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Research Article

Development of Enzymes and *In Vitro* Digestibility during Metamorphosis and Molting of Blue Swimming Crab (*Portunus pelagicus*)

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The work focuses on development of digestive enzymes (amylase, total protease, trypsin, and chymotrypsin) and activity ratio of trypsin to chymotrypsin (T/C ratio) for digestive efficiency and growth, in blue swimming crab (*Portunus pelagicus*) during metamorphosis and molting. Specific activities of all enzyme parameters studied were associated with growth during metamorphosis, while only those of trypsin and T/C ratio were associated during molting cycle where trypsin and chymotrypsin specific activities associated with consumption rate with especially high levels during late intermolt and early premolt stages. About 50% increased weight gain was observed with at least double increased T/C ratio at the end of molting period, compared to the stages prior to molting. Growth of carapace would be more significant after finishing molting. Carapace width gain and T/C ratio were highest at the first crab stage. Studies of *in vitro* protein digestibility of different feed raw materials indicated that *Artemia*, Rotifer, and *Moina* are the best for larval stages. Otherwise, the use of shrimp feed and *Artemia* flake could be the alternatives. Incorporating of cassava meal into the feed formula for early adult stage (juvenile) could be an advantage. The proteins from animals are more beneficial for adult crab culture than the proteins from plants and bacteria. The digestible quality of dietary protein is very important during larval stages, while the protein level of diet is more important during adult stages with fully developed digestive enzymes.

1. Introduction

Blue swimming crab, *Portunus pelagicus*, is a native marine species throughout the Indo-West Pacific region. It is one of the most significant fishery resources for local fishermen in Thailand. Unfortunately, the crab population and size are drastically declined due to overfishing and environmental deterioration. Therefore, raising *P. pelagicus* for substituting wild stock production is in demand and will be a necessary operation in the future. To promote commercial culture of *P. pelagicus*, a development of efficient commercial feeds is needed. Nevertheless, the basic scientific information needed for feed formulation is limited. The breakthrough in production of *P. pelagicus* feed will not be possible unless their digestive process and ability to hydrolyze, absorb, and assimilate nutrients are well understood. The development of digestive enzymes during metamorphosis could provide good indications for future diet formulations for different growth stages (i.e., [1–5]).

In decapod crustaceans, the herbivorous decapod larvae adapt to low food energy values with high enzyme activity levels, rapid food turnover, and low assimilation efficiency, while the carnivorous larvae exhibit low levels of enzyme activity but compensate by extending retention time of high-energy food to maximize assimilation efficiency [6]. This is similar to the observations that high trypsin activity and low amylase activity observed in copepods were related to the low protein and high carbohydrate content of phytoplankton [7, 8]; trypsin activity decreased and amylase activity increased in shrimp larvae, *Litopenaeus vannamei*, because zooplankton contains a pool of readily digestible protein and low carbohydrate content [9]. This is partly concurred with the indications that amylase and protease activities were modulated by the composition of the diet [10] and increased with respective increases in dietary glucides and proteins [11]. Moreover, the levels of trypsin specific activity have been found to relate to diet consumption [12] or protein consumption [13]. The different responses in the enzyme activity levels could be due to variations in the levels of food supply of whether it was under food-saturating condition or not and also due to differences in digestibility quality of the diets as well as the animal growth stages where the levels of enzyme development varied. However, amylase activity was not related to starch ingestion while trypsin activity showed significant correlation with protein ingestion [7, 10], indicating that protein digestibility is the key factor for determining food quality, as also observed by Areekijseree et al. [3] and Supannapong et al. [4]. The main digestive enzymes in crustaceans have been investigated, such as amylase and protease in mud crab, *Scylla serrata* [14] and spiny lobsters, *Panulirus interruptus* [15], *P. argus* [16], and *Jasus edwardsii* [17]; protease in langostilla, *Pleuroncodes planipes* [18]; trypsin in shrimp, *Penaeus monodon* [19]; chymotrypsin in shrimp, *P. vannamei* [20]; and both trypsin and chymotrypsin in crayfish, *Cherax quadricarinatus* [21]. Development of amylase and protease has also been studied in *P. setiferus* [22]. However, this information is still lacking in *P. pelagicus*.

The present study is a part of a project with the ultimate goal to establish feed formulae suitable for culture of *P. pelagicus* at different stages via digestive enzyme information. This study is emphasized on the main digestive enzymes amylase, total protease, trypsin, and chymotrypsin during metamorphosis and a molting period. The conditions for the analyses of the enzyme activities are based on the preliminary study on characteristics of the digestive enzymes in *P. pelagicus* [23]. The activity ratio of trypsin to chymotrypsin (T/C ratio) is also studied, as it has been shown to associate with feed efficiency [24–28] and fish growth rate [13, 24, 27–33]. Moreover, the *in vitro* digestibility of protein in different feed raw materials for future feed formulation is also studied using crude enzyme extracts from different growth stages of *P. pelagicus*. The *in vitro* digestibility technique could differentiate feed raw materials as well as formulated feeds [3, 4, 26, 34] that will be suitable for each developmental stage of *P. pelagicus*. The work could provide new knowledge on development of digestive enzymes and protein digestive ability, which is a prerequisite for future development of feed formulae for culture of *P. pelagicus*.

2. Materials and Methods

2.1. Animal Husbandry

2.1.1. Metamorphosis Study. The healthy female broodstock *P. pelagicus* (12–14 cm in external carapace width) with heart beating stage was obtained from the coastal zone of Chonburi province, Thailand. The broodstocks were held for spawning in 500-L fiberglass tanks at the laboratory of Aquatic Science Department, Burapha University. The healthy hatched larvae were transferred to new 500-L fiberglass tanks (6,000 individuals per tank) with a density of 15 larvae L^{-1}. The zoea 1 (Z1), zoea 2 (Z2), zoea 3 (Z3), zoea 4 (Z4), megalopa (Mp), and first crab (C) stages were fed, respectively, with Rotifer *Brachionus plicatilis*, early hatched *Artemia* sp., subadult *Artemia* sp., Artemia flake, and commercial shrimp feed with 35% protein.

Approximately 1,000 first crab larvae from the hatchery were raised at 50 individuals per m^2 in 5 m^3 rectangular concrete tanks for 1 to 4 months (1M–4M). They were fed with a commercial shrimp feed with 35% protein for months 1 and 2 and with 30% protein for months 3 and 4, with 14% lipid and 30% carbohydrate.

An open system was used for culturing. Cleaned seawater using 25 ppm calcium hypochlorite was daily exchanged at 20% by volume for Z1 to Z2 stages and 30–60% for Z3 to C and adult stages. Seawater quality throughout the experimental period was maintained at 26-27°C and pH 7.8–8.2 with salinity 28 and total alkalinity 120–140 mg L^{-1}.

2.1.2. Molting Study. Wild adult *P. pelagicus* of 78.1 ± 11.9 g with 8.4 ± 0.2 cm in external carapace width obtained from the coastal area of Chonburi province were acclimatized in a 2 m^3 concrete tank for 5 days. They were fed twice daily with chopped fresh marine fish. The molting stage of *P. pelagicus* was examined by light microscope. The stages of early postmolt (A), postmolt (B), intermolt (C1), and late intermolt (C2) were determined by the degree of thickness of cuticle. The stages of early premolt (D1), mid premolt (D2), late premolt (D3), and very late premolt (D4) were determined by the degree of epidermal withdrawal from the old cuticle and the development of new setae on the new cuticle [35].

2.2. Sample Preparation. All samples were collected in triplicate, and the crabs were anaesthetized by low temperature (on ice) before sampling. The whole body of crab larvae or hepatopancreas of adults were each pooled to 5 g per sample. Enzymes were extracted by homogenizing the sample with 1 : 3 (w/v) of 50 mM Tris buffer pH 7.0 on ice. The homogenate was centrifuged at 15,000 ×g at 4°C for 30 min. The upper lipid layer was discarded and the supernatant was then kept at −80°C for further determinations of specific activities of α-amylase, total protease, trypsin, and chymotrypsin. The protein content of the crude enzyme extracts was determined according to Lowry et al. [36].

Table 1: Biochemical composition (%) of feed raw materials for *in vitro* protein digestibility studies.

Raw materials	Moisture	Protein	Lipid	Fiber	Ash	Carbohydrate
For larval stages (Z1–C)						
Chlorella sp.	4.5	42.0	6.2	7.9	25.4	14.0
Tetraselmis sp.	2.3	38.0	4.6	19.2	19.4	16.5
Brachionus plicatilis (Rotifer)	5.6	36.8	5.5	1.7	22.7	27.7
Artemia sp. (early hatched)	1.3	52.2	18.9	14.8	9.7	3.1
Artemia sp. (adult)	1.4	56.4	11.8	12.1	13.4	4.9
Moina sp.	4.2	40.4	4.4	1.1	1.8	48.1
Lucifer sp.	3.6	50.1	4.8	1.8	3.1	36.6
Spirulina sp.	4.2	46.4	7.0	9.0	10.0	23.4
Artemia flake	1.3	55.7	20.0	11.0	11.4	0.6
Shrimp feed	10.3	46.7	2.8	11.1	1.3	27.8
For adult stages (1M–4M)						
Fish meal	6.8	61.0	8.6	1.0	21.0	8.4
Poultry meal	5.5	64.0	13.0	2.2	14.4	6.4
Wheat gluten meal	9.7	46.2	1.3	2.5	0.7	49.3
Corn gluten meal	8.6	62.0	1.6	3.4	1.6	31.4
Soybean meal	12.0	46.0	6.2	6.2	6.3	35.3
Cassava meal	12.0	2.0	0.3	1.7	1.5	94.5
Rice bran meal	10.7	12.0	15.0	7.1	7.1	58.8
Broken rice meal	12.0	6.6	1.6	0.7	0.3	90.8
Yeast protein meal	9.1	42.0	0.6	0.6	4.6	51.1
Bacterial protein meal (Bio-pro 480)	9.0	42.2	1.5	0.4	6.1	49.8

2.3. Determinations of Enzyme Specific Activities

2.3.1. α-Amylase. The activity of α-amylase was determined by measuring the increase in reducing sugar by the hydrolysis of α-*D*(1,4)-glycosidic bond in polysaccharides of starch solution, using 3,5-dinitrosalicylic acid (DNS) method according to Areekijseree et al. [37] based on Bernfeld [38], using maltose as standard. The 100 mM phosphate buffer pH 7 and temperature 50°C were chosen, as the most suitable condition for α-amylase activity of *P. pelagicus* according to Chamchuen et al. [23]. The enzyme extracts were diluted 1 : 50 (v/v) with the buffer before use. The amylase specific activity is defined as μmol maltose produced min^{-1} mg protein^{-1}.

2.3.2. Total Protease. Total protease activity was determined by measuring the increase in cleaved short chain polypeptides using azocasein as substrate, according to Areekijseree et al. [37] modified from García-Carreño [18]. The 100 mM phosphate buffer pH 7 and temperature 60°C were chosen, as the most suitable conditions for total protease activity of *P. pelagicus* according to Chamchuen et al. [23]. The enzyme extracts were diluted 1 : 2 (v/v) with the buffer before use. Total protease specific activity is defined as U mg protein^{-1}, whereas the unit (U) is the increase in absorbance at 440 nm min^{-1}.

2.3.3. Trypsin and Chymotrypsin. Trypsin and chymotrypsin activities were determined by measuring the initial increasing of *p*-nitroaniline [28]. The respective specific substrates BAPNA (benzoyl-*L*-arginine-*p*-nitroanilide) and SAPNA (*N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide) were used. The 100 mM phosphate buffer pH 9 was chosen with the temperature of 60°C or 40°C, as the most suitable condition for trypsin or chymotrypsin activity of *P. pelagicus*, respectively, according to Chamchuen et al. [23]. The enzyme extracts were diluted 1 : 2 (v/v) with the buffer before use. Both trypsin and chymotrypsin specific activities are defined as μmol *p*-nitroaniline produced min^{-1} mg protein^{-1}.

2.4. In Vitro Digestibility of Dietary Protein. Crude enzyme extracts of *P. pelagicus* from the different growth stages (zoea, megalopa, first crab, and adult) and from wild adults (5–7 cm carapace width) were dialyzed overnight against 50 mM Tris-HCl buffer pH 8.2 before use for determining *in vitro* digestibility. Freeze-dried diets of 20 different raw materials were used as substrates: 10 raw materials for *in vitro* protein digestibility study using crude enzyme extracts from the larval stages (Z1–C) and 10 raw materials for the study using crude enzyme extracts from the adult stages (1M–4M) and wild adults with carapace width of 5–7 cm. The biochemical compositions of the feed raw materials are shown in Table 1. The *in vitro* digestibility of protein in the feed raw materials using crude enzyme extracts was determined according to the method described by Thongprajukaew et al. [34], modified from Rungruangsak-Torrissen et al. [26] and Areekijseree et al. [3]. The *in vitro* protein digestibility was expressed as μmol *DL*-alanine equivalent g^{-1} of dried raw material trypsin activity^{-1}.

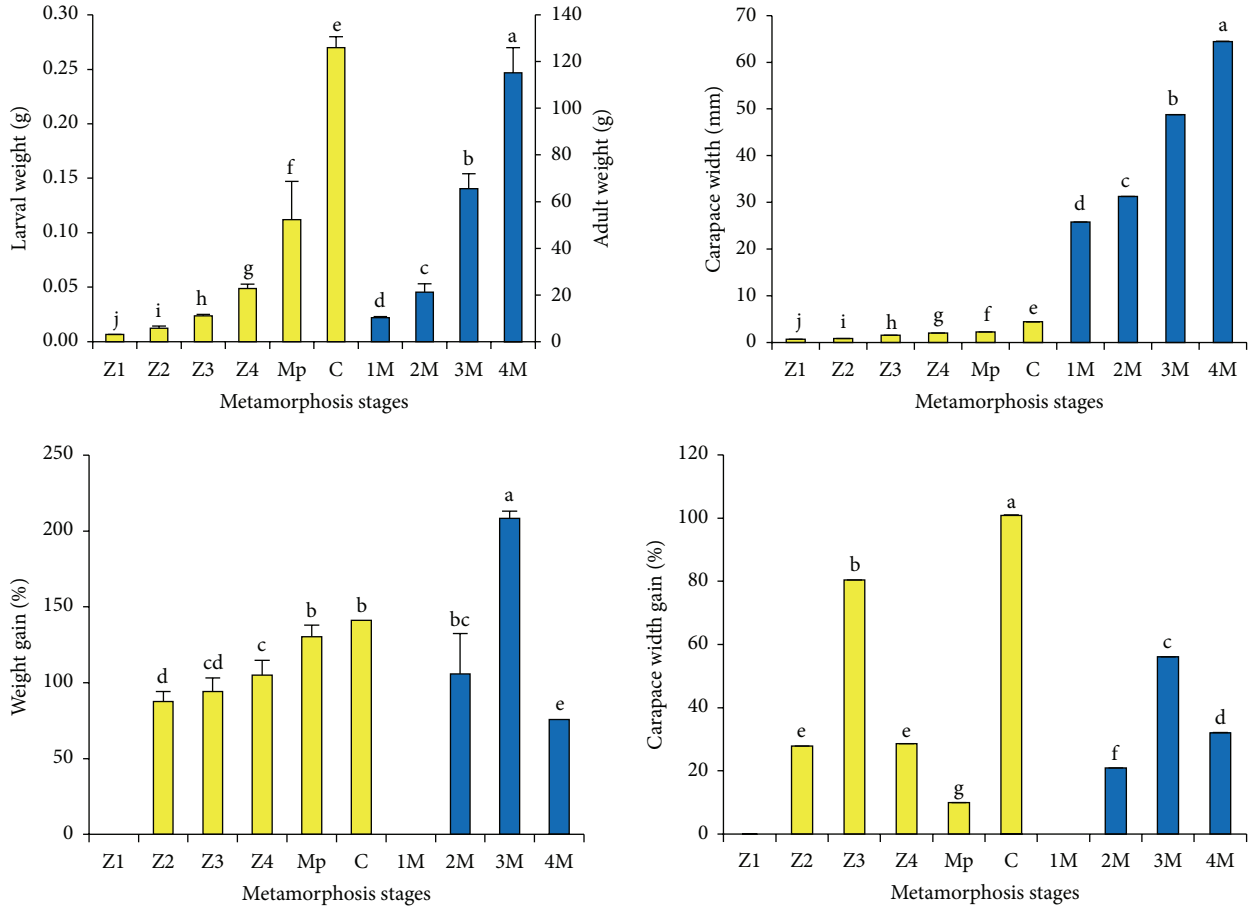

Figure 1: Growth in weight and carapace width of *P. pelagicus* during metamorphosis, at different larval stages (zoea, Z1–Z4; megalopa, Mp; first crab, C) and different adult stages (1M–4M). Vertical bars indicate ± SEM ($n = 3$–5). Interval % gain of weight and carapace width could not be calculated at 1M stage because no measurement was performed prior to this stage. The bars with different superscripts are significantly different ($P < 0.05$).

Table 2: Experimental periods of *P. pelagicus* during metamorphosis at different larval stages of zoea (Z1–Z4), megalopa (Mp), first crab (C), and different adult stages (1M–4M).

Metamorphosis stage	Z1	Z2	Z3	Z4	Mp	C	1M	2M	3M	4M
Day after hatching	1	4	7	10	13	18	48	78	108	138

3. Results

3.1. Metamorphosis Study

3.1.1. Growth. The ages in days after hatching of *P. pelagicus* at different stages during metamorphosis are shown in Table 2. The weight of *P. pelagicus* increased during the whole metamorphosis experiment of 138 days after hatching from larva Z1 until adult 4M stage, while % weight gain between each stage increased corresponding with weight only during the first 18 days after hatching from Z1 until C stage (Figure 1). Among all stages studied, % weight gain was significantly highest at the 3M stage and lowest at the 4M stage ($P < 0.05$, Figure 1). Carapace width also increased during the whole metamorphosis experiment from Z1 until 4M stage, while % carapace width gain varied among the different stages (Figure 1). Carapace width gain could be ranked as C > Z3 > 3M > 4M > Z2, Z4 > 2M > Mp ($P < 0.05$, Figure 1).

3.1.2. Development of Digestive Enzymes. Developmental levels of specific activity of different digestive enzymes and T/C ratio of *P. pelagicus* are shown in Figure 2. Specific activities of all studied enzymes were higher during adult stages, compared to earlier stages ($P < 0.05$). Generally, the levels of amylase and total protease seemed to increase with age, while those of trypsin and chymotrypsin were decreasing from Z1 to C stage and increasing with fluctuated levels during adult stages. The digestive efficiency T/C ratio, on the other hand, showed a systematic pattern of gradually increasing values during larval stages (Z1–C) and gradually decreasing values during adult stages (1M–4M).

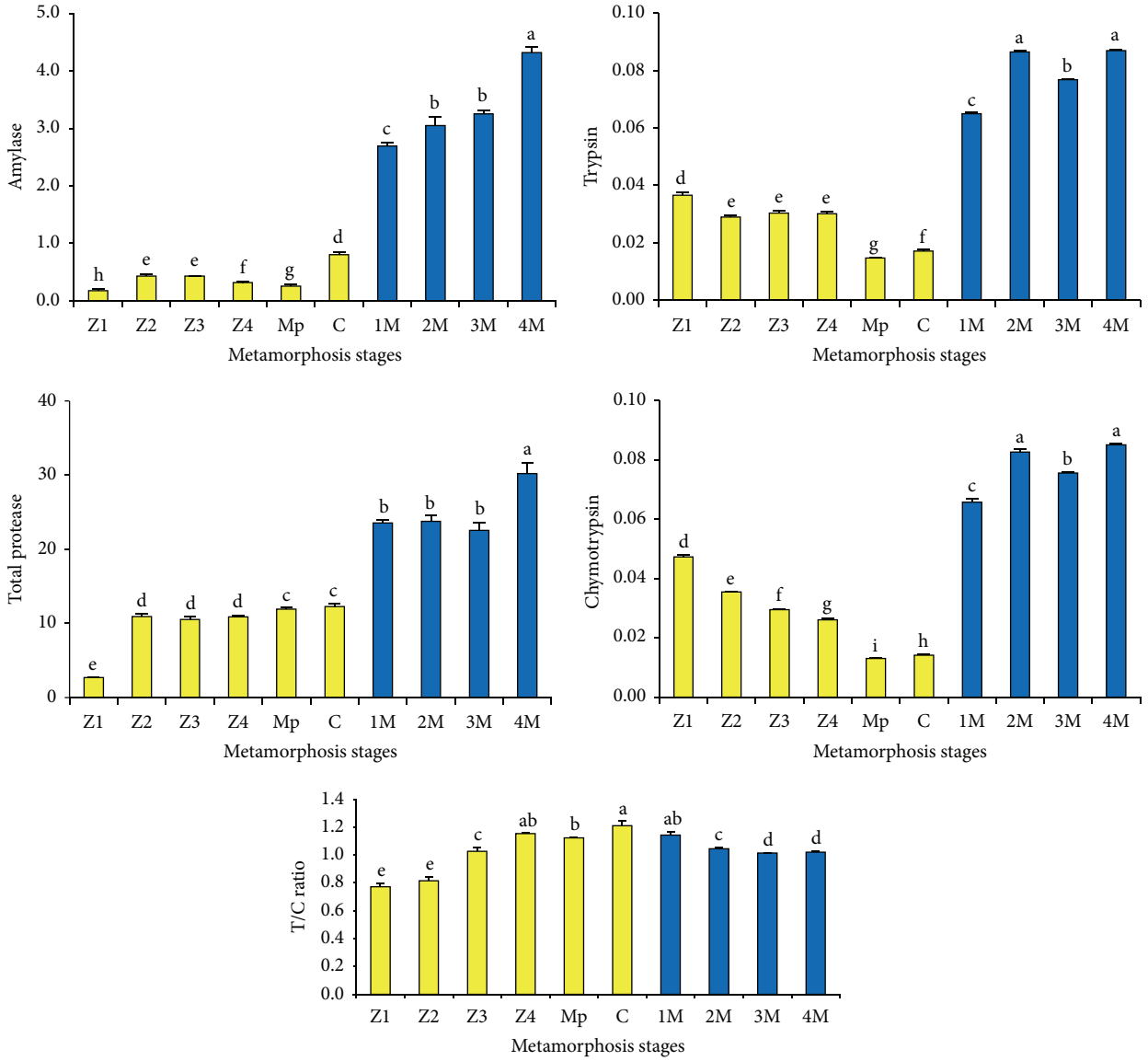

Figure 2: Specific activities of digestive enzymes, amylase (μmol maltose min^{-1} mg protein^{-1}), total protease (U mg protein^{-1}), trypsin and chymotrypsin (μmol p-nitroaniline min^{-1} mg protein^{-1}), and activity ratio of trypsin to chymotrypsin (T/C ratio) of *P. pelagicus* during metamorphosis, at different larval stages (zoea, Z1–Z4; megalopa, Mp; first crab, C) and different adult stages (1M–4M). Vertical bars indicate ± SEM ($n = 5$). The bars with different superscripts are significantly different ($P < 0.05$).

Table 3: Experimental periods and fresh feed consumption rate (% of total weight) of adult *P. pelagicus* at various stages during a molting period; early postmolt (A), postmolt (B), intermolt (C1), late intermolt (C2), early premolt (D1), mid premolt (D2), late premolt (D3), and very late premolt (D4).

Molting stage	A	B	C1	C2	D1	D2	D3	D4
Experimental day	1	3	8	15	22	27	32	35
Consumption rate	—	5	10–15	15–20	15–20	10	5	1-2

3.2. Molting Study. The experimental period and consumption rate at each stage during a molting period of *P. pelagicus* are shown in Table 3. Growth and development of the different digestive enzymes including the T/C ratio during molting cycle are shown in Figure 3. A gradually increasing weight (Figure 3) was observed during 35 days of molting period with highest consumption rate at C2 and D1 stages and lowest consumption rate at D4 stage (Table 3). Amylase specific activity gradually increased and was highest at D2 stage and then decreased to the lowest level at D4 stage (Figure 3). Total

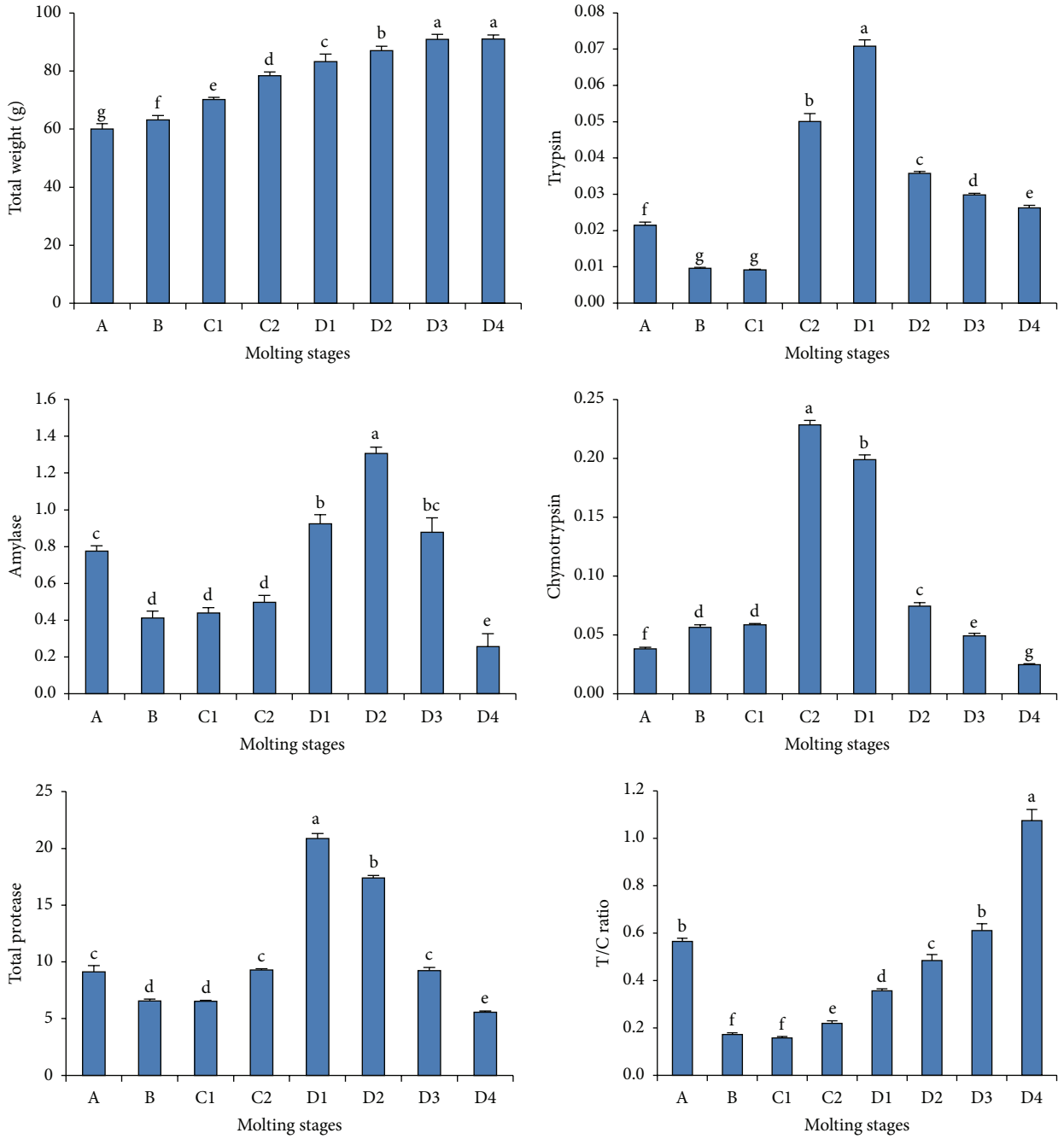

FIGURE 3: Total weight and specific activities of digestive enzymes, amylase (μmol maltose min^{-1} mg $protein^{-1}$), total protease (U mg $protein^{-1}$), trypsin and chymotrypsin (μmol *p*-nitroaniline min^{-1} mg $protein^{-1}$), and activity ratio of trypsin to chymotrypsin (T/C ratio) of adult *P. pelagicus* during a molting period; early postmolt (A), postmolt (B), intermolt (C1), late intermolt (C2), early premolt (D1), mid premolt (D2), late premolt (D3), and very late premolt (D4). Vertical bars indicate ± SEM (n = 3–5). The bars with different superscripts are significantly different ($P < 0.05$).

protease specific activity gradually increased and was highest at D1 stage and then decreased to the lowest level at D4 stage (Figure 3). Trypsin specific activity increased and was highest at D1 stage and decreased thereafter with the levels still higher than the B and C1 stages (Figure 3). Chymotrypsin specific activity was highest at C2 stage and decreased thereafter to the lowest level at D4 stage (Figure 3). The digestive efficiency T/C ratio, on the other hand, showed an interesting systematic pattern of gradually increasing values during the whole molting period from B to D4 stages, irrespective of

Table 4: Correlation coefficient (r) between different parameters studied during metamorphosis from zoea until adult *P. pelagicus*. Significant correlations are indicated in big bold numbers ($P < 0.05$).

Parameters ($n = 30$)	Weight	% weight gain*	Carapace width	% carapace width gain*	Trypsin	Chymotrypsin	T/C ratio	Total protease
Weight	1							
% weight gain*	0.180	1						
Carapace width	**0.941**	0.288	1					
% carapace width gain*	0.016	**0.483**	−0.009	1				
Trypsin	**0.753**	0.119	**0.901**	−0.165	1			
Chymotrypsin	**0.727**	0.011	**0.865**	−0.242	**0.985**	1		
T/C ratio	0.014	**0.572**	0.045	**0.492**	−0.134	−0.295	1	
Total protease	**0.778**	**0.404**	**0.910**	0.075	**0.849**	**0.778**	0.225	1
Amylase	**0.852**	0.288	**0.973**	0.017	**0.940**	**0.899**	0.059	**0.954**

*$n = 27$ because interval % gain could not be calculated at 1M stage because no measurement was performed prior to this stage.

Table 5: Correlation coefficient (r) between different parameters studied during a molting period of adult *P. pelagicus*. Significant correlations are indicated in big bold numbers ($P < 0.05$).

Parameters	Weight	% weight gain	Carapace width	% carapace width gain	Trypsin	Chymotrypsin	T/C ratio	Total protease
Weight ($n = 24$)	1							
% weight gain ($n = 24$)	0.189	1						
Carapace width ($n = 24$)	**0.497**	0.078	1					
% carapace width gain ($n = 24$)	0.077	0.351	**0.702**	1				
Trypsin ($n = 40$)	**0.453**	0.023	0.130	−0.063	1			
Chymotrypsin ($n = 40$)	0.106	0.358	0.043	−0.117	**0.783**	1		
T/C ratio ($n = 40$)	**0.535**	**−0.496**	0.138	−0.159	0.030	**−0.465**	1	
Total protease ($n = 40$)	0.296	−0.011	0.150	0.111	**0.791**	**0.508**	−0.124	1
Amylase ($n = 40$)	0.266	0.036	0.238	0.152	0.347	0.070	−0.073	**0.714**

the specific activity levels of trypsin and chymotrypsin (Figure 3). Before finishing molting, significantly higher weight and T/C ratio value were observed (stage D4: 91.1 ± 1.4 g and 1.07±0.05) compared to the beginning period (stage A: 60.1 ± 1.8 g and 0.56 ± 0.01) ($P < 0.05$, Figure 3).

3.3. Relationships among Different Parameters

3.3.1. During Metamorphosis. The correlation coefficients among different parameters studied during development of *P. pelagicus* are shown in Table 4. Weight correlated with carapace width, and % weight gain correlated with % carapace width gain ($P < 0.05$). Specific activities of all studied enzymes were correlated, and they also correlated with both weight and carapace width ($P < 0.05$). The specific activity of total protease also correlated with % weight gain, while the T/C ratio correlated with both % weight gain and % carapace width gain ($P < 0.05$).

3.3.2. During Molting Cycle. The correlation coefficients among different parameters studied during a molting period of wild adult *P. pelagicus* are shown in Table 5. Weight correlated with carapace width. Percent carapace width gain did not correlate with % weight gain but correlated with carapace width ($P < 0.05$). Specific activities of trypsin, chymotrypsin, and total protease were correlated, and amylase specific activity only correlated with total protease specific activity ($P < 0.05$). Among the studied enzymes, only trypsin specific activity and T/C ratio correlated with weight ($P < 0.05$). The T/C ratio also inversely correlated with % weight gain and chymotrypsin specific activity ($P < 0.05$).

3.4. In Vitro Protein Digestibility

3.4.1. During Larval Stages (Z1–C). Among the 10 raw materials studied, live *Artemia* sp. and Rotifer (*Brachionus plicatilis*)

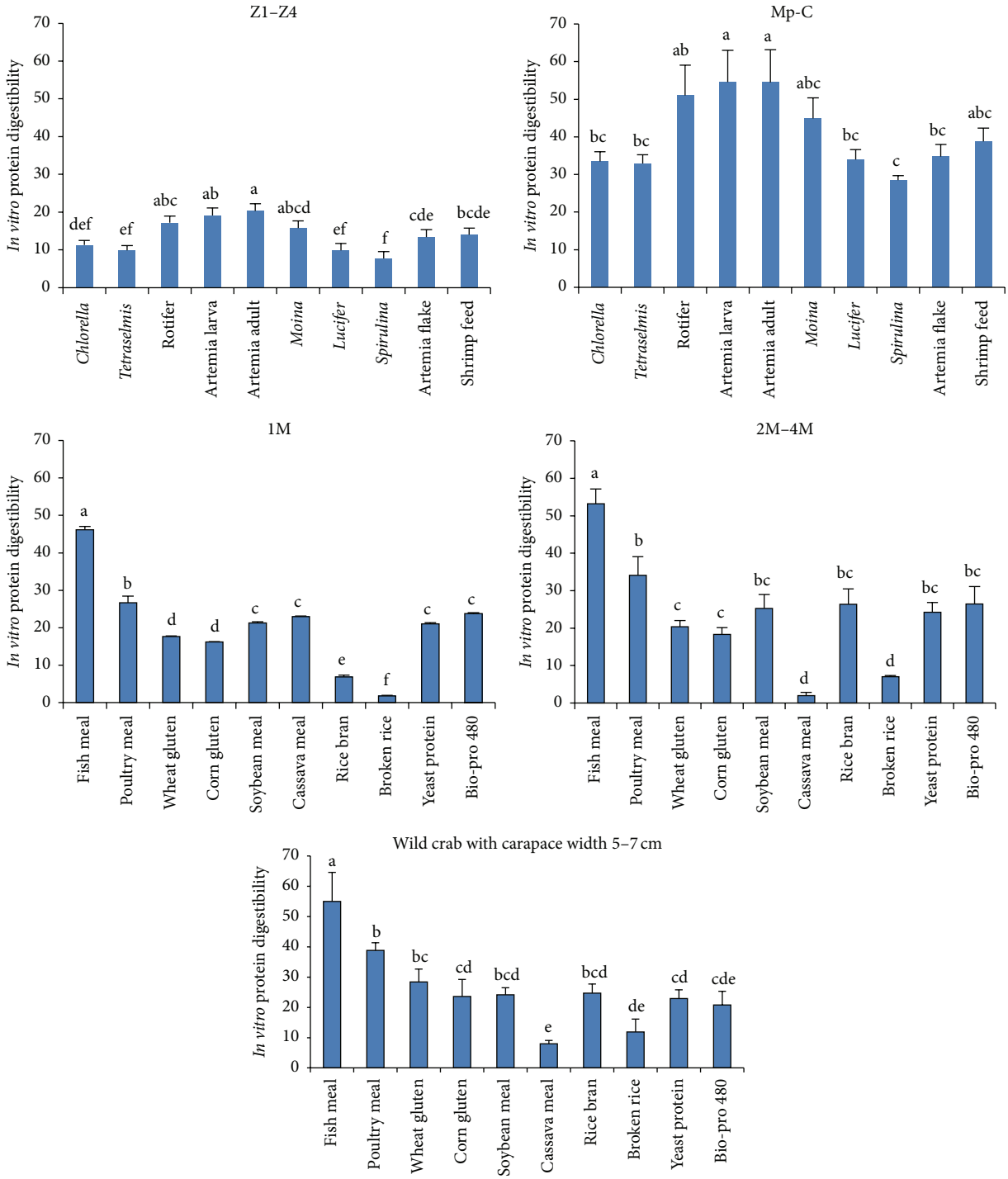

FIGURE 4: *In vitro* protein digestibility (μmol *DL*-alanine equivalent g^{-1} of dried raw material trypsin activity^{-1}) of different feed raw materials using crude enzyme extracts from different stages of cultured *P. pelagicus* (zoea, Z1–Z4; megalopa, Mp; first crab, C; adult stages, 1M–4M) and wild adult *P. pelagicus*. The bars with different superscripts are significantly different ($P < 0.05$).

are the most suitable diets for these early stages of *P. pelagicus*, followed by *Moina* sp., shrimp feed, Artemia flake, *Chlorella* sp., *Tetraselmis* sp., *Lucifer* sp., and *Spirulina* sp. (Figure 4). Similar relative digestibilities among the raw materials were observed among all larval stages (Z1–C), but they could be much better utilized at Mp and C stages than zoea stages (Figure 4). There was no correlation between % protein in the raw materials (Table 1) and *in vitro* protein digestibility levels using crude enzyme extracts from these larval stages ($R^2 = 0.008$, $n = 60$, $P > 0.49$).

3.4.2. During Adult Stages (1M–4M). Among the 10 raw materials studied, fish meal is the best raw material followed by poultry meal (Figure 4). The relative digestibilities among the raw materials were generally similar for all stages (1M–4M), except for the early adults at 1M stage that could utilize cassava meal much better than rice bran meal, while the adults at 2M–4M stages could utilize rice bran meal much better than cassava meal (Figure 4). Wild adult crabs with carapace width 5–7 cm showed similarity in utilizing these raw materials as the cultured adults at 2M–4M stages (Figure 4). The meals from wheat gluten, corn gluten, soybean, yeast protein, and bacterial protein showed moderate protein digestibilities, while broken rice meal generally showed a low protein digestibility, for adult stages (Figure 4). There was a significant correlation between % protein in the raw materials (Table 1) and *in vitro* protein digestibility levels using crude enzyme extracts from adult stages in cultured crabs ($R^2 = 0.407$, $n = 40$, $P < 0.0001$), as well as in wild adult crabs ($R^2 = 0.423$, $n = 30$, $P < 0.0001$).

4. Discussion

4.1. Growth and Development of Digestive Enzymes. Among the digestive enzymes studied, only the specific activities of chymotrypsin ($P = 0.005$) and trypsin ($P = 0.083$) and probably total protease ($P = 0.174$) were associated with consumption rate (Figure 3 and Table 3). Although the specific activities of all the enzymes studied were associated with growth of *P. pelagicus* during metamorphosis (Table 4), only trypsin specific activity and the T/C ratio were associated with growth during molting cycle (Table 5). This has made trypsin specific activity and T/C ratio the suitable factors for growth studies in terms of weight, while the specific activities of chymotrypsin and trypsin and probably total protease are the suitable indicators for diet consumption rate ([13, 39], present work) and/or protein consumption rate [13, 39]. During growth, specific activities of both trypsin and chymotrypsin increased and decreased about the same time resulting in different T/C ratio levels, whereas increases in the T/C ratio values were dependent on decreases in specific activity of chymotrypsin (Figures 2 and 3), as described by Rungruangsak-Torrissen et al. [30]. This is also supported by an observed significant negative relationship between T/C ratio and chymotrypsin specific activity (Table 5). The T/C ratio and % weight gain were correlated positively during metamorphosis (Table 4), but negatively during molting cycle (Table 5) because of decreasing % weight gain at later molting stages probably due to decreasing of consumption rate, energy loss for osmoregulation system during late premolt stage, and loss weight from exuvia. Moreover, % carapace width gain was dependent on carapace width but neither of these carapace growth parameters correlated with any enzymes specific activities during molting cycle (Table 5). This indicates the importance of increasing weight during molting cycle, where carapace width would increase according to the weight, and trypsin specific activity and T/C ratio are the key factors (Table 5). Since there was no significant correlation between T/C ratio and carapace growth during molting cycle (Table 5), the growth of carapace would be more significant after finishing a molting cycle. Since the T/C ratio values associate with growth rates over a period of 1-2 months [31], the significantly high T/C ratio level at the end of molting cycle (D4 stage, Figure 3) should probably also indicate a high carapace growth after finishing molting. During metamorphosis, carapace gain (Figure 1) and T/C ratio (Figure 2) were highest at the first crab stage. It is interesting to note that the T/C ratio was associated with growth rate (% weight gain and % carapace width gain) during metamorphosis (Table 4), while it was associated with the animal weight during molting cycle (Table 5). The crab especially gained weight during a molting period showing an increase of about 50% weight gain with at least double increase in T/C ratio at the end of the molting cycle, compared to the stages prior to molting (Figure 3). In accordance with the T/C ratio values (Figures 2 and 3), the growth rates during metamorphosis were increasing at larval stages and decreasing at adult stages (Figure 1), while the weight was gradually increasing during molting with a significant weight gain at the end of molting period (Figure 3).

Decreases in trypsin and chymotrypsin specific activities during larval development were observed in *P. pelagicus* (Figure 2) as well as in *P. monodon* [40]. Minimum activities of amylase and total protease before molting were observed in crab *P. pelagicus* (Figure 3) as well as in crab *Carcinus maenas* [41]. At D1 and D2 stages, maximum activities were found in amylase and total protease in crab *P. pelagicus* (Figure 3); in nucleotide pyrophosphatase in crab *Callinectes sapidus* [42]; and in total protease, trypsin, chymotrypsin, amylase, and carboxypeptidases A and B in shrimp *L. vannamei* [43]. In crab *P. pelagicus* (Figure 3), the specific activities of trypsin and chymotrypsin were high at C2 and D1 stages when the crab had highest consumption rates (Table 3). Variations in digestive enzymes during different successive growth phases of various invertebrates have been observed and depending on the digestibility quality and availability of the food consumed as well as the levels of digestive development during metamorphosis [7, 22, 40, 44, 45], whereas the enzymes specific activity levels were not always the good indices for digestion and growth. Our experiences have shown that, regardless of the specific activity levels of trypsin and chymotrypsin, the digestive efficiency T/C ratio is the important reliable factor that correlates with feed efficiency [24–28] and animal growth at any stages during the life cycle ([13, 24, 27–33], present work). Moreover, the T/C ratio values in fish oocytes could indicate the levels of oocyte growth and maturation rate in fish, independent on the specific activity levels of trypsin-like and chymotrypsin-like in the oocytes [32].

4.2. In Vitro Digestibility of Dietary Protein during Metamorphosis. Several authors [1–5, 27] have pointed out that digestive enzyme activities could be indices for estimating diet formulations for different ontogenetic stages. *In vitro* digestibility technique using an extract of crude digestive enzymes

for studying dietary protein quality has been developed [46–48]. The technique has also been used for investigation in invertebrates [3, 4, 49]. Among *in vitro* digestibility studies of different main nutrients (protein, carbohydrate, and lipid), protein digestibility is apparently the key factor determining food quality followed by carbohydrate digestibility as the second factor [3, 4], while lipid digestibility has never been found to associate with feed efficiency and the quality of the lipid itself [3]. The level of *in vitro* protein digestibility is directly dependent on the biochemical structure of dietary protein, whereas increased protein digestibility is related to increased contents of reactive sulphydryl (SH) group and decreased levels of disulphide (S–S) bond [26]. In order to compare the *in vitro* protein digestibility values resulting from different crude enzyme extracts, the values have to be standardized by trypsin activity [26, 28]. The works have also been reviewed in Rungruangsak-Torrissen [39].

The *in vitro* protein digestibility of different feed raw materials using crude enzyme extracts from different stages of the crab *P. pelagicus* and standardized by trypsin activity for comparisons is shown in Figure 4. During larval stages, the Mp and C stages showed higher digestibilities among the feed raw materials than zoea stages, showing live *Artemia*, Rotifer, and *Moina* being among the best foods for these larvae. Apart from live foods, the use of shrimp feed and Artemia flake could be the alternatives. For adult stages, the digestibilities among the feed raw materials were generally similar between cultured and wild adults, whereas the early adult 1M stage could utilize cassava meal better than later stages. Incorporation of cassava meal into the feed formula for 1M stage (juvenile) could be an advantage. The meals from animals (fish and poultry) are beneficial for the culture of *P. pelagicus*, as they showed better protein quality than the proteins from plants and bacteria. The significant correlation between the levels of % protein in the feed raw materials (Table 1) and the *in vitro* protein digestibility (Figure 4) observed during adult stages, but not during larval stages, indicated that the digestible quality of dietary protein is very important during the larval stages, while the levels of protein in the diet are more important at adult stages when the digestive enzymes are fully developed, as earlier reported [3, 4].

5. Conclusions

(1) Specific activities of α-amylase, total protease, trypsin, and chymotrypsin were associated with growth during metamorphosis.

(2) The T/C ratio was associated with growth rate (% gain) during metamorphosis.

(3) Trypsin specific activity and T/C ratio were related with growth during molting cycle.

(4) Trypsin and chymotrypsin specific activities associated with consumption rate with especially high levels during late intermolt and early premolt stages.

(5) Carapace width gain and T/C ratio were highest at first crab stage.

(6) *Artemia*, Rotifer, and *Moina* were the best feed for larval stages, while shrimp feed and Artemia flake could be the alternatives.

(7) Cassava meal could be used in the feed formula for juvenile stage, and fish and poultry meals are beneficial for adult stage of *P. pelagicus*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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