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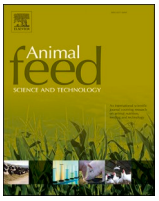

Dietary methionine and choline mediate mobilisation of plasma lipids to improve feed efficiency for the diets of giant grouper (*Epinephelus lanceolatus*)

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ABSTRACT

Giant grouper (*Epinephelus lanceolatus*) has become an increasingly popular candidate for aquaculture due to its high growth rates, disease resistance, and high production value. Despite these positive attributes its potential as an aquaculture species is limited by extensive knowledge gaps in its specific nutrient requirements needed for appropriate feed formulation. Methionine, an essential nutrient in feed ingredients, plays an important role in fish nutrition. Its complex metabolism suggests that its requirement may be influenced by other nutrients, such as the vitamin-like compound choline. This study aimed to investigate the interaction between dietary methionine and choline in regulating plasma lipid mobilisation, and how this affects feed efficiency in juvenile giant grouper. Two series of diets were tested: one with a constant methionine and varying choline levels, and another with a fixed choline (2.71 mg/g) and increasing levels of methionine. Both methionine and choline affected FCR, which appears to have been mediated by the utilisation of energy substrates, with shifts in circulating low-density lipoproteins (LDL), and triglycerides (TAG). These results appear to be related to energy derived from improved mobilisation of lipids to growing tissue, resulting in improved feed efficiency with increasing dietary methionine and choline. From this investigation, it is recommended that juvenile *E. lanceolatus* diets are supplemented with both dietary methionine and choline no less than 12.7 mg/g and 4.3 mg/g, respectively. Given that these results demonstrate that choline and methionine levels alter lipid mobilisation, they raise the potential to capture the growth and efficiency effects of higher lipid levels than previously found optimal for this species.

1. Introduction

Giant grouper (*Epinephelus lanceolatus*) has become an increasingly popular candidate for aquaculture due to its rapid growth rate, disease resistance and high production value (Dennis, 2020; Dennis et al., 2020). Despite its favourable attributes, aquaculture of this species is still in its relative infancy. Consequently, there is little species-specific nutritional research available to inform effective feed formulation.

Formulated aquafeeds allows diet optimisation to maximise growth and feed performance (Cooney et al., 2021) provided that key nutrients and nutrient interactions are understood. The most critical nutrient requirements to define are those that are in poor supply in

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common raw materials relative to their likely requirement values.

Methionine has been identified as a key limiting nutrient for giant grouper feeds, with requirement values recently elucidated (Candebat et al., 2023). In addition to its role in forming proteins, methionine is involved in various transmethylation pathways responsible for hepatic health and lipotropic function (Chu et al., 2014; Liao et al., 2014; Nunes et al., 2014; Espe et al., 2016; Zhou et al., 2016; Chen et al., 2023). It is also involved in carnitine synthesis, which is essential for β-oxidation of fatty acids, linking methionine to lipid metabolism and energy production (Shekhawat et al., 2013; Espe et al., 2023; Wang et al., 2023). Methionine insufficiency can cause liver dysfunction, characterised by increased liver size (hepatosomatic index) and decreased availability of dietary lipids for growth Irm et al., 2021). Methionine's involvement in the mobilisation of lipids from the liver is supported by a variety of other compounds, including the vitamin-like nutrient choline (Espe et al., 2016).

Choline is a component of phospholipids that are crucial for cell membrane formation and structure but also as well as for the emulsification and transportation of lipids through the circulatory system (Corbin and Zeisel, 2012; Chandler and White, 2017). Choline also regenerates methionine through its methyl-donor function, via betaine, and their requirements are therefore expected to be interrelated. Supplementation of choline to help regenerate methionine and improve lipid utilization has been suggested in species including yellow tail kingfish (*Seriola lalandi*) (Liu et al., 2021), seabream (*Sparidentex hasta)* (Kumar et al., 2012), and Atlantic salmon (*Salmo salar*) (Espe et al., 2016). Given that methionine and choline have separate yet interrelated roles, it remains unclear whether choline supplementation will affect the methionine requirement or whether choline addition will provide additional growth and physiological benefits. Additionally, previous studies have shown that dietary methionine and choline in feed formulations can improve growth indices, including feed efficiency (Geng et al., 2023). However, it remains unclear whether this relationship holds true

Table 1
Formulation and composition of raw materials in experimental diets, including increasing methionine and choline levels, crude protein and lipid contents, ash, and moisture content.

Ingredient	Experimental Diets							
	Diet 1 (7.10 mg/g Met, 2.87 mg/g Cho)	Diet 2 (9.30 mg/g Met, 2.72 mg/g Cho)	Diet 3 (10.90 mg/g Met, 2.68 mg/g Cho)	Diet 4 (12.3 mg/g Met, 1.36 mg/g Cho)	Diet 5 (12.5 mg/g Met, 2.05 mg/g Cho)	Diet 6 (12.5 mg/g Met, 2.60 mg/g Cho)	Diet 7 (12.4 mg/g Met, 3.62 mg/g Cho)	Diet 8 (12.7 mg/g Met, 4.3 mg/g Cho)
Fish meal, 65% CP[a]	20	20	20	20	20	20	20	20
Wheat gluten meal, 70% CP[g]	15	15	15	15	15	15	15	15
Lupin seed meal, 39.5% CP[a]	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Soy Protein Concentrate[a]	22	22	22	22	22	22	22	22
Blood meal[a]	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Canola oil[d]	3.33	3.33	3.33	3.33	3.33	3.33	3.33	3.33
Fish oil[a]	3	3	3	3	3	3	3	3
Wheat (whole)[b]	19.74	19.74	19.74	19.98	19.86	19.74	19.51	19.28
Vitamin Premix[a,e]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
L-Lysine[a]	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67
DL-Methionine[c]	0	0.20	0.40	0.60	0.60	0.60	0.60	0.60
Choline chloride[h]	0.23	0.23	0.23	0	0.11	0.23	0.46	0.69
L-glycine[a]	0.7	0.49	0.29	0.09	0.09	0.09	0.09	0.09
Mineral Premix[a,f]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Vitamin E-50[i]	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Yttrium oxide[j]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Proximate Composition								
Crude Protein	51.25	51.87	52.50	51.25	53.75	53.12	53.12	53.75
Crude Lipid	10.72	10.13	11.40	10.80	10.34	10.69	10.71	11.34
Ash	5.7	5.9	5.8	5.7	5.9	5.9	5.6	6
Moisture Content	9.66	6.50	6.60	8.96	7.15	8.05	8.97	5.89

[a] Skretting, Australia, Cambridge Tasmania.
[b] Feed 2 Go, Townsville, Australia.
[c] Evonik, Essen, Germany.
[d] Gold Sunset, Davies Collison Cave Pty Ltd, NSW, Australia.
[e] Composition (g/kg unless otherwise stated): Biotin, 1; Folic Acid,5; Niacin, 45; Pantothenic acid, 10; Pyridoxine, 10; Riboflavin, 20; Thiamin,10; Vitamin B12, 0.05; Vitamin C, 150; Vitamin A, 3000 IU/g; Vitamin D, 2400IU/kg; Vitamin K (menadione), 10; Inositol, 250; Antioxidant, 15.
[f] Composition (g/kg): Magnesium, 59.4; Copper, 1; Iron,8; Manganese, 5; Selenium, 0.02; Zinc, 20; Iodine, 0.8; Cobalt, 0.1; Ash, 700;Moisture, 20.
[g] Manildra, Gladesvale, NSW, Australia.
[h] JujiaBiotechnology, China
[i] RovimixE-50, DSM, Heerlen, Netherlands.
[j] Sigma-Aldrich,Burlington, USA.

for *E. lanceolatus* and whether it offers similar benefits for feed efficiency and growth.

Therefore, the aim of this study is to evaluate the interaction between dietary methionine and choline in regulating plasma lipid mobilisation to enhance feed efficiency in *E. lanceolatus*.

2. Material and methods

2.1. Fish collection and experimental design

Juvenile *E. lanceolatus* were obtained from the Company One hatchery in Cairns, Queensland. Prior to the experiment, the fish were on-grown in a 2000 L holding tank for five weeks and fed a commercial marine fish feed (Nova FF, Skretting, Cambridge, Tasmania).

The experiment was conducted using a recirculating aquaculture system (RAS) comprising twenty-four 500 L tanks, with water quality parameters maintained at optimal levels: flow rate of 800–900 L/h, temperature at 27 ± 1°C, salinity at 29.9 ± 1.2 ppt, dissolved oxygen > 6 mg/L, total ammonia nitrogen < 0.5 ppm, nitrite nitrogen < 0.5 ppm, and pH at 8 ± 0.5. Before stocking, feed was withheld for 24 hours. Fish (60.15 ± 0.84 g) were anesthetized using Aqui-S (20 mg/L), individually weighed, measured, and randomly assigned to tanks at a stocking density of 20 fish per tank.

Diets were randomly allocated to triplicate tanks and hand-fed to slight excess of satiation daily for 45 days. Uneaten feed was collected using a swirl separator, dried at 100°C until it reached a constant weight, and used in feed intake calculations.

2.2. Feed formulation and manufacture

A dose-response design was applied, using eight isonitrogenous (52.57 ± 0.36 %), isolipidic (10.76 ± 0.26 %), and isoenergetic (21.96 ± 0.07 MJ/kg) diets to assess the effects of varying methionine and choline levels (Table 1). Diets 1–3 and Diet 6 contained 2.71 ± 0.05 mg/g of choline and 7.1, 9.3, 10.9, and 12.5 mg/g methionine, respectively, while Diets 4–8 contained 12.48 ± 0.06 mg/g methionine and 1.3, 2, 2.6, 3.6, and 4.3 mg/g of choline, respectively. The formulation of the dietary treatments was aligned with known grouper nutritional requirements (Williams, 2009; Yeh et al., 2015; Yong et al., 2020; Lin et al., 2022; Nankervis et al., 2022). Methionine replaced glycine on an isonitrogenous basis, while choline was provided as choline chloride (70 %), replacing wheat in the formulation.

2.2.1. Feed processing

All dry ingredients were milled using an SR 300 Rotor Beater Mill (Retsch, Haan, Germany) with a 750 μm screen. Ingredients were blended in an A200 planetary mixer (Hobart, Troy, Ohio, USA) for 10 minutes before adding approximately 25 % water by mass. The mixture was extruded into 3 mm pellets using a 35 mm single-screw extruder (Telford Smith Engineering, Dandenong, Victoria, Australia) at three-barrel temperature zones (100°C, 110°C, 120°C), achieving a final melt temperature of ~120°C. Pellets were dried in a TD-700F Thermoline Scientific dehydrating oven at 60°C to a moisture content of 7.72 ± 0.48 %. Dried pellets were oil-coated in a cement mixer for even absorption and stored at −18°C until use.

2.2.2. Nutrient analysis

Feed and faecal samples from each diet were analysed for total choline content at Macquarie University using the methods of Liu et al. (2021). Methionine content was determined through amino acid analysis, which involved hydrolysis in 6 M hydrochloric acid at 110°C for 24 hours, following the procedure of Cohen and Michaud (1993). Ash content was measured gravimetrically after combustion in a muffle furnace at 500°C overnight, while yttrium content was quantified using inductively coupled plasma mass spectrometry (ICP-MS). Nitrogen content was assessed using a Costech elemental analyser (Costech Analytical Technologies, Valencia, USA) and converted to protein content with a 6.25 multiplier. Total lipid content was analysed externally at Symbio Laboratories, Brisbane, using the Soxhlet method (Willson et al., 2010).

2.3. Response variable

To minimize postprandial effects, the final feeding before sampling was scheduled 19–21 hours prior to sampling. Fish were euthanized using Aqui-S (400 mg/L), weighed to the nearest 0.01 g, and measured to the nearest millimetre (mm).

2.3.1. Growth and feeding response

The following growth and feeding parameters were calculated:

$$\text{Survival Rate } (SR;\ \%) = \left\{ \frac{\text{No. individuals at the end of the experiment}}{\text{No. of individuals at the start of the experiment}} \right\} \times 100$$

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{Total Feed Consumed (g)}}{\text{Total Weight Gained (g)}}$$

$$\text{Specific Growth Rate } (SGR;\ \%) = \left\{ \frac{\text{In Final Weight (g)} - \text{In Initial Weight (g)}}{\text{Experimental Period (days)}} \right\} \times 100$$

$$\text{Hepatosomatic Index } (HSI;\ \%) = \left\{ \frac{\text{Liver Weight (g)}}{\text{Body Weight (g)}} \right\} \times 100$$

$$\text{Feed intake per fish } (\text{g} \Big/ \text{fish}) = \frac{\text{Total feed consumed (g)}}{\text{Number of fish}}$$

$$\text{Feed Intake as \% Body Weight} \Big/ \text{day (FI \% BW d}^{-1}) = \left\{ \frac{\text{(Feed Intake (g)/(Final Weight (g) + Initial Weight (g)) / 2)}}{\text{Experimental Period (days)}} \right\} \times 100$$

$$\text{Condition Factor (K)} = \frac{(100 \times \text{Weight (g)})}{\text{Length (cm)}^3}$$

$$\text{Apparent Digestibility Coefficients (ADC) \%} = (1 - ((\frac{\%\ nutrient\ in\ faeces}{\%\ nutrient\ in\ diet}) \times (\frac{\%\ marker\ in\ diet}{\%\ marker\ in\ faeces}))) \times 100$$

$$\text{Digestible nutrient Intake (g/day/kg BW)} = \textit{Daily nutrient intake (g/day)} * \textit{nutrient ADC(\%) /geom. body weight (kg)}$$

2.3.2. Plasma biochemistry and liver proximate composition

For plasma biochemistry, blood was collected from the caudal vein of six randomly selected fish per tank using syringes preflushed with 10 % EDTA solution. Plasma was separated via centrifugation and stored at –18°C until analysis.

Regarding liver proximate composition, fish livers were dissected, removed, and weighed to the nearest 0.01 g. For proximate analysis, ten livers per tank were pooled, stored at −18°C, and transferred to –80°C for 24 hours before freeze-drying. Lipid content analysis was performed by Symbio Laboratories, Brisbane, using the Soxhlet method (Willson et al., 2010).

Faecal matter was stripped from the hindgut of all fish, pooled per tank, and stored at –18°C, then transferred to –80°C for 24 hours before freeze-drying. Yttrium content in the freeze-dried faecal samples was analysed using ICP-MS.

2.4. Statistical analysis

Data were analysed separately for each dataset to assess differences between diets with increasing choline and sufficient methionine, or with increasing methionine and constant choline supplementation.

Normality and homogeneity of variance were checked using Shapiro Wilks and Levene's test respectively prior to analysis. Differences between diets were then determined using one-way ANOVA, followed by a Tukey post hoc test when significant differences were observed. If the data did not meet the assumptions for ANOVA, a Kruskal-Wallis test was performed, followed by a Wilcoxon Signed Rank Test where significant differences occurred. Data exploration and statistical analysis were performed using the statistical software R and RStudio desktop version 2022.07.0 + 548.

3. Results

3.1. Growth and feeding response

The experiment achieved a 100 % SR across all treatments. Dietary methionine and choline levels did not affect weight gain (WG), specific growth rate (SGR) or the hepatosomatic index (HSI) in this study ($P > 0.05$, Table 2). Feed intake (g/fish, and % body weight

Table 2
Biometric performance of juvenile giant grouper (*E. lanceolatus*) from diets containing increasing levels of methionine and a constant choline level. Data are presented as mean ± standard error. Different superscript letters within the same row indicate significant differences ($P < 0.05$) based on one-way ANOVA and Tukey's post hoc test.

Parameter	Diet 1 (7.10 mg/g Met, 2.87 mg/g Cho)	Diet 2 (9.30 mg/g Met, 2.72 mg/g Cho)	Diet 3 (10.90 mg/g Met, 2.68 mg/g Cho)	Diet 6 (12.50 mg/g Met, 2.60 mg/g Cho)
Initial weight (g)	60.35± 0.66	59.69±0.76	59.83±0.30	60±0.64
Initial length (mm)	149.7± 0.68	149.92±0.32	149.84±0.72	148.62±0.82
Final weight (g)	190.7± 5.49	192.94±3.14	188.21±2.24	186.85±4.56
Final length (mm)	200.9± 1.82	204.15±0.69	201.2±1.81	200.23±1.74
Weight gain (%)	130.34± 4.97	133.25±2.82	133.25±2.82	126.85±4.23
Specific growth rate	2.55± 0.04	2.61±0.03	2.53±0.03	2.51±0.05
Feed intake (g/fish)	106.09±2.87^{ab}	109.61±2.55^{a}	99.51 ± 2.75^{b}	96.57 ± 2.40^{b}
Feed intake (% body weight/day)	1.88± 0.05^{ab}	1.93±0.02^{a}	1.78±0.02^{b}	1.74±0.02^{b}
Feed conversion ratio	0.81± 0.02	0.82±0.00	0.78±0.01	0.76±0.02
Condition factor (K)	2.35± 0.06	2.26±0.03	2.3±0.03	2.32±0.03
Hepatosomatic index (HSI)	5.02± 0.20	5.03±0.12	4.98±0.04	4.98±0.11

per day) decreased with increasing doses of methionine and choline ($P < 0.05$, Tables 2 and 3). An increase in choline decreased relative feed intake by 10.11 % between the highest 4.30 m/g, Diet 8) and lowest (1.36 mg/g, Diet 4) dietary choline levels. Similarly, the highest methionine dose (12.50 mg/g, Diet 6) resulted in a 7.45 % drop in relative feed intake compared to the lowest methionine dose (7.10 mg/g, Diet 1). An increase in dietary choline resulted in the lowest FCR of 0.74, with significant differences between the highest (4.30 mg/g, Diet 8) and lowest (1.36 mg/g, Diet 4) dietary choline levels. Increasing choline to 4.30 mg/g decreased FCR by 12.95 % (Table 3). Increase dietary methionine and resulted in a decrease in FCR from 0.81 ± 0.02–0.76 ± 0.02 and a *P*-value approaching significance ($P = 0.05$). The Apparent Digestibility Coefficients (ADC) showed that dietary methionine had a digestibility of approximately 95 %, while choline was nearly 100 % digestible (Table 4). Similarly, Digestible Nutrient Intake (DNI) of methionine and choline varied among treatments, with higher dietary levels leading to greater absorption and utilization of these nutrients (Table 5).

3.2. Plasma biochemistry and liver proximate composition

The liver lipid content ranged from 14.27 ± 0.52–17.07 ± 2.07 % between treatment means and was not significantly affected by dietary methionine or choline content ($P > 0.05$) (Table 6).

From the plasma biochemistry data, only alanine transaminase (ALT), low-density lipoprotein (LDL), from the choline diet series met the assumptions for ANOVA. The increase in methionine did not affect ALT and although LDL produced a significant *P* value, there was no consistent trend in the data (Table 7). The remaining analytes were subjected to a Kruskal Wallis test, which revealed significant differences in glucose and triglyceride (TAG) levels between diets (Table 7). Similarly, a *P*- value < 0.05 was produced from the glucose and TAG data, however, no clear trend was observed either result.

All plasma biochemical results from the diets with increasing levels of choline were subjected to a Kruskal-Wallis test (Table 8). Only Glucose produced a significant *P*-value ($P < 0.05$); however, there was no noticeable discernible trend in the response (Table 8).

4. Discussion

The interrelationship between methionine and choline and their influence on regulating plasma lipid mobilisation to improve feed efficiency and growth for *E. lanceolatus* was investigated in this study. Increased levels of both methionine and choline resulted in an increase in circulating substrates that indicate higher energy availability for metabolism, which improved the feed efficiency of the fish in this study. The previous methionine recommendation for this species was ~15 mg/g (Candebat et al., 2023) for optimal growth and feed performance; however, this study resulted in further improved feed efficiency with the addition of choline at 4.30 mg/g at this methionine recommendation. The highest level of methionine and choline tested support the most efficient production.

In a satiety-feeding scenario, such as this study, fish have the ability to control their feed intake; this study suggests that the fish consumed greater amounts of marginally deficient diets to compensate for insufficiencies in methionine or choline. This significantly reduced feed intake with unaffected growth, resulting in improved feed efficiency as represented by lower FCR. Increasing methionine to 12.7 mg/g and choline to 4.3 mg/g decreased FCR by 7.3 %, highlighting how increased methionine and choline can improve the utilisation of metabolic pathways involved in the breakdown of diets for energy, consistent with results previously reported for Nile tilapia (Nugroho et al., 2020) and channel catfish; (Wu and Davis, 2005).

In the present study, an increase in methionine to 12.7 mg/g and choline at 4.30 mg/g resulted in increased successful transportation of TAG into the plasma, potentially contributing to the improved feed-growth response. This increase in TAG also suggests that methionine and choline supplementation allows for more efficient metabolism and use of dietary lipids in *E. lanceolatus* diets.

While hepatic health was a consideration in this investigation due to concerns over the potential adverse effects of poorly formulated feeds (Nankervis et al., 2022), the present study did not directly assess liver health or steatosis. Although hepatic steatosis

Table 3
Biometric performance of juvenile giant grouper (*E. lanceolatus*) fed diets containing increasing levels of choline and a constant methionine level. Data are presented as mean ± standard error. Different superscript letters within the same row indicate significant differences ($P < 0.05$) based on one-way ANOVA and Tukey's post hoc test.

Parameter	Diet 4 (12.3 mg/g Met, 1.36 mg/g Cho)	Diet 5 (12.5 mg/g Met, 2.05 mg/g Cho)	Diet 6 (12.5 mg/g Met, 2.60 mg/g Cho)	Diet 7 (12.4 mg/g Met, 3.62 mg/g Cho)	Diet 8 (12.7 mg/g Met, 4.3 mg/g Cho)
Initial weight (g)	60.3±0.21	60.51±0.84	60±0.64	59.64±0.71	59.88±0.59
Initial length (mm)	122.06±30.73	150.55±0.76	148.62±0.82	149.42±1.02	149.55±0.53
Final weight (g)	190.35±4.13	186.53±2.27	186.85±4.56	184.52±3.81	188.92±7.43
Final length (mm)	203.93±1.17	199.01±0.76	200.23±1.74	200.37±1.73	201.83±2.50
Weight gain (%)	130.05±4.33	126.02±1.90	126.85±4.2	124.87±3.28	129.04±7.39
Specific growth rate	2.54±0.05	2.49±0.03	2.51±0.05	2.5±0.03	2.54±0.09
Feed intake (g/fish)	105.95±2.88[a]	96.22 ± 2.04[b]	96.57±2.40[b]	95.41 ± 1.85[b]	94.98±2.21[b]
Feed intake (% body weight/day)	1.88±0.03[a]	1.73±0.01[b]	1.74±0.02[b]	1.74±0.03[b]	1.69±0.04[b]
Feed conversion ratio	0.82±0.01[a]	0.76±0.01[ab]	0.76±0.02[ab]	0.76±0.01[ab]	0.74±0.01[b]
Condition factor (K)	2.23±0.04	2.36±0.04	2.32±0.03	2.29±0.04	2.29±0.01
Hepatosomatic index	5.3±0.34	5.16±0.02	4.98±0.11	5.07±0.14	4.98±0.00

Table 4
Apparent Digestibility Coefficient (ADC) means of juvenile giant grouper (*E. lanceolatus*) from treatment groups with increasing dietary methionine and choline inclusion.

Amino acid	Diet 1 (7.10 mg/g Met, 2.87 mg/g Cho)	Diet 2 (9.30 mg/g Met, 2.72 mg/g Cho)	Diet 3 (10.90 mg/g Met, 2.68 mg/g Cho)	Diet 4 (12.3 mg/g Met, 1.36 mg/g Cho)	Diet 5 (12.5 mg/g Met, 2.05 mg/g Cho)	Diet 6 (12.5 mg/g Met, 2.60 mg/g Cho)	Diet 7 (12.4 mg/g Met, 3.62 mg/g Cho)	Diet 8 (12.7 mg/g Met, 4.3 mg/g Cho)
Methionine	92.57	95.60	94.80	96.10	93.53	93.10	95.06	95.01
Choline	99.47	99.30	99.33	98.83	99.14	99.33	99.63	99.61
Cysteine	86.11	87.86	86.39	87.70	82.12	80.56	86.26	84.73
Serine	92.40	93.63	92.11	92.82	90.39	89.19	92.59	91.70
Arginine	94.85	95.82	94.74	95.04	93.56	92.67	95.14	94.60
Glycine	91.98	93.08	90.82	91.19	87.80	86.55	90.74	89.59
Aspartic Acid	89.61	91.83	89.39	90.21	86.66	84.92	90.06	89.24
Glutamic Acid	96.10	96.99	96.20	96.57	94.93	94.59	96.32	96.08
Threonine	89.31	91.16	89.20	90.21	86.81	85.76	90.09	88.87
Alanine	91.10	92.68	90.62	91.41	88.28	87.27	91.34	90.63
Proline	94.94	95.85	94.82	95.38	93.35	92.73	95.05	94.48
Lysine	92.75	94.24	92.37	93.08	90.55	89.46	92.88	92.56
Tyrosine	92.81	94.25	92.96	93.45	91.12	90.48	93.18	92.46
Valine	90.66	92.26	90.10	90.75	87.50	86.28	90.92	89.93
Isoleucine	91.91	93.46	92.14	92.95	90.20	89.72	92.83	91.88
Leucine	92.54	93.70	91.94	92.52	90.03	88.93	92.62	92.05
Phenylalanine	93.12	94.14	92.75	93.21	91.17	90.18	93.43	92.90

has previously been linked to overproduction of TAG levels relative to oxidation (Alves-Bezerra and Cohen, 2018), no differences in liver fat deposition were found in this study. These results coincide with the common roles of methionine and choline and the fact that choline was supplemented in the methionine-deficient diets in this study. Therefore, choline supplementation appears to mask some of the impacts of methionine deficiency and provides more robust formulation criteria. Increasing methionine levels in these diets also tended to decrease plasma lactate dehydrogenase (LDH), an enzyme responsible for converting lactate to pyruvate and is a widely used indicator of a stress response (Peres et al., 2013, 2014). It has previously been suggested that a decrease in LDH was due to a decline in metabolic rate and glycolytic processes, as this enzyme catalyses the oxidation of pyruvate to lactate during glycolysis (Agrahari et al., 2007). The concurrent increase in TAG and LDL with the decrease in LDH in the present study suggests that sufficient methionine results in a shift from metabolising carbohydrates for energy into metabolising lipids for energy. These results support the idea that the supplementation of methionine can help maintain hepatic health and increase the utilisation of dietary lipids by grouper.

The high ADC values observed for methionine (95 %) and choline (100 %) suggest that both nutrients are highly digestible and efficiently utilized. This aligns with the observed improvements in feed efficiency, as higher dietary levels resulted in greater nutrient intake and utilization (Candebat et al., 2023). The increased DNI of methionine and choline across treatments further supports their role in enhancing energy metabolism. Their efficient digestion likely facilitated their metabolic functions, including lipid mobilisation and improved FCR. The near-complete digestibility of choline may explain its greater influence on feed efficiency compared to methionine, as higher dietary choline levels were associated with improved nutrient utilization and energy availability (Gao et al., 2016; Shi et al., 2020).

Despite improved feed performance and increased circulating energetic substrates due to methionine and choline supplementation, there was no response in the growth parameters measured in this study. An increase in feed efficiency, which was not reciprocated in growth, may have been due to the conservative levels of lipids (10.76 $\pm$ 0.26 %) that remained the same in each diet. Such conservative lipid levels are in line with reported recommendations for *E. lanceolatus* (Nankervis et al., 2022); however, the current results indicate that the inclusion of 12.7 mg/g of methionine and 4.30 mg/g of choline result in more efficiently utilise the dietary lipids, suggesting that future *E. lanceolatus* diets may be improved with higher levels of lipids, which in return if adequately metabolised, may promote growth (Craig and Gatlin III, 1997; Tseng and Hwang, 2008; Chandler and White, 2017). Increasing dietary lipid inclusion promotes a growth response in some fish species (Vergara et al., 1999; Gómez-Requeni et al., 2013); however, when lipids are in excess, it leads to poor liver health (Cai et al., 2017; Obeid and Herrmann, 2009; Espe et al., 2010; Hansen et al., 2020). Consequently, it is recommended that further research is conducted to determine the dietary lipid threshold for this species to improve feed and growth performance while maintaining hepatic health.

5. Conclusion

In summary, the optimal feed formulation for juvenile giant grouper, *E. lanceolatus*, as a result of this study, is 12.7 mg/g of methionine and 4.3 mg/g of choline, respectively. This formulation improved feed efficiency, by reducing feed intake and FCR, likely due to enhanced utilisation of energy substrates, as indicated by shifts in circulating LDH and TAG levels. It is important to note that dietary methionine was 95 % digestible, while choline was nearly 100 % digestible in this study. When applying these results to raw material matrices with lower digestibility, higher supplementation of methionine and choline may be necessary to compensate for nutrient losses. Further research is recommended to establish threshold levels for these compounds and to investigate the effects of

Table 5
Amino acid digestible intake (g/day/kg/BW) means of juvenile giant grouper (*E. lanceolatus*) from treatment groups with increasing dietary methionine and choline inclusion.

Amino acid	Diet 1 (7.10 mg/g Met, 2.87 mg/g Cho)		Diet 2 (9.30 mg/g Met, 2.72 mg/g Cho)		Diet 3 (10.90 mg/g Met, 2.68 mg/g Cho)		Diet 4 (12.3 mg/g Met, 1.36 mg/g Cho)		Diet 5 (12.5 mg/g Met, 2.05 mg/g Cho)		Diet 6 (12.5 mg/g Met, 2.60 mg/g Cho)		Diet 7 (12.4 mg/g Met, 3.62 mg/g Cho)		Diet 8 (12.7 mg/g Met, 4.3 mg/g Cho)	
Methionine	0.86	± 0.02	1.19	± 0.01	1.28	± 0.01	1.53	± 0.02	1.40	± 0.01	1.40	± 0.02	1.42	± 0.02	1.42	± 0.02
Choline	0.37	± 0.01	0.36	± 0.00	0.33	± 0.00	0.17	± 0.00	0.24	± 0.00	0.31	± 0.00	0.43	± 0.01	0.50	± 0.01
Cysteine	0.82	± 0.02	0.84	± 0.01	0.79	± 0.01	0.81	± 0.01	0.74	± 0.01	0.70	± 0.01	0.76	± 0.02	0.71	± 0.02
Histidine	1.69	± 0.04	1.82	± 0.02	1.66	± 0.01	1.69	± 0.03	1.55	± 0.01	1.50	± 0.02	1.59	± 0.03	1.59	± 0.03
Serine	2.68	± 0.07	2.86	± 0.03	2.65	± 0.02	2.70	± 0.04	2.49	± 0.02	2.42	± 0.03	2.51	± 0.04	2.50	± 0.03
Arginine	3.09	± 0.08	3.34	± 0.03	3.09	± 0.02	3.13	± 0.05	2.93	± 0.03	2.87	± 0.03	2.95	± 0.03	2.95	± 0.03
Glycine	3.33	± 0.08	3.31	± 0.03	2.81	± 0.02	2.64	± 0.04	2.40	± 0.02	2.35	± 0.03	2.47	± 0.05	2.45	± 0.05
Aspartic Acid	4.48	± 0.11	4.78	± 0.05	4.41	± 0.03	4.43	± 0.07	4.02	± 0.04	3.97	± 0.05	4.23	± 0.15	4.22	± 0.14
Glutamic Acid	12.82	± 0.32	13.53	± 0.14	12.61	± 0.09	12.74	± 0.20	11.77	± 0.11	11.69	± 0.14	11.99	± 0.03	12.03	± 0.02
Threonine	2.05	± 0.05	2.21	± 0.02	2.03	± 0.01	2.07	± 0.03	1.89	± 0.02	1.85	± 0.02	1.95	± 0.03	1.93	± 0.03
Alanine	2.75	± 0.07	2.94	± 0.03	2.70	± 0.02	2.75	± 0.04	2.49	± 0.02	2.47	± 0.03	2.59	± 0.05	2.58	± 0.04
Proline	4.07	± 0.10	4.32	± 0.04	4.04	± 0.03	4.13	± 0.06	3.76	± 0.04	3.72	± 0.04	3.84	± 0.04	3.81	± 0.04
Lysine	3.56	± 0.09	3.80	± 0.04	3.48	± 0.02	3.53	± 0.06	3.24	± 0.03	3.21	± 0.04	3.34	± 0.02	3.36	± 0.02
Tyrosine	1.50	± 0.04	1.67	± 0.02	1.54	± 0.01	1.54	± 0.02	1.44	± 0.01	1.42	± 0.02	1.45	± 0.03	1.44	± 0.03
Valine	2.78	± 0.07	2.99	± 0.03	2.74	± 0.02	2.79	± 0.04	2.52	± 0.02	2.48	± 0.03	2.64	± 0.03	2.62	± 0.03
Isoleucine	2.28	± 0.06	2.48	± 0.03	2.27	± 0.02	2.33	± 0.04	2.12	± 0.02	2.10	± 0.02	2.20	± 0.06	2.18	± 0.05
Leucine	4.81	± 0.12	5.14	± 0.05	4.73	± 0.03	4.83	± 0.08	4.41	± 0.04	4.34	± 0.05	4.55	± 0.04	4.53	± 0.03
Phenylalanine	2.95	± 0.07	3.15	± 0.03	2.92	± 0.02	2.97	± 0.05	2.74	± 0.03	2.69	± 0.03	2.80	± 0.01	2.79	± 0.01

Table 6
Liver lipid content of juvenile giant grouper (*E. lanceolatus*) from diets containing increasing methionine and choline levels.

	Diets containing Increasing Methionine Levels				
Parameter	**Diet 1 (7.10 mg/g Met, 2.87 mg/g Cho)**	**Diet 2 (9.30 mg/g Met, 2.72 mg/g Cho)**	**Diet 3 (10.90 mg/g Met, 2.68 mg/g Cho)**	**Diet 6 (12.50 mg/g Met, 2.60 mg/g Cho)**	
Liver lipid (%)	15.73 ± 0.50	15.80 ± 0.72	16.17 ± 0.38	15.57 ± 0.78	
	Diet containing Increasing Choline Levels				
Parameter	**Diet 4 (12.30 mg/g Met, 1.36 mg/g Cho)**	**Diet 5 (12.50 mg/g Met, 2.05 mg/g Cho)**	**Diet 6 (12.50 mg/g Met, 2.60 mg/g Cho)**	**Diet 7 (12.40 mg/g Met, 3.62 mg/g Cho)**	**Diet 8 (12.70 mg/g Met, 4.30 mg/g Cho)**
Liver lipid (%)	17.07 ± 2.07	14.37 ± 0.66	15.57 ± 0.78	14.27 ± 0.52	15.80± 0.25

Table 7
Plasma biochemistry analysis of juvenile giant grouper (E. lanceolatus) from diets containing increasing levels of methionine with choline at 2.71 ± 0.05 mg/g, data presented as mean diet values including ± standard error, significance (sig.) indicates by * when $P < 0.05$ and * * when $P < 0.01$. Statistical significance was determined using the Kruskal-Wallis test followed by the Wilcoxon Signed Rank test.

Parameter	**Diet 1 (7.1 mg/g Met, 2.87 mg/g Cho)**	**Diet 2 (9.3 mg/g Met, 2.72 mg/g Cho)**	**Diet 3 (10.9 mg/g Met, 2.68 mg/g Cho)**	**Diet 6 (12.5 mg/g Met, 2.6 mg/g Cho)**	**sig.**
Glucose (mmol/L)	4.36 ± 0.76	4.62 ± 0.37	5.41 ± 0.29	3.6 ± 0.56	*
ALT (U/L)	1606.98 ± 238.99	1671.11 ± 117.96	1474.7 ± 160.92	1903.88 ± 75.64	0.05
AST (U/L)	64.08 ± 7.67	32.68 ± 5.98	36.11 ± 8.80	41.78 ± 18.99	
Albumin (g/dL)	7.97 ± 0.41	7.52 ± 0.31	6.67 ± 0.44	7.61 ± 0.49	
Cholesterol (mmol/L)	1.74 ± 0.20	1.76 ± 0.19	1.85 ± 0.17	1.75 ± 0.04	
TAG (mmol/L)	0.73 ± 0.08	0.76 ± 0.01	0.71 ± 0.06	0.88 ± 0.06	*
HDL (mmol/L)	0.66 ± 0.09	0.69 ± 0.11	0.69 ± 0.07	0.58 ± 0.08	
LDL (mmol/L)	0.54 ± 0.03	0.53 ± 0.06	0.66 ± 0.06	0.61 ± 0.02	* *
LDH (U/L)	982.71 ± 126.46	529.33 ± 189.82	487.77 ± 169.43	327.39 ± 39.41	

TAG; triglycerides, ALT; alanine transaminase, AST; aspartate transaminase, HDL: high density lipoproteins, LDH: lactate dehydrogenase, LDL: low density lipoproteins

Table 8
Plasma biochemistry analysis of juvenile giant grouper (*E. lanceolatus*) from the diets containing increasing levels of choline with methionine at 12.48 ± 0.07 mg/g, data presented as mean diet values including ± standard error, significance (sig.) indicates by * when $P < 05$, based on a non-parametric Kruskal-Wallis test followed by the Wilcoxon Signed Rank test.

Parameter	**Diet 4 (12.3 mg/g Met, 1.36 mg/g Cho)**	**Diet 5 (12.5 mg/g Met, 2.05 mg/g Cho)**	**Diet 6 (12.5 mg/g Met, 2.6 mg/g Cho)**	**Diet 7 (12.4 mg/g Met, 3.62 mg/g Cho)**	**Diet 8 (12.7 mg/g Met, 4.3 mg/g Cho)**	**sig.**
Glucose (mmol/L)	4.95 ± 0.69	4.07 ± 0.57	3.6 ± 0.56	4.96 ± 0.10	4.59 ± 0.33	*
ALT (U/L)	1629.65 ± 74.17	1376.61 ± 165.29	1903.88 ± 75.64	1416.97 ± 72.41	1519.62 ± 146.71	
AST (U/L)	63.63 ± 24.45	66.11 ± 7.76	41.78 ± 18.99	67.72 ± 29.24	29.45 ± 4.46	
Albumin (g/dL)	7.78 ± 0.12	7.92 ± 0.39	7.61 ± 0.49	7.94 ± 0.65	7.05 ± 0.33	
Cholesterol (mmol/L)	1.84 ± 0.11	1.97 ± 0.11	1.75 ± 0.04	2.11 ± 0.08	1.92 ± 0.15	
TAG (mmol/L)	0.77 ± 0.04	0.97 ± 0.12	0.88 ± 0.06	0.87 ± 0.06	1.16 ± 0.21	
HDL (mmol/L)	0.62 ± 0.05	0.6 ± 0.06	0.58 ± 0.08	0.72 ± 0.04	0.74 ± 0.12	
LDL (mmol/L)	0.52 ± 0.04	0.65 ± 0.07	0.61 ± 0.02	0.78 ± 0.07	0.58 ± 0.05	
LDH (U/L)	545.59 ± 234.77	564.72 ± 118.41	327.39 ± 39.41	538.29 ± 131.68	430.29 ± 97.57	

TAG; triglycerides, ALT; alanine transaminase, AST; aspartate transaminase, HDL: high density lipoproteins, LDH: lactate dehydrogenase, LDL: low density lipoproteins

increasing lipid levels on the improved feed formulation.

Ethics statement

This study was conducted with approval from the James Cook University Animal Ethics Committee (A2713).

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CRediT authorship contribution statement

Butler Grace: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Candebat Caroline:** Supervision, Project administration, Investigation. **Das Simon Kumar:** Writing – review & editing, Data curation. **Nankervis Leo:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Leo Nankervis reports financial support was provided by Australian Centre for International Agricultural Research. Reports a relationship with that includes:. Has patent pending to. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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