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Effects of different dietary protein sources on the immunological and physiological responses of marron, *Cherax cainii* (Austin and Ryan, 2002) and its susceptibility to high temperature exposure

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ABSTRACT

A two phased feeding trial was conducted to evaluate the effects of alternative protein sources on the immunophysiological responses of marron. During the phase I, marron were fed with five alternative protein supplemented diets for 90 days, while in phase II, the same marron were exposed to elevated temperature (30 °C) and their immunophysiological responses were investigated post exposure. Five isoproteic (crude protein 30%) and isoenergetic diets were prepared by containing fishmeal, poultry by-product meal, feather meal, lupin meal, and meat and bone meal as the main protein source. A hundred and fifty juvenile marron (*Cherax cainii*) of the average weight 9.09 ± 0.21 g were randomly distributed into 15 tanks (three replicates per feeding treatments). In the Phase I, general immune response parameters, such as, total haemocyte count (THC), proportion of hyaline cells, neutral red retention time (NRRT), phagocytic rate (PR), heamolymph bacteraemia, and condition indices of marron were investigated. The highest ($P < 0.05$) THC among dietary protein sources was obtained in marron fed with PbM at the end of experiment. Marron fed with FeM protein sources resulted in the highest survival rate followed by PbM fed group. Longer microvilli length (3.83 ± 0.18 μm) was demonstrated in marron fed with PbM diet. Diets containing FM and PbM protein sources revealed significantly ($P < 0.05$) lower number of microvilli/group than diets containing FeM and LM. The results demonstrated that different dietary protein sources in the marron diets did not detect significant ($P > 0.05$) change of the condition indices throughout the experiment period, however highest Hiw and Hid was recorded in marron fed with PBM at day 45. The PR of marron fed dietary protein from PbM did not change significantly after temperature exposure. Increased NRRT, PR and haemolymph bacteraemia was observed with dietary feeding of FM at the end of the trial. However, results revealed that PbM could be an alternative protein source for culture of marron as reflected in terms of increased THC, longer microvillus length and improved susceptibility to high temperature exposure. Overall, result could serve as useful baseline data in developing cost effective potential diets for marron aquaculture.

1. Introduction

As disease outbreaks are still continuing to be the major threat to the aquaculture industry, the use of conventional antibiotics to eliminate pathogens have been significantly reduced [1]. Simultaneously, the concert for depleting fishmeal supply is on the rise [2,3] which constitutes one of the major protein ingredients in crustacean aquaculture. In past, studies have demonstrated the potential of total or partial substitution of fishmeal with alternative protein sources in crustaceans [4] but with variable success. Previous research outcomes revealed that several plant and animal based-protein sources have the potential to substitute fishmeal protein in the diets of some decapod

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crustaceans [5,6]. Furthermore, the increase of non-fishmeal inclusion in the diets was observed to have detrimental effects on the physiological responses of signal crayfish, *Pascifastacus leniusculus* [7,8] and black tiger prawn, *Penaeus monodon* [9]. However, no studies have investigated the changes in the immunophysiological responses of the marron fed diets containing alternative protein sources to fishmeal.

Marron, *Cherax cainii*, an esteemed aquaculture commodity; are an iconic freshwater species to Western Australia [10,11] which generated AUD 1.5 million per year through its farming [12]. Over and above, the significant increase in commercial demand of marron has led to boost the research on improvement of immune responses to tackle down the disease outbreaks. Innate immunity in crayfish is similar to other crustaceans, which involves circulating blood cells or haemocyte to eliminate both pathogen and particles from the body through cellular activity [13–15]. Several attempts to improve innate immunity have been reported in previous studies in crayfish [16–18] and in tiger prawn [19]. The immunophysiological responses measured by alterations in total haemocytes counts (THC), differential haemocyte counts (DHC), neutral red dye retention technique (NRRT), phagocytic rate (PR), bacteraemia and condition indices could be used as indicators of immune competence of several crustaceans [16,20,21], including marron, *C. tenuimanus* [17] in order to understand the underlying mechanism of improved health status. Apart from this, research investigations on improvement of the clinical signs of the digestive system has also been carried out to evaluate the health status of cray fish [22,23].

Marron have gained significant attention as a potential candidate species for prospective growth of aquaculture owing to its large harvest size (up to 2 kg), higher price, non-burrowing behavior, simple life cycle and ease of live transport [10,11,24]. Consistent efforts are in progress to translocate crayfish species from natural habitat to other locations for globalization, therefore the effects of high temperature on physiological responses necessitates further investigations. Rouse & Kartamulia (1992) investigated the temperature tolerance of marron, *Cherax tenuimanus* before its domestication process in the US [10]. Numerous investigations on effects of temperature in crayfish physiological responses have been carried out [25–31]. However, the effects of temperature exposure on the immunophysiological responses of marron following supplementation with different dietary protein source are yet to be revealed.

Therefore, the aim of this study was to evaluate the efficacy of dietary protein sources on the immunophysiological responses and gut micrograph of marron. In addition, the susceptibility to the high temperature exposure of marron was delineated in terms of immunophysiological responses.

2. Material and methods

2.1. Test diets

Five isoproteic (crude protein 30%) and isoenergetic diets were prepared by containing fishmeal, poultry by-product meal, feather meal, lupin meal, and meat and bone meal as the main protein source. All the ingredients for experimental diets were supplied by Specialty Feeds Pty. Ltd, Western Australia. The diets were formulated using Feed LIVE software version 1.52 from Live Informatics Company Limited, Thailand (Table 1).

To obtain finer ingredients, the raw materials were weighed, grounded and sieved through a 100 μm mesh strainer. All ingredients were then mixed and added with a distilled water to increase the moisture level to facilitate the pelletizing process. The dough was then processed using a pellet extruder, and dried overnight at 60 °C in the oven. The protein content of feeds was guaranteed by altering the formulation until meeting the minimum value of ± 30%. All experimental diets were stored in labelled plastic containers in the feed storage unit until used.

Table 1
Feed ingredients and proximate composition (% dry weight).

	Diets				
	FM	PbM	LM	FeM	MbM
Ingredients (% dry weight)					
Fish Meal[a]	30.15	0.00	0.00	0.00	0.00
Feather Meal[a]	0.00	0.00	0.00	22.00	0.00
Lupin Meal[a]	0.00	0.00	36.40	0.00	0.00
Poultry by-Product Meal[a]	0.00	29.50	0.00	0.00	0.00
Meat and Bone meal[a]	0.00	0.00	0.00	0.00	24.30
Soybean Meal [b]	10.80	10.80	21.00	15.65	16.60
Wheat (10 CP) [b]	48.13	50.88	28.67	48.31	43.13
Corn/wheat starch[b]	4.80	4.80	4.01	5.50	5.50
Betacaine[b]	1.20	1.20	3.50	1.02	3.50
Cod liver oil[b]	4.20	2.10	3.45	4.80	4.00
Calcium carbonate[b]	0.02	0.02	0.02	0.02	0.02
Ascorbic Acid[b]	0.05	0.05	0.05	0.05	0.05
Vitamin premix[b]	0.15	0.15	0.15	0.15	0.15
Cholesterol[b]	0.25	0.25	0.25	0.25	0.25
Casein[b]	0.25	0.25	2.50	2.25	2.50
Proximate composition (g Kg^{-1} dry weight basis)					
DM%[c]	89.07	87.86	88.46	87.85	87.26
Ash%	8.24	4.05	6.21	3.18	10.13
GE MJ/kg	18.63	18.57	18.59	18.39	18.34
CP%	30.13	30.88	30.84	30.46	30.18
Lipid%[c]	7.17	7.53	7.26	7.16	7.63
Fiber%[c]	1.22	1.35	1.29	1.07	1.27

DM (Dry matter); GE (Gross energy); MJ (Mega joule); CP (Crude protein).
[a] Glen Forest Specialty Feeds, Western Australia.
[b] Talloman, Freemantle, Western Australia.
[c] Data were obtained from database.

2.2. Experimental animals and design

Two hundred marron juveniles 0 + years old, average wt. (9.09 ± 0.21 g) were procured from Aquatic Resource Management Pty. Ltd., Manjimup, Western Australia and transported to Curtin Aquatic Research Laboratory (CARL), Technology Park, Curtin University, Western Australia. Marron juveniles were then acclimated for two weeks in three cylindrical holding tanks (200 l capacity), which were equipped with continuous aerations and an adequate number of hides in the form of PVC pipes. Each acclimation tank was equipped with eight PVC pipes (55 mm in diameter, 150 mm long) and a filtration unit. Marron were fed with a commercial marron diet (Glenn Forrest, Australia) with a composition of 28% crude protein, 9% crude fat and 5% crude ash, calcium (1.5%), phosphorous (1%), and digestion energy 11 MJ/kg at 3% of body weight during the acclimation period.

Survived, marron juveniles were selected and transferred into100 L cylindrical plastic tanks (80 cm diameter and 20 cm height) in an outdoor purposed built experimental area at CARL. A continuous air supply was provided and medium pressure was set on in each tank to ensure the proper oxygen supply during feeding experiment. Freshwater in each tank was continuously filtrated using fluval 205 filters (Hagen, USA) at a rate of approximately 2 L min^{-1}. An automatic submersible heater (Sonpar®, Model: HA-100, China) was set at 20 °C in each tank to maintain constant temperature. Each tank was stocked with 10 marron juveniles and was kept individually in a modified 750 mL plastic container to provide shelter and avoid cannibalism during the molting. In total, 15 experimental tanks were used as all treatment groups were triplicated. Marron were fed with five different diets (Table 1) at the rate of 3% of body weight, once per day for 90 days. This feeding rate was considered to be above the satiation feeding level for marron [16]. Before next feeding, uneaten feeds and faeces were siphoned out and sufficient freshwater was replenished to avoid water quality degradation. Physico-chemical characteristics of water were analyzed in terms of total ammonia and nitrite using calorimeter PR 1890, USA, weekly basis; temperature and pH using a digital pH/mV/C meter, Cyberscan

pH300, Eutech instruments Singapore, daily basis; and dissolved oxygen was measured using a digital DO meter SM600, Milwaukee, Romani, daily basis [18]. All the immune parameters such as THC, proportion of hyaline cell % (DHC), bacteraemia and condition indices were analyzed at the commencement of the experiment (0 day), in the middle (45th day) and at the termination of the experiment (90^{th} day) while NRRT and PR were determined on the commencement (0^{th} day) and termination (90^{th} day) of the experiment during Phase-I.

2.3. Total and differential haemocyte count

Prior to the hemolymph sampling, two sets of fifteen 1.5 mL and 0.2 mL tube were labelled. The 0.2 mL tubes were loaded with 50 μL of anticoagulant containing 0.5 M NaCl, 0.1 M Glucose, 0.03 M Trisodium Citrate, 0.026 M Citric Acid, and 0.01 M Disodium EDTA for THC analysis. Hemolymph samples were withdrawn by puncturing a sterile syringe (23G) into the fifth walking leg. Samples were then transferred into labelled 1.5 mL tube. Using a 200 μL micropipette, a 50 μL of hemolymph was transferred into 0.2 mL microtube for THC analysis. Another two sets of 50 μL of hemolymph were smeared into microscope slide for DHC analysis and inoculated to the nutrient agar for bacterial load analysis.

THC was determined using a stage haemocytometer (Reichert, USA) under 100 x magnification. Haemocytes were counted in both grids and the mean was used as the THC prior to the haemocyte calculation, samples were mixed thoroughly using vortex (IKA MS1 Minishaker, USA). Total haemocyte counts were calculated using following equation;

$$\text{THC} = (\text{cells counted} \times \text{dilution factor} \times 1000) / \text{volume of the grid}(0.1\ \text{mm}^3).$$

The DHC was determined by measuring the proportion of hyaline cells in the sample as described previously [32]. Fifty microliter of hemolymph was smeared onto a glass microscope slide, then air dried and fixed in 70% methanol for 10 min. The fixed smears were stained with May-Grunwald and Giemsa stains for 10–15 min each and then mounted with coverslips. The proportion of hyaline cells were determined by counting approximately 200 cells on each slide using the microscope (Motic, Taiwan) with 100-fold magnification employing the following equation:

$$\text{DHC} = (\text{Number of different haemocyte cells type} / \text{Total haemocyte cells counted}) \times 100$$

2.4. Neutral red retention time (NRRT) assay

The neutral red retention time (NRRT) assay was determined by using an established protocol [33] with some modifications. The neutral red stock solution was prepared by dissolving 20 mg of neutral red powder in 1 mL dimethyl sulphoxide. NRRT working solution was prepared by diluting 10 μL of stock solution in 5 mL sterile saline water.

Prior to the haemolymph collection, a sterile 1.5 mL microtube was filled with 200 μL of sterile saline water. A similar amount of fresh haemolymph was diluted into the microtube and mixed thoroughly. A 50 μL of aliquot was then smeared into Poly-L-lysine glass slide (Thermo fisher Scientific, US). Mounted-slide was then incubated in a light proof chamber for 15 min with a temperature of around 10 °C to allow the haemolymph to adhere. Neutral red working solution (50 μL) was then added to the haemolymph smear and covered with a 20 × 40 mm cover slip before being returned to the incubation chamber. The NRRT was determined by observing the portion of haemocyte which had lost neutral red dye from its lysosomes every 15 min. Time taken for 50% of the cells to lose dye was recorded as the end point of the assay.

2.5. Phagocytic rate

The effects of the feeding experiment on the phagocytic rate of marron were assessed based on an established protocol [34,35]. Fresh hemolymph (40 μL) was smeared on a Poly-L-Lysine glass slide (Thermofisher Scientific, US). The slides were then incubated to dry haemolymph. Meanwhile, zymosan (Sigma Aldrich, Z4250) working solution was prepared by dissolving 0.0125 g of zymosan powder in 25 mL of sterile seawater. Dry haemolymph samples in glass slide were then treated with a similar amount of zymosan working solution (40 μL) and air dried. Samples were treated using a 10% formaldehyde solution (sea water solvent) for 20 min. Subsequently, the glass slides were transferred to Giemsa solution for 20 min of incubation for cell staining. Phagocytic rate was determined using the following equation:

$$\text{Phagocytic rate(PR)} = (\text{phagocytic haemocyte/total adhered haemocyte}) \times 100.$$

2.6. Bacterial loads

The bacterial load in the haemolymph (bacteraemia) on the initial (0^{th} day), middle (45th day) and at the end of the experiment (90^{th} day) was analyzed following a standard protocol [36] with minor modification by counting the total number of colony per mL of cultured haemolymph on nutrient agar. Individual drops of the aliquot were placed into nutrient agar plates and the lawn inoculated. For each marron, 3 drops of haemolymph were tested for bacterial load. Then inoculant were incubated at 25 °C for 24 h and CFUs were determined for each drop. Total cfu/mL haemolymph was counted on the basis of a total volume of 50 μL of each drop. The bacteraemia in the haemolymph were categorised into a fixed scale from 1 (0–19 cfu/mL) to 10 (180–199 cfu/mL) and the rank 11 was used for "too numerous for an accurate count". The method of bacterial load analysis in the water was similar to haemolymph.

The bacterial load in the intestine was determined following the established protocol [18] at the end of the experiment. Before removing gut, the marron were rinsed with distilled water and then with 70% alcohol. Further marron were washed with sterilised distilled water to eliminate the external bacteria. Gut sample (1 g) was ground thoroughly in the test tube and diluted into 1 mL of phosphate buffer saline, and then centrifuged briefly. The supernatant (5μ) was lawn inoculated to tryptone soya agar. The plates were incubated for 24 h at 25 °C. The bacterial loads were ranked from 1 (1–250 cfu/mL) to 10 (2501–3000 cfu/mL) and the rank 11 was used for "too numerous for an accurate count. The method of bacterial load analysis of feed sample was similar to intestine.

2.7. Condition indices

Condition indices analysis were performed at the initial, middle, and at the end of the feeding experiments. The tail muscle and hepatopancreas from individual marron of each replicates were weighed and measured to calculate organosomatic indices and moisture content as described by Ref. [37] using the following equations:

$$\text{Wet hepatosomatic indices}(Hi_w) = H_w \times 100/BW$$

$$\text{Wet tail muscle indices}(TMiw) = TM_w \times 100/BW$$

$$\text{Dry hepatosomatic indices}(Hi_d) = H_d \times 100/BW$$

$$\text{Dry tail muscle indices}(TMid) = TM \times 100/BW$$

$$\text{Hepatopancreas moisture}(HM\%) = (H_w - H_d)/H_w$$

$$\text{Tail muscle moisture}(TM\%) = (TW_w - TW_d) \times 100/H_w$$

Where BW: wet body weight; H_w: hepatopancreas wet weight; TM_w: tail

muscle wet weight; H_d: hepatopancreas dry weight; TM_d: tail muscle dry weight; $H_{d:}$ hepatopancreas dry weight. Hepatopancreas and tail muscle samples were dried in the oven at 90 °C for 24 h for dry hepatosomatic and tail muscle analysis.

2.8. Growth performance and survival rate

All marron juveniles were counted and weighed at the commencement and termination of the feeding trial. Growth parameters of marron juveniles were assessed using the following formula during the experimental period:

$$\text{Weight Gain}(\%) = 100 \times (W_t - W_0)/W_0$$

$$\text{Specific growth rate}(SGR)(\%) = 100 \times (\ln W_t - \ln W_0)/t$$

W_t is the weight of marron at measurement (t) and at the commencement (W0),where t is experimental period (day).

$$\text{Feed conversion ratio}(FCR) = \text{mass of feed consumed/wet weight gain}$$

$$\text{Survival rate } \% = 100 \times (n_t/n_0)$$

where n_t is the number of live marron at (t) days and n_0 is the number of marron initially stocked.

2.9. Gut micrograph

Five marron from five randomly selected tanks within each group were dissected on day 90, and the midgut samples were prepared for scanning electron microscopy (SEM) exposure following the standard protocol [38]. Midgut samples were soaked in 3% glutaraldehyde containing 0.1 M cacodylate buffer for overnight. Subsequently samples were washed in cacodylate buffer and distilled water for three consecutive changes for 5 min. Further samples were immersed in 2% OsO_4 for 2 h followed by three washes in distilled water for 5 min, and then dehydrated using a series of 50%, 75% and 95% ethanol solutions for 5 min before three final washes in 100% ethanol for 5 min. The samples were dried by washing in a series of 50%, 75% and 100% (twice) hexamethyldisilizane (HMDS) in ethanol solutions for 5 min. All processed samples were dried at room temperature and mounted on a stub using carbon tape, coated with gold and viewed under a pressure scanning electron microscope (SEM, model Phillips XL 30, FEI, Hillsboro, OR, USA). Internal digestive tract was assessed under 5000 X magnification for distribution and densities. The images obtained from SEM were used to calculate the number of midgut microvilli by counting and averaging microvilli on each slide (n = 3) using digital imaging software (Adobe Photoshop CC 2015, Adobe System Incorporated, USA).

2.10. Exposure to high temperature – phase II

In phase II, the same marron were exposed to elevated temperature (30 °C) and their immunophysiological responses were investigated post exposure. The temperature challenge started on day 110 of the feeding experiment. Water temperatures in the tank were gradually increased to 30 °C within three days with the help of automatic submersible heater and maintained for 5 days. The feeding rate and frequency were maintained similar to phase-I and uneaten food and faeces were removed prior to next feeding. The effects of temperature on the immunophysiological responses of marron juveniles were determined at both pre and post exposure in terms of conditions indices, total haemocyte count, differential haemocyte count (proportion of hyaline cells%), and phagocytic rate.

2.11. Statistical analysis

All data were analyzed using parametric analysis in SPSS version 22.0 software. The normality and homogeneity of the data was also checked prior to SPSS analysis. A data transformation was required when the data was not homogeneous. The results were presented as means and SE (standard error). The significant difference from each treatment was determined using randomized block design and ANOVA (analysis of variance). Least significant difference post hoc tests were performed to determine any significant differences of the marron fed with different diets. All significant tests were performed at $P < 0.05$ level.

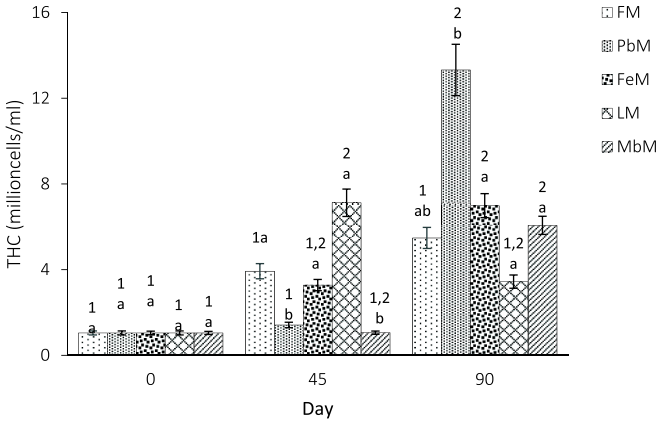

Fig. 1. Total haemocyte count (means ± SE; million cells/mL) of marron fed with diets containing FM, PbM, FeM, LM, and MbM over time periods. Data with different alphabets ($^{a, b}$) represent significantly different at $P < 0.05$ among the treatments. Data with different numerals ($^{1, 2}$) represent significantly different at $P < 0.05$ over time periods.

3. Result

3.1. Immune competence

The highest ($P < 0.05$) THC among dietary protein sources was obtained in marron fed with PbM at the end of experiment (Fig. 1). The mean of DHC (hyaline cells %) on marron fed with LM was significantly ($P < 0.05$) higher on day 45, while the mean hyaline cell % of marron fed with MbM and PbM did not change significantly ($P > 0.05$) over any sampling period, however the mean of hyaline cell % was increased significantly ($P < 0.05$) in marron fed with FM, FeM, and LM at 90th day (Fig. 2).

The remarkable ($P < 0.05$) decreasing pattern of NRRT was observed in all treatment diets over the time period. Although there was

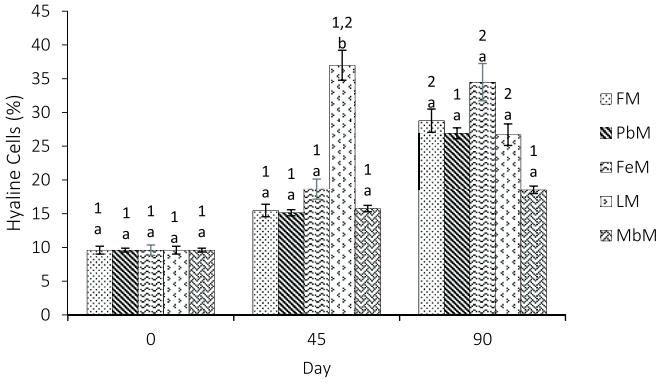

Fig. 2. Hyaline cells (means ± SE; %) of marron fed with FM, PbM, FeM, LM and MbM over time periods. Data with different alphabets ($^{a, b}$) at the top of bar represent significantly different at $P < 0.05$ among the treatments. Data with different numerals ($^{1, 2}$) at the top of bar represent significantly different at $P < 0.05$ over time periods.

Table 2
Weight gain % (mean ± SE, %), survival rate (mean ± SE, %), specific growth rate (SGR) and feed conversion ratio (FCR) of marron fed with different protein sources at the end (90d) of the trial.

Growth parameters	Dietary protein sources				
	FM	PbM	FeM	LM	MbM
Weight gain (%)	28.53 ± 4.35[a]	25.45 ± 3.45[a]	21.55 ± 3.38[a]	32.20 ± 4.82[a]	33.59 ± 5.52[a]
Survival rate (%)	90.00 ± 0.58	96.67 ± 0.33	100.00 ± 00	90.00 ± 0.58	83.33 ± 1.20
SGR (g/day %)	0.27 ± 0.02[a]	0.25 ± 0.04[a]	0.21 ± 0.03[a]	0.31 ± 0.07[a]	0.32 ± 0.04[a]
FCR	2.94 ± 0.26[a]	2.47 ± 0.31[a]	3.79 ± 0.46[a]	2.78 ± 0.62[a]	2.26 ± 0.45[a]

Data in the similar row with different superscript ([a, b]) represent significant difference at $P < 0.05$.
Note: FM (Fish meal); PbM (Poultry by product meal); FeM (Feather meal); LM (Lupin meal); MbM (Meat and bone meal).

Table 3
Phagocytic rate (PR) (means ± SE; %), and neutral red time retention (NRRT) (means ± SE; minute) of marron fed with protein sources.

Immune parameters	Day	Dietary protein sources				
		FM	PbM	FeM	LM	MbM
PR	0	$_1$10.99 ± 2.63[a]	$_1$10.99 ± 2.63[a]	$_1$10.99 ± 2.63[a]	$_1$10.99 ± 2.63[a]	$_1$10.99 ± 2.63[a]
	90	$_2$42.14 ± 6.76[a]	$_1$26.52 ± 1.80[a,b]	$_1$13.96 ± 0.76[b]	$_1$12.14 ± 0.97[b]	$_2$23.89 ± 6.81[a,b]
NRRT	0	$_1$123.75 ± 8.07[a]	$_1$120.75 ± 7.07[a]	$_1$128.75 ± 11.07[a]	$_1$126.75 ± 16.07[a]	$_1$121.75 ± 9.07[a]
	90	$_2$65.34 ± 13.23[a]	$_2$55.67 ± 13.23[a]	$_2$40.63 ± 10.04[a]	$_2$55.54 ± 13.23[a]	$_2$35.29 ± 5.02[a]

Data in the similar row with different superscript ([a, b]) represent significant difference at $P < 0.05$.
Data in the similar column with different subscript ($_{1,\ 2}$) represent significant difference at $P < 0.05$.
Note: FM (Fish meal); PbM (Poultry by product meal); FeM (Feather meal); LM (Lupin meal); MbM (Meat and bone meal).

no significant ($P > 0.05$) difference in the NRRT among the treatment groups, but marron fed with FM resulted in the higher NRRT than other treatment diets at the end of the feeding trail (Table 3). The phagocytic rate (PR) increased over the experiment period, however, the marron fed with FM and MbM diets displayed significantly ($P < 0.05$) higher PR. At the end of the feeding experiment, the highest PR among the treatment groups was observed in marron fed with FM ($P < 0.05$). The lowest PR at the same time was achieved by marron fed with FeM and LM (Table 3).

3.2. Growth performance and survival rate

Weight gain %, SGR and FCR of marron juvenile fed with five different protein diets were not significantly different ($P > 0.05$) at the end of the experiment. The highest ($P > 0.05$) wt gain % and SGR of marron was recorded in the group fed with MbM protein while FCR was lowest ($P > 0.05$) among other treatments groups (Table 2). The diets containing FeM protein resulted on the lowest SGR (0.21 g/day). Survival of marron at the end of the trial fed with five different protein sources were not significantly different ($P > 0.05$). Marron fed with feather meal (FeM) protein sources resulted on the highest survival rate followed by PbM fed protein source, whereas lowest marron survival rate was recorded in the group fed with MbM containing diet (Table 2).

3.3. Bacterial loads

The bacterial loads in the water, diets, and intestine of marron fed with different dietary protein sources were the same ($P > 0.05$) (Table 5). After 45 days of the feeding experiment, marron fed with FM had the highest ($P < 0.05$) bacterial loads in the haemolymph, whereas the lowest ($P < 0.05$) bacterial load was observed in PbM and FeM fed groups. The bacterial loads in haemolymph of marron fed with FM decreased significantly ($P < 0.05$), while non-significant ($P > 0.05$) change was noticed in the treatment groups fed with LM and MbM at the end of the feeding experiment. The bacterial loads in haemolymph of the marron fed with LM was significantly higher ($P < 0.05$) than those marron fed with diet containing FM and FeM at the end of the feeding trial (Table 5).

3.4. Condition indices

No significant differences ($P > 0.05$) were observed in the HM, TM, Hid, TMiw, and TMid of marron at the initial, middle and final of feeding experiment among different treatment diets (Table 4). Similarly, Hiw of all treatment diets at the initial and the final was not significantly different ($P > 0.05$). However, in the middle of the experiment (day 45), marron fed with PbM had the highest Hiw and the lowest Hiw was achieved by marron fed with FeM. The HM, Hiw, and Hid of marron fed over the experimental period were similar ($P > 0.05$) in almost all diets except the Hid of marron fed with PbM was significantly increased ($P < 0.05$) at day 45. The TM of marron in all treatments diets were increased significantly ($P < 0.05$) with respect to the time interval. In contrast, the significant reduction ($P < 0.05$) of the TMid was observed in all treatment diets over the experimental duration. Increasing trend of TMiw was noticed with consequent increase in the experimental duration in all the dietary treatment groups except with MbM.

SEM images of the interior surface of the midgut of marron fed with alternative protein sources were exhibited in Fig. 3. The microvilli size, number of microvilli/group, and microvilli density were significantly ($P < 0.05$) different among the dietary treatments at the end of feeding trail (Table 6). Longer microvilli length (3.83 ± 0.18 μm) was demonstrated in marron fed with PbM diet. The highest number of villi/group was achieved by marron fed with diet containing FeM and was similar to group fed with LM diet. Diets containing FM and PbM as protein sources revealed significantly ($P < 0.05$) lower number of microvilli/group than diets containing FeM and LM. The microvilli density of the marron fed with diet containing FeM was significantly denser ($P < 0.05$) than other treatment diets (Table 6).

3.5. Temperature challenge

The mean of total haemocyte count, hyaline cell percentage and phagocytic rate of marron after temperature exposure did not show any significant ($P > 0.05$) different among dietary protein sources (Table 7). However, marron fed with LM and MbM showed significantly ($P < 0.05$) lower proportion of hyaline cells % after temperature

Table 4
Hepatopancreas moisture content (HM%), tail muscle moisture content (TM%), wet hepatosomatic index (Hiw), dry hepatosomatic index (Hid), wet tail muscle indices (TMiw), dry tail muscle indices (TMid) of marron fed with different dietary protein sources. (means ± SE %).

	Day	Dietary protein sources				
		FM	PbM	FeM	LM	MbM
Wet wt	0	$_{1}8.34 \pm 0.18^{a}$	$_{1}9.43 \pm 0.62^{a}$	$_{1}8.77 \pm 12^{a}$	$_{1}8.85 \pm 0.23^{a}$	$_{1}10.09 \pm 0.42^{a}$
	45	$_{1}8.85 \pm 0.43^{a}$	$_{1}9.48 \pm 0.40^{a}$	$_{1}9.16 \pm 0.17^{a}$	$_{1}8.87 \pm 0.34^{a}$	$_{1}10.48 \pm 0.52^{a}$
	90	$_{2}10.72 \pm 0.40^{a}$	$_{2}11.83 \pm 0.46^{a,b}$	$_{2}10.66 \pm 0.41^{a}$	$_{2}11.70 \pm 0.53^{a,b}$	$_{2}13.48 \pm 0.65^{b}$
Index						
HM	0	$_{1}50.52 \pm 3.19^{a}$	$_{1,2}50.52 \pm 3.19^{a}$	$_{1}50.52 \pm 3.19^{a}$	$_{1}50.52 \pm 3.19^{a}$	$_{1}50.52 \pm 3.19^{a}$
	45	$_{1}32.07 \pm 3.19^{a}$	$_{1}33.50 \pm 2.92^{a}$	$_{1}31.52 \pm 4.97^{a}$	$_{1}33.24 \pm 5.66^{a}$	$_{1}40.05 \pm 1.44^{a}$
	90	$_{1}62.00 \pm 5.90^{a}$	$_{2}62.82 \pm 0.66^{a}$	$_{1}62.05 \pm 6.44^{a}$	$_{1}57.98 \pm 9.49^{a}$	$_{1}64.87 \pm 4.48^{a}$
TM	0	$_{1}22.28 \pm 0.92^{a}$	$_{1}22.28 \pm 0.92^{a}$	$_{1}22.28 \pm 0.92^{a}$	$_{1}22.28 \pm 0.92^{a}$	$_{1}22.28 \pm 0.92^{a}$
	45	$_{2}80.50 \pm 0.62^{a}$	$_{2}79.63 \pm 0.13^{a}$	$_{2}80.50 \pm 0.68^{a}$	$_{2}79.41 \pm 0.46^{a}$	$_{2}78.76 \pm 0.30^{a}$
	90	$_{3}92.42 \pm 10.85^{a}$	$_{3}92.09 \pm 1.29^{a}$	$_{3}90.09 \pm 1.55^{a}$	$_{3}92.01 \pm 1.26^{a}$	$_{3}92.16 \pm 0.91^{a}$
Hiw	0	$_{1}6.05 \pm 0.72^{a}$	$_{1}6.05 \pm 0.72^{a}$	$_{1}6.05 \pm 0.72^{a}$	$_{1}6.05 \pm 0.72^{a}$	$_{1}6.05 \pm 0.72^{a}$
	45	$_{1}6.31 \pm 0.37^{a,b}$	$_{1}7.78 \pm 0.77^{b}$	$_{1}4.83 \pm 0.46^{a}$	$_{1}6.83 \pm 0.44^{a,b}$	$_{1}6.19 \pm 0.12^{a,b}$
	90	$_{1}6.74 \pm 0.46^{a}$	$_{1}7.12 \pm 0.67^{a}$	$_{1}5.07 \pm 0.05^{a}$	$_{1}6.11 \pm 0.42^{a}$	$_{1}6.35 \pm 0.68^{a}$
Hid	0	$_{1}3.03 \pm 0.85^{a}$	$_{1}3.03 \pm 0.85^{a}$	$_{1}3.03 \pm 0.85^{a}$	$_{1}3.03 \pm 0.85^{a}$	$_{1}3.03 \pm 0.85^{a}$
	45	$_{1}5.39 \pm 0.63^{a}$	$_{2}6.67 \pm 0.95^{a}$	$_{1}4.79 \pm 0.77^{a}$	$_{1}6.67 \pm 1.26^{a}$	$_{1}6.03 \pm 1.12^{a}$
	90	$_{1}5.15 \pm 1.27^{a}$	$_{1,2}5.89 \pm 0.60^{a}$	$_{1}5.44 \pm 0.37^{a}$	$_{1}5.37 \pm 0.51^{a}$	$_{1}4.57 \pm 1.022^{a}$
TMiw	0	$_{1}11.60 \pm 0.24^{a}$	$_{1}11.60 \pm 0.24^{a}$	$_{1}11.60 \pm 0.24^{a}$	$_{1}11.60 \pm 0.24^{a}$	$_{1}11.60 \pm 0.24^{a}$
	45	$_{2}17.54 \pm 0.38^{a}$	$_{1,2}16.28 \pm 1.06^{a}$	$_{1}14.19 \pm 0.71^{a}$	$_{1,2}13.46 \pm 0.67^{a}$	$_{1}13.96 \pm 1.82^{a}$
	90	$_{2}17.92 \pm 1.60^{a}$	$_{2}19.44 \pm 2.14^{a}$	$_{2}17.31 \pm 0.74^{a}$	$_{2}15.54 \pm 1.01^{a}$	$_{1}16.23 \pm 0.61^{a}$
TMid	0	$_{3}9.02 \pm 0.29^{a}$	$_{3}9.02 \pm 0.29^{a}$	$_{3}9.02 \pm 0.29^{a}$	$_{3}9.02 \pm 0.29^{a}$	$_{3}9.02 \pm 0.29^{a}$
	45	$_{2}3.42 \pm 0.14^{a}$	$_{2}3.31 \pm 0.40^{a}$	$_{2}2.77 \pm 0.23^{a}$	$_{2}2.77 \pm 0.19^{a}$	$_{2}2.95 \pm 1.47^{a}$
	90	$_{1}1.48 \pm 0.28^{a}$	$_{1}1.73 \pm 0.35^{a}$	$_{1}1.91 \pm 0.24^{a}$	$_{1}1.39 \pm 0.16^{a}$	$_{1}1.44 \pm 0.10^{a}$

Data in the similar row with different superscript ($^{a, b}$) represent significant difference at $P < 0.05$.
Data in the similar column with different subscript ($_{1, 2}$) represent significant difference at $P < 0.05$.
Note: FM (Fish meal); PbM (Poultry by product meal); FeM (Feather meal); LM (Lupin meal); MbM (Meat and bone meal).

exposure. Moreover, phagocytic rate of marron fed with diets containing FM, LM and MbM were significantly lower ($P < 0.05$) after temperature exposure.

The hepatopancreas moisture, tail muscle moisture, wet hepatosomatic index, wet & dry tail muscle index were not significantly ($P > 0.05$) different among dietary protein sources both before and after temperature exposure. However, dry hepatosomatic index of marron juvenile fed with PbM, FeM and LM increased significantly ($P < 0.05$) after temperature exposure (Table 8).

4. Discussion

Dwindling trend of marine fish capture coupled with heavy consumption by animal feed industry have led tremendous pressure on crustacean aquaculture that impelled researchers to hunt for partial or total replacement of fish meal (FM), the most important and expensive protein ingredients used in aquafeeds [3]. Therefore, various cheaper alternative protein sources to fishmeal, from both animal and plant sources needs to be evaluated to reduce heavy reliance on fishmeal for sustainable crustacean aquaculture. Further, no studies have investigated changes in the immunophysiological responses of marron fed diets containing alternative protein sources to fishmeal. Therefore, current study evaluated the effects of practical diets incorporated with different alternative protein sources of fishmeal on immunophysiological responses of marron.

The availability and capability of circulated haemocyte (THC) in crustaceans has been used as crucial parameters for their defense system [39]. Moreover, the improved immunity of crayfish were assessed in a number of previous studies employing THC [19,22,36,40]. Jussila et al. (1997) reported the association of the THC and the physical conditions of western rock lobster, *Panulirus cygnus* and found that moribund and stressed western rock lobster had significantly lower THC compared to a normal animal [41]. Similarly, the THC was also strongly associated with the haemocyte clotting time in the same

Table 5
Bacterial loads/bacteraemia of haemolymph, water, diets and intestine of marron juveniles. (means ± SE %).

	Day	Dietary protein sources				
		FM	PbM	FeM	LM	MbM
Haemolymph	0	$_{1}1.67 \pm 0.33^{a}$	$_{1}1.67 \pm 0.33^{a}$	$_{1}1.67 \pm 0.33^{a}$	$_{1}1.67 \pm 0.33^{a}$	$_{1}1.67 \pm 0.33^{a}$
	45	$_{2}11.00 \pm 0.0^{b}$	$_{1}1.50 \pm 0.41^{a}$	$_{1}3.50 \pm 1.22^{a}$	$_{1,2}4.00 \pm 1.63^{a,b}$	$_{2}9.00 \pm 1.63^{a,b}$
	90	$_{1}3.00 \pm 1.15^{a}$	$_{1}6.33 \pm 2.60^{a,b}$	$_{1}5.33 \pm 0.88^{a}$	$_{2}7.67 \pm 3.33^{b}$	$_{1,2}7.00 \pm 2.33^{a,b}$
Water	90	1.67 ± 0.33^{a}	3.33 ± 0.33^{a}	2.67 ± 0.67^{a}	3.00 ± 1.00^{a}	6.00 ± 2.08^{a}
Feeds	90	3.00 ± 0.57^{a}	5.00 ± 1.15^{a}	3.00 ± 1.20^{a}	2.50 ± 0.33^{a}	3.50 ± 0.88^{a}
Gut	90	11.00 ± 0^{a}	11.00 ± 0^{a}	8.00 ± 1.73^{a}	11.00 ± 00^{a}	10.00 ± 1.73^{a}

Data in the similar row with different superscript ($^{a, b}$) represent significant difference at $P < 0.05$.
Data in the similar column with different subscript ($_{1, 2}$) represent significant difference at $P < 0.05$.
Note: FM (Fish meal); PbM (Poultry by product meal); FeM (Feather meal); LM (Lupin meal); MbM (Meat and bone meal).

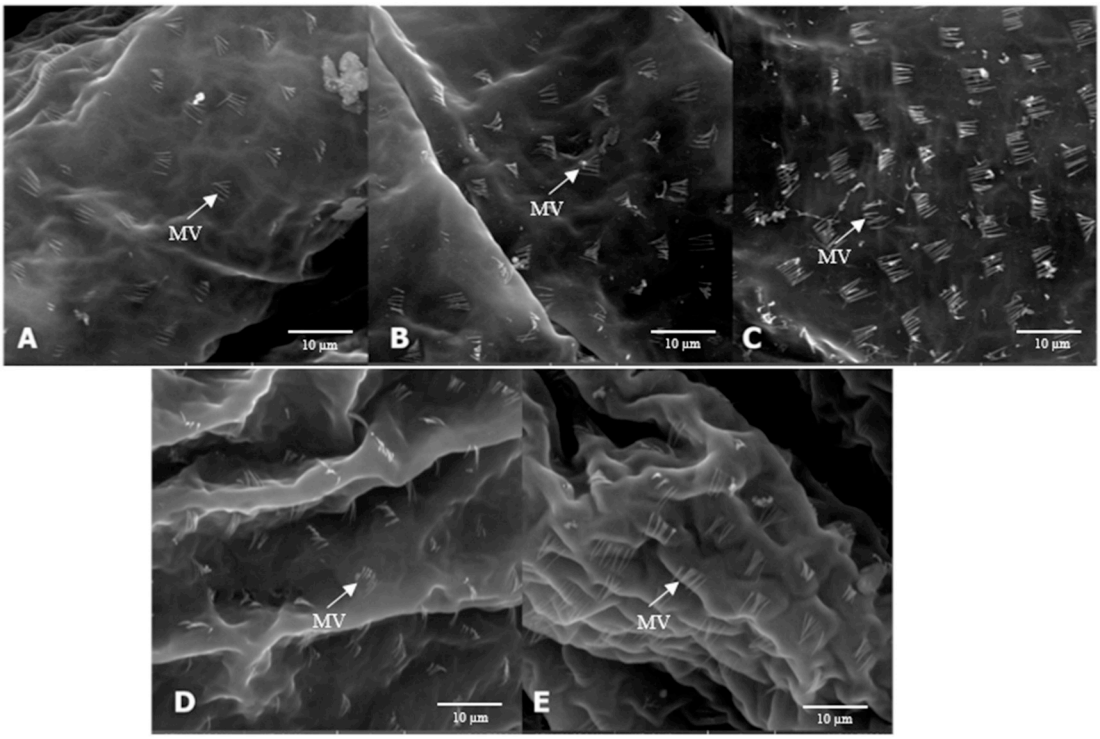

Fig. 3. Comparisons of mid gut micrograph of marron fed with diets containing dietary protein sources after 90 days of feeding trial. Fish meal (A), Poultry by-product meal (B), Feather meal (C), Lupin meal (D), and Meat and bone meal (E). MV; microvilli.

species, which is linked to the stress level [42]. In the current study, the dietary protein sources demonstrated an implications on the THC of marron, indicating that replacing fishmeal with PbM in the marron practical diet increased the THC significantly. Higher THC in marron fed dietary PbM could be attributed due to presence of essential amino acid profile close to that of FM which could account for good performance of PbM. The suitability of PbM as a substitute for fishmeal in fish diets has been reported [43,44]. Although Fuertes et al. (2013) suggested the optimum inclusion of PbM upto 45% in the practical diets of signal crayfish [7], however the inclusion level of PbM upto 100% has been reported on red claw crayfish without impairing growth performance [45]. Therefore, dietary PbM holds the ability to stimulate the health status of marron.

NRRT assay has been widely used to evaluated the effect of environment [33,35], pathogen [46] and pollutants exposure [34,47] on the health status of marine bivalve. In crustacean, NRRT was used to assess the health status of marron fed with different concentration of prebiotic supplementation [48]. Research findings proved that NRRT is a powerful tool to assess the stress level through lysosomal activity [49,50]. The reduced NRRT results demonstrate that the dietary protein sources increased the stress level of marron over the time period. However, FM protein source in the diet seems to induce better influence on marron indicating that diet containing FM were less stressed than the other treatments.

In crayfish haemocyte, hyaline cells play an important role during the phagocytosis reaction to eliminate foreign substances [39] including bacteria and pathogen [51]. The proportion of phagocytic cells in the haemocyte or phagocytic rate (PR), has been successfully used as tools to assess the immune response of several marine bivalves [33,35,52,53]. However, little is known about the application of this method on freshwater crayfish. McKay & Jenkin (1970) reported the effect of vaccination on the phagocytic activity of crayfish, *Parachaeraps bicarinatus* [40]. Increased PR in the dietary FM fed group of present study can be correlated with the higher content of essential amino acid of FM than other alternative protein sources. Earlier research revealed that the fishmeal replacement with alternative protein sources negatively influenced the growth performance of juvenile crayfish [7,8,54]. The current study emphasize that the fishmeal replacement not only affects the physiological responses such as condition indices, but also the immune system of marron.

The lower bacterial load in the haemolymph at the beginning, and then increase in the middle (45th day) of feeding trial can be associated with the bacterial development due to feeding of alternative protein diets. Although the feeding frequency did not affect the bacterial

Table 6
Lenght of individual microvilli (mean ± SE µm), number of microvilli/group (mean ± SE), microvilli density per 100 µm^2 (mean ± SE) of marron juveniles at the end of feeding trial.

Measurements	Dietary protein sources				
	FM	PbM	FeM	LM	MbM
Length of microvilli (µm)	2.81 ± 0.95^{a}	3.83 ± 0.18^{b}	3.68 ± 0.16^{a,b}	3.08 ± 0.28^{a,b}	2.99 ± 0.10^{a,b}
Number of microvilli/group	5.03 ± 0.30^{a}	5.35 ± 0.34^{a}	9.52 ± 0.61^{c}	8.58 ± 0.41^{b,c}	6.85 ± 0.01^{a,b}
Villi density/100 µm^2	14.00 ± 1.52^{a}	14.67 ± 0.67^{a}	24.66 ± 1.33^{b}	16.00 ± 1.73^{a}	14.67 ± 0.33^{a}

Data in the similar row with different superscript ($^{a, b}$) represent significant difference at $P < 0.05$.
Note: FM (Fish meal); PbM (Poultry by product meal); FeM (Feather meal); LM (Lupin meal); MbM (Meat and bone meal).

Table 7
Total haemocyte count (means ± SE; million cells/mL), hyaline cells (means ± SE; %), and phagocytic rate (means ± SE; %) of marrons before (BF) and after (AF) temperature exposure.

Immune parameters	Time	Dietary protein sources				
		FM	PbM	FeM	LM	MbM
THC	BF	$_{1}2.24 \pm 0.90^{a}$	$_{1}2.14 \pm 0.84^{a}$	$_{1}4.14 \pm 0.96^{a}$	$_{1}4.22 \pm 1.04^{b}$	$_{1}1.93 \pm 0.22^{a}$
	AF	$_{1}3.55 \pm 1.06^{a}$	$_{1}3.05 \pm 1.27^{a}$	$_{1}3.09 \pm 1.99^{a}$	$_{1}3.51 \pm 1.97^{a}$	$_{1}3.54 \pm 1.19^{a}$
Hyaline cells	BF	$_{1}28.72 \pm 5.90^{a}$	$_{1}26.91 \pm 6.13^{a}$	$_{1}34.49 \pm 2.08^{a}$	$_{1}35.20 \pm 3.57^{b}$	$_{1}26.63 \pm 0.71^{a,b}$
	AF	$_{1}15.47 \pm 7.08^{a}$	$_{1}7.27 \pm 3.78^{a}$	$_{1}9.26 \pm 0.48^{a}$	$_{2}7.79 \pm 1.11^{a}$	$_{2}8.48 \pm 1.06^{a}$
PR	BF	$_{1}24.13 \pm 4.52^{a}$	$_{1}18.47 \pm 3.69^{a}$	$_{1}20.83 \pm 8.18^{a}$	$_{1}23.38 \pm 3.22^{a}$	$_{1}20.06 \pm 6.07^{a}$
	AF	$_{2}7.20 \pm 2.97^{a}$	$_{1}10.01 \pm 4.11^{a}$	$_{1}10.82 \pm 2.30^{a}$	$_{2}8.30 \pm 2.66^{a}$	$_{2}15.03 \pm 0.71^{a}$

Data in the similar row with different superscript ($^{a, b}$) represent significant difference at $P < 0.05$.
Data in the similar column with different subscript ($_{1, 2}$) represent significant difference at $P < 0.05$.
Note: FM (Fish meal); PbM (Poultry by product meal); FeM (Feather meal); LM (Lupin meal); MbM (Meat and bone meal).

density [55], the supplementation of probiotic significantly reduced the bacteraemia in haemolymph [18]. Sang & Fotedar (2010a, 2010b) reported the similar pattern of the decrease in bacteraemia following prebiotic administration [17,23]. The bacterial loads in haemolymph of the marron fed with LM was significantly higher than FeM and FM fed group at the end of trial which indicates that immune capacity of the animals has declined and thus possibly can result in increased susceptibility to infections [21]. Further, our results indicated that the bacterial load of water, feeds, and intestines were not different among the treatments. As the identification of bacteria to species level was not performed in the current study, the abundance of bacteria in the haemolymph cannot be determined whether it's beneficial or harmful to marron health.

Remarkable increase in the PR of marron fed was observed with diet containing FM while lowest was observed with LM diet at the end of the trial Therefore, the number of bacterium in the haemolymph has a strong association with the number of phagocytic cells in the haemolymph. A number of studies agreed that the defense mechanism to attack pathogens or foreign substances in crustaceans was highly dependent on the circular haemocyte through melanisation, phagocytosis, nodule formation and cytotoxicity [13–15,39]. Thus, confirming that although the bacterial load increased in the middle of the experiment, it can be reduced later on by promoting the phagocytic cells in haemolymph. Hence, FM dietary protein sources performed better than others.

Condition indices have been used to evaluate the stress condition in marron [18,23,56,57], western king prawn *Penaeus latisulcatus* [21,58] and black tiger prawn [59]. Fotedar (2004) used the hepatopancreas and tail muscle index of marron to evaluate the effect of dietary protein and lipid sources [37]. Similarly, condition indices have been used to assess the effectiveness of probiotic [18], and prebiotic supplementation [23] on marron juveniles. All of the experimental diets displayed a significant increase of initial, middle and final TM over the experimental period. The similar pattern was observed in TMiw except for marron fed with MbM dietary protein. In contrast, TMid decreased significantly over the experimental duration in all treatment diets. This indicates that the dietary protein sources combined with the isolation method in the present feeding trial increased the stress of marron both nutritionally and environmentally, as the marron used in this experiment were initially cultured in a pond abundant in aquatic vegetation and phytoplankton. Research has shown that physical water condition as an environmental factor that affects the physiological conditions of crustacean [58]. Previous investigations conducted by Jussila & Evans (1996) reported the size of tank also affected the growth of marron [20].

In addition, the HM% and tail muscle indices can be used as stress indicator in marron as the substitution of dietary protein sources in marron diet resulted in higher stress level that can be observed through the significant increase in HM% [37]. Although there were no significant differences in the mean of HM, TM, Tid among the treatment diets in the present study, the Hiw of marron fed PbM was higher than other diets on 45th day indicating that marron were stressed at that time.

Table 8
Hepatopancreas moisture content (HM%), tail muscle moisture content (TM%), wet hepatosomatic index (Hiw), dry hepatosomatic index (Hid), wet tail muscle indices (TMiw), dry tail muscle indices (TMid) of marron before and after temperature exposure (means ± SE %).

Index	Time	Dietary protein sources				
		FM	PbM	FeM	LM	MbM
HM	BF	$_{1}74.12 \pm 3.18^{a}$	$_{1}74.08 \pm 0.66^{a}$	$_{1}79.93 \pm 3.03^{a}$	$_{1}79.21 \pm 2.23^{a}$	$_{1}77.52 \pm 2.25^{a}$
	AF	$_{1}75.36 \pm 1.87^{a}$	$_{1}77.01 \pm 1.17^{a}$	$_{1}71.80 \pm 2.65^{a}$	$_{1}74.36 \pm 0.97^{a}$	$_{1}75.59 \pm 1.88^{a}$
TM	BF	$_{1}81.56 \pm 0.88^{a}$	$_{1}82.11 \pm 0.87^{a}$	$_{1}82.74 \pm 1.03^{a}$	$_{1}80.48 \pm 1.11^{a}$	$_{1}82.10 \pm 0.34^{a}$
	AF	$_{1}77.77 \pm 2.91^{a}$	$_{1}79.63 \pm 1.73^{a}$	$_{1}81.35 \pm 1.22^{a}$	$_{1}82.61 \pm 0.96^{a}$	$_{1}80.11 \pm 1.40^{a}$
Hiw	BF	$_{1}6.35 \pm 0.49^{a}$	$_{1}5.50 \pm 0.77^{a}$	$_{1}5.16 \pm 0.46^{a}$	$_{1}5.18 \pm 0.33^{a}$	$_{1}4.63 \pm 0.81^{a}$
	AF	$_{1}5.33 \pm 0.28^{a}$	$_{1}5.19 \pm 0.16^{a}$	$_{1}4.57 \pm 0.65^{a}$	$_{1}5.71 \pm 0.39^{a}$	$_{1}4.90 \pm 0.64^{a}$
Hid	BF	$_{1}1.61 \pm 0.08^{a}$	$_{1}1.42 \pm 0.20^{a}$	$_{1}1.04 \pm 0.21^{a}$	$_{1}1.08 \pm 0.16^{a}$	$_{1}1.01 \pm 0.15^{a}$
	AF	$_{1}3.02 \pm 0.78^{a}$	$_{2}4.67 \pm 0.62^{a}$	$_{2}3.65 \pm 0.22^{a}$	$_{2}4.25 \pm 0.29^{a}$	$_{1}2.58 \pm 0.85^{a}$
TMiw	BF	$_{1}20.18 \pm 3.21^{a}$	$_{1}16.67 \pm 0.81^{a}$	$_{1}14.70 \pm 0.87^{a}$	$_{1}16.44 \pm 2.73^{a}$	$_{1}13.71 \pm 1.77^{a}$
	AF	$_{1}14.92 \pm 1.58^{a}$	$_{1}14.56 \pm 0.86^{a}$	$_{1}16.86 \pm 1.07^{a}$	$_{1}15.00 \pm 1.34^{a}$	$_{1}16.16 \pm 1.78^{a}$
TMid	BF	$_{1}3.66 \pm 0.39^{a}$	$_{1}2.97 \pm 0.16^{a}$	$_{1}2.54 \pm 0.25^{a}$	$_{1}3.15 \pm 0.37^{a}$	$_{1}2.46 \pm 0.36^{a}$
	AF	$_{1}3.26 \pm 0.34^{a}$	$_{1}2.98 \pm 0.40^{a}$	$_{1}3.16 \pm 0.41^{a}$	$_{1}2.59 \pm 0.16^{a}$	$_{1}3.26 \pm 0.56^{a}$

Data in the similar row with different superscript ($^{a, b}$) represent significant difference at $P < 0.05$.
Data in the similar column with different subscript ($_{1, 2}$) represent significant difference at $P < 0.05$.
Note: FM (Fish meal); PbM (Poultry by product meal); FeM (Feather meal); LM (Lupin meal); MbM (Meat and bone meal).

Some previous studies on dietary glutamine supplementation had revealed to increase the mucosal fold length and microvilli height in gastro intestinal tract (GIT) of jian carp [60], juvenile hybrid sturgeon [61] channel cat fish [62] and hybrid striped bass [63]. Positive influence of dietary glutamine and arginine on measurements of enterocyte, microvillus and fold heights were noticed in GIT of red drum [64]. Recent study demonstrated increased villi length, width and length to width ratio on black sea trout fed with diet containing 50% protein and found to be higher than those fed with other diets [65]. Consistent with this, the current study also indicated that the different dietary protein sources affected the size, number of villi/group, and villi density.

The highest number of villi/group was obtained by marron fed with FeM (9.52 ± 0.61), which is slightly higher (8.85 ± 0.61) than the previous result achieved by Sang & Fotedar (2010b) [23]. No parallel report is available to substantiate the present findings on number of villi in the marron gut. Furthermore, the present study also imply that the longest microvilli size was observed on the marron fed PbM (3.83 ± 0.18 μm) which is novel findings on the length of microvilli and thus cannot be compared with previous work on marron or crayfish. However, the microvilli length measurement has been established following prebiotic feeding in pacific white shrimp, *Litopenaeus vannamei* [66] and in marron on dietray organic selenium supplementation [67]. In addition, longer villus height of gut in the diet of marron fed with PbM can be correlated with better balance of essential (arginine) and non-essential amino acids (glutamine), more absorptive surface area for better nutrient utilization, and improved immune function.

Temperature has been considered as a crucial environmental factor that plays an important role in growth and survival of crayfish [10,25,68–70], and thus they can respond to environmental changes by altering their behavior and/or internal physiology [26]. High temperature exposure led to reduction in the mean molt interval in swamp crayfish, *Procambarus clarkii* [26]. The optimum condition for marron to survive was reported to be 25 °C [10]. In addition, it was reported that although marron survived throughout the year in earthen ponds, the optimum temperature of marron under semi intensive farming scale was 24 °C with the maximum growth [71]. In the present study, the temperature exposure at 30 °C for five days had non-significant effects on the conditions indices of marron juveniles. All of condition indices were similar among the different dietary sources both before and after high temperature exposure indicating that physiological response was unaffected.

In terms of immune response parameters, high temperature exposure did not significantly change the THC both among the dietary protein sources and over the experimental period. However, the dietary inclusion of LM and MbM resulted in the significantly lower proportion of hyaline cells following temperature exposure. In addition, the PR of marron fed dietary protein from PbM and FeM did not change significantly after temperature exposure. To corroborate our finding non-significant differences in immunological parameters, including, phenoloxidase activity (PO) and respiratory burst activity were observed with dietary inclusion of PBM in *Macrobrachium nipponense* [72] and PBM was found to be optimum in terms of keeping both survival rate and fish welfare in *Dicentrarchus labrax* [73]. Similarly FeM (15% replacement of FM protein) in diets of juvenile *Pacifastacus leniusculus* was used without impairing growth rate [8]. The result in the present study suggests that the dietary protein sources from PbM and FeM in the diet of marron reduced the negative effects of rising temperature as reflected in terms of similar THC, hyaline cells percentage and PR both before and after high temperature exposure. This might be correlated due to presence of superior essential amino acid (arginine, lysine and methionine) balance of PBM and FeM compare to the other protein sources. Therefore, both dietary protein sources can be used in order to improve the susceptibility of marron to high temperature exposure.

In conclusion, the overall results demonstrated that immunological responses in terms of NRRT, PR and bacterial load performed better with dietary feeding of FM at the end of the trial. However, results revealed PbM could be an alternative protein source for development of marron aquaculture as reflected in terms of increased THC, longer microvillus length and improved susceptibility to high temperature (30 °C) exposure. This result will serve as useful baseline data in developing potential diets for marron aquaculture and to promote further investigation on the marron health status.

Conflicts of interest statement

Authors declare that the research was conducted without any commercial or financial relationships that could be interpreted as a possible conflict of interest.

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