

Successful nursing of *Caridina cantonensis* larvae with Ca-alginate microencapsulated diet in the first feeding

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Abstract: The effect of dietary microencapsulated diet (MED), artificial diet (ATD; approximately 28% protein) and *Artemia* shell-free (ART) on growth performance, survival rate, diet digestibility, protein synthesis capacity, enzyme activities of the larvae shrimp were investigated. The experiment was conducted in 3 treatments with 5 replicates. The 1 day post hatched (1 dph) shrimp were then fed twice daily with experimental diets for 60 days. The results of this study indicated that the larvae fed with the MED showed a higher ($P < 0.05$) growth performance in term of total weight, specific growth rate, average daily gain and survival rate than the larvae fed with ART and ATD. Concerning the experimental diet digestibility, MED was reported to have higher protein and carbohydrate digestibility than ART and ATD in both the 15 and 60 days larvae (15 and 16 dph) ($P < 0.05$). Regarding, protein synthesis capacity, muscle RNA concentration in the shrimps fed with MED ($16.33 \pm 1.15 \mu\text{g mg}^{-1}$ muscle) was much higher than the other treatments ($P < 0.05$). Similarly, the shrimps fed with MED over 60 days had the highest protein synthesis capacity (RNA/protein ratio) ($P < 0.05$). However, the results of muscle protein concentration showed no difference between the treatments. ($P > 0.05$). Enzyme activities were also focused on, the results indicated that the protease, amylase and lipase activity of the larvae fed with the microencapsulated diet was much higher than the other experimental diets ($P < 0.05$).

Keywords: alginate, microencapsulated diet, growth performance, *Caridina cantonensis*

Introduction

The requirement to reduce live feed for fish and crustacean species at some or all stages of larvae culture is still in high demand (D'Abtamo *et al.*, 2006). This is because of the high cost of live feed production, such as rotifer and newly hatched of *Artemia*. Global supplies of *Artemia* cysts are renowned for their fluctuations in availability, price, and nutrient composition (Kolkovski *et al.*, 2000). Another reason is the increase of pathogens in the live feed. Kumaran and Citarasu (2016) reported that *Artemia* can act as a reservoir or carrier for bacterial pathogens such as *Vibrio*, *Erwinia*, *Micrococcus*, *Staphylococcus* and *Bacillus*. As a result, the search for formulated diets to support growth and development of larvae from first feeding is necessary. A number of studies in the development of micro diets for aquatic animal larvae have been reported (Yufera *et al.*, 2005; D'Abtamo *et al.*, 2006; Kolkovski *et al.*, 2000; Helen Holme *et al.*, 2006; Chu and Ozkizilick, 1999; Baskerville-Bridges *et al.*, 2000; Fernandezdiaz *et al.*, 2006; Guthrie and Rust, 2000). For the production of micro diets, Ca-alginate diet is considered to be one of the appropriate method. In this study,

the microencapsulated diets were produced in two steps. The steps consist of emulsion and internal gelation: ionic crosslinking using alginate solution. The emulsion step was prepared by using the water in oil (W/O) emulsion technique. (Ponzelet *et al.*, 1995) and the ionic crosslinking solution was prepared by mixing the raw materials in a sodium alginate solution. The exchange sodium ion between the sodium alginate solution and divalent cation was the main factor causing gelled calcium alginate in the starting process. The alginate shell entrapped the raw material solution after adding the sodium alginate solution with the materials into the emulsion. Then the alginate solution was mixed with the calcium chloride resulting in phase inversion in the emulsion, occurring from the surface inward. In this process, the mixture between calcium source and sodium alginate was necessary as it stimulated Ca ions delivery and sequential gelation reaction. It was observed that the lower the pH value, the higher the stimulation. After process completion, the raw materials were entrapped in a sponge-like matrix of Ca-alginate (Ponzelet *et al.*, 1992, Yufera *et al.*, 2005; Chan *et al.*, 2002).

Regarding *Caridina cantonensis* larvae, the small atyid shrimps are widely found in tropical clean freshwater areas, including rivers, streams, lakes reservoirs, mountain creeks, pools, rice fields and irrigation canals (Lai and shy, 2009). *Caridina* is a kind of atyid Asian fresh waters shrimp (160 species in Asia and; 83 species in China) (Yam and Dudgeon, 2005). The *Caridina cantonensis* larvae, widely known as red bee shrimp is one of the most popular ornamental shrimp for the aquarium trade in Thailand. From the survey, it was found that shops selling red bee shrimps apportioned price by gender, the male shrimp price was 100 to 15,000 Baht, while the female shrimp price was 100 to 20,000 Baht per shrimp. The price of shrimp depended also on other factors such as lustre, colour pattern, health and so forth. However, at the present time red bee shrimp culture in Thailand is in a difficult situation as it is faced with a problem involving low survival rate. It is the result of farmers preference for using live feed for the culture and nursing larvae but live feed has several limitations. For example, live feed residues in the tank are often a problem for water quality management, the larvae shrimp usually moult frequently and consequently there is a need to provide adequate feed. This can be difficult if nursing with live feed and it is also difficult to ensure a standard feed regime is maintained. Moreover, there are only a few studies on the appropriate micro diets for this specie. Drawing from what has been described, the study on microencapsulated diet in order to support growth and development of red bee shrimp and eradicate live feed for larvae culture has become the objective of this study.

Materials and methods

Microencapsulated diet preparation

The micro diets were prepared according to a modified method of internal gelation from Yufera *et al.* (2005). An emulsion of rice bran oil 800 ml and soy lecithin 40 g were mixed with continuous stirring for 20 min. The process began by preparing 200 ml of sodium alginate solution (5% W/V) (Product no. A2033; Sigma-Aldrich Pte Ltd, Singapore). Then, 100g of the feed ingredients were weighted and mixed with the sodium alginate solution. After that 100 ml of 4.6 % calcium citrate was added. The sodium alginate solution with the feed ingredients was mixed, then put into the emulsion with a homogenizer and mixed at 500 rpm for 8 minute at room temperature (approximately 25°C). Acetic acid solution represent-

ing 4% (v/v) of the total oil volume was added to the emulsion with continuous stirring, 500 rpm for 8 minutes reaction time. The formed microparticles were allowed to settle, and the supernatant oil was removed by pouring. The microparticles were dispersed in a calcium chloride solution (0.7% w/v) for 5 min and then in a Tween 80 solution (1%) for 5 min (repeat). The capsules were washed with freshwater in order to remove debris and freeze-dried.

The feed ingredients consisted of 25% fish meal, 12% squid meal, 25% shrimp meal, 15% Spirulina powder, 10% maltodextrin, 4% tuna fish oil, 3% soy lecithin, 2% soya oil, 1% vitamin premix, 1% mineral premix and 2% vitamin C, dry weight.

Larvae shrimp culture

The microencapsulated diet was tested with *Caridina cantonensis* larvae. The experiment was conducted in 3 treatments with 5 replicates, observations were over 60 days under 12-h light/12-h dark cycle. The 1 day post hatched (1 dph) shrimp larvae were randomly distributed into 15 aquaria (10 cm width × 10 cm length × 25 cm height) at a density of 20 shrimps per aquarium (8000 shrimp/m³). The shrimp were then fed twice daily at 09.00 and 15.00 h. with

- 1) *Artemia* shell-free (INVE Aquaculture, INVE Thailand. LTD),
- 2) Microencapsulated diet (particles size 180-249 µm) and
- 3) Artificial diet (approximately 28 % of protein).

The water parameters were monitored at 3 days intervals until the end of the test. The parameters measure were water temperature (thermometer), conductivity (conductivity meter), pH (pH meter), dissolved oxygen (azide modification), total alkalinity (phenolphthalein methyl orange indicator), total hardness (EDTA titration), and total ammonia nitrogen (phenate method) (APHA-AWWA-WPCF, 1998).

Observing microencapsulated diet consumption

To ensure the red bee shrimp larvae were consuming the MED. Fluorescein isothiocyanate–dextran (λ_{ex} 492 nm; λ_{em} 518 nm) microspheres (Product no. 46946; Sigma-Aldrich Pte Ltd, Singapore) were used as an inert marker in the experimental diets. The microspheres were easily visible with a fluorescent microscope and could be quantified by visual observation. The fluorescent microspheres (1%w/w) were mixed into a maltodextrin binder solution prior to adding to the dry dietary ingredients. The marked micro diets were prepared following the method

mention above and immediately freeze-dried to prevent settling of the micro diet. The diets were observed under a microscope to ensure uniform mixing of the FITC-dextran marker. Then, the red bee shrimp after fed marked micro diets for 60 day were randomly selected from the tank and viewed under a fluorescent microscope.

Sampling and data analysis

Physical and chemical properties of microencapsulated diet

The size distribution of the dry microparticles was determined by using a sieve to separate the particles into groups. There were six classes: < 50 μm , 50-179 μm , 180-249 μm , 250-499 μm , 500-710 μm and >710 μm . The microencapsulated diet for the experiments was determined in triplicate for chemical composition (crude protein, lipid, fibre, ash and moisture) by proximate analysis according to the methods of AOAC (2005).

The morphology of the freeze-dried microcapsules was characterized by using scanning electron microscopy. The microcapsules were directly mounted on the SEM specimen stubs with conductive silver paint, followed by a gold coated for 120s and observed by using a Quanta 450 FEI and cam scan scanning electron microscope operated at 10 KV.

Experimental diets digestibility

Fifteen and sixty day old red bee shrimps digestibility (15 and 60 dph) after feeding the experimental diets was determined. The whole body of the shrimp was homogenized on ice without adding any of the buffer solution. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant collected below the lipid layer, then kept at -20°C until investigated for protein content (Lowry et al., 1951) and *in vitro* digestibility including enzyme specific activities of protease, amylase and lipase.

In vitro digestibility of the experimental diet was investigated by using an enzyme extract from the whole body of 15 and 60 day old red bee shrimp (15 and 60 dph) according to the method modify from Rungruangsak-Torrissen et al. (2002). *In vitro* digestibility was performed in approximately 30 mg of the sample and 30 ml of the buffer pH added, then mixed thoroughly. The mixture was incubated for 24 h. at 25 °C, the sample was run in triplicate. Before performing digestion analysis, 1.5 ml of each mixture was collected as the control, immediately heated at 100°C for 5 min to inactivate the enzyme and frozen at -80

°C for later determination of the control.

The protein digestibility was determined by measuring the reactive amino group by using Ninhydrin assay (Nankervis and Southgate, 2009). A solution 1.0 ml of undigested control (0h) or the digested mixture (24 h) were mixed thoroughly with 1.0 ml of cd-ninhydrin reagent. The mixture was incubated at 84°C for 5 minutes and rapidly cooled on ice. The supernatant was measured at 507 nm and the concentration of the reaction amino group was calculated by using Tyrosine as the standard. The *in vitro* digestibility of protein was expressed as mg Tyrosine/mg sample.

The carbohydrate digestibility was determined by measuring the increase of reducing sugar by using DNS assay. A solution of 250 μl digested mixture and 250 μl dinitrosalicylic acid (DNS) was heated in boiling water for 5 min and cooled down to room temperature (25°C). The absorbance was measured at 540 nm and then compare with the maltose standard curve. The *in vitro* digestibility of carbohydrate was expressed as mg maltose/mg sample.

Growth performance and feed utilization

Efficiency of the experimental diets was evaluated on the basis of larval growth after 60 days of the experiment. The parameters were calculated as the total body length, total body weight, specific growth rate, average daily growth, feed intake, feed efficiency, feed conversion ratio and survival rate.

Protein synthesis capacity assay

Growth depends upon faster synthesis than degradation of protein. The rate of protein synthesis is determined by the efficiency with which the existing components of the ribosome cycle make protein and by the quantity of the components that are present. The tissue content of RNA is taken as an index of the capacity of synthesis and efficiency is expressed as the amount of protein formed per amount of RNA over a certain time period (Morgan and Beinlich, 1997). The protein synthesis capacity was determined by using the method from Sunde et al. (2001). White muscle samples of 100 mg with 1 ml of TRIzol® (Thermo Fisher Scientific, MA, USA) added were mixed with an Omni-Rupter 250 ultrasonic homogenizer (OMNI International Inc.) for 10 min at room temperature (25°C), then 0.2 ml of chloroform was added. After centrifugation at 5,000× g for 10 min, the solution was separated, the higher phase contained the RNA, while the lower phase contained

the soluble protein fraction; 200 µl of the RNA and protein phase which were transferred to separate tubes. Then, 1.5 ml of isopropanol was added for precipitation and centrifuged at $5,000 \times g$ for 10 min.; 0.5 ml of 96% ethanol was added twice in order to wash the pellets and then centrifuged at $5,000 \times g$ for 10 min. After drying the particles at 55°C for 20 min, the RNA pellets were dissolved with 1 ml 0.1M sodium acetate (pH 5) and heated for 10 min at 55°C . The protein pellets were added to 1 ml 1% SDS and dissolved by heating for 10 min at 55°C . The RNA concentration was measured at 260 nm and protein concentration was measured at 280, expressing the result according to the following formula:

The RNA concentration = $\text{OD}_{260} \times \text{dilution} \times 40$ (ug/mL)

The protein concentration = $\text{OD}_{280} \times \text{dilution} \times 2.1$ (mg/mL)

Enzyme activity

Protease is the general digestive scheme for protein, where protein is broken down to polypeptides by protease, polypeptides are broken down into free amino acids by peptidases (Halver and Hardy, 2002). The protease activity was determined by using the modified casein method (Pan *et al.*, 2005). Crude enzyme (20µl) was incubated with 250 µl of 2% casein substrate dissolved in the various pH buffers 2-12. The reaction mixture was incubated for 10 minutes at room temperature (25°C) and then the reaction stopped by adding 1 ml of 1.2 M trichloroacetic acid (TCA). The control (blank) was prepared by mixing the crude enzyme with TCA to denature the enzymes before adding the substrate (Areekijsee *et al.*, 2004). The reaction solution was mixed and stood for 15 minutes at room temperature (25°C) to complete the reaction. TCA was added and after that centrifuged at 10,000 rpm for 5 minutes. The supernatant was transferred to a test tube then 1 ml of 0.4N NaOH added and incubated at 40°C for 10 minutes. After incubation, 200 µl of 50% Folin reagent was added to the solution and then incubated at room temperature (25°C) for 10 minutes. Total protease activity was measured at absorbance 660 nm. The product tyrosine was determined by means of Folin-hydroxybenzene. One unit of protease activity (U mg^{-1} protein) was defined as 1 mM of tyrosine liberated by hydrolysing casein in 1 min. The protein concentration of the enzyme extracts was determined by using the Lowry method using bovine serum albumin as the standard protein.

Amylase is hydrolysis of complex carbohydrate

extracellular in the stomach, intestine and ceca in association with membrane-linked hydrolysis in the anterior intestine by a variety of carbohydrate. The products of this hydrolysis are polysaccharides and monosaccharide (sugars) (Halver and Hardy, 2002). Amylase specific activity was determined by the increase of reducing sugar (maltose) from the hydrolysis of α -D (1, 4) bond in polysaccharides, and stained with 3,5-dinitrosalicylic acid (DNS) (Areekijsee *et al.*, 2004). Total amylase activity was determined by using the Bernfeld (1955) method. Starch was used as the substrate by boiling 1% soluble starch for 10 min in the various buffers pH 2-12 and 60 µl of 6 mM NaCl. Crude enzyme extract 20 µl was added to 250 µl of the substrate, then incubated at room temperature (25°C) for 5 minutes. The reaction was stopped by adding 250 µl of 1% DNS, after that heated in a boiling water bath for 5 minutes, cooled down and 1.5 ml of distilled water added. The control (blank) was prepared by adding DNS reagent before the crude enzyme. Amylase activity was measured by using the absorbance at wavelength 550 nm with a spectrophotometer. One unit of amylase activity (U mg^{-1} protein) was defined as 1 mM of glucose per min per mg protein.

Lipase is the general digestive scheme for lipids is extracellular hydrolysis of lipids in the stomach and intestine by a variety of lipase and colipases, lipase cleaves the fatty acids from triglycerides and phospholipase cleaves fatty acids from phospholipid (Gisbert *et al.*, 1999; Kuzmima and Gelman, 1997). Lipase specific activity was measured according to Markweg *et al.* (1995). p-nitrophenylpalmitate (pNPP) was used as the substrate. Crude enzyme extract 20 µl was added to 100 µl of 0.1M p-nitrophenylpalmitate (pNPP) substrate and 880 µl of the various pH buffers 2-12 then incubated at room temperature (25°C) for 30 minutes and the reaction stopped by adding 400 µl of 0.1M Na_2CO_3 and then centrifuged at 4000 rpm for 10 minutes. The supernatant was collected and measured at absorbance 420 nm by spectrophotometer. The control (blank) was prepared by mixing the crude enzyme with Na_2CO_3 to denature the enzymes before adding the substrate. One unit of lipase activity (U mg^{-1} protein) was defined as 1 mM of p-nitro phenol per min per mg protein.

Statistical analysis

Mean and standard deviation of the results were calculated. Statistical analysis at 95% significance level was determined by using analysis of variance

(ANOVA), and multiple comparisons were analysed by the Duncan Multiple Range Test (Zar, 1996).

Results

Physical and chemical properties of microencapsulated diet

The particle size distribution of this producing method is shown in Figure 1. The diameter distribution of the dry particles ranged from 50 to larger than 710 μm , with $21.89 \pm 0.87\%$ between 50 and 179 μm , $22.78 \pm 1.81\%$ between 180 and 249 μm , $52.27 \pm 1.00\%$ between 250 and 499 μm , $1.60 \pm 0.13\%$ between 500 and 710 μm and $1.46 \pm 0.05\%$ of the particles larger than 710 μm . The diet showed the highest particle size between 250 and 499 μm ($P < 0.05$). Concerning the chemical composition, the microencapsulated diets with particle size from 50 to 500 μm consisted of 48.63 - 52.64% crude protein, 11.19 - 13.19% crude lipid, 4.96 - 5.38% crude fibre, 5.22-6.93% crude ash and 22.13-28.34% carbohydrate (Tab. 1). The particle size between 50 and 179 μm showed higher crude protein than other sizes ($P < 0.05$). The morphology of microencapsulated diet process is shown in Figure 1. The microencapsulated diet was dried by using the freeze-dry technique. It had irregular shape with wrinkles.

Larvae shrimp culture

Experimental diets digestibility

According to the study, the results of *in vitro* experimental diets digestibility were as follows (Tab. 2). The protein digestibility of enzyme extracted from 60 and 15 day old shrimps (60 and 15 dph) was significantly different among the three experimental diets. ATD and MED were reported to have a higher protein digestibility than ART ($P < 0.05$). Carbohydrate digestibility was also studied at the same time. MED was shown to have the highest carbohydrate digestibility in both ages, followed by ART and ATD.

Growth performance and feed utilization

The effect of the diets on growth performance and feed utilization in red bee shrimp was assigned in 3 treatments with 5 replicates. The treatment was controlled with *Artemia* shell-free (ART) and the other two treatments of microencapsulated diets (MED) and artificial diet (ATD). Growth performance and feed utilization of the red bee shrimp after feeding the experimental diets over 60 days are shown in Table 3.

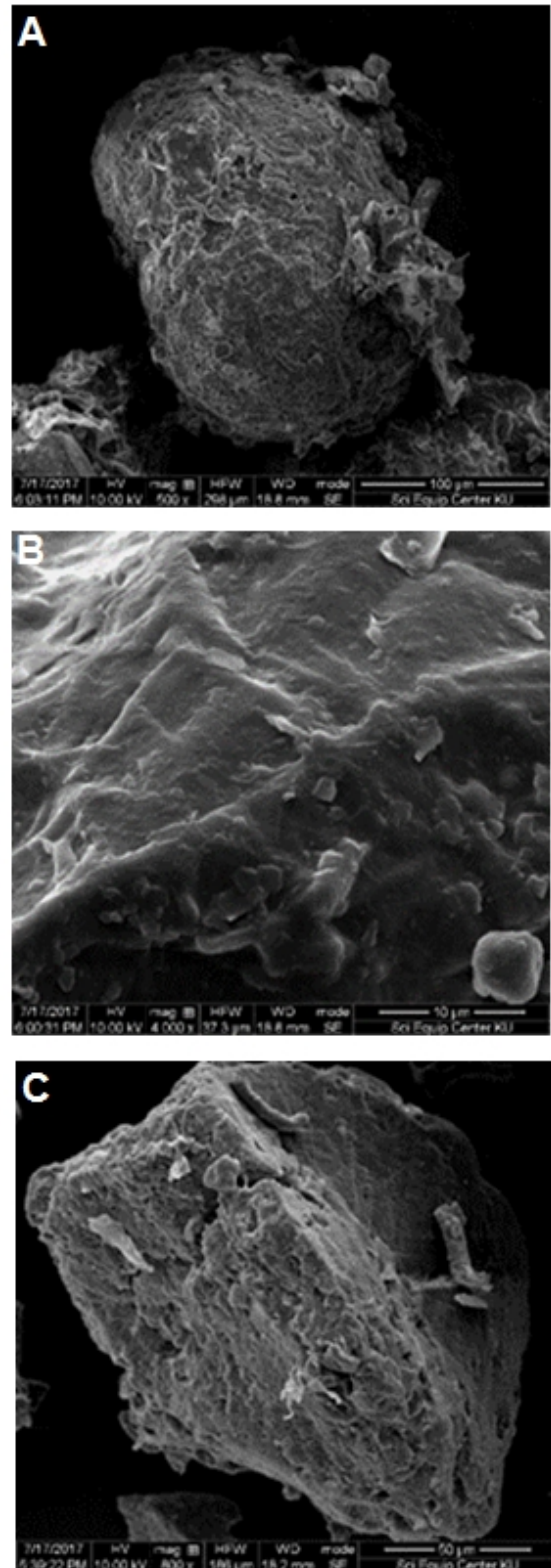


Fig. 1: SEM photomicrograph showing the microencapsulated diet after freeze-dried (A), surface area (B), cross section (C).

The results indicated that there were significant differences in growth performance in term of final weight, weight gain, specific growth rate, average

Tab. 1: Chemical composition of microencapsulated die in each class of size.

| Dietary Components (%) | Diets diameter (µm) | | | | | P-value |
|------------------------|---------------------|-------------------|------------------|---------|------|---------|
| | 50-179 | 180-249 | 250-499 | 500-710 | >710 | |
| Crude protein | 52.64b (1.46) | 48.63a (1.43) | 49.85a (0.75) | | | 0.020 |
| Crude lipid | 13.02 (1.47) | 13.19 (0.91) | 11.19 (2.16) | | | 0.308 |
| Crude fiber | 5.30 (0.51) | 4.96 (0.18) | 5.38 (1.05) | nd | nd | 0.738 |
| Crude ash | 6.90b (0.42) | 6.93b (0.72) | 5.22a (0.46) | | | 0.014 |
| Carbohydrate | 22.13a (1.20) | 26.29ab (2.74) | 28.34b (2.11) | | | 0.030 |

Values in each column with different letters have significant differences ($P < 0.05$). Data are presented as means (SD).

Tab. 2. Experimental diets digestibility of experimental diets by digestive enzyme of red bee shrimp.

| Experimental Diets | Protein ¹ | | Carbohydrate ² | |
|--------------------|----------------------|------------------|---------------------------|-----------------|
| | 60 dph | 15 dph | 60 dph | 15 dph |
| ART | 21.10a (2.39) | 7.07a (1.46) | 9.06ab (0.48) | 4.72a (0.83) |
| MED | 37.77b (2.31) | 15.24b (0.65) | 10.85b (0.97) | 7.32b (0.75) |
| ATD | 33.76b (5.68) | 11.54b (3.50) | 8.80a (1.14) | 3.31a (0.56) |
| P-value | 0.004 | 0.012 | 0.065 | 0.001 |

1) mg Tyrosine/g digested sample, 2) mg maltose/g digested sample
- Values in each column with different letters have significant differences ($P < 0.05$). Data are presented as means (SD).

Tab. 3: Growth performance, survival rate and feed utilization of red bee shrimp after feeding with experimental diets over 60 days.

| Parameters | Experiment | | | P-value |
|-------------------------------------|------------------|-------------------|------------------|---------|
| | ART | MED | ATD | |
| Weight (mg/shrimp) | 6.42a (0.14) | 7.41b (0.29) | 6.88a (0.40) | 0.004 |
| Length (cm/shrimp) | 0.74 (0.03) | 0.78 (0.05) | 0.71 (0.04) | 0.094 |
| Survival rate (%) | 66.66a (5.44) | 81.66b (11.38) | 65.00a (6.38) | 0.034 |
| Specific growth rate (%/day) | 3.09a (0.04) | 3.33b (0.06) | 3.21a (0.94) | 0.003 |
| Average daily growth (g/shrimp/day) | 0.10a (0.00) | 0.12b (0.00) | 0.11a (0.00) | <0.001 |
| Feed intake: FI (mg/shrimp/day) | 2.92 (0.35) | 2.54 (0.21) | 3.21 (0.47) | 0.074 |
| Feed efficiency: FE | 0.32ab (0.04) | 0.35b (0.03) | 0.28a (0.01) | 0.037 |
| Feed conversion ratio: FCR | 3.50 (0.41) | 3.04 (0.25) | 3.51 (0.11) | 0.077 |

- Values in each column with different letters have significant differences ($P < 0.05$). Data are presented as means (SD).

daily gain, survival rate and feed utilization ($P < 0.05$). Drawing from the results, total length, feed intake and feed conversion ratio of the red bee shrimp were not significantly different. The growth performance and survival rate of the red bee shrimp fed with MED was higher than the other treatments ($P < 0.05$). The final weight of the red bee shrimp fed with ART, MED and

ATD over 60 days were 6.42 ± 0.14 , 7.41 ± 0.29 and 6.88 ± 0.40 mg/shrimp respectively. The highest survival rate was the shrimp fed with MED ($81.66 \pm 11.38\%$), followed by shrimp fed with ART ($66.66 \pm 5.44\%$) and then ATD ($65.00 \pm 6.38\%$). To ensure the red bee shrimp larvae were consuming the MED fluorescein isothiocyanate-dextran microspheres were used as an inert marker in the experimental diets. After six hours, the red bee shrimp fed with the fluorescently labelled diet were observed. It exhibited as a gleam in the intestine (Fig 2).

Protein synthesis capacity

Muscle RNA concentration in the shrimp fed with MED ($16.33 \pm 1.15 \mu\text{g mg}^{-1}$ muscle) was much higher than the other treatments ($P < 0.05$). Similarly, the shrimp fed with MED over 60 days had the highest protein synthesis capacity (RNA/protein ratio) ($P < 0.05$). The protein synthesis capacity of the shrimp fed with ART, MED and ATD were 14.54 ± 3.14 , 32.79 ± 1.21 and $12.93 \pm 4.03 \mu\text{g mg}^{-1}$ respectively. However, the results of muscle protein concentration showed no difference among the treatments. ($P > 0.05$). The mean values and standard deviation of the measured RNA and protein synthesis parameters are summarized in Table 4.

Enzyme activity

Enzyme activities of red bee shrimps fed experimental diets over 60 days are shown in Table 4. The results indicated that there were significant differences in protease, amylase and lipase activity ($P < 0.05$). When compared with the other experimental diets, the protease activity of the red bee shrimp showed the highest activity ($P < 0.05$) after fed MED (0.83 ± 0.03 U/mg protein) over 60 days. The lowest protease activity was found in the red bee shrimp fed ART (0.64 ± 0.02 U/mg protein) ($P < 0.05$). In the study of amylase, the

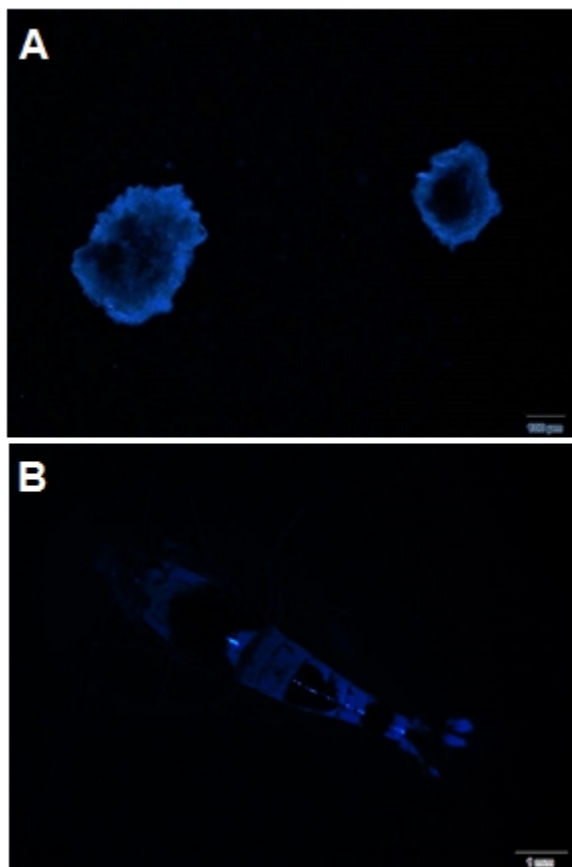


Fig. 2: Fluorescence microphotographs of fluorescently labelled microencapsulated diet (A) and the digestive tract of a red bee shrimp after 6 hr. feeding on fluorescently labelled microencapsulated diet (B).

Tab. 4: Protein synthesis capacity and enzyme activity of red bee shrimp larvae after fed with experimental diets over 60 days.

| Parameters | Experiment | | | P-value | |
|------------|---|------------------|-------------------|------------------|--------|
| | ART | MED | ATD | | |
| A | RNA ($\mu\text{g mg}^{-1}$) | 7.339a (1.52) | 16.33b (1.15) | 6.33a (2.51) | 0.001 |
| | Protein (mg mg^{-1}) | 0.49 (0.05) | 0.50 (0.02) | 0.52 (0.01) | |
| | Protein synthesis Capacity ($\mu\text{g mg}^{-1}$) | 14.54a (3.14) | 32.79b (1.21) | 12.93a (4.03) | |
| B | Protease | 0.64a (0.02) | 0.83b (0.03) | 0.63a (0.04) | <0.001 |
| | Amylase | 2.24a (0.09) | 3.30c (0.15) | 2.46b (0.14) | |
| | Lipase | 85.56a (7.76) | 120.83c (8.66) | 97.69b (5.29) | |

A: Protein synthesis capacity, B: Enzyme activity (Unit/ mg protein)
 - Values in each column with different letters have significant differences ($P < 0.05$). Data are presented as means (SD).

red bee shrimp fed MED (3.30 ± 0.15 U/mg protein) showed higher amylase activity than the other treatments ($P < 0.05$). Similar to the protease and amylase activity, when the lipase activity was analysed the shrimp fed MED (120.83 ± 8.66 U/mg

protein) showed higher lipase activity than the other treatments ($P < 0.05$).

Discussion and conclusion

From the experimental diets digestibility, it showed that the older shrimp had more diet digestion efficiency than the younger shrimp. *In vitro* assays, the study provided information relating to these effects. The information is considered to be useful for the evaluation of proteins used in feed for fish (Grabner 1985; Dimes *et al.* 1994) and shrimp (Lan and Pan 1993; Ezquerro 1997 *et al.* 1998). The study demonstrated that crude enzyme extracted from the whole bodies of the red bee shrimp larvae could break down the microencapsulated diet very well. Thus, protein and carbohydrate digestibility in the microencapsulated diets proved to be the highest. In contrast to the red bee shrimp larvae, California halibut larvae (*Paralichthys californicus*) showed higher *in vitro* protein digestibility of the live feed than other diets (Martinez-Montano and Lazo, 2012). From this study, the 15-day red bee shrimp had lower protein and carbohydrate digestibility than the 60-day shrimp. This is probably due to the poorly developed digestive capacity of the larvae (Kolkovski, 2001). Regarding the diet digestibility of larvae, *in vivo* studies of digestibility revealed that it was not easy to focus on digestibility performance in small larvae, this was due to technical difficulties involving the quantification of ingestion and faeces collection. For these reasons, *in vitro* digestibility was therefore analysed in this study. The *In vitro* digestibility method is known as a very useful tool for examining larvae diets. It is also accepted as a good method of reducing the number of dietary treatment tests in growth-trial studies.

Results of this study brought out the fact that successful culture of red bee shrimp larvae depends on maintaining a stable environment with good water quality. It was proven that persistent feeding of the micro-particulate diets was important for the larvae since micro-particle diets have been developed for feeding larvae in particular. (Walford *et al.* 1991; Jones *et al.* 1993; Yufera *et al.* 1996). It was evident from many studies of micro-particle diets that poor growth was often found to be the result. Only a few successful attempts to feed larvae of marine fish on artificial diets from first feeding have been found. (Rosch and Applebaum, 1985; Walford *et al.*, 1991; Robin *et al.*, 2003; Yúfera *et al.*, 2003). From the results mentioned above, at first feeding the red bee shrimp larvae ingested and broke down the MED very

well. The shrimp larvae fed with MED alone and continuously after hatching showed the highest growing until the end of the experiment when compared with the shrimp larvae fed with ART and ATD. However, the red bee shrimp larvae fed with MED showed a different growth performance and survival pattern when compared with marine fish larvae fed with alginate-micro-diet (Fernandez-Diaz et al. 2006; Yufera et al., 2005). Yufera et al. (2005) reported that when larvae of *Sparus aurata* and *Solea senegalensis* were fed with an *Artemia* control treatment, the growth and survival rate is higher than when fed with an alginate micro-diet. In this matter, Fernandez-Diaz et al. (2006) examined the alginate micro diet in Senegal sole (*Solea senegalensis*). They found that the fish fed with *Artemia* have the highest growth rate. Besides alginate-microencapsulated diets, many kinds of micro-diet such as micro-bound diets, cross-linked protein-walled micro-diets, lipid-walled micro-diets, micro-coated diets and complex micro-particles diets are widely studied in aquatic animals (Langdon., 2003; Kelly et al., 2000; Kovalenko et al., 2002; D'Abramo et al., 2006; Holme et al., 2006; Genodepa et al., 2004). Genodepa et al (2004) observed mud crab larvae (*Scylla serrata*) fed with micro-bound diets. They found that the larvae fed with a combination of 25% micro-bound diets and 75% *Artemia* showed the highest survival rate among all treatments. In contrast to the above studies, another study provided different results. The study of freshwater prawn larvae (*Macrobrachium rosenbergii*) indicated that the survival rate when fed with micro-bound diets was higher than when fed with *Artemia* (Kovalenko et al., 2002). African catfish larvae (*Clarias gariepinus*) fed on a micro-diet were also studied (Appelbaum and Mcgreen, 1998). The result of this study, however, is consistent with the result of red bee shrimp larvae. According to the present report, dry micro-diets promoted a higher growth rate than live diets *Artemia* nauplii (Appelbaum and Mcgreen, 1998).

In this study, the RNA concentration and RNA/protein ratio showed a positive correlation with growth performance. It provided particular facts which were consistent with previous reports in juvenile Atlantic cod (*Gadus morhua*) and rainbow trout (Houlihan et al., 1993; Foster et al., 1993a; Suresh and Sheehan, 1998). However, this trend of RAN concentration and RNA/protein ratio is different from the report in Atlantic salmon (*Salmo salar* L.) (Sunde et al., 2001). White muscle RNA concentration and

RNA/protein ratio are the two parameters, among several biochemical parameters, that have been employed to find the correlation between growth rate and nutritional status. A higher RNA/protein ratio is an indicator of higher protein synthesis in the animal (Sunde et al., 2001). This is due to the fact that RNA coordinates the transfer of encoded information between DNA and ribosomes. Further, it varies as a function of the protein synthesis rate (Brachet, 1960; Otta and Landry, 1984).

At first feeding, the digestive system of an animal is not fully functional. Thus, the diets at the larvae stage are significantly important to the digestive system and also the digestive enzyme (Kolkovski, 2001). From the results of this report, the larvae shrimps fed with MED without live feed supplementation showed the highest enzyme activity when compared with ART and ATD. The results are in contrast to those reported in Asian redbtail catfish larvae *Mystus nemurus* (Kamarudin et al., 2011) and Japanese flounder larvae *Paralichthys olivaceus* (Ji et al., 2013). According Ji et al. (2013) significantly higher protease, amylase, lipase, and trypsin activities are found in Japanese flounder larvae fed with *Artemia* than larvae fed with a micro-diet. In the same way, Asian redbtail catfish showed the highest enzyme activities in larvae fed with a combination of live feed micro-diets. The main reason supporting the results is many species of larvae may not possess enough digestive enzyme to digest dry feed completely. At first feeding, tryptic (Baragi and Lovell, 1986) and lipolytic enzymes (Ozkizilcik et al., 1996) of striped bass larvae showed that enzymes from the live feed can help fish larvae digest and stimulate enzyme secretion. Dabrowski (1982) suggested that, there are not sufficient enzymes for digesting non-living diets in most small aquatic animals. The exogenous enzymes from live feed, therefore, has been suggested as the key component in assisting the digestive process of fish and crustacean larvae (Dabrowski, 1982; Jones et al., 1993; Kamarudin et al., 1999; Kolkovski et al., 1997). However, the reasons given by the above-mentioned studies seem not to relate with the red bee shrimp larvae in this study, as it is proven that replacing live feed with dry micro diets for sixty days after hatching the red bee shrimp is successful and able to demonstrate an improved growth rate.

A number of previous studies showed it was not possible to replace *Artemia* with micro-particle diets in larvae feed (Kamarudin et al., 2011; Ji et al., 2013; Kumlu and Jones, 1995). There are contrary to that

found in this study. Nevertheless, the use of alginate-microencapsulated diets might have been possible in red bee shrimp larvae. Perhaps it is because this experiment used raw materials as the components that passed through the digestibility study process. Other reasons are the control of particle size and modification of the nutrient composition. It is observed that many factors such as composition and digestibility of dietary protein, nutritional status of the animal including individual variations in feed utilization also lead to effects on the growth rate and nutrient consumption in animal (Rungruangsak Torrissen and Male, 2000). The higher growth performance and survival rate of larvae fed with micro diet without supplemental live food in this study demonstrates that exogenous digestive enzymes from *Artemia* nauplii are not essential for the digestion of formulated diets of the shrimp larvae because the autolytic enzymes originating from prey contribute minimally to digestion (Cahu et al., 1995; Kurokawa et al., 1997; Garcia-Ortega et al., 1998; Kolkovski et al. (2000). The success of a microencapsulated diet for larval culture of red bee shrimp establishes the foundation to investigate its potential application to larval culture of other species of crustaceans currently being raised on *Artemia* nauplii.

Kolkovski et al. (2013) remarked that little attention has been focused on the process of feeding micro-diets. This also includes feeding systems, specific design to deal with very small particles as in micro-diet particles, dispersion of particles in the water column, feeding strategies (continuous vs periodic), water hydrodynamics and the interaction between the diet particle and the larvae in the rearing tank.

In conclusion, the results of this study suggest that the microencapsulated diet is able to support growth performance and enzyme activities in *Caridina cantonensis* larvae. Nevertheless, the improvement of formulated diets and modified nutrient content for the larvae shrimp should be further investigated. In addition, the laboratory success needs to be repeated and evaluated under large-scale conditions for commercial production.

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