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Physiological responses of smooth marron *Cherax cainii* fed various protein sources substituting with larvae oil of black soldier fly *Hermetia illucens*

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Abstract. *Dao TTT, Fotedar R, Howieson J. 2024. Physiological responses of smooth marron* Cherax cainii *fed various protein sources substituting with larvae oil of black soldier fly* Hermetia illucens. *Asian J Agric 8: 104-115.* A ten-week feeding experiment was conducted to examine the impact of using Black Soldier Fly Larvae (BSFL) oil as a substitute for Fish Oil (FO) on the growth and health of smooth marron (*Cherax cainii* Austin & Ryan, 2002). Six diets were formulated with varying protein and lipid sources: D1 (fish meal [FM] + FO + canola oil), D2 (FM + BSFL oil), D3 (poultry by-product meal + BSFL oil), D4 (defatted BSFL meal + BSFL oil), D5 (lupin meal [LM] + BSFL oil), and D6 (LM + FO + BSFL oil). Marron was randomly distributed among the dietary treatments, which had three replicated tanks per treatment. Growth, immune response, and organosomatic indices were measured. Marron fed on lupin meal and BSFL oil (D5) exhibited reduced growth and immune response. Marronfed D2, D3, and D4 exhibited significantly higher protease activity compared to other diets. Feed water stability was highest in D1 and D2, and shortest in D5 and D6. The hepatosomatic index and hepatopancreas moisture content varied among diets, but there were no significant differences in tail muscle indices. In conclusion, BSFL oil can replace FO in animal protein-based diets, but FO is still needed when lupin is the main protein source.

Keywords: Black soldier fly larvae oil, fish oil, growth performance, smooth marron *Cherax cainii,* various protein sources

Abbreviations: BSFL oil: Black Soldier Fly Larvae oil, DBSFLM: Deffated Black Soldier Fly Larvae Meal, FM: Fish Meal, FO: Fish Oil, LM: Lupin Meal, and PBM: Poultry By-product Meal

INTRODUCTION

Lipids are an essential energy source for growth and development in aquatic animals (Kayama et al. 1980; Abramo 1989; Molina-Poveda 2016) and serve as the primary organic reserve for regulating the physiological metabolism of several crustacean species (Lim et al. 1997; Zhang et al. 2013), including freshwater crayfish (Hernández-Vergara et al. 2003; Li et al. 2011; Chen et al. 2021a, 2022a). Traditionally, aquadiets have utilized Fish Meal (FM) and Fish Oil (FO) manufactured from wild-caught fish as sources of proteins and lipids because of their high nutritional value. However, the escalation of overfishing poses a significant threat to many wild fish populations (Nugroho and Nur 2018). Rising demand and limited availability have increased the prices of FM and FO, resulting in a growing interest in sustainable alternatives for aquafeeds (Xiao et al. 2018; Hodar et al. 2020).

Both plant- and animal-based proteins can be important dietary components for freshwater crayfish. However, the utility of plant-based proteins is limited by imbalanced amino acids, inferior palatability, and the presence of anti-nutritional factors (Gatlin III et al. 2007; Samtiya et al. 2020), and they may change the intestinal microflora structure of aquatic animals and contribute to digestive tract inflammation (Merrifield et al. 2011; Hu et al. 2019). Poultry By-product Meal (PBM) is a terrestrial animal by-product with excellent nutritional value (Cruz-Suárez et al. 2007). Studies have demonstrated the efficacy and performance of PBM in raising redclaw crayfish (*Cherax quadricarinatus*) (Saoud et al. 2008), signal crayfish (*Pacifastacus leniusculus*) (Fuertes et al. 2013), and smooth marron (*Cherax cainii,* Austin and Ryan, 2002) (Siddik et al. 2020).

Insect meal, particularly from the larvae of the black soldier fly (*Hermetia illucens)* larvae (BSFL) has garnered interest as another alternative to FM (Tran et al. 2015; Benzertiha et al. 2020) because of its rapid reproductive cycle, substantial biomass yield, and efficient conversion of protein and lipids (Wang and Shelomi 2017; Surendra et al. 2020). However, diets with a high percentage of BSFL have been blamed for decreased growth, alterations to the composition of whole-body nutrient profiles (Chen et al. 2022b), and changes to the intestinal structure of shrimp (Chen et al. 2021b). Defatted black soldier fly larvae meal (DBSFLM) has an increased protein content (56.9%) (Spranghers et al. 2017; Wang and Shelomi 2017), and decreased fat content (4.8%) (Zozo et al. 2022). DBSFLM is characterized by a threefold increase in essential amino acid content and a 2.6-fold increase in lauric acid content compared to FM (Tippayadara et al. 2021; Li et al. 2022).

BSFL oil is rich in saturated fatty acids, with the most prevalent being lauric acid (21-49% of the total fatty acids) and myristic acid (5.1-9.4%) (Nogales-Mérida et al. 2019). The utilization of DBSFLM and BSFL oil to replace FM and FO has been investigated in various fish species, such as rainbow trout (*Oncorhynchus mykiss*), tongue sole (*Cynoglossus semilaevis*), Atlantic salmon (*Salmo salar*), and Siberian sturgeon (*Acipenser bareii*) (Dumas et al. 2018; Belghit et al. 2019a; Rawski et al. 2020; Li et al. 2022). Feed containing up to 75% DBSFM was a suitable diet for Jian carp (*Cyprinus carpio* var. *Jian*) (Li et al. 2017). In barramundi (*Lates calcarifer*) feeds containing 30% DBSFM or less did not negatively impact growth and enhanced the immune capability of the fish (Hender et al. 2021). The consumption of BSFL oil resulted in decreased fat storage in the intestine and smaller intraperitoneal adipocytes in juvenile carp (Li et al. 2016; Xu et al. 2021). However, there is a lack of research on freshwater crayfish.

Smooth marron (*C. cainii*), is a popular farmed freshwater crayfish in Western Australia. There is a lack of general nutritional knowledge about this species and the dietary role of various plant and animal ingredients that provide protein and lipid sources. To date, various ingredients have been used in the marron diet (Fotedar 2004; Foysal et al. 2019; Saputra et al. 2019; Siddik et al. 2020; Saputra and Fotedar 2021) with inconclusive outcomes or a lack of publications, but little is known about the effects of using BSFL meal as a source of protein and lipids in the marron diet. This research is the first attempt to assess growth performance, including molt parameters, immune response, and hemolymph physiology, in marronfed on different protein sources that replace FO with BSFL oil. This study also examines whether dietary BSFL can influence the efficacy of various protein sources in the marron diet.

MATERIALS AND METHODS

Feed composition and preparation

The six isonitrogenous and isolipidic diets were formulated as shown in Table 1. Six-day-old BSFL (Future Green Solutions, Western Australia) were reared in plastic trays containing minced carp and discarded grain for six days. BSFL were dehydrated in an oven for 48 h at 60°C. Subsequently, BSFL were finely ground into powder with a coffee grinder (Sunbeam Café Series, EMM0500BK). BSFL oil was extracted through a cold-pressing process following the methodology described by Matthäus et al. (2019). All dry ingredients were mixed prior to the addition of FO and enough distilled water to create a dough. This dough were passed through a mincer to obtain wet strands with a diameter of 2 mm that were subsequently dehydrated in an oven at 60°C for 24 h. The dry strands were broken into pieces 3 mm long and stored in sealed, labeled bags in a cool room kept at 4°C. Feed proximate analysis was conducted according to the methodology described by the Association of Official Analytical Chemists (AOAC) (AOAC 2005).

Table 1. Composition and proximate analysis of experimental diets (% dry matter)

Ingredients	Experimental diets					
	D1	D2	D3	D4	D5	D6
FM*	**42.00**	**41.00**	0.00	0.00	0.00	0.00
DBSFLM	0.00	0.00	0.00	**52.60**	0.00	0.00
PBM	0.00	0.00	**42.00**	0.00	0.00	0.00
Lupin Meal	0.00	0.00	0.00	0.00	**66.00**	**67.00**
Wheat	33.00	33.00	30.80	23.00	7.60	7.00
Corn/wheat starch	11.00	11.00	11.00	11.00	11.00	11.00
Cholesterol	0.50	0.50	0.50	0.50	0.50	0.50
Canola oil	**2.00**	0.00	0.00	0.00	0.00	0.00
Cod liver oil	**4.00**	0.00	0.00	0.00	0.00	**3.00**
BSFL oil	0.00	**7.00**	**8.20**	**5.40**	**7.40**	**4.00**
Vitamin premix	0.30	0.30	0.30	0.30	0.30	0.30
Vitamin C	0.10	0.10	0.10	0.10	0.10	0.10
Dicalcium phosphate	0.10	0.10	0.10	0.10	0.10	0.10
Lecithin – soy	3.00	3.00	3.00	3.00	3.00	3.00
Barley	4.00	4.00	4.00	4.00	4.00	4.00
Proximate composition (% dry matter)						
Crude protein (%)	31.41±0.40	31.34±0.41	32.78±0.48	32.81±0.49	32.16±0.04	32.33±0.47
Crude lipid (%)	13.34±0.14	13.25±0.34	14.38±0.37	13.54±0.83	13.16±0.60	13.50±0.66
Moisture (%)	8.14±0.02	8.88±0.03	8.49±0.01	7.75±0.05	9.78±0.52	9.08±0.02
Ash (%)	10.57±0.04	9.27±0.71	7.16±0.13	7.74±0.04	4.31±0.02	4.13±0.02

Note: D1: Fish meal and fish oil; D2: Fish meal and black soldier fly larvae oil; D3: Poultry by-product meal and black soldier fly larvae oil; D4: Defatted black soldier fly larvae meal and black soldier fly larvae oil; D5: Lupin meal and black soldier fly larvae oil; and D6: Lupin meal, black soldier fly larvae oil and fish oil. *Fish meal contains 58.55% crude protein and 9.46% crude lipid; Defatted black soldier fly larvae meal contains 49.44% crude protein and 10.41% crude lipid; Poultry by-product mea contains 62.75% crude protein and 15.10% crude lipid; Black soldier fly larvae oil contains 13.84% crude protein and 85.00% crude lipid; Lupin meal contains 41.47% crude protein and 9.01% crude lipid

Experimental design

Marron with an average initial weight of 2.22±0.04 g were acquired from Blue Ridge Marron, Manjimup, Western Australia (-34°14'27.60"S, 116°08'45.60"E) and transported to Curtin Aquatic Research Laboratory (CARL) at Technology Park, Western Australia. Marron were placed in holding tanks (300 L) and fed a commercial diet (Specialty Feeds Company, Perth, Western Australia) containing 28% protein and 9% lipid for two weeks of acclimation. Dead marron were removed during and immediately after acclimation. The surviving 450 individuals were randomly stocked into 18 tanks (six dietary treatments with three replications per treatment). Each tank had a capacity of approximately 300 L, a diameter of 100 cm, and a height of 40 cm. The tanks were fitted with an external canister filter Astro® AS-2212 that provided continuous aeration. Marron were housed individually in labeled 1000 mL plastic containers (150×110×70 mm) to avoid cannibalism and to facilitate the collection of individual molt-related data. Containers had small holes to ensure water exchange with the main tank.

For a total of ten weeks, marron were fed once each day during the dark hours on an amount equivalent to 3% of the total marron biomass. Leftover feed and feces were removed daily before the next feeding commenced. Every two weeks, 50% of the total water volume was exchanged. Water quality parameters, including dissolved oxygen (DO), pH, temperature, and total ammonia, were assessed once a week using a multi-parameter meter (YSI, USA), and an API ammonia NH_3/NH_4 kit (Aquarium Pharmaceuticals™ API). The natural photocycle of marron was reversed by maintaining 12 h of darkness during the day and using artificial light in the dark laboratory. During the experiment, marron mortality and post-molt weight were recorded daily when they occurred.

Growth performance

At the end of the experiment, marron were weighed individually using a Mettler PM4600 DeltaRange® electronic scale. Orbital carapace length was determined by measuring the distance from the mid-posterior of the carapace to the stem of the eyestalk using a vernier caliper. Survival rate was calculated by recording the number of deceased marron in each tank. Molt increments and intermolt periods were determined by the number of molted marron, their post-molt weights, and the time intervals between two successive molts for each tank. Growth performance parameters, including specific growth rate (SGR; % per day), weight gain (WG; %), biomass increment (BI; %), survival rate (%), and molting rate (MR; %), were calculated using the formulas described by Jussila and Evans (1998) and Saputra and Fotedar (2021).

$$\text{Feed conversion rate } (FCR) = 100 \times \text{Total feed given}/(\text{Total Final weight} - \text{Initial weight})$$

$$\text{Molt increment } (MI) = 100 \times (Wmolt_{n+1} - Wmolt_n)/Wmolt_n$$

$$\text{Intermolt period } (Tim) = Date_{n+1} - Date_n$$

$$\text{Molting rate } (MR) = 100 \times (\text{Number of molted marron}/\text{Total number of marron})$$

Where:
N: the number of molts
W(g): weight of the marron

Total and differential hemocyte count

Hemolymph was extracted from 18 marron (one marron per tank), from the fifth pereopod using a 1 mL sterile syringe (27-gauge). The syringe held 0.2 mL of anticoagulant solution (Tulsankar et al. 2022). An aliquot of 50 µL was analyzed with a Neubauer-improved hemocytometer (Paul Marienfeld GmbH & Co.KG, Germany) to calculate the Total Hemocyte Count (THC). Cells were observed in both grids using a Leica DM2500 microscope at 20X magnification.

To perform a differential hemocyte count, one drop of diluted hemolymph was spread onto a glass microscope slide and left to dry in the open air. The slides were fixed in 70% methanol for 5 min. Following this, the slides were immersed in a May-Grünwald stain solution, followed by a Giemsa stain solution for ten minutes per stain. Granular Cells (GCs), Hyaline Cells (HCs), and Semi-Granular Cells (SGCs) were identified following the methodology described by Sang et al. (2009). The percentages of GCs, HCs, and SGCs were calculated by counting 200 cells on each slide. The formulas were described by Sang et al. (2009).

$$THC = (\text{Hemocyte cells counted} \times \text{Dilution factor} \times 1{,}000)/0.1\ mm^3$$

$$GC\ (\%) = 100 \times \left(\frac{\text{Number of granular cells}}{\text{Total hemocyte cells counted}}\right)$$

$$HC\ (\%) = 100 \times \left(\frac{\text{Number of hyaline cells}}{\text{Total hemocyte cells counted}}\right)$$

$$SGC\ (\%) = 100 \times \left(\frac{\text{Number of semi} - \text{granular cells}}{\text{Total hemocyte cells counted}}\right)$$

Lysozyme activity assay

Hemolymph was sampled from 18 marron (one marron per tank) with a sterile syringe (27-gauge) containing an anticoagulant solution (hemolymph-anticoagulant ratio= 1:1). Diluted hemolymph samples were placed into 1.5 mL Eppendorf tubes and stored at -80°C for further analysis. Lysozyme activity was measured through a turbidimetric assay following the procedure outlined by Tulsankar et al. (2022), with some adjustments. A bacterial suspension was prepared using a sample of 5 mg of *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) mixed with 20 mL of Phosphate-Buffered Saline (PBS). The 100 µL duplicated hemolymph samples were pipetted into a 96-well plate. After incubation at room temperature (25°C) for 15 min, 100 µL aliquots of the bacterial suspension were added to the wells and thoroughly mixed. The 96-well plate was placed in an MS212 reader (TiterTek Plus, Austria), and the absorbance was recorded at 450 nm every 2 min over a 20-min period. Lysozyme activity was presented as EU/mL.

Protease activity assay

One marron from one tank was randomly chosen to collect a hepatopancreas sample, which was stored at -80°C for protease assays. A sample of 0.3 g hepatopancreas was weighed and homogenized in 3 mL of PBS. The homogenate was centrifuged at 10,000 x g at 4°C for a duration of 20 min. After centrifuging, the supernatant was used to measure protease activity, and tissue debris at the bottom was discarded. Protease activity was determined

using the commercial Protease Assay Kit (Thermo Scientific™ Pierce™, USA). In summary, 5 mL of BupH™ borate buffer was used to dissolve a sample of 10 mg of lyophilized succinylated casein to prepare the succinylated casein solution. In a 96-well microplate, 100 µL of the succinylated casein solution was added to each well, followed by 50 µL of the supernatant samples in duplicate. The microplate was incubated for 20 min at room temperature before 50 µL of 2,4,6-trinitrobenzene sulfonic acid (TNBSA) working solution was added to each well. After an additional 20-min incubation period at room temperature, the absorbance was recorded at 450 nm using an MS212 reader (TiterTek Plus, Austria). Protease activity was presented as U/mg protein.

Condition indices

One marron from each tank was used to measure the organosomatic indices using the method outlined by Fotedar (1998). Marron tail muscle and hepatopancreas were removed and weighed to calculate the wet tail muscle index (Tiw) and wet hepatosomatic index (Hiw). The same tail muscle and hepatopancreas were dried in an oven at 80°C for 24 h to determine dry tail muscle index (Tid) and dry hepatosomatic index (Hid). The moisture content of tail muscle (TM) and hepatopancreas (HM) was determined using the following formulae:

$$TM = 100 \times (WTwet - WTdry)/WTwet$$
$$HM = 100 \times (WHwet - WHdry)/WHwet$$
$$Tiw = WTwet \times 100/W$$
$$Hiw = WHwet \times 100/W$$
$$Tid = WHdry \times 100/W$$
$$Hid = WHdry \times 100/W$$

Where:
W: marron weight (g)
WT: weight of tail muscle (g)
WH: weight of hepatopancreas (g)

Feed water stability test

Pellet water stability was evaluated using the method outlined by Sudaryono (2001). Approximately 4 g of feed pellets from each test diet were placed into glass beakers filled with 50 mL of tap water, with three replicates per diet. The pellets were subjected to immersion times of 30 min, 60 min, 2 h, and 8 h. After the allocated water immersion time, the water was siphoned off, and the remaining pellets were dried at 60ºC for 24 h, followed by cooling in a desiccator and recording sample weights. Feed stability was determined by calculating the percentage of dry matter loss for each diet after immersion.

Statistical analysis

Results are presented as the mean ± the Standard Error (SE). Prior to analysis, the Shapiro–Wilk test and Levene's test were used to assess the normality and homogeneity of the data. Data was transformed to normal before performing the least significant difference analysis. Statistically significant variations in parameters were determined by using a one-way ANOVA and Tukey's HSD post hoc test with significance defined as P values < 0.05. Data were examined using SPSS 26.0.

RESULTS AND DISCUSSION

Water quality parameters

No significant difference (P>0.05) was observed in water quality parameters across tanks (Table 2). The temperature (18.39 to 18.89°C), pH (7.04 to 7.41), DO (6.59 to 7.10 mg/L), and ammonia NH_3/NH_4^+ concentration (<0.050 mg/L) remained within the optimum ranges specified for culturing marron (Mills et al. 1994).

Growth performance, intermolt period, and survival rate

The mean wet body weight of marron increased from 2.22±0.04 to 3.37±0.08 g, and mean carapace length increased from 14.28±0.23 to 17.89±0.20 mm. Mean final body weight differed significantly by diet, but there was no difference in carapace length among the treatments (Table 3).

Marron fed D1 exhibited the highest WG, which was significantly higher than those fed other diets, with the exception of D4 (Table 4). Marron fed D1 had the highest BI and SGR. By contrast, the lowest values were recorded in D5. There was no statistically significant difference (P>0.05) in marron FCR among the diets. The survival rate for marron was greater than 97% regardless of diet. However, marron fed D5 exhibited the lowest MI. Tim and MR were not influenced by diet.

Immune parameters and digestive enzyme activity

Marron fed D1 and D4 had the highest lysozyme activity (P<0.05), followed by D2, D3, and D6, while marron fed D5 exhibited significantly lower lysozyme activity (Figure 1.A). A similar trend was observed in THC among treatments. Marron fed D5 and D6 had the lowest THC among the groups (Figure 1.B). There were no significant variations (P>0.05) in the proportion of granular or hyaline cells (Figures 1.C-D), although marron fed D1 had the lowest proportion of semi-granular cells and marron fed D6 the highest (Figure 1.E). Protease activity in marron fed D2, D3, and D4 was significantly higher (P<0.05) than in marron fed D5 (Figure 1.F).

Condition indices

There were no significant differences (P>0.05) in the Hiw of marron among diets (Table 5). Marron fed D1 and D3 had significantly lower Hid than those fed D2 and D4. The opposite trend was observed in HM; marron fed D1 had the highest HM, while marron fed D2 exhibited the lowest value. There were no significant differences observed in Tiw, Tid, or TM among diets.

Table 2. Water quality parameters of treatment tanks (mean of the three replicates ±SE)

Water quality	Treatment					
	D1	D2	D3	D4	D5	D6
Temperature (°C)	18.59±0.24	18.57±0.28	18.40±0.17	18.39±0.15	18.89±0.17	18.65±0.12
pH	7.04±0.09	7.41±0.05	7.32±0.06	7.30±0.14	7.16±0.18	7.34±0.14
DO (mg/L)	6.95±0.30	6.59±0.43	6.86±0.00	7.10±0.10	6.64±0.32	6.81±0.09
Ammonia NH_3/NH_4^+ (mg/L)	0.025±0.00	0.037±0.012	0.037±0.012	0.025±0.025	0.025±0.000	0.025±0.000

Note: D1: Fish meal and fish oil; D2: Fish meal and black soldier fly larvae oil; D3: Poultry by-product meal and black soldier fly larvae oil; D4: Defatted black soldier fly larvae meal and black soldier fly larvae oil; D5: Lupin meal and black soldier fly larvae oil; and D6: Lupin meal, black soldier fly larvae oil and fish oil

Table 3. Body weight and length per marron before and after the feeding trial

Diet treatments	Weight (g)		Orbital carapace length (mm)	
	Initial	Final	Initial	Final
D1	2.35±0.07	3.90±0.10b	14.48±0.28	18.86±0.32
D2	2.41±0.05	3.48±0.14ab	14.15±1.34	17.63±0.35
D3	2.12±0.04	3.11±0.10a	14.40±0.35	17.58±0.13
D4	2.19±0.02	3.39±0.22ab	14.38±0.15	18.05±0.81
D5	2.11±0.06	3.16±0.10a	14.28±0.62	17.92±0.61
D6	2.14±0.19	3.18±0.21a	14.00±0.55	17.34±0.43
P values	0.198	0.034	0.996	0.384

Note: Mean value (±SE) of three replicates. Letters (a and b) in the same column indicate statistically significant differences, as assessed by a one-way ANOVA followed by a Tukey's HSD post hoc test ($P<0.05$). D1: Fish meal and fish oil; D2: Fish meal and black soldier fly larvae oil; D3: Poultry by-product meal and black soldier fly larvae oil; D4: Defatted black soldier fly larvae meal and black soldier fly larvae oil; D5: Lupin meal and black soldier fly larvae oil; and D6: Lupin meal, black soldier fly larvae oil and fish oil

Table 4. Growth parameters, intermolt period, and survival rate of marron by diet

Parameter	Treatment					
	D1	D2	D3	D4	D5	D6
WG (%)	77.12±4.27b	56.13±3.25a	53.09±4.75a	61.52±3.09ab	50.74±4.09a	54.02±3.55a
SGR (%/day)	0.66±0.02b	0.51±0.01ab	0.49±0.05ab	0.56±0.03ab	0.48±0.08a	0.52±0.06ab
BI (%)	64.36±3.06b	46.21±3.45ab	45.59±2.95ab	52.38±4.48ab	42.59±4.57a	49.85±5.66ab
FCR (%)	2.66±0.51	3.35±0.41	2.88±0.09	3.03±0.37	2.79±0.26	2.99±0.17
Survival (%)	98.66±1.33	97.33±1.33	98.66±1.33	98.66±1.33	98.66±1.33	98.66±1.33
MI (%)	37.35±1.93b	32.20±6.09ab	26.15±1.46ab	32.33±1.58ab	22.45±1.24a	29.30±1.62ab
Tim (days)	36.46±1.13	39.74±1.35	43.76±2.18	42.19±2.70	46.29±2.06	45.18±2.19
MR (%)	89.33±7.05	81.33±1.33	82.66±4.80	92.00±2.30	76.00±10.06	78.66±9.61

Note: Mean value (±SE) of three replicates. Letters (a and b) in the same row indicate significant differences, as assessed by one-way ANOVA followed by Tukey's HSD post hoc test ($P<0.05$). D1: Fish meal and fish oil; D2: Fish meal and black soldier fly larvae oil; D3: Poultry by-product meal and black soldier fly larvae oil; D4: Defatted black soldier fly larvae meal and black soldier fly larvae oil; D5: Lupin meal and black soldier fly larvae oil; and D6: Lupin meal, black soldier fly larvae oil and fish oil. WG: weight gain; SGR: specific growth rate; BI: biomass increment; FCR: feed conversion ratio; MI: molt increment; Tim: intermolt period; and MR: molting rate

Table 5. Condition indices in marron by diet at the end of the feeding experiment

Condition indices	Treatment					
	D1	D2	D3	D4	D5	D6
HM (%)	79.33±0.71c	69.10±1.30a	76.45±1.39bc	70.65±1.26ab	74.44±1.83abc	76.52±1.32bc
TM (%)	83.14±0.82	81.43±0.50	82.22±0.75	82.38±0.55	82.82±0.30	83.06±0.84
Hiw (%)	5.92±0.49	6.95±0.35	7.67±0.84	7.17±0.53	6.30±0.13	7.00±0.28
Hid (%)	1.26±0.15a	2.15±0.15c	1.28±0.18a	2.09±0.10bc	1.53±0.08abc	1.50±0.08ab
Tiw (%)	24.50±0.37	27.31±1.54	24.79±0.71	24.48±0.96	24.04±0.46a	24.28±0.81
Tid (%)	4.13±0.26	5.08±0.41	4.54±0.37	4.44±0.36	4.34±0.31	4.11±0.26

Note: Mean value (±SE) of three replicates. Letters (a, b, and c) in the same row indicate significant differences, as assessed by a one-way ANOVA followed by a Tukey's HSD post hoc test ($P<0.05$). D1: Fish meal and fish oil; D2: Fish meal and black soldier fly larvae oil; D3: Poultry by-product meal and black soldier fly larvae oil; D4: Defatted black soldier fly larvae meal and black soldier fly larvae oil; D5: Lupin meal and black soldier fly larvae oil; and D6: Lupin meal, black soldier fly larvae oil and fish oil. HM: hepatopancreas moisture content; TM: tail muscle moisture content; Hiw: wet hepatosomatic index; Hid: dry hepatosomatic index; Tiw: wet tail muscle index; and Tid: dry tail muscle index

Table 6. Water stability (% dry matter weight loss) by diets and immersion time

Period	D1	D2	D3	D4	D5	D6
30 min	$_{1}8.79\pm0.11^{a}$	$_{1}8.85\pm0.20^{a}$	$_{1}10.80\pm0.04^{bc}$	$_{1}10.16\pm0.45^{ab}$	$_{1}12.39\pm0.58^{d}$	$_{1}12.24\pm0.18^{cd}$
60 min	$_{1}10.21\pm0.81^{ab}$	$_{1}8.99\pm0.15^{a}$	$_{2}11.87\pm0.13^{b}$	$_{12}11.25\pm0.20^{b}$	$_{2}16.13\pm0.21^{c}$	$_{2}14.25\pm0.74^{c}$
2 h	$_{1}10.31\pm0.20^{a}$	$_{2}10.35\pm0.03^{a}$	$_{3}12.52\pm0.03^{b}$	$_{2}12.56\pm0.0.13^{b}$	$_{2}17.31\pm0.21^{d}$	$_{2}14.92\pm0.29^{c}$
8 h	$_{2}13.42\pm0.13^{a}$	$_{3}13.49\pm0.26^{a}$	$_{4}15.29\pm0.16^{b}$	$_{3}15.45\pm0.35^{b}$	$_{3}19.39\pm0.09^{c}$	$_{3}18.66\pm0.26^{c}$

Note: Mean value (±SE) of three replicates. A one-way ANOVA and a multiple comparisons Tukey's HSD post hoc test determined significant differences at P < 0.05. Superscript letters (a, b, c) in the same row denote significantly different means for different test diets (*P*<0.05). Subscript numbers (1,2,3 and 4) indicate significantly different means at different time periods (*P*<0.05). D1: Fish meal and fish oil; D2: Fish meal and black soldier fly larvae oil; D3: Poultry by-product meal and black soldier fly larvae oil; D4: Defatted black soldier fly larvae meal and black soldier fly larvae oil; D5: Lupin meal and black soldier fly larvae oil; and D6: Lupin meal, black soldier fly larvae oil and fish oil

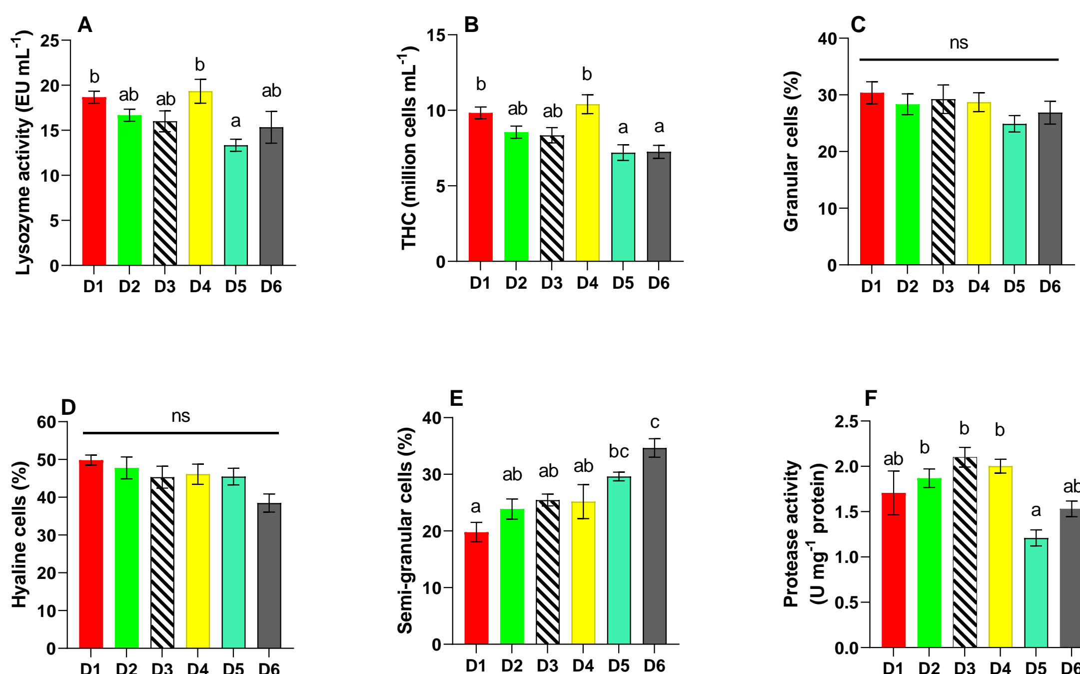

Figure 1. Immune responses of marron by diet, as measured by A. Lysozyme activity, B. THC, C. Granular cells, D. Hyaline cells, E. Semi-granular cells, and F. Protease activity. The mean value (±SE) of three replicates is shown. Bars with different letters denote significant differences (*P*<0.05) as assessed by a one-way ANOVA followed by a Tukey's HSD post hoc test. THC: Total Hemocyte Count; D1: Fish meal and fish oil; D2: Fish meal and black soldier fly larvae oil; D3: Poultry by-product meal and black soldier fly larvae oil; D4: Defatted black soldier fly larvae meal and black soldier fly larvae oil; D5: Lupin meal and black soldier fly larvae oil; and D6: Lupin meal, black soldier fly larvae oil and fish oil

Feed water stability

The percentage of dry matter loss ranged from 8.79-12.39% during the first 30 min and increased significantly after 8 h of post-water immersion (13.42-19.39%) in all diets (Table 6). D1 and D2 were the most stable diets, with no difference in dry matter loss from the first 30 min to 2 h of immersion. By contrast, D5 and D6 had the least stable pellets; their dry matter weight loss increased significantly (*P*<0.05) from 12.39% to 19.39% with increasing water immersion duration.

Discussion

This is the first study to evaluate the impacts of using BSFL oil as a replacement for FO in marron raised on different protein sources. The results show that BSFL oil can be an alternative lipid source for FO when marron are fed animal protein sources such as FM, PBM, and DBSFM; however, FO is still required when LM is the sole dietary protein source. These results align with Wang et al. (2021), who found no adverse impacts on the growth of white-leg shrimp (*Litopenaeus vannamei*) when up to 60% of FM was replaced with DBSFLM. Positive growth outcomes for crayfish species fed a diet of PBM have been described in *C. quadricarinatus* (Saoud et al. 2008; Garza de Yta et al. 2012; Eroldoğan et al. 2022), red swamp crayfish (*Procambarus clarkii*) (Yang et al. 2022), and *P. leniusculus* (Fuertes et al. 2013). When combined with a protein source of animal origin, marron fed BSFL oil meet

essential fatty acid requirements. Studies have shown that incorporating BSFL oil into the diets of fish has no negative effects on growth (Li et al. 2016; Hender et al. 2021; Sudha et al. 2022). Hender et al. (2021) demonstrated that feeding *L. calcarifer* a diet with 30% BSFL protein and BSFL oil resulted in similar growth to FM- and FO-based diets. Similarly, Xu et al. (2021) reported that diets containing BSFL oil enriched with n-3 Highly Unsaturated Fatty Acids (HUFAs) improved growth and health status in mirror carp (*C. carpio* var. *specularis).* In the present study, D2-fed marron exhibited similar growth to D1, suggesting that BSFL oil can replace FO and canola oil in marron diets. FO mixed with canola oil is the most common oil combination in the marron industry because the addition of canola oil decreases the total feed cost. While FO is rich in essential omega-3 fatty acids (Alhazzaa et al. 2018), the exclusive use of FO can be prohibitively expensive and it is not readily available in large quantities. By contrast, canola oil is more cost-effective and still delivers most of the essential fatty acids because of its high concentration of unsaturated fatty acids, particularly oleic acid and linoleic acid (El-Nakhlawy and Shiboob 2011).

The D5 diet resulted in the lowest WG, SGR, and BI in marron, similar to our earlier findings, as described in Molina-Poveda et al. (2013) and Dao et al. (2024) (unpublished), who found reduced growth and digestibility in *L. vannamei* fed a diet containing 75-100% LM as a substitute for FM. The decreased growth may be due to the presence of anti-nutritional factors, low digestibility, and imbalanced amino acids in an LM-based protein diet (Sudaryono et al. 1999b; Glencross 2001). Moreover, the lack of n-3 HUFAs and high lauric acid content in the BSFL diet may have contributed to the observed effects. Xu et al. (2021) found that feeding *C. carpio* var. *specularis* on diets containing 50% or more n-3 HUFA-enriched BSFL oil resulted in improved growth and immune responses. In contrast, Li et al. (2016) showed that *C. carpio* var. *Jian* fed a diet containing BSFL oil not enriched with n-3 HUFAs exhibited no significant differences among diets, suggesting that the addition of n-3 HUFA was responsible for the improved growth. Additionally, animal sources of protein, such as FM, PBM, and DBSFM, meet all the amino acid requirements of marron, whereas lupin protein fails to do so. The amino acids lysin and methionine are probably the most limited in LM (Lim and Dominy 1991; Nunes et al. 2014), but these acids have not been analyzed in this study. Studies have shown that supplementing diets with methionine and lysin can improve the growth of *P. clarkii* (Tan et al. 2018), black tiger shrimp (*Penaeus monodon*) (Nwanna et al. 2019), and kuruma shrimp (*Marsupenaeus japonicus*) (Alam et al. 2005). However, when FO is added to a lupin-based diet, the synergetic effects of essential fatty acids available in FO appear to overcome the lack of essential amino acids in LM. Another possible explanation is that the fatty acids available in FO can only suppress the need for amino acids requirements in marron fed animal proteins. However, this explanation warrants further research.

In addition, the poor growth of marron fed D5 could be related to the low MI and high Tim (22.45% and 46.29 days, respectively), as the growth rate of crustaceans can be reflected in molt frequencies and molt increments (Jussila and Evans 1996; Reynolds 2002). Tim values in this study ranged from 36.46 to 46.29 days, which is similar to previous research by Pattikawa and Wenno (2014), who reported Tim values from 30.58 to 45.50 days when marron were reared at 19-25°C. As ambient water temperature increases, the molt number also increases (Morrissy 1990; Rouse and Kartamulia 1992). For example, marron did not molt when maintained in temperatures below 12°C, but the intermolt period increased to 2-3 months at 22-24°C. The water temperature in this study was maintained at 18.40-18.89°C, which could explain the higher intermolt periods.

The percentage of pellet dry matter loss varied significantly among the treatments, which could be linked to marron growth. Sudaryono et al. (1999a) demonstrated that pellet water stability decreased significantly with an increased level of LM in *P. monodon* diets because LM contains high fiber, including non-starch polysaccharides (Abraham et al. 2019), which could reduce the water stability of the pellets. Consistent with these findings, a study on *P. monodon* revealed that diets containing 71% LM exhibited the lowest water stability (Sudaryono et al. 1995). Jussila and Evans (1998) reported that marron fed stable pellets had a shorter Tim and higher WG compared to those fed unstable pellets. In the present study, diets containing LM (D5 and D6) had the lowest water stability. The percentage of dry matter loss increased from 12% after 30 min to 19% after 8 h, which aligns with the results of Sudaryono et al. (1995) and Sudaryono (2001). The feeding behavior of marron means that they ignore the remains of disintegrated pieces from the unstable pellets. Hence, low feed ingestion resulting from poor water stability could lead to the decreased growth seen in marron fed D5. D1 and D2 had the most stable pellets, followed by D3 and D4, while Saputra and Fotedar (2021) reported FM pellets were less stable than diets containing PBM and LM.

Although marron fed a diet that combined LM and BSFL oil (D5) exhibited decreased growth, their growth rate increased when FO was included along with BSFL oil (D6), indicating that the beneficial synergetic effects of the essential fatty acids in FO can mitigate the deficiency of essential amino acids in LM-based protein. Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LOA), and α-linolenic acid (LNA), some of the fatty acids found in FO, are important for growth and reproduction (Elovaara 2001, 2009; Araújo et al. 2019). The dietary requirements for essential fatty acids vary among species and are influenced by environmental conditions, such as freshwater, estuarine, or marine habitats (Glencross 2009). For example, marine shrimp species have higher polyunsaturated fatty acids (PUFAs) requirements than freshwater species (Kanazawa et al. 1977). Lim and Dominy (1997) reported that juvenile *L. vannamei* fed a diet containing menhaden oil, a source of EPA and DHA, exhibited significantly higher growth rate than those fed diets based on soybean oil, canola oil, or linseed oil. However, other studies found increased growth

rate in *C. quadricarinatus* fed a diet containing soybean oil, similar to the diets containing FO and linseed oil (Li et al. 2011; Chen et al. 2021a). Linseed oil and soybean oil, which have abundant LOA and LNA (Thompson et al. 2010), likely have the appropriate nutritional values to satisfy the essential fatty acid requirements of *C. quadricarinatus* (Thompson et al. 2010; Chen et al. 2021a).

The high survival rates of more than 97% in this study, with no differences among the dietary treatments, were similar to the results of a previous study by Saputra and Fotedar (2021). The high survival rate is likely explained by the lack of post-molt cannibalism because the marron were separated into individual compartments. The FCR ranged from 2.66 to 3.35, which is similar to the FCR values obtained in a previous study on marron fed various protein sources, ranging from 2.26 to 3.79 (Saputra and Fotedar 2021). Similarly, the SGR and WG (0.48-0.66% per day and 50.74-77.12%, respectively) were comparable to previous results from Saputra et al. (2019). However, the SGR values were lower than those reported by Fotedar (2004), which ranged from 0.89 to 1.12% per day. This difference in growth can be attributed to the different rearing conditions, as Fotedar (2004) raised marron in ponds abundant with natural food. By contrast, the present study was conducted under laboratory conditions. In this study, the marron were individually housed in small plastic containers. Confined rearing environments are known to compromise growth, as confirmed by Jussila and Evans (1996), who found that increasing the volume of the sump tanks in intensive crayfish culture systems improved growth performance and biomass production. Increased water volume facilitates enhanced natural food production, which seems to be even more important for the growth of marron than the feed protein composition. Chen et al. (2021a) reported that when *C. quadricarinatus* were fed different lipid sources, SGRs of 0.93-1.39% per day and WGs of 68.5-118% were achieved. Similarly, *P. clarkii* fed a diet that included 30% protein and 7% lipid exhibited an SGR of 3.14% per day and a WG of 483.38% (Xu et al. 2013). These investigations confirm that crayfish growth rates are species-specific and that marron exhibit the lowest growth rates among farmed crayfish species (Rigg et al. 2020; Haubrock et al. 2021).

Hemocytes are crucial for the health of crustaceans, and the total hemocyte count and differential hemocyte count are key indicators for assessing immune system function (Söderhäll 2016). Hemocyte composition differs among species (Hose et al. 1990). Inmarron, hyaline cells are the most abundant circulating hemocytes, followed by granular cells and semi-granular cells (Prastowo et al. 2020). In *C. quadricarinatus*, the predominant circulating hemocytes are semi-granular cells and granular cells, which normally represent 65-85% and 15-35% of the hemocyte population, respectively (Li et al. 2018). In this study, different dietary protein and lipid sources significantly impacted lysozyme activity and hematological parameters. Marron fed D1 and D4 showed the highest lysozyme activity and THC, which aligns with Foysal et al. (2021) who reported marron fed BSFL exhibited an increased THC. Nogales-Mérida et al. (2019) suggest that BSFL not only promotes growth but also acts against pathogenic bacteria due to biologically active peptides, such as antimicrobial peptides (AMPs) (Park et al. 2014, 2015), chitin, and chitosan (Caligiani et al. 2018). These peptides have antioxidant properties and are capable of stimulating innate immune responses in crayfish. In addition, lauric acid, the predominant fatty acid in BSFL oil, has exhibited potential antimicrobial activity (Lieberman et al. 2006; Park et al. 2014). The *L. vannamei* fed DBSFLM showed increased survival rates after exposure to *Vibrio parahaemolyticus* (Richardson et al. 2021).

Digestive enzyme activity can also impact marron growth. The highest values of protease activity were observed in D3 and D4, as animal-based protein is more easily digested than plant-based protein (Wang et al. 2021). Irrespective of digestibility, the ingestion rate of marron is less dependent on various dietary protein, and lipid sources as marron are omnivores and can ingest a variety of food sources, including aquatic plants, algae, insects, other crustaceans, and detritus from their natural environment (Mills et al. 1994; Fotedar 2004). Chitinase enzymes present in the digestive system of marron (Ceccaldi 1989) can break down the chitin of BSFL into smaller particles that are easier to digest (Tzuc et al. 2014), which increases its digestibility. Furthermore, chitin has been found to positively influence molting and digestion, as insects are a natural component of crustacean diets (Ceccaldi 1989; Zhang et al. 2014). Incorporating 20% chitin into the diet of freshwater prawn (*Macrobrachium tenellum*) enhanced their growth rate (Santos-Romero et al. 2017). By contrast, marron fed D5 exhibited the lowest protease activity, likely due to the alkaloids and proteinase inhibitors in LM. These anti-nutritional compounds are known to bind and inhibit digestive enzymes in the gastrointestinal tract (Council 2011), which results in reduced nutrient absorption and poor growth. Additionally, high quantities of soluble non-starch polysaccharides and oligosaccharides in LM-based diets (Sinha et al. 2011) could alter gastric emptying, gut physiology, and morphology. Molina-Poveda et al. (2013) found that *L. vannamei* fed diets composed of 75-100% LM exhibited significantly decreased ingestion rates and low weight gain.

The hepatopancreas is vital for absorbing nutrients and storing energy. It is highly sensitive to variations in nutrient availability and capable of quickly adjusting to fluctuations in nutritional conditions. For example, a study on *C. quadricarinatus* revealed that the hepatopancreas lipid and protein content decreased when crayfish experienced prolonged starvation Calvo et al. (2013). Hepatosomatic indices are good indicators of the nutrient status of various crayfish species (Jussila 1997, 1999). High Hiw and Hid indicate better nutrient conditions. In the present study, marron fed D2, a dietary combination of FM and BSFL oil (D2), had significantly higher Hid and lower HM than marron fed a diet based on FM and FO (D1), which suggests higher nutrient reserves in the hepatopancreas. This difference can be explained by the highly sensitive reactions of the hepatopancreas to nutritional deprivation and other overarching stressors. Similar results were observed in farmed marron, which have higher

hepatosomatic indices than wild marron (Jussila 1997). Farmed marron are fed more intensively than wild marron subject to fluctuations in the availability of natural foods. As a result, farmed marron exhibit increased energy storage in hepatopancreases (McClain 1995a,b), as shown by higher hepatopancreatic and tail muscle indices. Jussila and Evans (1998) and Jussila (1999) also reported that non-fed marron had lower hepatosomatic indices and higher HM than fed marron, which supports the finding that food deprivation affects the energy reserves of marron. Non-fed *C. quadricarinatus* were observed to have significantly lower hepatosomatic and tail muscle indices than fed crayfish during a 50-day starvation trial (Calvo et al. 2013). Marron tend to use energy reserves in the hepatopancreas to maintain their metabolism during periods of food deprivation. Therefore, starvation induced a decline in Hiw (Jones and Obst 2000). Furthermore, lauric acid is primarily used as an energy source over longer fatty acids and has been shown to reduce lipid storage (Belghit et al. 2019b). Dietary BSFL oil has also been shown to decrease lipid storage in the hepatopancreas of *C. carpio* var. *specularis* (Xu et al. 2021) and *S. salar* (Belghit et al. 2019a). Tail muscle index is another condition index that reflects the health status of marron (Jussila 1999). The test diets in this study did not alter the tail muscle indices (Tiw and Tid) or TM in marron, which is contradictory to the findings of Fotedar (2004), who reported that marron fed a diet including FM and FO had higher Tiw, but Hiw and Hid were similar among marron fed other diets. The difference may be due to different culturing environments or the duration of the study. The study by Fotedar (2004) was conducted in cultured ponds for a duration of 12 months, while the present study was conducted over a shorter period of ten weeks, which may be insufficient to see any changes to the tail muscle ofmarron. Past research has shown that chronic stress is reflected in the tail muscle before the hepatopancreas (Fotedar 1998).

In conclusion, marron fed animal-protein sources showed higher growth performance than marron fed a diet based on LM with BSFL oil, which exhibited reduced growth. However, supplementing this diet with FO improved the growth of marron. The combination of animal-based protein and BSFL oil also improves immunity and digestive enzyme activity. The hepatosomatic index and HM of marron are affected by the variation of protein and lipid sources among diets, while tail muscle index and TM are not. Marron shows acceptable growth and health performance when their diet includes animal protein sources along with oil extracted from BSFL.

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