

## Reproductive performance of seahorse, *Hippocampus barbouri* (Jordan and Richardson 1908) in control condition

Nur F.A.H.<sup>1</sup>; Christianus A.<sup>1,2\*</sup>; Muta Harah Z.<sup>2</sup>; Ching F.F.<sup>3</sup>;  
Shapawi R.<sup>3</sup>; Saad C.R.<sup>2</sup>; Senoo S.<sup>3</sup>

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### Abstract

*Hippocampus barbouri* is one of the seahorse species found in shallow water of Malaysia. It is used as known as a global trade species in ornamental fish industry. To date, there is no documented report on seahorse aquaculture especially for *H. barbouri* in Malaysia even in Southeast Asia region. Seahorse aquaculture should be considered as an alternative source of seahorses to reduce the pressure on wild population. Therefore, this study was conducted to establish suitable techniques for broodstock maintenance and reproduction by focusing on culture system and feeding. *Hippocampus barbouri* were maintained and bred successfully in a controlled culture system. Minimum water depth required for the spawning of *H. barbouri* is 38 cm. Best reproductive performances was observed in broodstock fed with post-larvae shrimp. However, frozen mysid can also be used in the culture of *H. barbouri*. The minimal requirement of n-3 and n-6 fatty acids for the reproduction of *H. barbouri* was  $5.13 \pm 0.04$  % and  $14.83 \pm 0.10$  % respectively.

**Keywords:** *Hippocampus barbouri*, Culture system, Feeding

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1-Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

2-Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

3-Borneo Marine Research Institute, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia.

\*Corresponding author's email: annie@upm.edu.my

## Introduction

Seahorses belong to the Syngnathidae family includes pipefishes, pipehorses and seadragons (Lourie *et al.*, 1999). Harvesting seahorses to fulfill the high demands for traditional Chinese medicine (TCM) and marine aquarium trade resulted in the decline of the wild populations ( Vincent, 1996; Lourie *et al.*, 1999; Foster and Vincent, 2004). Seahorses also an icon for issues related to incidental by-catch and habitat loss (CITES, 2002). Therefore, seahorses are among the fourteen bony fishes listed in Appendix II of the Convention on the International Trade of Endangered Species of Wild Fauna and Flora (CITES, 2002) and Red List of International Union for Conservation of Nature as Threatened Species (IUCN, 2006).

Ten species of seahorses were found in Malaysia, this includes *Hippocampus barbouri* (Lim *et al.*, 2011). Its shallow water habitat in Malaysia exposed this species to human activities (Choo and Liew, 2004). As a global trade species, *H. barbouri* is the most common species kept in aquarium due to its uniqueness in appearance and attractive color variation from white, pale yellow to pale brown (Kuitert, 2000; Koldewey and Martin-Smith, 2010; Olivotto *et al.*, 2011).

Seahorse aquaculture can be considered as an alternative source in order to reduce the pressure on wild population (Payne and Rippingale, 2000; Lin *et al.*, 2007; Faleiro *et al.*, 2008). However, production of seahorse

in captivity is still at infancy stage with husbandry problems and high juvenile mortalities (Vincent, 1996; Planas *et al.*, 2008). In the process to develop of suitable techniques for broodstock maintenance and reproduction, special focus was given to culture system and feeding (Planas *et al.*, 2008). Culture system is an important factor particularly to maintain water quality in order to provide favorable conditions for seahorse broodstock (Koldewey, 2005; Planas *et al.*, 2008).

Based on previous study, diets for seahorse broodstock have great influence on gonad development and brood size (Wong and Benzie, 2003; Sheng *et al.*, 2006). Adult *Artemia* were regularly given to seahorse broodstock in captivity compared to other variety of food items, including mysid shrimp, shrimps and amphipods either as live or frozen diet (Woods and Valentino, 2003; Dzyuba *et al.*, 2006; Lin *et al.*, 2006; Lin *et al.*, 2007; Buen-Ursua *et al.*, 2015). Providing a diet that fulfill nutritional requirement is considered as a great challenge in seahorse aquaculture.

Success reproduction is crucial to ensure the sustainability of seahorse aquaculture (Payne, 2003; Lin *et al.*, 2007). To date, no report on seahorse aquaculture in Malaysia and the Southeast Asian region (Koldewey and Martin-Smith, 2010). Therefore, this study was conducted to establish breeding technology for *H. barbouri* which will contribute to the development of seahorse aquaculture.

## Materials and methods

### *Acquisitions of seahorse*

Adult seahorses were bought from fishermen at Semporna coast of Sabah, Malaysia. These seahorses were conditioned for 1 month before being used for breeding trials. Tilapia fry and shrimp postlarvae were used as feed during this conditioning period. Only healthy seahorses were used for all experiments.

### *Culture system*

Breeding experiments were conducted at three different locations, Borneo Marine Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia (BMRI); Department of Aquaculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia (DoA) and Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia (IBS). Different culture systems were used in each location for the conditioning and breeding of wild *H. barbouri*.

In BMRI, broodstock were conditioned in one tonne black circular polyethylene tank (H=0.8m). This conditioning tank was connected with another tank of similar size as a filtration unit filled with hard coral as filter media. Airlift technique was used to circulate water from one tank to the other. For breeding tank, similar system as conditioning tank was set using square fiberglass tank (0.4×0.4×0.4m).

In DoA, plastic tanks (0.55×0.25×0.33m) were used for both conditioning and breeding of *H.*

*barbouri*. Each tank was equipped with Classica® Crystal Hang-on Filter (EF-087). Sponge and sintered glass Biohome® Plus were used as filter media. In IBS, rectangular fiberglass tanks (0.4×0.4×0.5m) were used with setup similar to BMRI. Glass tanks (0.4L×0.3W×0.4H m) were used as breeding tank in IBS. Each of these tanks was equipped with hang-on filter (with sponge and Biohome as filter media), similar to the setup at DoA. Figure 1, 2 and 3 shows the front view of both conditioning and breeding tank set up in BMRI, DoA and IBS respectively. In each conditioning and breeding tank, plastic chain tied to a sinker served as holdfast for seahorses.

After conditioning for 1 month, each pair of broodstock with average standard length (SL)  $12.040 \pm 0.459$  cm was transferred into breeding tank. Standard length was determined by measuring the length from the tip of the tail to the mid-point of the cleithral ring plus the length from the tip of the snout to the mid-point of the cleithral ring (Lourie *et al.*, 1999). Seahorses were blotted using filter paper before being weighed to get the wet weight, WW (Job *et al.*, 2002). Data on reproductive performance was collected only after two weeks of introduction into breeding tank. This experiment was carried out for 3 months.

Prior to usage, seawater undergoes serial filtration (cartridge pore size of 5, 1 and  $0.1 \mu\text{m}$ ) then passed through Atman® UV Sterilizer (Model UV-11W) with water flow rate of 10 l/min.

Broodstock was fed twice daily, *ad libitum* with frozen Hikari® Bio-Pure Mysis Shrimp at 0930 and post-larvae of white shrimp and red tilapia fry at 1530. Faeces and excess feed were siphoned daily before and after feeding. Water depth in the tank was maintained throughout the experimental period. YSI Professional Plus Multi-Parameter and HACH® DR/2400 Portable Spectrophotometer were used to measure dissolved oxygen, DO (ppm), temperature (°C), salinity (ppt), pH, ammonia (ppm), nitrate (ppm), and nitrite (ppm) twice a week throughout this experiment.

#### *Feeding experiment*

Experiment conducted at IBS used similar breeding tank set up as in the previous experiment. After one month conditioning, each pair of broodstock with average SL and WW ( $12.100 \pm 0.424$  cm and  $5.551 \pm 0.322$  g) were introduced into each breeding tank. Broodstock were fed with adult *Artemia* (AA), frozen Hikari® Bio-Pure Mysis Shrimp (FM), post-larvae of white shrimp (PLS) and fry of red tilapia (TF) twice daily at 0930 and 1530.

Diets were rinsed few times with freshwater and blotted using filter paper to measure the WW of the diets prior fed to the seahorse broodstock. After an hour of feeding, excess diets were siphoned, blotted using filter paper and weighted to determine daily food intake of seahorses broodstock. Sampling for weight of broodstock was conducted

twice a week. Food consumption in the percentage of body weight (% BW) was estimated by measuring the amount of food intake per total weight of seahorse individual per day (individual/seahorse/day). Experiment was carried out for three months. Quantity of diets fed to seahorse was estimated based on the data of food consumption. Throughout this study, water quality was monitored and data on reproductive performance were recorded.

#### *Diet preparation*

Newly hatched *Artemia* nauplii from Bio Marine *Artemia* cysts were grown in circular fiberglass tank with vigorous aeration at stocking density 200/l. *Artemia* nauplii were fed with rice flour and *Spirulina* powder (Josens) until reach adult stage (total length, TL: 5-10mm). Adult *Artemia* was enriched with cod liver oil for twelve hours prior to feeding.

Both PLS and TF bought from fish farms once a month were maintained in one tonne rectangular fiberglass tank. The average TL of PLS (the tip of rostrum to the tip of telson) and TF (the tip of closed mouth to the extended tip of the caudal fin) that were fed to seahorse broodstock were  $13 \pm 2$  mm and  $6 \pm 2$  mm respectively. Prior to use, TF fry fed with newly hatched *Artemia* nauplii *ad libitum* for five minutes. Freshwater was used to defrost and clean FM. All diet were rinsed with filtered freshwater and seawater for 3 times before being fed to the

broodstock. Samples of three diets were stored at  $-20^{\circ}\text{C}$  for proximate analysis and fatty acid profiling.

#### *Data collection*

Data collected were used to calculate the weight gain (WG) and specific growth rate (SGR) during the experiment.  $\text{WG}(\%) = (W_f - W_i) / W_i \times 100$  and  $\text{SGR}(\%) = ((\ln W_f - \ln W_i) / t) \times 100$  where,  $W_f$  is the final weight (g),  $W_i$  is the initial weight (g), SL is the standard length (cm), and t is the duration (d).

To measure the reproductive performance of seahorse, data on the occurrence of spawning, unsuccessful spawning (when all of unfertilized eggs found at tank bottom), pregnancy period (number of days male incubated the fertilized eggs until giving birth), brood size (number of newborn juvenile seahorse), size of newborn juvenile (SL and WW), number of unfertilized eggs (from unsuccessful spawning or some of unfertilized eggs aborted during spawning), number of aborted eggs (fertilized eggs being aborted from brood pouch during pregnancy) and number of premature juvenile were recorded. The newborn juvenile seahorses were cultured for two weeks. Survival was recorded to determine the effect of broodstock diet on juvenile quality.

Unfertilized eggs found at the bottom of the tanks were siphoned and counted. Eggs diameter was measured using ocular micrometer fits in eyepiece of Leica<sup>®</sup> DM500 Compound Microscope. Volume of the unfertilized eggs and

yolk sacs was estimated based on length (L) and width (W) measures, using the volume of an ovoid,  $V = \frac{1}{6} \times \pi L W W$

(Faleiro *et al.*, 2008).

#### *Proximate Analysis*

Proximate analysis (protein, lipid, ash and moisture) of diet samples were conducted according to standard methods AOAC.

#### *Fatty acid profiling*

Fatty acid profiling was carried out respectively after total lipid determination. Lipid from sample was extracted using Soxhlet extraction apparatus. Fatty acid methyl esters (FAME) were injected into a capillary column SUPELCO SP-2380 (100m fused silica, 0.25mm internal diameters) installed in a HP-5890 Series II Plus gas chromatograph. Peaks were identified by comparison with standard FAME as an internal standard.

#### *Data analysis*

Data were presented as mean  $\pm$  standard deviation (SD). Data collected during the feeding experiment were analysed using one way of Analysis of Variance (ANOVA) and Tukey test to determine the significant difference between the treatments. Correlation coefficient of reproductive performance to selected biochemical parameters were analyzed using correlation test at  $\alpha = 0.05$ . All statistical analyses were carried out using SAS 9.4

## Results

### *Culture system*

Different culture systems used at different location depend on the availability of materials (tank, filter, filter media). In this study, adult *H. barbouri* were maintained and bred in captive conditions using different culture system. However, gas bubble disease (GBD) occurs frequently in air-lift culture system at BMRI. Conditioning of *H. barbouri* in DoA with high stocking density resulted in highest level of ammonia, nitrate and nitrite compared to other locations. Table 1 shows the comparison in tank set up and water quality data between the three different locations.

Prior to spawning, courtship behaviour was observed mostly early morning for all types of culture systems. Males initiated the courtship by brightening their body coloration, pushing their belly forwards to extend the brood pouch, hooked the tail of female and swim in pair. Upon successful spawning, female will transfer eggs into brood pouch of male. Transfers of eggs were observed to be unsuccessful in GBD male or when the water depth is less than 0.3m.

Breeding tank set up in IBS with water depth 0.38m recorded the least occurrences of unsuccessful eggs transfer. As for water quality, lowest ammonia ( $0.04 \pm 0.03$  ppm), nitrate ( $1.15 \pm 0.40$  ppm) and nitrite ( $0.02 \pm 0.02$  ppm) level were recorded for the breeding tank set up in IBS. Table 2 shows the comparison on breeding tank

set up and data on reproductive performance between different locations. Besides the variation of tank set up, feed may influence the reproductive performance of seahorse. Therefore, the next experiment was conducted to determine the actual effects of provided diet on the reproductive of *H. barbouri*.

### *Feeding experiment*

Post-larvae of white shrimp (PLS) was the most preferred feed since broodstock of *H. barbouri* consumed  $9.81 \pm 0.77\%$  by body weight of PLS daily, which was significantly higher ( $p < 0.05$ ) compared to AA ( $5.80 \pm 0.14\%$ ), FM ( $5.83 \pm 0.37\%$ ) and TF ( $4.11 \pm 0.28$ ). Broodstock fed with PLS recorded significantly higher ( $p < 0.05$ ) of WG ( $21.30 \pm 0.67\%$ ) and SGR ( $2.76 \pm 0.08\%$ ) compared to broodstock fed with other diets (Table 3).

Broodstock fed on PLS and FM have the highest number ( $p < 0.05$ ) of spawning occurrences (Table 4). However, with regards to brood size, broodstock fed on PLS produced largest size ( $p < 0.05$ ) juveniles (SL:  $0.95 \pm 0.05$ ; WW:  $0.005 \pm 0.001$ ) and highest number of juvenile ( $384 \pm 76.37$  pieces). Broodstock fed with AA recorded significantly lowest ( $p < 0.05$ ) brood size producing significantly higher ( $p < 0.05$ ) number of premature eggs ( $21.5 \pm 3.54$  eggs). These eggs were fertilized eggs that have been aborted before completely develop into juvenile. Highest number of abnormal juvenile

was produced by broodstock fed on AA (24.5±7.78 pieces) or TF (27.5±10.61 pieces). Abnormal juvenile was characterized by short snout and incompletely developed of dorsal fin rays which lead to swimming inability. Male of *H. barbouri* in all treatment incubated the fertilized eggs for 14 days before give birth the newborn juvenile.

Unsuccessful spawning occurs to the broodstock fed with AA when female *H. barbouri* failed to transfer eggs into male brood pouch. No unsuccessful spawning occurs in the broodstock fed with other diets. Significantly higher numbers of unfertilized eggs (55±4.24 eggs) was found at the tank bottom consist of broodstock fed with AA compared to the broodstock fed on TF (5±0 eggs), FM (4±0 eggs) and PLS (3±0 eggs) respectively. Shape of unfertilized eggs varies from rod, teardrop to round. Unfertilized eggs consist of yolk surrounded by orange-yellow oil droplets. Broodstock fed on

PLS produced significantly highest ( $p<0.05$ ) volume of eggs and yolk which were 5.36±0.72  $\mu\text{L}$  and 4.67±0.67 $\mu\text{L}$  respectively compared to the other treatment.

Proximate compositions for each type of diet were presented in Table 5. Percentage of protein was highest (66.51±0.48%) in PLS and TF (65.60±1.98%). Significantly highest fat (17.19±0.31%) and energy (227.00±0.00kJ) were recorded in TF. Carbohydrate (29.03±2.31%) and ash (25.38±1.02%) content was significantly highest in AA.

Fatty acid profiles of different diet were recorded in Table 6. Palmitic acid (C16:0) was significantly highest ( $p<0.05$ ) in AA. Mono unsaturated fatty acid (MUFA) especially oleic acid (C18:1-n9) was significantly higher ( $p<0.05$ ) in FM. Significantly highest percentage of total n-3 (16.70±0.04%) and n-6 (24.92±0.20%) fatty acid was observed in TF and PLS respectively.

**Table 1: Data during conditioning broodstock of *Hippocampus barbouri* in three different locations.**

		<b>BMRI</b>	<b>DoA</b>	<b>IBS</b>
Water depth		0.75 m	0.30 m	0.48 m
Tank	Size (m)	$\pi \times 0.75 \times 0.80$	$0.55 \times 0.25 \times 0.33$	$0.4 \times 0.4 \times 0.5$
	Colour	Black	(L × W × H) Transparent	(L × W × H) Blue
	Material	Polyethylene tank	Plastic tank	Fiberglass tank
Filtration	System	Air-lift	Hang-on filter	Air-lift
	Media	Hard coral	Sponge & Bio-home	Hard coral
Stocking density		1 ind/ 80l	1 ind/ 10l	1 ind/ 18l
Feeding		frozen Hikari® Bio-Pure Mysis Shrimp, red tilapia fry & post-larvae of white shrimp		
Water parameter	DO (ppm)	7.46 ± 0.20	4.94 ± 0.34	7.25 ± 0.11
	Temperature (°C)	27.65 ± 0.64	26.78 ± 0.53	28.03 ± 1.44
	Salinity (ppt)	31.84 ± 0.38	30.58 ± 0.52	30.89 ± 0.53
	pH	8.93 ± 0.20	7.95 ± 0.21	7.98 ± 0.18
	Ammonia (ppm)	0.10 ± 0.06	0.20 ± 0.09	0.04 ± 0.03
	Nitrate (ppm)	2.07 ± 0.35	4.03 ± 2.03	1.15 ± 0.40
	Nitrite (ppm)	0.06 ± 0.03	0.16 ± 0.07	0.02 ± 0.02

**Table 2: Data on breeding of *Hippocampus barbouri* at three different locations.**

		BMRI	DoA	IBS	
Water depth		0.35 m	0.30 m	0.38 m	
Tank	Size (m)	0.4L × 0.4W × 0.4H	0.55L × 0.25W × 0.33H	0.4L × 0.3W × 0.4H	
	Colour	Blue	Transparent	Transparent	
	Material	Fiberglass tank	Plastic tank	Glass tank	
Filtration	System	Air-lift	Hang-on	Hang-on	
	Media	Hard coral	Sponge & Bio-home	Sponge & Bio-home	
Stocking density		1 ind/ 28l	1 ind/ 20l	1 ind/ 22l	
Feeding		frozen Hikari® Bio-Pure Mysis Shrimp, red tilapia fry & post-larvae of white shrimp			
Water parameter	DO (ppm)	7.41 ± 0.16	5.25 ± 0.23	5.80 ± 0.07	
	Temperature (°C)	27.88 ± 0.85	27.03 ± 0.24	29.17 ± 0.98	
	Salinity (ppt)	31.88 ± 0.38	29.83 ± 0.92	30.47 ± 0.80	
	pH	7.98 ± 0.15	7.76 ± 0.17	8.16 ± 0.10	
	Ammonia (ppm)	0.10 ± 0.04	0.13 ± 0.02	0.04 ± 0.01	
	Nitrate (ppm)	1.23 ± 0.43	2.38 ± 0.42	1.58 ± 0.52	
	Nitrite (ppm)	0.03 ± 0.02	0.05 ± 0.02	0.02 ± 0.01	
	Reproductive performance	Spawning occurrence	13	10	20
		Unsuccessful spawning	2	2	-
		Incubation period	14-15 days	14-15 days	13-14 days
Brood size		54.77 ± 5.183	26.70 ± 9.129	59.05 ± 4.947	
Unfertilized eggs		93	114	2	
Juvenile size	SL: 0.83 ± 0.02 cm	SL: 0.81 ± 0.03 cm	SL: 0.85 ± 0.02		
	WW: 0.004 ± 0.001 g	WW: 0.004 ± 0.001 g	WW: 0.005 ± 0.001 g		
No. of premature	-	23	-		

**Table 3: Data on food consumption, weight gain (WG) and specific growth rate (SGR) of *Hippocampus barbouri* fed on different feed.**

Treatment	Amount of feed consumed		WG (%)	SGR (%)
	Wet weight (g)	Percentage by body weight (%)		
Adult <i>Artemia</i>	0.30 ± 0.01 <sup>b</sup>	5.80 ± 0.14 <sup>b</sup>	9.28 ± 0.28 <sup>b</sup>	1.27 ± 0.04 <sup>b</sup>
Frozen mysids	0.32 ± 0.01 <sup>b</sup>	5.83 ± 0.37 <sup>b</sup>	12.29 ± 1.74 <sup>b</sup>	1.65 ± 0.22 <sup>b</sup>
Post larvae shrimp	0.55 ± 0.02 <sup>a</sup>	9.81 ± 0.77 <sup>a</sup>	21.30 ± 0.67 <sup>a</sup>	2.76 ± 0.08 <sup>a</sup>
Tilapia fry	0.23 ± 0.02 <sup>c</sup>	4.11 ± 0.28 <sup>c</sup>	10.88 ± 0.82 <sup>c</sup>	1.48 ± 0.11 <sup>c</sup>

Different superscript letters shows significant differences between treatment at  $p < 0.05$ .

**Table 4: Data on reproductive performance of *Hippocampus barbouri* fed with different feed.**

	Adult <i>Artemia</i>	Frozen Mysids	Post Larvae Shrimp	Tilapia Fry
Spawning occurrence	2 ± 0 <sup>c</sup>	5 ± 0 <sup>a</sup>	5.5 ± 0.71 <sup>a</sup>	3.5 ± 0.71 <sup>b</sup>
Unsuccessful spawning	1 ± 0	-	-	-
Brood size	74 ± 11.31 <sup>c</sup>	211 ± 2.83 <sup>b</sup>	384 ± 76.37 <sup>a</sup>	143 ± 26.87 <sup>bc</sup>
Unfertilized eggs	55 ± 4.24 <sup>a</sup>	4 ± 0 <sup>b</sup>	3 ± 0 <sup>b</sup>	5 ± 0 <sup>b</sup>
No. of premature	21.5 ± 3.54 <sup>a</sup>	11 ± 0 <sup>b</sup>	-	7 ± 0 <sup>b</sup>
No. of abnormal	24.5 ± 7.78 <sup>a</sup>	7.5 ± 0.71 <sup>b</sup>	-	27.5 ± 10.61 <sup>a</sup>
Egg volume (µL)	1.60 ± 0.30 <sup>c</sup>	2.52 ± 0.41 <sup>b</sup>	5.36 ± 0.72 <sup>a</sup>	2.02 ± 0.31 <sup>c</sup>
Yolk volume (µL)	0.98 ± 0.09 <sup>c</sup>	1.75 ± 0.42 <sup>b</sup>	4.67 ± 0.67 <sup>a</sup>	1.39 ± 0.14 <sup>b</sup>
Juvenile size (SL, cm; WW, g)	SL: 0.73 ± 0.03 <sup>b</sup>	SL: 0.79 ± 0.04 <sup>b</sup>	SL: 0.95 ± 0.05 <sup>a</sup>	SL: 0.77 ± 0.03 <sup>b</sup>
	WW: 0.003 ± 0.000 <sup>c</sup>	WW: 0.004 ± 0.000 <sup>b</sup>	WW: 0.005 ± 0.001 <sup>a</sup>	WW: 0.004 ± 0.000 <sup>b</sup>
Juvenile survival (%)	76.67 ± 7.64 <sup>c</sup>	91.67 ± 2.89 <sup>b</sup>	99.00 ± 1.00 <sup>a</sup>	81.67 ± 2.89 <sup>c</sup>

Different superscript letters shows significant differences between treatment at  $p < 0.05$ .

SL = standard length; WW = wet weight.

**Table 5: Data on proximate composition of different feed.**

Treatment	Moisture (%)	Percentage by dry matter (%)				Energy (kJ)
		Protein	Fat	Carbohydrate	Ash	
Adult <i>Artemia</i>	92.73 ± 0.04 <sup>a</sup>	33.28 ± 3.31 <sup>c</sup>	12.31 ± 0.03 <sup>b</sup>	29.03 ± 2.31 <sup>a</sup>	25.38 ± 1.02 <sup>a</sup>	110.91 ± 2.96 <sup>d</sup>
Frozen mysids	91.42 ± 0.00 <sup>b</sup>	60.14 ± 2.15 <sup>b</sup>	4.72 ± 0.09 <sup>c</sup>	21.56 ± 1.49 <sup>b</sup>	13.58 ± 0.58 <sup>b</sup>	134.00 ± 0.00 <sup>c</sup>
Post larvae Shrimp	87.24 ± 0.05 <sup>d</sup>	66.51 ± 0.48 <sup>a</sup>	3.68 ± 0.13 <sup>d</sup>	11.67 ± 0.62 <sup>c</sup>	18.14 ± 0.01 <sup>c</sup>	185.00 ± 0.00 <sup>b</sup>
Tilapia fry	87.84 ± 0.14 <sup>c</sup>	65.60 ± 1.99 <sup>ab</sup>	17.19 ± 0.31 <sup>a</sup>	7.10 ± 2.07 <sup>d</sup>	10.12 ± 0.23 <sup>d</sup>	227.00 ± 0.00 <sup>a</sup>

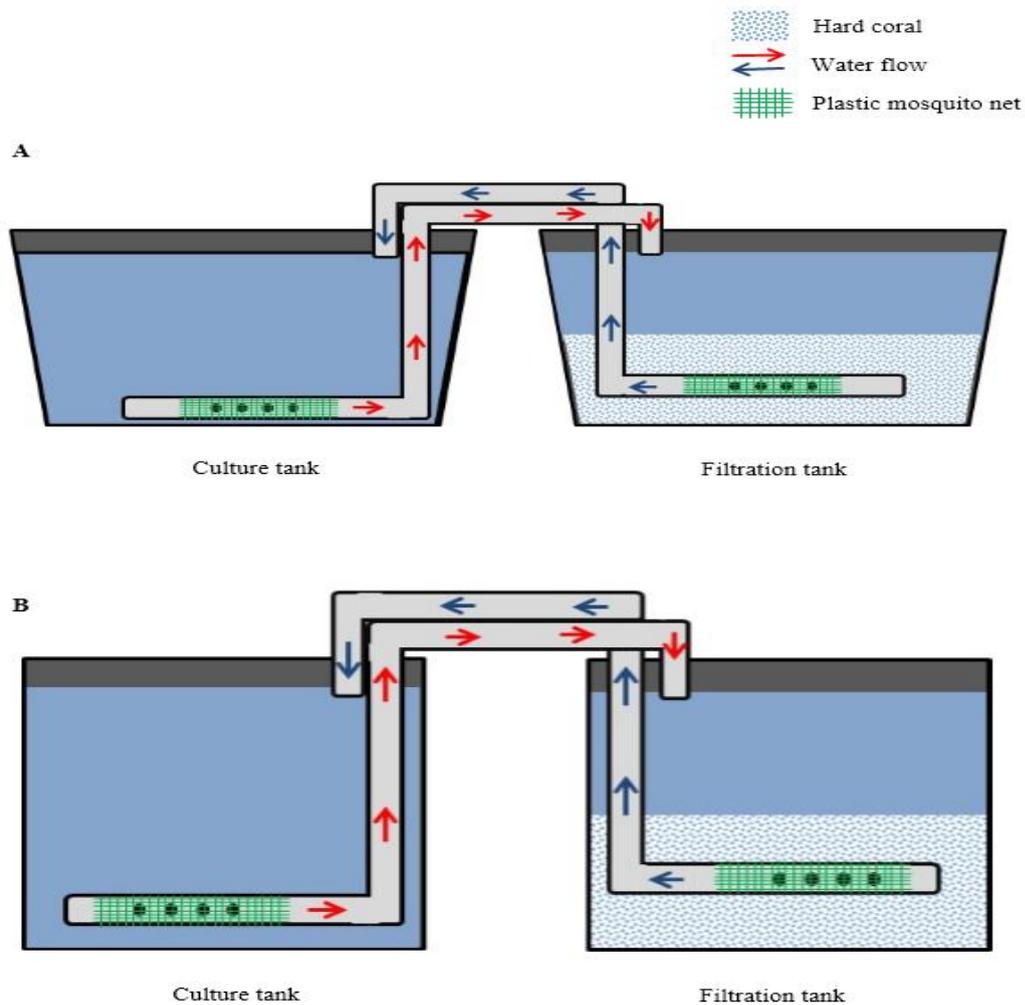
Different superscript letters shows significant differences between treatment at  $p < 0.05$ .

**Table 6: Data on fatty acid profile of different feed.**

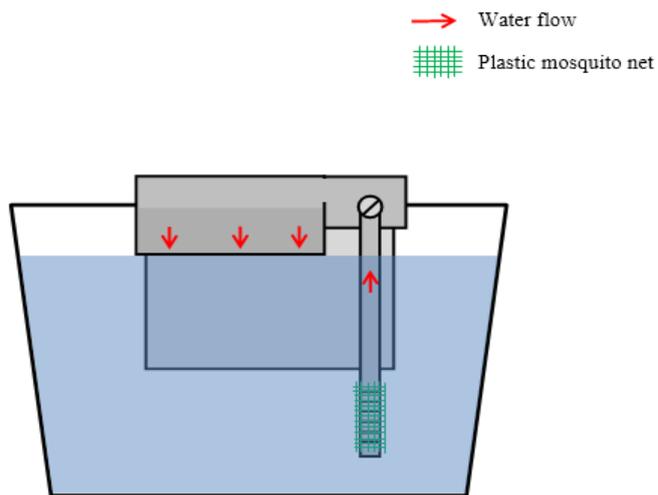
Fatty acid	Treatment			
	Adult <i>Artemia</i>	Frozen mysids	Post larvae shrimp	Tilapia fry
Myristic acid, C14:0	7.97 ± 0.01 <sup>a</sup>	3.64 ± 0.01 <sup>b</sup>	1.59 ± 0.03 <sup>d</sup>	2.32 ± 0.04 <sup>c</sup>
Palmitic acid, C16:0	40.66 ± 0.01 <sup>a</sup>	30.14 ± 0.00 <sup>b</sup>	18.21 ± 0.16 <sup>c</sup>	30.25 ± 0.08 <sup>b</sup>
Palmitoleic acid, C16:1n-7	5.03 ± 0.01 <sup>b</sup>	3.61 ± 0.08 <sup>d</sup>	5.76 ± 0.07 <sup>a</sup>	4.44 ± 0.04 <sup>c</sup>
Stearic acid, C18:0	14.71 ± 0.01 <sup>c</sup>	18.22 ± 0.19 <sup>a</sup>	16.16 ± 0.08 <sup>b</sup>	12.43 ± 0.07 <sup>d</sup>
Oleic acid, C18:1n-9	21.57 ± 0.03 <sup>c</sup>	24.46 ± 0.04 <sup>a</sup>	19.94 ± 0.02 <sup>d</sup>	23.51 ± 0.03 <sup>b</sup>
Linoleic acid, C18:2n-6	4.90 ± 0.04 <sup>d</sup>	14.61 ± 0.08 <sup>b</sup>	22.63 ± 0.23 <sup>a</sup>	8.04 ± 0.04 <sup>c</sup>
Linolenic acid, C18:3n-3	1.49 ± 0.03 <sup>c</sup>	1.88 ± 0.05 <sup>b</sup>	2.71 ± 0.02 <sup>a</sup>	0.58 ± 0.01 <sup>d</sup>
Arachidonic acid, C20:4n-6 (ARA)	0.00 ± 0.00 <sup>c</sup>	0.23 ± 0.02 <sup>b</sup>	2.29 ± 0.04 <sup>a</sup>	2.32 ± 0.04 <sup>a</sup>
Eicosapentaenoic acid, C20:5n-3 (EPA)	1.80 ± 0.06 <sup>b</sup>	1.27 ± 0.04 <sup>c</sup>	5.99 ± 0.04 <sup>a</sup>	5.73 ± 0.05 <sup>a</sup>
Docosahexaenoic acid, C22:6n-3 (DHA)	1.89 ± 0.01 <sup>c</sup>	1.99 ± 0.05 <sup>c</sup>	4.72 ± 0.03 <sup>b</sup>	10.40 ± 0.01 <sup>a</sup>
n-3	5.17 ± 0.08 <sup>c</sup>	5.13 ± 0.04 <sup>c</sup>	13.42 ± 0.06 <sup>b</sup>	16.70 ± 0.04 <sup>a</sup>
n-6	4.90 ± 0.04 <sup>d</sup>	14.83 ± 0.10 <sup>b</sup>	24.92 ± 0.20 <sup>a</sup>	10.35 ± 0.00 <sup>c</sup>
n-3: n-6	1.06 ± 0.02 <sup>b</sup>	0.35 ± 0.01 <sup>d</sup>	0.54 ± 0.01 <sup>c</sup>	1.62 ± 0.01 <sup>a</sup>
DHA: EPA	1.05 ± 0.03 <sup>c</sup>	1.57 ± 0.02 <sup>b</sup>	0.79 ± 0.00 <sup>d</sup>	1.82 ± 0.02 <sup>a</sup>
DHA: ARA	0.00 ± 0.00 <sup>d</sup>	8.77 ± 0.88 <sup>a</sup>	2.07 ± 0.02 <sup>c</sup>	4.50 ± 0.08 <sup>b</sup>

Different superscript letters shows significant differences between treatment at  $p < 0.05$ .

n-3 =omega 3; n-6 =omega 6.



**Figure 1: Front view of A, conditioning tank and B, breeding tank in BMRI. PVC pipe ( $\varnothing = 5\text{cm}$ ) and air-lift technique were used to circulate water from one tank to another.**



**Figure 2: Front view of both conditioning tank and breeding tank in DoA. Hang-on with sponge and sintered glass Biohome® Plus were used to filtered water.**

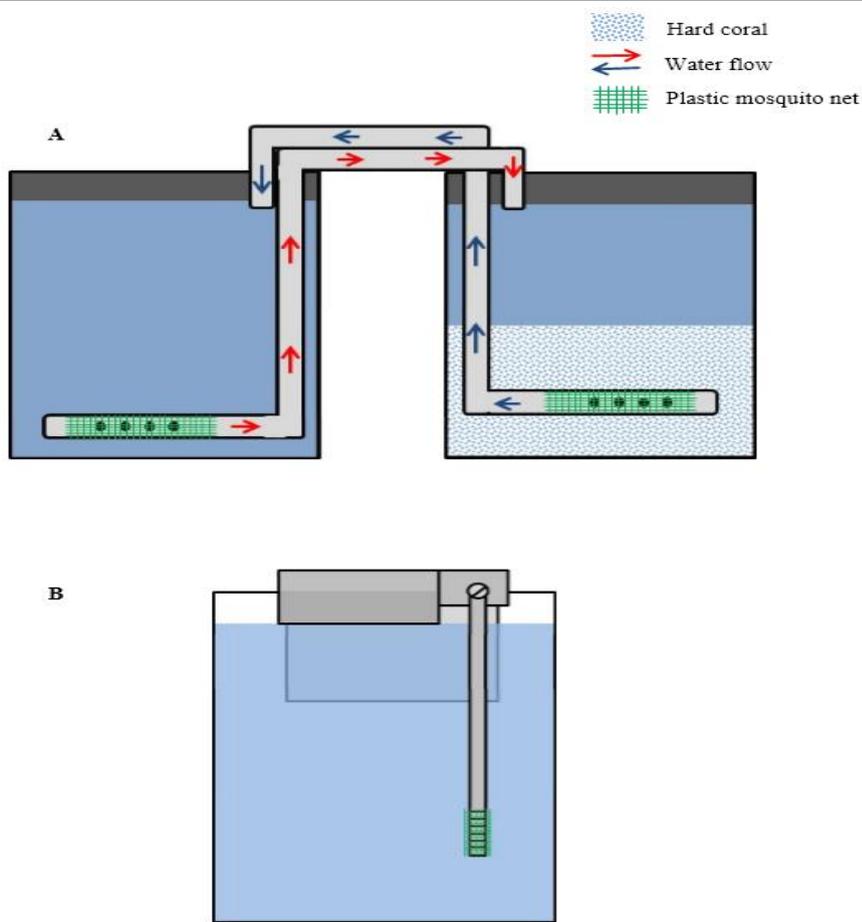


Figure 3: Front view of A; conditioning tank and B; breeding tank in IBS.

### Discussion

Development of suitable techniques especially the physical factor is very important for broodstock maintenance and reproduction (Planas *et al.*, 2008). Culture system is the physical key factor to provide favourable conditions with minimal resources (Duarte *et al.*, 2011; Blanco *et al.*, 2014). Based on this study, *H. barbouri* can be maintained in different culture system. However, there are some factors constraining the successful spawning of *H. barbouri* in captive conditions. Unsuccessful spawning in the present study frequently relates to the occurrence of GBD in seahorse cultured

in air-lift system creates water movement as well as supplemental aeration to minimize the consumption of resources (Loyless and Malone, 1998). However, it causes the presence of air bubble in culture system and increases the occurrence GBD in seahorses (Reinemann, 1987; Planas *et al.*, 2008).

Super saturation of gas especially nitrogen and oxygen typically related with the application of air-lift technique (Parker *et al.*, 1984). This super saturation become the causative agent for GBD in seahorse, whereby gas entrapment in the brood pouch, subcutaneous emphysema on the tail

segment or hyperinflation of swim bladder (Koldewey, 2005; Planas *et al.*, 2008; Koldewey and Martin-Smith, 2010). During this study, air-lift system recorded the highest dissolved oxygen, to near saturation level as suggested by Masser *et al.* (1999).

Tank height is another restrictive factor to the successful breeding of *H. barbouri*. It is important to ensure water depth is sufficient to enable successful eggs transfer to the male brood pouch during spawning process (Woods, 2003; Koldewey, 2005). *Hippocampus abdominalis* requires tank depth of at least 90 cm to facilitate the egg transfer (Sobolewski, 1997). However, high water level will cause difficulty in tank maintenance. Therefore, it is important to determine minimum water depth required to ensure successful eggs transfer during seahorse spawning. Breeding tank with different water depth was tested in this study. All seahorse broodstocks spawned successfully in tank with 38 cm depth in IBS. Hence, *H. barbouri* required at least 38 cm water depth for successful eggs transfer.

Adequate of nutrients is one of the main factors influencing the spawning outcome of teleost fish (Izquierdo *et al.*, 2001). Variety of feeds were given to seahorse broodstock in captivity which include adult *Artemia*, mysid shrimp, amphipods and shrimps, given as live or frozen (Woods and Valentino, 2003; Dzyuba *et al.*, 2006; Lin *et al.*, 2007; Palma *et al.*, 2012). In this study, TF was selected as feed for seahorse

broodstock in this study due to its ready supply. Furthermore, Garcia *et al.* (2012) reported the use of fish larvae as feed for adult *H. barbouri*.

Broodstock of *H. barbouri* fed on PLS shows the best reproductive performance with high numbers of spawning occurrences and brood size. Coincidentally, it was the most preferred feed by the seahorses as compared to the other feeds. Based on optimum foraging theory, seahorse prefer to consumed caridean shrimp with lowest energy expenditure required (Anderson Jr, 2000; Felício *et al.*, 2006). In addition, structure of syngnathid eyes made them adaptable to the mobility and carotenoid-rich prey (Collin and Collin, 1999). Similar to previous finding, adult *H. barbouri* prefers to consumed FM compared to the highly mobile AA and TF (Felício *et al.*, 2006).

Quality and quantity of feed give a major influence on brood size, which affects their gonad development and sperm quality (Wong and Benzie, 2003; Foster and Vincent, 2004; Lin *et al.*, 2007). According to Otero-Ferrer *et al.* (2012) the utilization of live feed resulted in better growth and gonad development of adult seahorses. However, in this study broodstock fed with FM showed better reproductive performance (in terms of spawning occurrence, brood size and juvenile survival) as compared to broodstock fed with live feed such as AA and TF. This condition may due to the higher

percentage of FM consumed by *H. barbouri* as compared to AA and TF.

Composition of n-3 HUFA, especially DHA considered as the major dietary requirement for successful reproduction in marine fishes by affecting steroidogenesis, spermiation and other reproductive parameters (Izquierdo *et al.*, 2001). DHA plays an important role in cells membrane by regulating the integrity and function besides being an important component of phosphoglycerides in gonad and juvenile (Izquierdo *et al.*, 2001). Its deficiencies can cause decreases in fecundity, lower fertilization rates, embryo deformities and poor larval quality (Izquierdo *et al.*, 2001; Otero-Ferrer *et al.*, 2012). In contrast, the present study, broodstock *H. barbouri* fed on PLS (which lower in n-3 contents) compared to TF, shows better reproductive performance evident with bigger brood size with no abnormality on newborn juveniles. Low n-3 content in live feed may be sufficient to compensate the dietary requirement for seahorse (Otero-Ferrer *et al.*, 2012). In addition, low n-3 content also found in eggs of *H. guttulatus* (Planas *et al.*, 2008)

Composition of protein in feed also affects the reproductive performance of marine fish (Izquierdo *et al.*, 2001; Buen-Ursua *et al.*, 2015). For example, the reduction in protein composition reduced egg viability in seabass (Cerdá *et al.*, 1994). Often freshwater fish contained higher n-6 compared to n-3 (Steffens, 1997). However, some

amphidromous marine fish contained higher n-6 compared to n-3 (Usman, 2014). In East Malaysia, *H. barbouri* usually found together with *H. kuda* at the estuaries or near the river mouths (Choo and Liew, 2004). Therefore, *H. barbouri* may be an amphidromous marine fish that requires more n-6 compared to n-3. Highest percentage of protein and n-6 found in PLS compared to other feed offered in this study may likely be the reason of better reproductive performance of *H. barbouri*.

*H. barbouri* can be maintained and bred in captive condition in Malaysia. However, presence of bubble gas in culture system should be avoided since it can be a causative agent for GBD in seahorse. Minimum water depth required for successful spawning of *H. barbouri* is 38 cm. Based on this study, the best diet for *H. barbouri* broodstock is PLS, obvious with the best reproductive performance. However, FM can also be used in the culture of *H. barbouri*. The minimal requirements of n-3 and n-6 fatty acids for reproduction of *H. barbouri* are  $5.13 \pm 0.04\%$  and  $14.83 \pm 0.10\%$  respectively. Further study on bioavailability and n-6 requirements for *H. barbouri* should be conducted.

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