

Optimal Dietary Protein and Lipid Levels for Juvenile Yellowstripe Goby (*Mugilogobius Chulae*), a Proposed Laboratory Fish

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Abstract: *Mugilogobius chulae* is a small euryhaline or estuarine goby recently proposed as an ideal species for use in ocean environmental toxicology studies. However, the nutritional requirements of *M. chulae* remain unknown. Eight experimental diets at one of four protein levels (35%, 40%, 45%, or 50%) and two lipid levels (5% or 10%) were formulated to investigate the effects of differing dietary protein and lipid levels on the growth performance, body composition and liver morphological condition of *M. chulae*. Juvenile gobies (1760 individuals, 0.011 ± 0.001 g fish⁻¹) were randomly allotted to 32 tanks (four replicates of each of the eight treatments) and fed twice a day for 9 weeks. Weight gain (*WG*) and specific growth rate (*SGR*) increased significantly with increasing dietary protein from 35% to 45% ($P < 0.05$), and further increases, from 45% to 50%, caused a decline in both of these values. However, *WG* and *SGR* were not affected by dietary lipid concentration ($P > 0.05$). Whole-body crude lipid and linoleic acid (C18:2n-6) contents in juvenile *M. chulae* increased significantly with increasing dietary lipid content ($P < 0.05$). Although hepatosomatic index (*HSI*) and viscerosomatic index (*VSI*) did not increased significantly with increasing dietary lipid content ($P > 0.05$), and no pathological changes were observed in the liver, lipase activity significantly decreased ($P < 0.05$). The dietary protein level driving optimal *WG* and *SGR* also led to the highest trypsin activity. Thus, 45% crude protein and 5% lipid is sufficient to ensure good growth performance in juvenile *M. chulae*, and this diet appears to have no substantial adverse effects.

Keywords: *Mugilogobius chulae*, Nutrient Requirement, Optimal Growth, Body Composition, Morphometry, Laboratory Fish

1. Introduction

Mugilogobius chulae (Perciformes: Gobiidae; Smith, 1932) is a small groundfish distributed broadly throughout the coastal waters in the western Pacific from Japan to the Philippines and Malaysia [1, 2]. This species has been proposed as ideal for laboratory studies because it is small and sexually dimorphic, has a short breeding cycle (2 weeks) and high reproductive rate (1000~3000 eggs each time), and is suitable for environmental monitoring [3-5]. Preliminary studies indicated that *M. chulae* is amenable to laboratory

culture, and several studies on its biology have since been carried out [6, 7]. It is known that *M. chulae* can be reared without live food over 30 days of age (d) [8] and that growth performance during the transitional period (from 80–120 d) is promoted by the use of a commercial feed [9]. However, more information is required to facilitate the widespread use of *M. chulae* in the laboratory.

To date, studies on the nutritional requirements of fishes have mostly focused on economically important species, and

few studies have examined small-bodied laboratory fishes, particularly those belonging to estuarine or marine taxa, which represent a promising new avenue for laboratory research. The only species for which a considerable number of nutritional studies have been carried out is the zebrafish *Danio rerio* (Hamilton); however, even in this model species, there is no consensus on the optimal nutritional demand, and no significant effects of crude protein (32%–75%) or lipid (8%–16%) on specific growth rate have been observed [10]. More studies have focused on the effect of different diets on growth performance in zebrafish. Zebrafish larvae can be reared without live food, and furthermore, good growth performance has also been observed upon feeding with a compound diet [11, 12]. No protein sparing effect was observed in the growth characteristics of swordtails *Xiphophorus helleri* (Poeciliidae; Heckel) when lipid concentration was increased at lower protein levels [13]. Additionally, no specific dietary requirements have been observed in marine medaka, *Oryzias melastigma* (McClelland) and three-spined stickleback, *Gasterosteus aculeatus* (Linnaeus). To date, only a few unsystematic studies have been carried out on commercial giant gobies. The marble goby *Oxyeleotris marmorata* (Bleeker) has a limited ability to utilize dietary lipid [14], and only a moderate amount of ammonia is produced after feeding [15]. Both dried *Artemia* and *Artemia*-based feeds can be used to feed goby fingerlings of the species *Pseudapocryptes elongatus* [16]. However, these results have little value for *M. chulae* because dietary nutritional requirements may vary with species, body size, and aquaculture environment. Gobies, particularly small species, have only recently been cultured as laboratory models for environmental monitoring; accordingly, the nutritional requirements of similar goby species have not been investigated.

Although it is possible to feed *M. chulae* live diets comprising such taxa as *Artemia* (Leach) or *Brachionus*

(Pallas) throughout their lifespan, these diets carry the risk of pathogens and parasites [17] and, also lack some essential nutrients [18, 19]. For this reason, formulated feeds containing known levels of nutrients may be beneficial for laboratory fish. Moreover, a nutritionally well-defined fed would be useful in some toxicological experiments in which a formulated diet is needed [20, 21]. Thus, understanding the nutritional requirements of *M. chulae* is essential for its long-term husbandry.

The aim of this study was thus to evaluate the effects of dietary protein and lipid levels and the interaction between these two factors on the growth performance, body composition and liver morphological condition of juvenile *M. chulae* and thereby to provide reference data for the standardized management of this species in a laboratory setting.

2. Materials and Methods

2.1. Ingredients and Diets

Ingredients and diet proximate compositions were determined, and the results are provided in Table 1. Diets were formulated to contain one of four different protein levels (35%, 40%, 45% and 50%) and one of two lipid levels (5% and 10%) for a total of eight different diets. Diet ingredients were weighed, ground (particle size 0.3 mm), and mixed, and fish oil, soybean oil, and distilled water were added. Pellets were prepared (diameter: 0.8 mm) using a pelleting machine (XL Extrude Granulator; Zhi Yang Machinery Equipment Co., Changzhou, China) and dried in an oven at 70°C. Pellets were then crushed to one of three particle sizes corresponding to fish age (0.3–0.5 mm for fish < 60 d; 0.6–0.8 mm for fish age 90–120 d; and 0.8 mm for fish > 120 d), sieved, and stored in a freezer at -20°C until use.

Table 1. Formulation and proximate composition (dry matter basis, g kg⁻¹) analysis of eight diets.

Diets	P35L5	P35L10	P40L5	P40L10	P45L5	P45L10	P50L5	P50L10
Ingredients								
Fish meal [†]	450	450	550	550	650	650	750	750
Soybean meal [†]	150	150	150	150	150	150	150	150
Fish oil [‡]	20	20	15	15	10	10	5	5
Soybean oil	0	50	0	50	0	50	0	50
Potato flour	320	260	220	160	120	60	20	0
Vitamin primix [§]	10	10	10	10	10	10	10	10
Mineral primix [¶]	10	10	10	10	10	10	10	10
Choline chloride	2	2	2	2	2	2	2	2
CMC ^{§§}	20	20	20	20	20	20	20	20
Cellulose microcrystalline	18	28	23	33	28	38	33	3
Chemical composition								
Crude protein	334.12	335.03	414.20	411.12	443.23	456.30	534.21	525.01
Crude Lipid	47.04	105.22	54.01	108.12	57.01	103.23	58.02	110.12
Crude Fibre	2.93	8.52	6.20	6.42	5.43	6.32	5.11	9.32
Moisture	114.03	81.20	71.21	69.30	76.22	68.62	75.10	54.21
Ash	116.32	118.42	139.23	135.02	155.11	152.00	172.33	173.02
NFE ^{††}	385.56	341.61	315.15	270.02	263.00	213.53	155.23	128.32
Gross energy (kJg ⁻¹)	18.32	19.61	18.67	19.96	18.68	19.88	19.06	20.10

Diets	P35L5	P35L10	P40L5	P40L10	P45L5	P45L10	P50L5	P50L10
C18:3n-3 ^{‡‡}	0.19	2.30	0.32	2.42	0.27	1.81	0.42	2.81
C18:2n-6 ^{‡‡}	12.40	35.22	11.43	32.03	14.30	38.12	11.00	34.32
C22:6n-3 ^{‡‡}	3.81	4.42	3.80	4.41	3.94	3.93	4.02	2.72
C20:5n-3 ^{‡‡}	2.62	3.43	2.92	3.50	3.12	3.32	3.12	2.53

†Fishmeal (g kg⁻¹ dry weight): crude protein 640 g, crude lipid 42 g; salt and sand 19.6 g; Total Volatile Base-Nitrogen 0.42 g (Atlantic cod white fishmeal, Ensor, Alaska, USA).

¶ Soybean meal (g kg⁻¹ dry weight): crude protein 436 g, crude lipid 18 g, crude fiber 50 g (high-quality soybean meal, Sunflower, Foshan, China).

‡ Fish oil (g L⁻¹ volume): omega-3 PUFA 240 g, vitamin A 50 mg, docosahexenoic acid 120 g, eicosapentaenoic acid 80 g (cod fish oil, peter moller'stran, Axellus AS, Oslo, Norway).

§ Vitamin premix (IU kg⁻¹ or mg kg⁻¹ mix): vitamin A, 82000 IU; vitamin B₁, 1520 mg; vitamin B₂, 25 mg; vitamin B₆, 50 mg; vitamin B₁₂, 10 mg; vitamin C, 8000 mg; vitamin D₃, 3000 IU; vitamin E, 1000 IU; vitamin K₃, 30 mg; biotin, 5.0 mg; folacin, 40 mg; inositol, 800 mg; calcium pantothenate, 50 mg.

¶¶ Mineral premix (mg kg⁻¹ mix): NaCl, 500 mg; NaH₂PO₄·2H₂O 12500 mg; Mg₂SO₄·H₂O 7500 mg; KI, 0.8 mg; CuSO₄ 10 mg; Fe₂SO₄ 80 mg; ZnSO₄·7H₂O 50 mg; MnSO₄·4H₂O 60 mg; Ca (H₂PO₄)₂·H₂O 3000mg.

§§ CMC, carboxymethylcellulose (food-grade), Shanghai Shengguang Edible Chemicals Co., Shanghai, China.

††NFE (Nitrogen free extract) = 100 - (%crude protein +%crude lipid +%crude fiber +%ash).

‡‡ α -linolenic acid (C18:3n-3), linoleic acid (C18:2n-6), docosahexenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3) levels are expressed as g kg⁻¹ and detected by gaschromatographic mass spectrometry (GC-MS).

2.2. Fish and Experimental Design

All experimental procedures were carried out in accordance with Standardized Welfare Terms for the Zebrafish Community [22] and approved by the Institutional Animal Care and Use Committee (IACUC) of Guangdong Laboratory Animals Monitoring Institute (GLAMI; Guangzhou, China). Goby fry were obtained from a closed colony in the laboratory at GLAMI. Larvae were fed rotifers (0 – 30 d) and *Artemia* nauplii (30 d – 50 d) three or four times per day. Before the growth trial, 60 L tanks (90 cm × 45 cm × 15 cm; HP5B; Rodman plastic company, Guangzhou, China) were set up with circulating water systems and filters. Fish (50 d; n = 1760 individuals) of similar size (0.011±0.001 g) were randomly distributed into the tanks (eight treatment groups × four replicates for each group) and fasted for 24 h to empty the gut. Each group was then fed the corresponding experimental diet for 9 weeks (twice daily, at 9:00 and 16:00). Tanks were cleaned daily and 1/3 of the water was replaced with clean seawater. Water quality parameters, including temperature, salinity, pH, total ammonia concentration (090080; Guangdong huankai microbiological technology co. LTD, Guangzhou, China), and residual chlorine (090330; Guangdong huankai microbiological technology co. LTD, Guangzhou, China), were maintained at set values (24.0±1.6°C, 20.0±1.0 g L⁻¹, 7.4~8.0, ≤0.5 mg L⁻¹, and ≤0.05 mg L⁻¹, respectively), and monitored weekly. The experimental photoperiod was set at 12:12 h light: dark. Additionally, a control group (gobies of the same stage, fed *Artemia* nauplii under the same growth condition) was used to evaluate adverse effects.

2.3. Sample Collection and Analysis

Following the growth trial, juvenile gobies were fasted for 24 h, following which all fish from each tank were euthanized (using MS222 at 50 mg L⁻¹; Sigma-Aldrich, St. Louis, MO, USA) and counted to estimate survival, growth performance, and feed utilization parameters including survival rate (SR), weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER), and feed conversion ratio (FCR).

Fifteen fish were randomly sampled and stored at -20°C for whole-body composition analysis, four were used in fatty acid composition analysis, and three were used in histological studies. The rest of the gobies (14 – 20 individuals) from each tank were dissected to obtain samples of the liver and other viscera (intestines, spleen, gallbladder, etc.), which were measured and weighed to determine the hepatosomatic index (HSI) and viscerosomatic index (VSI); the livers were then used in an enzyme assay.

2.4. Chemical Analysis

The proximate composition of diets and whole-body samples were determined in triplicate according to standard methods proposed by the Association of Official Analytical Chemists [23]. Crude protein was measured using the Kjeldahl method on a Kjeldahl System (Buchi, Flawil, Switzerland). Crude lipid content was measured using the ether-extraction method in a Soxtec Solvent Extraction system (HT6; Foss, Hillerød, Denmark). Moisture was measured by oven drying at 105°C for 6 h until weight was constant. Ash was measured by combustion in a muffle furnace (SX2-4-10TZ; Bo Xun, Shanghai, China) at 600°C for 4 h.

To determine fatty acid composition, total lipids in each diet as well as that in individual fish (n = 4 fish per tank) were extracted via homogenization in chloroform and methanol (2:1 v/v) [24], methylated, and transesterified with a boron trifluoride-methanol complex. Fatty acids were separated and quantified using a gas chromatography mass spectrometer (Trace 1310 ISQ; Thermo Fisher, Waltham, MA, USA) equipped with a flame ionization detector and a fused silica capillary column (30 m × 0.25 mm; film thickness: 0.25 µm; TG-5MS; Thermo Fisher, Waltham, MA, USA). Helium was used as the carrier gas. The temperature was programmed to increase from 80 to 200°C at 10°C min⁻¹, from 200 to 250°C at 5°C min⁻¹, from 250°C to 270°C at 2°C min⁻¹, and to hold at 270°C for 3 min. The injector temperature was 290°C, and the detector temperature was 280°C. Fatty acids were identified by comparing standard fatty acid methyl esters using methyl nonadecanoate (Sigma-Aldrich, St. Louis, MO,

USA).

2.5. Liver Histological Studies

Liver samples used in the histological analysis were fixed in 1.01 g ml⁻¹ formaldehyde-phosphate solution (4%), dehydrated, and embedded in paraffin according to standard procedures. After being sectioned into 4- μ m thick slices using a microtome (Leica RM2235; Leica, Wetzlar, Germany), sections were stained using haematoxylin-eosin (HE; Sigma-Aldrich, St. Louis, MO, USA). Processed slides were then examined under a light microscope (Leica DM1000; Leica, Wetzlar, Germany) to measure fat accumulation in hepatocytes. Five microscope fields (100 hepatocytes per field) were used per slide. Maximum and minimum hepatocyte length and hepatocellular area were measured.

2.6. Enzyme Assay

Liver samples were placed in 1.5 mL plastic centrifuge tubes and homogenized in 10 volumes (v/w) with 0.65% physiological saline at 0°C using a tissue homogenizer (Ningbo Scientz Biotechnology, Ningbo, China). Tissue homogenates were then centrifuged for 10 min at 3000 g and 4°C, and the supernatant thus obtained was used in subsequent assays. Enzyme parameters were measured using commercial assay kits (Nanjing Jianchen Bioengineering Co., Nanjing, China) and a spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan). Activity levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined colorimetrically using test kits (C009-2, C010-2; Nanjing Jianchen Bioengineering Co., Nanjing, China) and were expressed in activity units (U) L⁻¹. AST and ALT activity units were defined as a decrease of 0.001 in absorbance min⁻¹ at 340 nm at 37°C. Lipase and trypsin activity levels were measured according to the procedures specified by the manufacturers of the respective assay kits (A054, A080-2; Nanjing Jianchen Bioengineering Co., Nanjing, China). Trypsin activity was measured using arginine ethyl ester as a substrate at 37°C. The lipase activity unit was measured as the concentration of lipase that caused the consumption of 1 μ mol substrate in 1 min. The trypsin activity unit was defined as the quantity of enzyme that caused absorbance to change by 0.03 min⁻¹. The soluble protein content of the liver homogenates was measured according to the methods specified by the

manufacturer of the Bicinchoninic acid (BCA) kit (A045-3, Nanjing Jiancheng Bioengineering Co., Nanjing, China). Bovine serum albumin was used as a protein standard.

2.7. Statistical Analysis

All data are expressed as means \pm standard deviation (SD). Significant differences between the parameters caused by differing levels of dietary protein or lipid were analyzed by a one-way ANOVA, and the interaction between the two were analyzed using a two-way ANOVA. Statistical analyses were carried out in SPSS 17.0 (IBM Corporation, Armonk, NY, USA), and the significance threshold was set at 0.05. In cases where ANOVA identified significant differences between groups, the groups contributing to that difference were identified using Tukey's HSD.

3. Results

3.1. Growth Performance, Feed Utilization and Body-Organ Indices

During the feeding trial, the survival rate of juvenile *M. chulae* ranged from 67.27 to 77.73% (Table 2), and no significant differences were found among treatments ($P > 0.05$). Significant improvement in *FBW* was observed when dietary protein was increased from 40 to 45% ($P < 0.05$; Table 2). Growth performance (as indicated by *WG* and *SGR*) and feed utilization parameters (as indicated by *FCR* and *PER*) were not affected by dietary lipid concentration, and furthermore, no significant interactions were observed between protein and lipid levels ($P > 0.05$; Table 2). *WG* and *SGR* increased significantly with increasing dietary protein from 35% to 45% ($P < 0.05$); however, further increases, from 45% to 50%, caused a decline in values of both of these parameters. *SGR* was highest in the fish fed diets containing 45% protein (Table 2). *FCR* significantly decreased with increasing protein level ($P < 0.05$), whereas *PER* was only affected when protein content exceeded 45% ($P < 0.05$; Table 2). Although significant interactions were observed between protein and lipid levels for juvenile *HSI* ($P < 0.05$), dietary protein and lipid contents had no effect on organ coefficients (*HSI*, *VSI*) in juvenile goby ($P > 0.05$ in all cases; Table 2).

Table 2. Growth performance and organ coefficient of juvenile goby fed different test diets for 9 weeks.

Diets	SR (%) [†]	IBW (g fish ⁻¹)	FBW (g fish ⁻¹)	WG (%) [‡]	SGR (%) [§]	FCR ^{‡‡}	PER [‡]	HSI (%) ^{§§}	VSI (%) ^{¶¶}
Control	87.62 \pm 7.46	0.011 \pm 0.001	0.23 \pm 0.02	2000.02 \pm 166.12	4.83 \pm 0.12	0.85 \pm 0.07	2.34 \pm 0.20	5.02 \pm 1.23	10.52 \pm 1.38
P35L5	67.27 \pm 5.30	0.012 \pm 0.001	0.17 \pm 0.01	1338.02 \pm 103.58	4.10 \pm 0.11	1.43 \pm 0.11	2.16 \pm 0.15	2.04 \pm 0.84	7.59 \pm 0.70
P35L10	68.18 \pm 5.82	0.010 \pm 0.001	0.16 \pm 0.02	1529.24 \pm 188.72	4.29 \pm 0.18	1.35 \pm 0.13	2.25 \pm 0.20	4.16 \pm 0.69	11.31 \pm 0.78
P40L5	73.64 \pm 8.18	0.011 \pm 0.000	0.18 \pm 0.01	1633.91 \pm 135.26	4.39 \pm 0.12	1.12 \pm 0.08	2.18 \pm 0.19	2.79 \pm 1.05	8.28 \pm 1.41
P40L10	77.73 \pm 7.86	0.010 \pm 0.001	0.16 \pm 0.01	1577.63 \pm 100.87	4.34 \pm 0.09	1.11 \pm 0.05	2.19 \pm 0.10	3.74 \pm 0.76	8.79 \pm 1.41
P45L5	75.00 \pm 6.48	0.011 \pm 0.001	0.19 \pm 0.01	1661.75 \pm 134.51	4.41 \pm 0.12	1.00 \pm 0.04	2.27 \pm 0.07	3.48 \pm 1.11	8.54 \pm 1.99
P45L10	74.55 \pm 7.82	0.012 \pm 0.001	0.21 \pm 0.02	1748.55 \pm 181.71	4.48 \pm 0.15	0.98 \pm 0.08	2.25 \pm 0.18	4.30 \pm 1.35	7.45 \pm 1.24
P50L5	73.18 \pm 8.76	0.011 \pm 0.001	0.18 \pm 0.01	1650.38 \pm 21.37	4.40 \pm 0.02	1.06 \pm 0.06	1.79 \pm 0.08	5.16 \pm 1.27	10.39 \pm 2.41
P50L10	70.45 \pm 10.47	0.010 \pm 0.000	0.16 \pm 0.01	1540.03 \pm 193.03	4.30 \pm 0.17	1.05 \pm 0.05	1.82 \pm 0.07	3.71 \pm 1.42	9.40 \pm 2.97
Means of main effects									
Protein									
350	67.73	0.011	0.17 ^a	1433.63 ^a	4.19 ^a	1.39 ^c	2.21 ^b	3.10	9.45
400	75.68	0.010	0.17 ^a	1605.77 ^{ab}	4.36 ^{ab}	1.12 ^b	2.18 ^b	3.27	8.54

Diets	SR (%) [†]	IBW (g fish ⁻¹)	FBW (g fish ⁻¹)	WG (%) [‡]	SGR (%) [§]	FCR ^{‡‡}	PER [¶]	HSI (%) ^{§§}	VSI (%) ^{¶¶}
450	74.77	0.011	0.20 ^b	1705.15 ^b	4.45 ^b	0.99 ^a	2.26 ^b	3.89	7.99
500	71.82	0.010	0.17 ^a	1595.21 ^{ab}	4.35 ^{ab}	1.05 ^{ab}	1.81 ^a	4.44	9.89
Lipid									
50	72.27	0.010	0.18	1571.01	4.32	1.15	2.10	3.37	8.70
100	72.73	0.011	0.17	1598.86	4.35	1.12	2.13	3.98	9.24
ANOVA (P-value)									
Protein	0.302	0.073	0.002	0.008	0.006	0.000	0.000	0.079	0.263
Lipid	0.887	0.052	0.226	0.587	0.585	0.280	0.600	0.125	0.463
Protein×Lipid	0.893	0.069	0.041	0.168	0.137	0.735	0.910	0.023	0.093

Values are means of quadruplicate groups and presented as mean ± standard deviation (SD); main effect means values in the same column with different superscripts are significantly different ($P < 0.05$); IBW: initial body weight, FBW: final body weight.

[†] Survival rate (SR) = final number of fish / initial number of fish × 100%.

[‡] Weight gain (WG) = $(W_f - W_i) / W_i \times 100\%$.

[§] Specific growth rate (SGR) = $[(\ln W_f - \ln W_i) / T] \times 100\%$.

^{‡‡} Feed conversion ratio (FCR) = total feed provided (g) / total wet weight gain (g).

[¶] Protein efficiency ratio (PER) = total wet weight gain (g) / protein intake (g).

^{§§} Hepatosomatic index (HSI) = liver weight (g) / live body weight (g) × 100%.

^{¶¶} Viscerosomatic index (VSI) = visceral weight (g) / live body weight (g) × 100%.

W_f: mean final weight; W_i: mean initial weight; T: total growth experimental days.

3.2. Body Proximate Composition

Whole-body protein content was not affected by dietary protein and lipid levels (Table 3). Whole-body lipid content in juvenile *M. chulae* increased significantly with increasing dietary lipid content ($P < 0.05$), whereas ash content significantly decreased ($P < 0.05$; Table 3). Furthermore, whole-body lipid content in juvenile goby was highest in the group fed 40% protein and 10% lipid (Table 3). However, whole-body moisture content did not differ between treatments ($P > 0.05$; Table 3).

Table 3. Whole-body proximate composition of juvenile goby fed different test diets for 9 weeks (g kg⁻¹).

Diets	Crude protein	Crude lipid	Crude moisture	Crude ash
Cnotrol	165.54±5.97	33.94±2.93	754.63±16.38	31.52±1.33
P35L5	165.50±8.21	31.48±1.79	766.51±15.60	35.52±0.90
P35L10	167.43±9.11	36.90±0.57	753.70±4.92	32.01±0.52
P40L5	159.65±3.53	31.93±1.33	754.02±10.11	33.30±1.82
P40L10	158.80±8.86	38.48±3.89	753.91±17.20	30.20±0.43
P45L5	165.70±9.11	32.20±2.47	777.21±14.02	33.71±0.92
P45L10	166.53±15.04	34.93±1.07	770.30±22.61	28.93±2.60
P50L5	161.98±8.79	30.25±1.73	757.63±11.10	29.52±1.80
P50L10	161.87±7.34	35.65±0.66	755.51±7.50	29.12±0.83
Means of main effects				
Protein				
350	166.65	34.19	760.11	33.76 ^c
400	159.23	35.20	753.97	31.75 ^{bc}
450	166.11	33.56	773.75	31.32 ^{ab}
500	161.93	32.95	756.57	29.32 ^a
Lipid				
50	163.21	31.46 ^a	763.84	33.01 ^b
100	163.66	36.49 ^b	758.35	30.06 ^a
ANOVA (P-value)				
Protein	0.354	0.157	0.095	0.000
Lipid	0.892	0.000	0.345	0.000
Protein×Lipid	0.991	0.280	0.863	0.083

Values are means of quadruplicate groups and presented as mean ± standard deviation (SD); main effect means values in the same column with different superscripts are significantly different ($P < 0.05$).

The whole-body fatty acid profile for juvenile fish is shown in Table 4. Whole-body linoleic acid (C18:2n-6) and γ -linolenic acid (C18:3n-6) significantly increased with increasing dietary lipid level ($P < 0.05$); moreover, palmitic acid (C16:0) and eicosapentaenoic acid (EPA, C20:5n-3) decreased with increasing dietary lipid content ($P < 0.05$). Significant changes were found in whole-body myristic acid (C14:0), myristoleic acid (C14:1), stearic acid (C18:0), oleic acid (C18:1n-9), eicosapentaenoic acid (EPA, C20:5n-3), and

docosahexaenoic acid (DHA, C22:6n-3) content at various levels of dietary protein; however, no definite trend was apparent. Only γ -linolenic acid (C18:3n-6) increased continuously with increasing protein level. Significant interactions were observed between protein and lipid levels in whole-body myristic acid (C14:0), myristoleic acid (C14:1), palmitoleic acid (C16:1), stearic acid (C18:0), linoleic acid (C18:2n-6), and docosahexaenoic acid (DHA, C22:6n-3) ($P < 0.05$).

Table 4. Whole-body fatty acid composition of juvenile goby fed different test diets for 9 weeks (% total fatty acids by weight).

Diets	Fatty acids										
	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1n-9	C18:2n-6	C18:3n-6	C20:4n-6	C20:5n-3	C22:6n-3
Control	4.02±0.50	0.55±0.06	31.23±1.62	6.53±0.10	9.98±0.50	23.23±0.66	10.20±1.10	1.15±0.41	0.05±0.01	0.73±0.05	0.05±0.02
P35L5	1.52±0.07	0.03±0.00	24.68±4.10	0.53±0.12	6.81±0.44	23.22±1.58	6.74±0.24	0.17±0.01	3.12±0.24	16.55±0.34	5.99±0.10
P35L10	0.95±0.23	0.02±0.01	15.82±3.85	0.40±0.02	4.99±0.62	22.69±0.37	27.53±1.29	0.20±0.00	3.05±0.07	11.44±1.07	9.63±0.70
P40L5	1.32±0.46	0.03±0.00	20.99±1.02	0.46±0.04	4.04±0.13	21.07±0.23	9.16±0.11	0.14±0.00	3.15±0.11	17.05±0.95	10.54±0.78
P40L10	1.65±0.14	0.03±0.00	20.56±0.53	0.56±0.11	4.33±0.57	21.97±1.32	24.47±2.10	0.23±0.02	3.23±0.18	12.23±0.44	7.86±0.45
P45L5	2.27±0.35	0.04±0.01	17.20±2.96	0.65±0.07	4.43±0.13	21.84±0.52	9.49±1.06	0.17±0.01	3.17±0.23	17.87±1.15	9.32±0.56
P45L10	1.77±0.03	0.05±0.00	16.12±4.17	0.48±0.02	3.44±0.26	23.33±0.15	24.84±1.90	0.27±0.04	3.41±0.43	13.49±0.78	7.76±0.33
P50L5	2.26±0.32	0.06±0.01	22.11±3.98	0.61±0.06	4.13±0.28	24.70±2.10	11.88±0.35	0.23±0.01	3.07±0.19	13.35±1.64	9.06±0.21
P50L10	1.36±0.60	0.02±0.00	16.80±0.66	0.55±0.04	4.95±0.09	22.10±0.12	25.37±1.12	0.27±0.05	3.05±0.08	11.27±0.23	8.25±0.32
Means of main effects											
Protein											
350	1.24 ^a	0.03 ^a	20.25	0.47	5.90 ^b	22.96 ^{ab}	17.14	0.19 ^a	3.09	13.99 ^b	7.81 ^a
400	1.49 ^{ab}	0.03 ^{ab}	20.78	0.51	4.19 ^a	21.52 ^a	16.82	0.19 ^a	3.19	14.64 ^{bc}	9.20 ^b
450	2.02 ^b	0.05 ^b	16.66	0.57	3.94 ^a	22.59 ^{ab}	17.17	0.22 ^{ab}	3.29	15.68 ^c	8.54 ^{ab}
500	1.81 ^b	0.04 ^b	19.46	0.58	4.54 ^a	23.40 ^b	18.63	0.25 ^b	3.06	12.31 ^a	8.65 ^b
Lipid											
50	1.84	0.04	21.25 ^b	0.56	4.85	22.71	9.32 ^a	0.18 ^a	3.13	16.20 ^b	8.73
100	1.43	0.03	17.33 ^a	0.50	4.43	22.52	25.55 ^b	0.24 ^b	3.19	12.11 ^a	8.37
ANOVA (P-value)											
Protein	0.004	0.000	0.136	0.043	0.000	0.044	0.091	0.001	0.366	0.000	0.001
Lipid	0.008	0.001	0.006	0.037	0.012	0.049	0.000	0.000	0.543	0.000	0.094
Protein ×											
Lipid	0.032	0.000	0.099	0.020	0.000	0.068	0.001	0.057	0.713	0.050	0.000

Values are means of quadruplicate groups and presented as mean ± standard deviation (SD); main effect means values in the same column with different superscripts are significantly different ($P < 0.05$).

3.3. Liver Histological Analysis of Juvenile Goby

Histological analysis indicated cytoplasmic vacuolation and nuclear displacement in the juvenile liver cells of all treatments, including in the control group (Figure 1). Clearly organized congestion was observed in fish fed the diet containing 50%

protein and 10% lipid (Figure 1 (h)). However, there was no significant difference in the degree of hepatocellular vacuolation between the treatment groups ($P > 0.05$). The pancreas retained structural integrity (Figures 1 (a), 1 (e)), and neither severe lipid infiltration nor necrosis was detected in the liver.

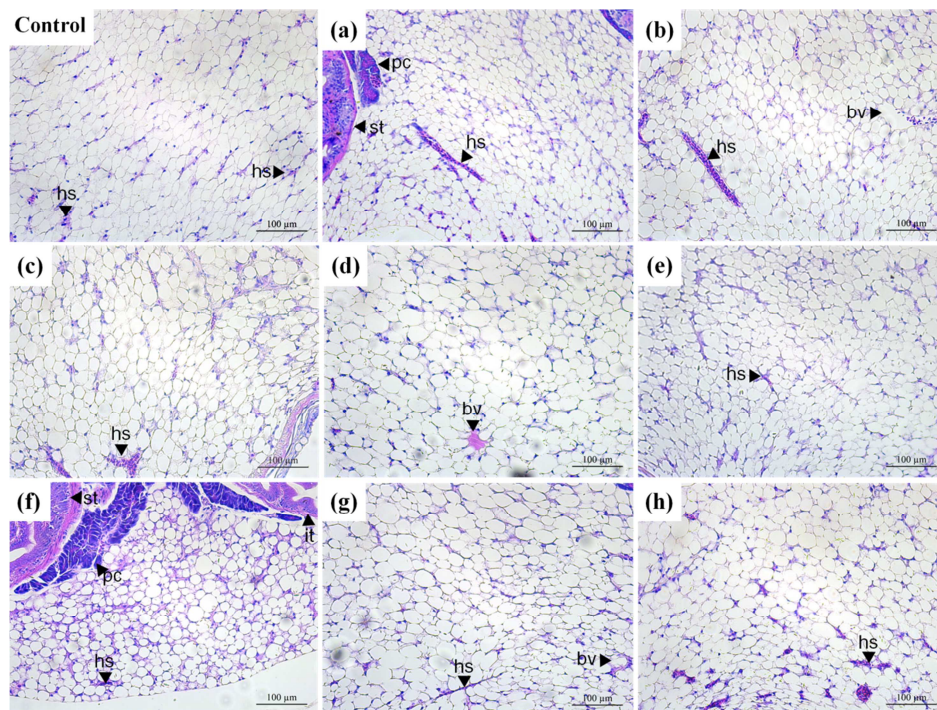


Figure 1. Liver morphology of juvenile yellowstripe goby (*M. chulae*) fed different experimental diets at the end of 9 weeks feeding trail (H&E, ×400). (a) ~ (h): treatment groups fed by diet P35L5, P35L10, P40L5, P40L10, P45L5, P45L10, P50L5 and P50L10; pc: pancreas, st: stomach, it: intestine, hs: hepatic sinusoid, bv: blood vessel; scale bar = 100 μm.

No significant morphological differences were detected in minimum hepatocyte length or minimum hepatocellular area between treatments ($P > 0.05$ for each; Table 5). Significant interactions were observed between protein and lipid levels in maximum hepatocyte length and maximum hepatocellular area ($P < 0.05$), and, furthermore, these were positively

affected by lipid content when the protein content was lower than 45%. Only maximum hepatocyte length and maximum hepatocellular area increased significantly when dietary protein was increased from 35% to 40% and 45% to 50% ($P < 0.05$).

Table 5. Morphometric values of hepatocytes from juvenile goby fed different test diets for 9 weeks.

Diets	Maximum hepatocyte length (μm)	Minimum hepatocyte length (μm)	Maximum hepatocellular area (μm^2)	Minimum hepatocellular area (μm^2)
Control	30.87 \pm 1.03	10.72 \pm 0.85	772.52 \pm 60.87	83.25 \pm 12.33
P35L5	23.72 \pm 1.10	10.55 \pm 0.93	440.07 \pm 40.60	90.77 \pm 16.08
P35L10	30.34 \pm 2.76	10.89 \pm 1.32	754.14 \pm 134.74	94.48 \pm 22.48
P40L5	30.23 \pm 2.49	11.64 \pm 0.88	743.44 \pm 121.39	110.21 \pm 15.12
P40L10	36.69 \pm 2.44	11.77 \pm 0.99	1045.22 \pm 144.23	114.48 \pm 17.99
P45L5	29.49 \pm 2.58	9.53 \pm 1.09	685.25 \pm 122.07	76.15 \pm 16.02
P45L10	31.34 \pm 1.50	10.86 \pm 1.07	782.49 \pm 77.39	95.26 \pm 17.22
P50L5	36.17 \pm 2.34	10.66 \pm 1.14	1034.61 \pm 137.49	89.79 \pm 18.72
P50L10	33.27 \pm 2.10	11.20 \pm 1.36	882.94 \pm 110.96	101.48 \pm 24.22
Means of main effects				
Protein				
350	27.03 ^a	10.72	597.10 ^a	92.62
400	33.46 ^{bc}	11.70	894.33 ^{bc}	112.34
450	30.42 ^{ab}	10.19	733.87 ^{ab}	85.70
500	34.72 ^c	10.93	958.78 ^c	95.63
Lipid				
50	29.91	10.60	725.84	91.73
100	32.91	11.18	866.20	101.42
ANOVA (P-value)				
Protein	0.000	0.155	0.000	0.116
Lipid	0.003	0.210	0.007	0.217
Protein \times Lipid	0.003	0.796	0.006	0.878

Values are means of quadruplicate groups and presented as mean \pm standard deviation (SD); main effect means values in the same column with different superscripts are significantly different ($P < 0.05$).

3.4. Liver Enzyme Activities Analysis

Different dietary lipid levels significantly affected the activity levels of AST, ALT, and lipase, albeit inconsistently (Table 6). Increases in lipid levels from 5% to 10% led to an increase in AST and ALT activity ($P < 0.05$ for each); however, lipase activity significantly decreased ($P < 0.05$). Trypsase activity increased significantly with increasing dietary protein

from 35% to 45% ($P < 0.05$), and further increases, from 45% to 50%, caused a decline ($P < 0.05$). The dietary protein level driving optimal growth performance also caused the highest trypsin activity (Table 6). A significant interaction between protein and lipid levels was detected for AST, ALT, lipase, and trypsin ($P < 0.05$; Table 6).

Table 6. Enzyme activities in liver of juvenile goby fed different test diets for 9 weeks.

Diets	AST (U L ⁻¹)	ALT (U L ⁻¹)	Lipase (U mg ⁻¹)	Trypsase (U mg ⁻¹)
Control	224.52 \pm 24.15	312.63 \pm 45.76	0.283 \pm 0.027	3827.66 \pm 423.52
P35L5	239.08 \pm 19.58	280.67 \pm 18.30	0.237 \pm 0.010	2140.05 \pm 201.79
P35L10	245.36 \pm 24.57	350.39 \pm 24.45	0.123 \pm 0.004	1823.04 \pm 88.03
P40L5	221.74 \pm 16.93	381.50 \pm 26.65	0.268 \pm 0.024	2807.95 \pm 162.92
P40L10	321.80 \pm 15.18	579.10 \pm 34.48	0.178 \pm 0.012	2192.06 \pm 217.15
P45L5	202.43 \pm 13.43	252.09 \pm 22.10	0.222 \pm 0.002	3470.45 \pm 171.72
P45L10	233.40 \pm 25.90	383.50 \pm 15.01	0.198 \pm 0.013	2471.02 \pm 180.52
P50L5	172.02 \pm 17.60	277.45 \pm 20.09	0.177 \pm 0.006	1042.96 \pm 210.45
P50L10	244.98 \pm 34.57	654.58 \pm 55.40	0.120 \pm 0.005	2951.33 \pm 332.12
Means of main effects				
Protein				
350	242.22 ^{ab}	315.53 ^a	0.180 ^b	1981.54 ^a
400	271.77 ^b	480.30 ^c	0.223 ^c	2500.01 ^b
450	217.91 ^a	317.80 ^a	0.210 ^c	2970.74 ^c
500	208.50 ^a	466.01 ^{bc}	0.148 ^a	1997.14 ^a
Lipid				
50	208.82 ^a	297.93 ^a	0.226 ^b	2365.35
100	261.39 ^b	491.89 ^b	0.155 ^a	2359.36

Diets	AST (U L ⁻¹)	ALT (U L ⁻¹)	Lipase (U mg ⁻¹)	Trypsase (U mg ⁻¹)
ANOVA (P-value)				
Protein	0.000	0.000	0.000	0.000
Lipid	0.000	0.000	0.000	0.944
Protein×Lipid	0.005	0.000	0.000	0.000

Values are means of quadruplicate groups and presented as mean±standard deviation (SD); main effect means values in the same column with different superscripts are significantly different ($P < 0.05$); AST: aspartate aminotransferase; ALT: alanine aminotransferase.

4. Discussion

4.1. High Dietary Lipid Level Is not Optimal for the Growth of Juvenile Yellowstripe Goby

In the present study, juvenile *M. chulae* fed diets containing 45% protein level had the highest *SGR* and *WG*; however, no significant improvement in growth (*SGR* and *WG*) and feed utilization (*FCR*, *PER*) were observed by increment of dietary lipids from 5% to 10%. Ideal *SR* (74.55 ± 7.82 and $75.00 \pm 6.48\%$) was also recorded at this protein level and was not affected by dietary lipid concentration. These results suggest that no improvement would be elicited by the inclusion of dietary lipids beyond the optimum level. It is well known that growth performance parameters themselves are not necessarily indicative of health because the diets may influence other physiological processes. However, previous studies do suggest that *M. chulae*'s growth is affected by the quantity and/or quality of dietary nutrients proposed for normal growth. Thus, this represents an important first step in understanding the nutritional requirements of this species.

The results of our proximate component analyses indicated that dietary lipid contributed significantly to fat deposition in juvenile *M. chulae*. This result agrees with those for marble goby, *O. marmorata* [14, 25], and those from other studies that increasing dietary lipid content may result in high fat deposition and fatty acid composition variability in the fish body [26, 27, 28]. These findings may indicate that *M. chulae* has a limited ability to utilize high amounts of lipid. In the current study, soybean oil was used in high lipid diets, which significantly increased the content of linoleic and linolenic acid, and this may be responsible for the increased body linoleic acid content. In general, higher levels of polyunsaturated fatty acid (PUFA), especially omega-3 fatty acids, could enhance growth in fish [29]. Our results support this explanation; furthermore, increasing dietary lipid resulted in extremely significant enhancement of whole-body linoleic acid content in juveniles. A similar result has also been observed in the juvenile hybrid grouper, *E. fuscoguttatus* × *E. lanceolatus* [28], and masu salmon, *Oncorhynchus masou* [30], but the opposite result has been observed in white seabass, *A. nobilis* [27]. This indicates that *M. chulae* had the capacity to selectively retain high linoleic acid concentrations.

Previous research has revealed that protein and lipid metabolism in fish, including synthesis and degradation, is primarily regulated by the liver [31, 32]. Morphological patterns can be altered by dietary lipid content; the energy associated with these molecules might also exceed the capacity of hepatic cells to process them [33]. Although *HSI* in juvenile *M. chulae* did not differ between different lipid

treatments, significant interactive effects were observed between protein and lipid levels for *HSI*. Thus, liver enzyme activities were used to evaluate the effects of dietary protein and lipid levels on body fat deposition. In the present study, liver lipase activity decreased as dietary lipid levels increased from 5% to 10%. Decreased lipase activity may lead to fat accumulation in hepatocytes and an increase in *HSI*. The dietary protein level driving optimal growth performance also caused the highest trypsin activity; however, trypsin activity declined significantly with increase in dietary lipid levels in this condition.

These findings indicate that juvenile yellowstripe goby has a limited ability to utilize high amounts of lipid, and that the inclusion of lipids in the diet at a level of 10% is not optimal for the growth of this species.

4.2. 45% Dietary Protein Level Is Optimal for Juvenile *M. Chulae*

In this study, the growth performance (as indicated by *WG* and *SGR*) of juvenile goby was improved as dietary protein levels increased from 35 to 45%, but further increase of dietary protein to 50% led to decreased growth performance. No significant difference was found in *FCR* when protein content exceeded 45%, whereas *PER* was significantly decreased. This suggested that no improvement would happen when dietary protein levels are beyond the optimum level. Based on growth and feed utilization parameters, the optimal protein content for juvenile *M. Chulae* is 45%, which is similar to that found for juvenile swordtails *X. helleri* [13]. However, under laboratory conditions, this value is higher than that for other small laboratory fishes such as zebrafish *D. rerio*, which have a dietary protein requirement for juvenile estimated at 37.6% for maximum *WG* [34]. Another study has shown that a diet with 32% crude protein is sufficient to meet the growth requirements of *D. rerio* [10]. In aquaculture condition, it would appear that a diet containing 47.5% protein is needed for the optimal growth of the marble goby *O. marmorata* [35]. Because of the considerable difference between marine and freshwater environments and the variation in metabolic rate due to body size, different species have differing nutritional requirements [36, 37]. Therefore, these results provide a preliminary foundation for further studies on the nutritional requirements of small gobies, especially *M. chulae*.

Although high correlation exists between dietary variables and liver conditions [32, 38], liver morphometry changes are difficult to observe in this species and cytoplasmic vacuolation was persistent in *M. chulae*, even in the control group. However, maximum hepatocyte length and maximum hepatocellular area increased significantly when dietary

protein was increased from 45% to 50%; furthermore, minor liver congestion was observed in fish fed a 50% protein diet at a 10% lipid level. This suggests that energy reserves of *M. chulae* are stored in the liver, and much high protein level influenced normal life activity. In this study, gobies fed 50% dietary protein exhibited higher ALT activities, as well as the lowest trypsin activity. Although increased levels of ALT and AST suggested protein catabolism at high diet protein levels, such high levels of ALT activity may indicate weakened or damaged liver function [39]. These results suggest that liver morphology and enzyme activity were suitable in fish fed the diet containing 45% protein and 5% lipid.

4.3. Dietary Protein and Lipid Level Driving Optimal Growth Performance Appears to Have no Substantial Adverse Effects

Natural diets (mainly *Artemia* sp.) were widely utilized to design experimental feeds for laboratory fish, such as *D. rerio* and *X. hellerii* [11, 12, 40]; furthermore, live feed appears to have beneficial effects on juvenile fish growth performance. Thus, the control group was used to evaluate adverse effects in this study. Although *M. chulae* fed *Artemia* nauplii is still have significantly better SGR and WG than all treatments fed artificial diets, gobies fed diets containing 45% protein and 5% lipid achieved acceptable growth performance; moreover, no significant difference in body composition were observed, especially in linoleic acid (C18:2n-6) composition. However, whole-body EPA (C20:5n-3), and DHA (C22:6n-3) content significant increased, these are critical for growth in many fish, including zebrafish [12, 41, 42]. Additionally, liver enzyme

activities (AST and trypsin) of juvenile gobies fed diets containing 45% protein and 5% lipid were very similar to those of the control group. Therefore, the dietary protein and lipid levels that drive optimal growth performance appear to have no substantial adverse effects.

5. Conclusion

Based on growth performance, body composition and liver condition, 45% crude protein and 5% lipid appears to be sufficient for good growth performance for juvenile *M. chulae*, and this diet appears to have no substantial adverse effects. These results provide a preliminary foundation for further studies seeking to develop artificial diets. To further characterize the effect of body lipid deposition on health and growth performance, future studies should focus on the genetic regulation by *M. chulae* of the metabolism of essential unsaturated fatty acids acquired through the diet.

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Appendix

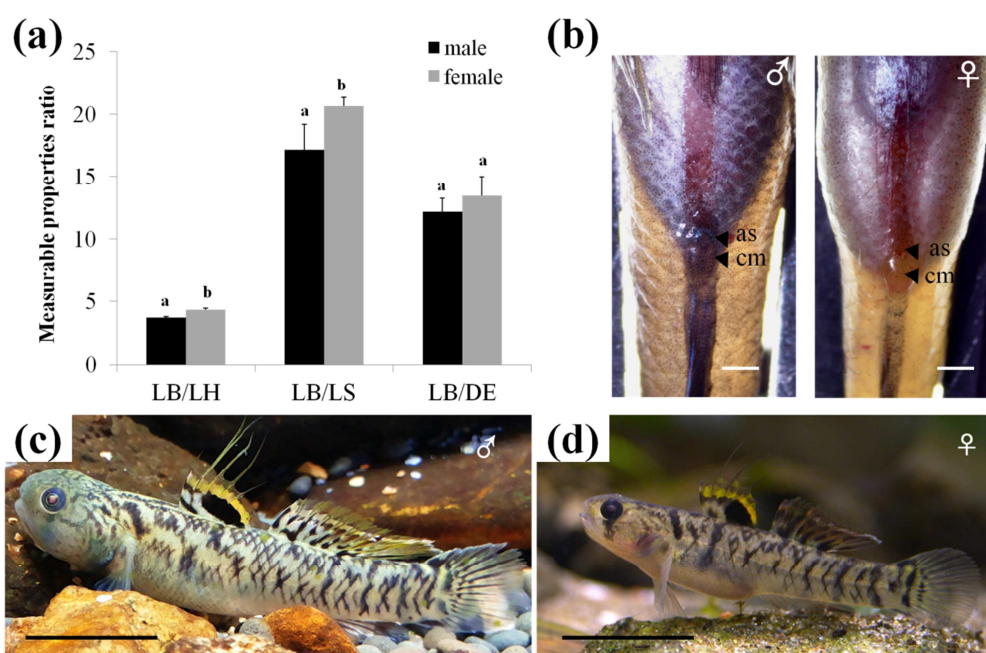


Figure A1. Typical characteristics of adult yellowstripe goby, *M. chulae*. (a) Measurable properties ratio of adult goby (200 d), LB: body length, LH: head length, LS: snout length, DE: distance between neighboring eyes; (b) Typical characteristics of cloacal orifice at maturity, black (male), white or light yellow (female), as: anus, cm: cloacal mastoid, bar = 1mm; (c) male, 200 d, 0.72 g in body weight and 3.27 cm in total length, scale bar = 1cm; (d) female, 200 d, 0.34 g in body weight and 2.70 cm in total length, scale bar = 1cm.

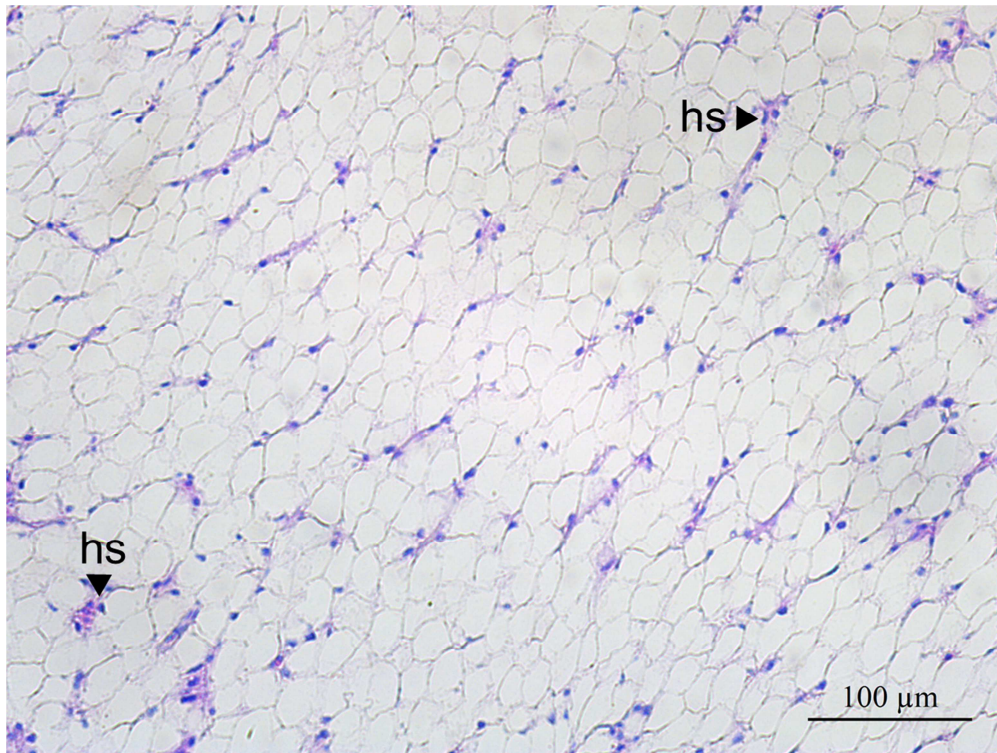


Figure A2. Liver morphology of yellowstripe goby (*M. chulae*) fed *Artemia nauplii* at 120 d (H&E, $\times 400$). hs: hepatic sinusoid, bar = 100 μm .

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