Hence, further validation using higher levels of digestible protein above P540 is likely required to demonstrate any potential protein-sparing effect from non-protein energy sources.

5. Conclusion

Juvenile Malabar snapper exhibits a high protein demand for growth. The digestible protein requirement for optimal growth was

estimated at 479.1 g kg^{-1} with a DP/DE ratio of 25.78 g MJ^{-1}. The estimated DP/DE ratio requirement for optimal protein deposition was 25.32 g MJ^{-1}. However, the DP requirement for optimal protein deposition could not be estimated. Above the acceptance level of digestible carbohydrates of 185.8 g kg^{-1}, dietary carbohydrate negatively affects fish growth performance and protein retention. In addition, no apparent protein-sparing effects from non-protein energy sources were observed in the range of DP levels investigated. In the absence of nutritional information for this species, an indirect approach was proposed to provide a crude estimate of amino acid requirements in conjunction with the estimation of dietary protein requirements. This indirect method is useful for suggesting EAA levels to be included in an actual dose-response trial. The nutritional requirements established in this study offer a valuable initial step toward developing species-specific diets for Juvenile Malabar snapper, which will enhance its aquaculture productivity. However, since the dietary carbohydrate levels were inversely proportional to the dietary protein contents in the diets, further studies using isonitrogenous diets and protein levels above requirement are warranted to determine the effects of carbohydrates and verify any potential protein-sparing action in Malabar snapper. Also, given that this study's carbohydrate and energy data were calculated instead of analysed, the results pertained to carbohydrate and energy warrant validation using analysed values.

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Ethical approval

All procedures related to the handling of fish were conducted in compliance with the guidelines using animals set by the National Advisory Committee on Laboratory Animal Research (NACLAR) for the care and use of animals for scientific purposes in Singapore and were approved by James Cook University Singapore's Institutional Animal Care and Use Committee under ethics approval number: 2021-A013.

CRediT authorship contribution statement

Si Yan Ngoh: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Xueyan Shen:** Writing – review & editing, Funding acquisition. **Tai Lok Chan:** Writing – review & editing, Investigation. **Leo Nankervis:** Writing – review & editing. **Katheline Hua:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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