Effect of Cultured *Artemia* on Growth and Survival of Juvenile *Hippocampus barbouri*

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ABSTRACT

Populations of seahorses (*Hippocampus* spp.) have been greatly reduced during recent decades due to over-exploitation, primarily from the ornamental fish and traditional Chinese medicine industries. Efforts at captive breeding have been made to reduce the dependence on wild-caught individuals, but to date, only some species have been bred successfully. The main obstacle in the culture of seahorses is the suitability of diets, as nutrient requirements of seahorses at different stages are not fully understood. Several studies have shown that *Artemia* enrichment improves the growth and survival of juvenile seahorses. This study determined the effect of feeding *Artemia* cultured with different media on growth and survival of *Hippocampus barbouri*. To prepare *Artemia* for the experiment, *Artemia* metanauplii were placed in cultured medium for 24 h, after sieved and rinsed, then another 30 min on respective cultured medium prior to feeding to seahorses. Five treatments were used: *Artemia* (A), *Artemia* with fresh *Chlorella* sp. (A+CF), *Artemia* with marine shrimp pellets (A+P), *Artemia* with *Chlorella* powder (A+CP) and *Artemia* with *Spirulina* powder (A+S). At the end of the experimental period, 74 days after birth, juveniles seahorse fed with diets A, A+P and A+S had significantly higher body length ($p<0.05$) (34.44±2.37 mm, 32.59±1.61 mm and 36.01±1.57 mm, respectively) than other treatments. A and A+S produced highest final weights (0.198±0.026 g and 0.221±0.057 g), while A+CF and A+CP produced lowest final weights. In terms of survival, diets A and A+S resulted in lowest ($p<0.05$) survival of 26.99 % as compared to 53.99 % in juveniles fed with A+CF, A+P and A+CP. To achieve better growth and higher survival, treatment A+P is highly recommended for rearing juvenile *H. barbouri*.

Keywords: *Artemia*, *Hippocampus barbouri*, Larval rearing

INTRODUCTION

Seahorse populations have been receiving attention lately due to reports of declining catch rates. The main reasons for the decline in the natural seahorse population is over-exploitation for use in traditional Chinese medicine (TCM) and for the ornamental fish trade (Vincent *et al*., 2011). *Hippocampus barbouri* exists in different color variations, from white, pale yellow to pale brown, as well as pale reddish to orange, with a striped snout. Such color patterns make this species one of the most attractive ornamental fishes. Seahorse populations with low densities and poor mobility amplify the threat level even more (Foster and Vincent, 2004). In order to prevent extinction of these seahorses, cultivation of this species has been practiced since the early 1990`s. Successful

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aquaculture of either food fish or ornamental fish has lessened impacts on wild populations, providing juvenile and marketable size fish year round. Thus, seahorse aquaculture has great potential to achieve goals of both long term sustainability and conservation (Murugan *et al*., 2009). The development of seahorse aquaculture has enabled the production of captive bred species for the ornamental fish trade. Captive bred seahorses have tremendous advantages as compared to those caught from the wild. However, problems arise in the effort to culture seahorses, with mass mortality at the juvenile stage. Two main factors, culture technique (Wilson and Vincent, 2000; Planas *et al*., 2008) and suitable feed (Sheng *et al*., 2006) influence the early developmental stages of seahorses.

Because seahorses lack a true stomach and digestion takes place in the intestine, they need to be fed frequently. Starvation may affect the internal organs or immune system, whereas stress will be reduced if the seahorses are fed regularly. Seahorses do not readily eat frozen foods such as mysids or *Artemia*. The movement of live food triggers these seahorses' predatory instinct to prey on it. Study by Woods (2002) reported that *H. abdominalis* often ingested whole prey items in one strike without breaking them; however due to their large size, shrimp are broken into smaller pieces during feeding. Seahorses are primarily visual carnivores, thus their culture mainly depends on live prey, specifically *Artemia*. Until to date there is no documented report on the successful commercial culture of seahorses using artificial foods. According to Olivotto *et al*. (2008), the weaning process for seahorse to accept frozen food are often very challenging and most cases to train the wild seahorse to ingest frozen food takes too long, therefore slowly lead to starvation and finally mortality to the seahorse. *Artemia* has been found as a suitable diet for a large and diverse group of organisms in the animal kingdom (Sorgeloos, 1980). As such, *Artemia* becomes the most convenient live food, since it can be hatched readily from commercially available cysts. Additionally, various sizes of *Artemia*, from nauplii to adults, are available in the market (Leger *et al*., 1986). *Artemia* is commonly used as live food for aquaculture species due to its small size, ease of preparation and availability throughout the year.

Microalgae plays an important role in aquaculture production, either directly or indirectly. It is used to supply to zooplankton, which later becomes the main diet of fish larvae (Ferreira *et al*., 2009). The main species of microalgae used in this way are *Chlorella* sp., *Isochrysis* sp., *Pavlova* sp., *Nannochloropsis* sp. and *Tetraselmis* sp. (Hemaiswarya *et al*., 2011). Feeding with *Artemia* cultured with *Spirulina* sp. resulted in better growth of juvenile *H. abdominalis* as compared to cod liver oil, DHA Protein Selco® and Super Selco® (Woods, 2003). Another natural source of enrichment is *Chlorella* sp., a single-celled plant (Ötleş and Pire, 2001). Selco® and cod liver oil are two commercial enrichments that can be used for the improvement of fatty acid content in *Artemia*. The results may vary depending on enrichment materials and target species. This study focused on the feeding aspect, whereby seahorse juveniles were fed with *Artemia* with different cultured diets. It is hoped that the findings of this study will contribute to the establishment of culture techniques for *H. barbouri* and *H. kuda*.

MATERIALS AND METHODS

Maintenance of broodstock and production of juvenile seahorses

Trials using *Hippocampus barbouri* were carried out at Hatchery unit, Institute of Bioscience, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. Mature F2 generation *H. barbouri* broodstock were produced at this location, and juvenile seahorses were produced for this study. Broodstock were conditioned in a laboratory with constant feeding. Seawater used in the study was filtered, treated with ultra-violet (UV) light, and stored in a reservoir tank with strong aeration. Pairs of mature seahorses displaying courtship behavior were placed in breeding tanks. Seahorse pairs were fed with a mixed diet comprised of post-larvae shrimp (*Penaeus vannamei*), frozen mysids (Hikari, USA), adult *Artemia*, fresh mysids (*Neomysis* sp.), and freshwater shrimp (*Macrobrachium* spp). Heavily pregnant male seahorses were monitored daily for abdominal contraction as a sign of giving birth. Upon release, newborn seahorses were

placed into a nursing tank. Hang-on suction filter tubing was covered with fine mesh netting to prevent accidental suction of juvenile seahorses into the filtration system. Stocking was at 60-80 newborns per tank. These newborns were stabilized and maintained in nursing tanks prior to feeding experiments.

Preparation of Artemia nauplii

Hatching of *Artemia* was carried out daily to provide newly-hatched nauplii to the newborn seahorse juveniles. *Artemia* cysts weighing 0.2 g were incubated in 2 L of seawater in a beaker with strong aeration. *Artemia* hatched approximately 24 h later with the release of nauplii. Harvesting was carried out by removing the aeration to allow the nauplii to congregate at the bottom of the beaker, while the cyst shells float to the surface. After five min, nauplii were siphoned from the beaker and ready to be used for feeding to newborn seahorses. Feeding was carried out to observed satiation at 9 AM, 1 PM and 6 PM. *Artemia* was used as live food for the juvenile seahorses, and were cultured in tanks to various sizes depending on the size of the seahorses.

Preparation of cultured Artemia

Culture of Artemia was carried out by using *Artemia* of nauplii stage (24 h after incubation) were cultured in separate 15 L aquarium tanks with different culture media for 24 h until they reached metanauplii (24 h after hatching). The procedure continued with further bioenhancement using the same respective treatment, lasting 30 min, then collected by 500 µm sieve before feeding to seahorse juveniles. Survival of *Artemia* metanauplii was approximately 50 %. The first treatment (A) was *Artemia* metanauplii only using low-cost growing medium to sustain the survival of *Artemia*. Fresh *Chlorella* (1 L) for the second treatment (CF) was obtained from SATREPS (microalgae production project in Institute of Bioscience, UPM, Serdang, Selangor, Malaysia) and used as culture media for *Artemia* metanauplii. The third treatment (P) included marine shrimp sinking pellets (Starfeed 7701, Malaysia) with 36 % protein content. The fourth culture media named treatment (CP) was *Chlorella* tablets (Yaeyama, Japan) ground into powder using mortar and pestle prior to use. Spirulina (Josens, Malaysia) with 72 % protein content was used as the fifth culture media (S). The three culture media in powder form (P, CP and S) were dissolved in filtered seawater (30 ‰) at 0.5 $g \cdot L^{-1}$ using electronic blender, prior to feeding it to *Artemia*. These preparations were repeated daily in order to prepare the same cultured *Artemia* for juvenile seahorses.

Feeding of Hippocampus barbouri

Hippocampus barbouri juveniles aged 14 days after birth (DAB) were used for experiments after they had passed the critical period (Nur *et al*., 2018) and had become more stable. The five treatments were used for feeding seahorse juveniles: *Artemia* (A), *Artemia* with fresh *Chlorella* sp. (A+CF), *Artemia* with pellets (A+P), *Artemia* with *Chlorella* powder (A+CP) and *Artemia* with *Spirulina* powder (A+S). Experimental tanks were prepared in advance, complete with hang-on filtration unit with biofilter material. Seahorses were randomly chosen and stocked at 10 juveniles per tank. Feeding of juvenile seahorses was carried out daily at 9 AM, 12 PM, and 4 PM. Excess *Artemia* metanauplii and waste were removed 1 h after feeding to avoid the accumulation of organic matter, which could increase ammonia concentration and bacteria. Height (mm) and weight (g) of seahorses were measured using vernier caliper and microbalance, respectively, at initiation of the trials, and then every 30 days for the 60-day study period. Survival was monitored daily and tabulated at the end of the experimental period.

Water quality parameters, data collection and analysis

Height (Ht) of seahorse was measured to the nearest millimeter (mm) from the tip of coronet to the tip of the outstretched tail, with the head held at right angles to the body following Lourie *et al*. (1999). Seahorse is very fragile at young age, thus should be handled with utmost care to lower the risk of mortality. Wet weight (Wt) was measured to the nearest gram (g) with seahorse gently dab on soft tissue to ensure water is absorb before taking

measurement. Data on final height (mm), weight (g) and survival of *H. barbouri* were analyzed statistically using Analysis of Variance (ANOVA) and Tukey test (post-hoc). Data of survival (%) were arcsine-transformed prior to ANOVA. Results are presented as mean±standard deviation (SD). All statistical analyses were performed using SPSS software (Version 21.0). Water parameters which include salinity, pH, ammonia, nitrite, nitrate, dissolved oxygen and temperature were measured once a week.

RESULTS

Growth and survival of Hippocampus barbouri fed with different diets cultured Artemia

Gestation period for *Hippocampus barbouri* was around 14 days. Evidence of a critical period with high mortality for *H. barbouri* was observed from birth until 12 to 13 DAB. Thus, the feeding experiment was initiated using 14 DAB juveniles of *H. barbouri*. At the end of the experimental period, juveniles at 74 DAB fed with diets A, A+P and A+S resulted in significantly higher final heights ($p<0.05$) of 34.44±2.37 mm, 32.59±1.61 mm and 36.01±1.57 mm, respectively, as compared to juveniles given diets A+CF and A+CP.

Juveniles at 74 DAB fed with diets A and A+S had higher ($p<0.05$) final weights than the other treatments, 0.198±0.026 g and 0.221±0.057 g, respectively. Meanwhile, the lowest ($p<0.05$) final weights were for juveniles fed with A+CF and A+CP. However, juveniles fed A+P (final weight of 0.175±0.023 g) did not significantly differ ($p>0.05$) from any of the other diets. Based on growth parameters, feeding of seahorse juveniles with diets, A, A+P and A+S produced higher final height and weight as compared to A+CF and A+CP. In terms of survival (Table 1), diets A and A+S resulted in lower ($p<0.05$) survival (26.99 %) than juveniles fed with A+CF, A+P and A+CP (53.99 %).

Growth in terms of height and weight of seahorse juveniles was measured two times, at 44 and 74 DAB during the 60-day experimental period. There was no significant difference ($p>0.05$) in height of seahorse juveniles at 44 DAB (Figure 1a). Similarly, for the weight of juveniles, no significant difference ($p>0.05$) was observed at 44 DAB among treatment groups (Figure 1b).

Water parameters for all treatment tanks during the 60 days experimental period were as shown Table 2. Dissolved oxygen and pH were between 4.2-6.0 $mg \cdot L^{-1}$ and 7.4-8.0 respectively. Water temperature and salinity in all experimental

Table 1. Initial and final height (mm) and wet weight (g), and survival of *Hippocampus barbouri* juveniles fed with five diets, after a 60-day experimental period.

Diets	Height (mm)		Weight (g)		Survival (%)
	Initial 14 DAB	Final 74 DAB	Initial 14 DAB	Final 74 DAB	
A	23.37±1.12^{a}	34.44±2.37^{a}	0.060±0.01^{a}	0.198±0.026^{a}	26.99^{b}
A+CF	23.65±1.63^{a}	26.83±1.36^{b}	0.060±0.01^{a}	0.115±0.015^{b}	53.99^{a}
A+P	23.77±2.11^{a}	32.59±1.61^{a}	0.060±0.01^{a}	0.175±0.023^{ab}	53.99^{a}
A+CP	23.63±2.10^{a}	28.11±2.52^{b}	0.060±0.01^{a}	0.128±0.035^{b}	53.99^{a}
A+S	23.84±1.34^{a}	36.01±1.57^{a}	0.060±0.01^{a}	0.221±0.057^{a}	26.99^{b}

Note: Different superscripts within the same column indicate significant difference ($p<0.05$); A: *Artemia*; A+CF: *Artemia* fed with fresh *Chlorella*; A+P: *Artemia* fed with marine shrimp pellets; A+CP: *Artemia* fed with *Chlorella* powder; A+S: *Artemia* fed with *Spirulina* powder; DAB: Days after birth

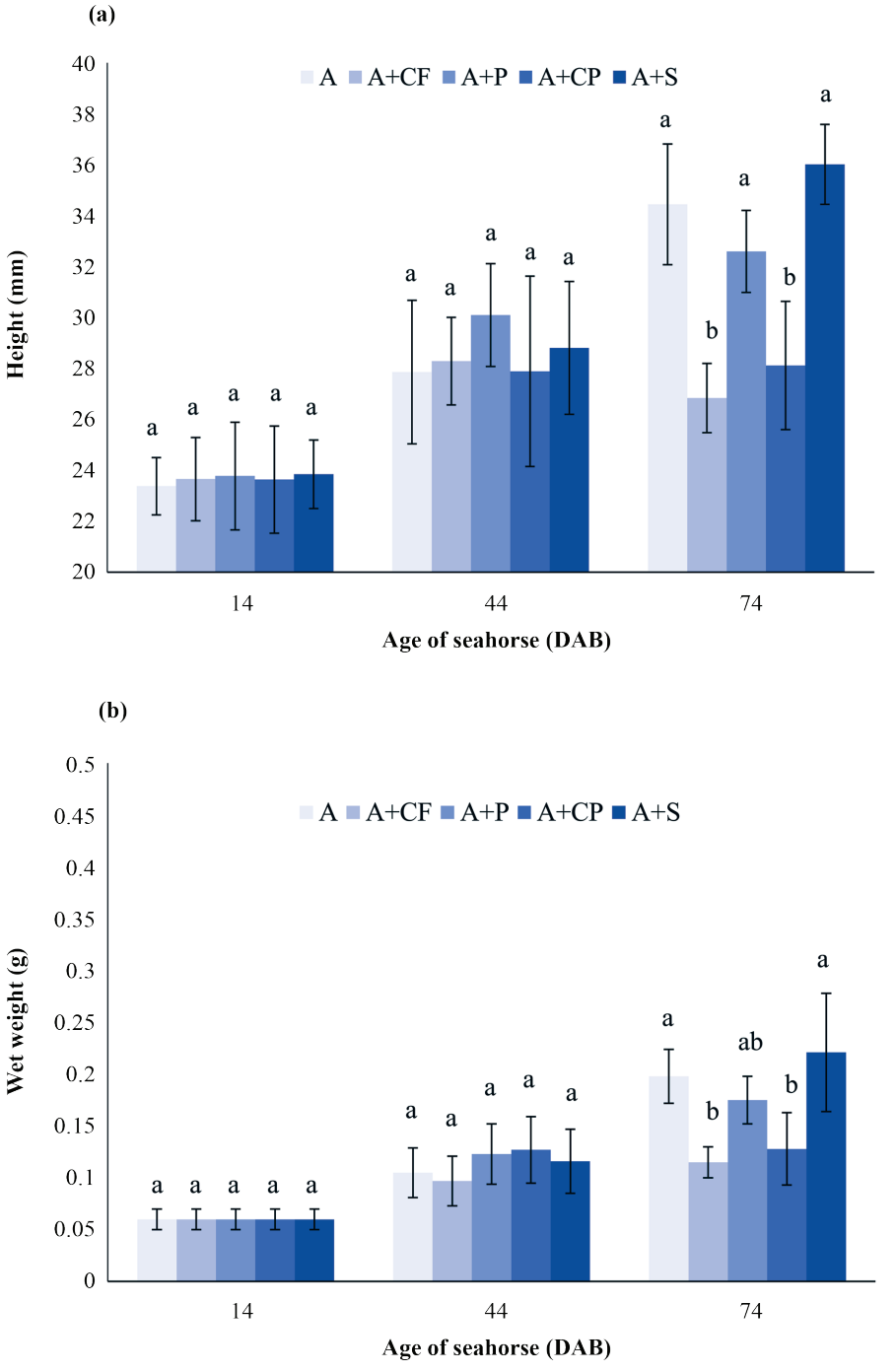

Figure 1. Height (a) and wet weight (b) of juvenile *Hippocampus barbouri* fed with five different diets: *Artemia* (A), *Artemia* fed with fresh *Chlorella* (A+CF), *Artemia* fed with pellet (A+P), *Artemia* fed with *Chlorella* powder (A+CP) and *Artemia* fed with *Spirulina* powder (A+S). Error bars indicate standard deviations. Different letters above each bar indicate significant difference ($p<0.05$).

Table 2. Water parameters during the 60 days culture period of *Hippocampus barbouri* juveniles fed with 5 different diets.

Water Parameters	Diets				
	A	A+CF	A+P	A+CP	A+S
DO ($mg \cdot L^{-1}$)	4.9-5.6	4.2-5.8	4.3-5.5	4.3-5.6	4.5-6.0
Temperature (°C)	27.5-29.8	27.7-29.6	27.2-29.5	27.5-28.9	27.9-29.9
pH	7.4-7.9	7.5-7.9	7.6-8.0	7.4-8.0	7.5-7.9
Ammonia ($mg \cdot L^{-1}$)	0-0.25	0-0.25	0-0.25	0-0.25	0-0.25
Nitrite ($mg \cdot L^{-1}$)	0-0.25	0-0.25	0-0.25	0-0.25	0-0.25
Nitrate ($mg \cdot L^{-1}$)	0-0	0-5	0-5	0-0	0-5
Salinity (‰)	30-32	30-32	29-33	29-31	30-32

Note: A: *Artemia*; A+CF: *Artemia* fed with fresh *Chlorella*; A+P: *Artemia* fed with pellet; A+CP: *Artemia* fed with *Chlorella* powder; A+S: *Artemia* fed with *Spirulina* powder.

tanks fluctuate within narrow range of 27.2-29.9 °C, and 29-32 ‰, respectively. Ammonia and nitrite in all experimental tanks were between 0-0.25 and 0-0.25 $mg \cdot L^{-1}$, respectively. While nitrate was 0 $mg \cdot L^{-1}$ in all the experimental tanks with juveniles fed diets A and A+CP, while slightly higher (0-5 $mg \cdot L^{-1}$) in tanks with juveniles fed diets A+CF, A+P and A+S.

DISCUSSION

Artemia were preferred by most of the aquaculture industry either for the food fish or the ornamental industry. As for the seahorse aquaculture, it becomes the best option of live prey due to the absence of teeth (Lourie, 2003) and also the ease of suiting the mouth size of seahorse as they are growing. Woods (2002) reported that the gut content in wild seahorse *Hippocampus abdominalis*, a big size seahorse (132-274 mm) consisted mainly of amphipod and Caridean shrimp. While the gut of *H. zosterae*, a small size seahorse (<35 mm) was consisted only of copepods (Tipton and Bell, 1988). Bigger seahorses may prefer larger prey. Thus there is a need to culture *Artemia* to supply suitable diets size for the early juvenile seahorse in captive conditoon. However, *Artemia* nauplii at 1-2 days after hatching will become less nutritious for seahorse juveniles.

Newly-hatched *Artemia* nauplii will rapidly develop into metanauplii within 6-8 h when kept at water temperature above 28 °C (Sorgeloos *et al*., 2001). A previous study by Benijts *et al*. (1976) showed that *Artemia* metanauplii contained lower fatty acids and lipid 24 h after hatching. Aside from that it is no doubt that metanauplii are larger in terms of length but weigh much less (20 % decrease) than newly-hatched *Artemia* nauplii. However, the efficiency of using metanauplii stage of *Artemia* is still unknown, and previous studies showed that more metanauplii need to be fed to cultured species in order to achieve the same food uptake (Benijts *et al*., 1976). Nevertheless, *Artemia* can be fed with certain nutrients, as they are non-selective feeders as studied by Dhont *et al*., 2009 reported *Artemia* at metanauplii stage are able to feed immediately when placed in culture medium. Thus in this study, culture of *Artemia* with different diets were being conducted. This investigation did not seek to define the exact nutrient components in the enrichment media or the specific amounts required for the optimal growth and survival of seahorses, but rather to determine the type of culture media for *Artemia*, that is suitable for seahorse juveniles. Even hormones can be added into the enrichment mixture for the enhancement of seahorse growth (Nur *et al*., 2018). Furthermore, the growth and survival may vary depending on the seahorse species when fed with different cultured *Artemia*.

Culture media used in this study are commonly available commercial products. The main enrichment medium initially is microalgae. The base of aquatic food chains is phytoplankton, either as fresh microalgae or microalgae-based products. Production of fresh microalgae using a non-sterile system may cause contamination in the culture, therefore microalgae-based products are used instead of fresh microalgae. Zooplankton relies on this phytoplankton as its natural food source (Muller-Feuga, 2000). Previous studies have shown that enrichment of zooplankton such as rotifers and brine shrimp with microalgae resulted in better growth and survival of seabass larvae (Zaki and Saad, 2010). As for feeding of seahorses, *H. guttulatus* with *Chlorella*-enriched *Artemia* resulted in 60 % survival of juveniles as compared to 0 % survival when fed with *Artemia* enriched with DHA Selco (Palma *et al*., 2011). Nutritional value of zooplankton can be improved through enrichment, not only with protein and energy but also vitamins and other essential nutrients, such as polyunsaturated fatty acids, PUFA (Hemaiswarya *et al*., 2011). Another report by Mélo *et al*., 2016, even though early juveniles *H. reidi* have a higher growth and survival not relative to higher ingestion rate but rather due to nutritional improvement of the diets supply to them. In the present study, juvenile seahorse fed with *Artemia* cultured using fresh microalgae, *Chlorella* did not give better growth, but was able to support better survival of *H. barbouri* juveniles.

Seahorse nutrition is still a relatively new focus of interest however in the feeding cycle of seahorses in their natural environment, phytoplankton only serves as food for most of their prey. In the wild, seahorses are considered a secondary consumer in the food chain. A study on the gut contents of *H. abdominalis* by Woods (2002) showed that this seahorse feeds on crustaceans, in particular, amphipods and zooplankton. As seahorses are carnivorous, they depend more on animal-based protein to support their growth. In the present study, enrichment of zooplankton using shrimp with high protein content was able provide nutrients to support growth and survival of *H. barbouri* juveniles. This indicates that protein might be one of the key factors in sustaining the juvenile seahorse, similar to other aquatic species. The optimal nutritional requirements even for the most commercialized *H. abdomialis* is still unknown (Woods, 2007). As this study did not show the exact requirement of juvenile seahorses, it is difficult to determine whether the *Artemia* enrichment media were fully absorbed by the seahorses. Based on this study, cultured of *Artemia* using plant-based protein, in this case, microalgae might not be sufficient to support optimum growth of seahorses.

Aquaculture involved the culture of aquatic organism, thus water quality become one of the major concern. Commonly measured parameters are temperature, dissolved oxygen (DO), salinity, pH and ammonia. The most critical is DO, whereby it restricts the survival of aquatic organism. DO levels below 2 $mg \cdot L^{-1}$ is considered hazardous for most aquatic organism. In this study, the DO levels in the experimental tanks for *H. barbouri* ranged from 4.2-6.0 are considered to be within acceptable limits for the normal living of aquatic organism (Boyd and Tucker, 2012; Bhatnagar and Devi, 2013). In this study, ranges of pH of in *H. barbouri* tanks were 7.4-8.0. According to Bhatnagar and Devi (2013), pH between 7.0-8.5 is ideal for fish. Dwiputra (2013) reported the optimum pH for the culture of *H. barbouri* is between pH 7 to 8. High fluctuation of pH may cause physiological stress, whereby after long period reduce the immune system thus become more susceptible to pathogen. In extreme cases, it may results in the mortality of aquatic organism (Boyd and Tucker, 2012), even worse for the sensitive seahorses. According to Wong and Benzie (2003), better growth of *Hippocampus whitei* was observed when cultured at high temperature of 26 °C. While study on *Hippocampus abdominalis* showed better growth of juvenile at a lower temperature of 18 and 21 °C. Nonetheless, the temperature ranges for these two seahorses cannot be used as benchmark, as other seahorse species may have different temperature tolerances. The specific temperature range should be according to the natural habitat of that particular seahorse species. Ambas (2009) reported the survival of *H. barbouri* juveniles at temperature range of 26-32 °C. In the present study, *H. barbouri* juveniles of 14 to 74 day after birth were able to thrive well with survival of 27-54 % at temperature

range of 27.2-29.9 °C. Hilomen-Garcia *et al*. (2003) reported the survival of *H. kuda* with short term exposure to salinity of 15 to 50 ‰. In this study, *H. barbouri* showed wide range of salinity tolerance of 29-33 ‰ as they are found marine habitat with salinity range of 28-32 ‰. Ammonia is a by-product from protein metabolism and bacterial decomposition of organic matter in the water. While nitrite is a product from the nitrification process of bacteria. These two parameters at high concentration are consider lethal to fish (Bhatnagar and Devi, 2013), therefore should be maintain at lowest level. Low fluctuations of ammonia, nitrite and nitrate were observed in the present study, with levels close to 0 $mg{\cdot}L^{-1}$. The highest concentrations of ammonia and nitrite at 0.25 $mg{\cdot}L^{-1}$ in some tanks show no detrimental effect on *H. barbouri* juveniles. Nitrate is less toxic compared to ammonia and nitrite. However, in fish culture, its level should not exceed 100 $mg{\cdot}L^{-1}$ (OATA, 2008). In comparison, the nitrite level of 0-5 $mg{\cdot}L^{-1}$ in the seahorse tanks in the present study is so much lower. In short, water quality tolerance for *H. barbouri*, DO should be above 4.2 $mg{\cdot}L^{-1}$, with temperature range of 27.2 to 29.9 °C, pH of 7.4 to 8.0, ammonia below 0.25 $mg{\cdot}L^{-1}$ and salinity between 29 to 32 ‰ for the successful culture.

CONCLUSION

Based on the findings of this study, *Artemia* cultured with marine shrimp pellets is able to support growth and survival of *Hippocampus barbouri*. Thus, it can provide a solution for the feeding of seahorse juveniles, the main bottleneck in seahorse aquaculture. *Hippocampus barbouri* showed ready acceptance to enriched *Artemia*. In addition, feeding using cultured *Artemia* can sustain the juvenile seahorse until two months of age, therefore providing a solution for the feeding of seahorse juveniles. The use of fish pellets as enrichment for *Artemia* metanauplii is highly recommended, since they contain balanced nutrients to support growth of fish. These enriched *Artemia* can also be used as a food for other aquatic species.

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