Influence of stocking density and temperature on the growth and survival of seahorse juveniles, *Hippocampus barbouri* (Jordan and Richardson, 1908)

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Abstract

Low survival of newborn seahorses hindered studies on the early life stage. Many important aspects contributing to the survival of newborn seahorses remained unknown. More research on juvenile stage is necessary to optimize the culture protocols. Stocking density may or may not affect fish growth and behavior. Effect of stocking density on the growth and survival of seahorses in the early stages had received much less attention. Temperature is decisive in the early development of fish, with great impact on primary and secondary production and consequently on growth, survival and feeding of juveniles. It affects metabolism rate of organisms which can greatly impact the growth and survival of seahorse particularly at juveniles' stage. Hence, effects of stocking density and temperature on the growth and survival of *Hippocampus barbouri* juveniles were studied. Three treatments of 0.3, 0.4 and 0.5 juv/L, respectively 6, 8, and 10 seahorse juveniles per tank, were tested for 12 weeks. Three temperature levels selected for this experiment were 25, 28 and 31°C for 4 weeks of experimental period. There was no significant different ($p>0.05$) on the growth of *H. barbouri* juveniles cultured under different stocking density. This may be due to the stocking densities employed in this study has not reached it maximum limit, hence no significance difference between treatments. Highest survival of 90% was recorded at highest stocking density of 10 juv/tank. Temperature between 25 and 28°C showed better growth of *H. barbouri* juveniles when compared to high temperature of 31°C. Survival of *H. barbouri* juveniles cultured under different temperature resulted in more than 80% for all treatments. These findings indicated that *H. barbouri* juveniles can withstand temperature between 25 and 31°C. To conclude, stocking density of 0.5 juv/L produced the best survival while at temperature between 25 and 28 °C produce better growth and survival of *H. barbouri*.

Keywords: *Hippocampus barbouri*, Temperature, Stocking density, Growth, Survival

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Introduction

Global demand for seahorses is currently high and prices have risen (Job *et al.,* 2006). Zhang *et al.* (2010) mentioned that there are more than 10 species of seahorses, which have been successfully reared in captivity such as *H. abdominalis, H. comes, H. erectus, H. kuda, H. reidi, H. subelongatus, H. trimaculatus and H. guttulatus* However, not all seahorse species perform similarly under culture conditions due to their species-specific effect (Olivotto *et al.,* 2011).

Seahorse culture is still a new industry (Woods, 2003; Lin *et al.*, 2007; Olivotto *et al.,* 2011) that needs to be studied. However, low survival of newborn seahorses hindered studies on the early life stage (Scaratt, 1995; Forteath, 1996, Martinez-Cardenas and Purser, 2012). Thus, many important aspects contributing to the survival of newborn seahorses remained unknown. In contrast, studies on sub adult seahorses showed low mortality throughout the experimental period (Woods, 2003; Wong and Benzie, 2003; Lin *et al.*, 2009; Olivotto *et al.*, 2011). Wong and Benzie (2003) mentioned the void essential, particularly on growth and survival of juveniles. Hence, more researches on juvenile stage are necessary to optimize the culture protocols (Lin *et al*., 2006).

One of the causes in early stage mortality of cultured fish could be their high susceptibility to crowding stress (Van der Salm *et al.*, 2004). Effect of stocking density on the growth and survival of seahorses in the early stages had received much less attention (Lin *et al.*, 2010). Stocking density may affect fish growth and behavior (Woods, 2003). Hence, it is important to determine the optimum stocking density of seahorse juveniles.

Environmental factors, mainly temperature, are decisive in the early development of fish, with great impact on primary and secondary production and consequently on growth, survival and feeding of juveniles (Blaxter, 1991). It affects metabolism rate of organisms that can greatly affect the growth and survival of seahorse particularly at juveniles' stage (James and Woods, 2001; Wong and Benzie, 2003). Optimum temperature which tolerable to larvae will optimize the use of energy for growth, however when temperature are significantly higher or lower than the optimum levels for the larvae, the energy gained from food will partly use for environmental adaptation or other physiological process (Ambas, 2009). The range of temperature tolerance in newborns seahorse and the implications of the temperature level are unknown (Planas *et al*., 2012). Therefore, the aim for this study is to determine suitable stocking density and best temperature for the growth and survival of *Hippocampus barbouri* juveniles.

Methodology

Experiment on stocking density was conducted at the Hatchery Unit of Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor and Malaysia. While experiment on

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temperature was carried out at Aquarium Tunku Abdul Rahman, Fisheries Research Institute, Batu Maung, Penang, Malaysia. *Hippocampus barbouri* juveniles used for each experiment were produced from a single brood. *Hippocampus barbouri* juveniles of 12 day after birth (DAB) were used for these two experiments. Sources of seawater were from Port Dickson, Negeri Sembilan, Malaysia and Batu Maung, Penang, Malaysia. Serial filtration using cartridge size of 5, 1 and 0.1 µm then UV sterilizer were carried out for seawater prior to usage. Experimental tanks were equipped with hang on filter (TIANRUN, TP 606 H) and holdfasts (made of nylon string tied with weight).

Feeding of H. barbouri juveniles

One gram of *Artemia* cyst from Bio-Marine (USA) was weighed using microbalance and placed inside a 2 L beaker. One liter of 30 ppt seawater was added, mixed with the cysts and aerated for 24 hours before harvesting the nauplii. Newly hatched *Artemia* nauplii were to seahorse juveniles to observed satiation at 0930 and 1530 hrs. Faeces were siphoned prior to each feeding.

Seahorse measurements and survival

Seahorse juveniles were measured at initiation and periodically during the experimental period. Length in term of height was measured following Lourie *et al*. (1999). This measurement is from the top of the coronet to the tip of the straightened tail, measured using a digital vernier caliper (Mitutoyo, Japan). Wet weight was taken using micro-balance (Sartorius BP 310 S and Mettler Toledo PB 303 L) after blot-dried with on paper towel. Survival of seahorse juveniles was observed and noted on daily basis.

Water quality parameter

YSI Professional Plus Multi-parameter, thermometer, hand refractometer and API® Saltwater Master Test Kit were used for *in-situ* measurement of pH, dissolved oxygen (DO), temperature (°C), salinity (ppt), ammonia (ppm), nitrate (ppm) and nitrite (ppm) once a week at 0900.

Data analysis

Data analysis was conducted using software SPSS version 21.0. Data of the experiments were analyzed using one-way of Analysis of Variance (ANOVA). Tukey test was used to determine the significant difference between treatments. Data of survival were transformed using Arcsine prior to ANOVA. Results of growth and survival were presented as mean ± standard deviation (S.D.).

Experiment 1: stocking density

Nine glass tanks with size of 42.5 x 22.5 x 23.0 cm filled with 20 L of seawater were used. Three treatments of 0.3, 0.4 and 0.5 juv/L, with corresponding number of 6, 8, and 10 seahorse juveniles per tank, were tested for 12 weeks. Each treatment was triplicated and used to test the effect of stocking density on the growth and survival of seahorse juveniles. Height and wet weight of each *H. barbouri* juveniles were taken at the initial and end of 12 weeks experimental period.

Experiment 2: temperature

Tanks with size of 24.5 x 40.5 x 24.5 cm were set in a laboratory with air conditioned throughout the study period. Thermostat (XiLONG, XL-282) was provided for each tank in order to maintain the temperature at required level. A preliminary experiment was conducted with the lowest and highest temperature tolerated by *H. barbouri* juveniles. At temperature below 25 and above 31°C, *H. barbouri* juveniles became weak, passive and within 15 minutes settled motionless at bottom of tank. Thus three temperature levels selected for this experiment were 25, 28 and 31°C. Each treatment was triplicated and contained 10 individual seahorse/tank. Height and weight of seahorse juveniles were recorded at the initiation of the experiment, and at 2 weeks interval throughout the study period.

Results

Stocking density

There were no significant differences (p>0.05) for both height and weight on the initial and end of experiment (Table 1). Initial height was recorded between 19.53 and 19.82 mm while initial weight was recorded between 0.025 and 0.027 g. Although there were no significant differences on growth, best height was at stocking density of 8 juv/tank with 48.98±5.80 mm, followed by 6 juv/tank with 48.17±5.52 mm and least height of 44.71±5.67 mm at stocking density of 10 juv/tank. While best weight of 0.369±0.142 g was recorded at lowest stocking density of 6 juv/tank, followed by stocking density of 8 and 10 juv/tank with weight of 0.362±0.120 and 0.274±0.110 g, respectively. As for survival, there was a significant different (p<0.05) between treatments. The highest survival of 90% (p<0.05) was at highest stocking density of 10 juv/tank, as compared to the other 2 treatments with survival of 58.5 and 50%, respectively.

All the water parameters measured (Table 2) were within acceptable range for the living of marine organisms. Temperature was between 27.6 and 28.74°C while salinity within 30.15 to 33.64 ppt. DO were between 4.82 and 5.61 mg/L; pH 7.85 to 7.93. Ammonia, nitrite and nitrate levels were at 0 ppm throughout the study period.

Temperature

The initial height and weight of *H. barbouri* juveniles were ranged between 18.10±1.63 and 18.63±1.43 mm and within 0.022±0.004 to 0.023±0.003 g, respectively. Both the initial height and weight were not significantly different (p>0.05) among treatments (Table 3). After 2 weeks, height of *H. barbouri* juveniles cultured at 25 and 28°C were significantly higher (p<0.05) than juveniles cultured at 31°C.

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Table 1: Growth and survival of *H. barbouri* juveniles cultured under different stocking density for 12 weeks culture period.

Stocking density (juv/tank)	Initial		Final		Survival (%)
	Height (mm)	Weight (g)	Height (mm)	Weight (g)	
6	19.82±1.34[a]	0.027±0.007[a]	48.17±5.52[a]	0.369±0.142[a]	58.34±11.79[b]
8	19.77±1.29[a]	0.025±0.006[a]	48.98±5.80[a]	0.362±0.120[a]	50.00±0.00[b]
10	19.53±1.42[a]	0.025±0.006[a]	44.71±5.67[a]	0.274±0.110[a]	90.00±0.00[a]

Mean ± S.D. with the same superscripts within the same column are not significantly different

Table 2: Water quality parameters for experiment on stocking density throughout the experimental period.

Water parameter	Stocking density (juv/tank)	Week					
		2	4	6	8	10	12
Temperature (°C)	6	28.44	28.31	28.74	28.6	28.47	28.59
	8	27.87	27.9	28.23	28.0	28.14	28.11
	10	27.72	27.6	27.81	28.2	28.15	28.23
Salinity (ppt)	6	30.15	31.64	31.58	32.47	33.23	32.51
	8	30.27	31.33	33.64	32.58	32.06	31.88
	10	30.27	31.48	32.83	32.41	33.59	32.14
pH	6	7.87	7.88	7.90	7.90	7.87	7.89
	8	7.84	7.75	7.79	7.89	7.82	7.81
	10	7.88	7.85	7.85	7.90	7.93	7.89
DO (mg/L)	6	5.08	4.84	4.82	4.97	5.24	5.18
	8	5.54	5.61	5.15	5.37	5.24	5.44
	10	5.23	4.94	5.33	4.89	5.47	5.26
Ammonia (ppm)	6	0	0	0	0	0	0
	8	0	0	0	0	0	0
	10	0	0	0	0	0	0
Nitrite (ppm)	6	0	0	0	0	0	0
	8	0	0	0	0	0	0
	10	0	0	0	0	0	0
Nitrate (ppm)	6	0	0	0	0	0	0
	8	0	0	0	0	0	0
	10	0	0	0	0	0	0

Table 3: Height and weight of *H. barbouri* juveniles cultured at different temperature throughout 4 weeks culture period.

Week	Height (mm)			Weight (g)		
	Temperature (°C)					
	25	28	31	25	28	31
0	18.10±1.63[a]	18.63±1.43[a]	18.60±1.19[a]	0.022±0.004[a]	0.023±0.003[a]	0.023±0.003[a]
2	27.93±1.84[a]	28.38±2.14[a]	26.48±1.96[b]	0.091±0.016[a]	0.091±0.019[a]	0.073±0.016[b]
4	34.41±2.06[a]	33.28±2.88[ab]	31.69±3.13[b]	0.157±0.028[a]	0.143±0.037[ab]	0.127±0.045[b]

Mean ± S.D. with the same superscripts within the same row are not significantly different (p>0.05)

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Similar observation on the weight, *H. barbouri* juveniles cultured at 25 and 28°C were significantly higher (p<0.05) than juveniles cultured at 31°C. At the end of the experimental period, it was observed that *H. barbouri* juveniles cultured at 25°C were significantly higher (p<0.05) in height (34.41±2.06 mm) and weight (0.157±0.028 g) as compared to *H. barbouri* juveniles cultured at 31°C with height of 31.69±3.13 mm and weight of 0.127±0.045 g. However, results was not significantly different (p>0.05) when compared to *H. barbouri* juveniles cultured at 28°C with height of 33.28±2.88 mm and weight of 0.143±0.037 g. Observation on *H. barbouri* juveniles showed higher tendency to develop gas bubble (Fig. 1) at the abdomen when cultured at 31°C.

Survival of *H. barbouri* juveniles (Table 4) in tanks throughout the experimental period at temperature of 25, 28 and 31°C decreased from 100 to 90±10.00 %, 100 to 96.67±5.77% and 100 to 96.67±5.77%, respectively. The highest survival of *H. barbouri* juveniles at the end of the experimental period was 90±10.00% at 25°C, followed by 86.67±5.77% at 31°C, then 83.33±5.77% at 28 °C. Overall, survival for *H. barbouri* juveniles cultured between 25 and 31 °C was more than 80%. As for water parameters (Table 5), salinity fluctuated slightly between 30 and 31.67 ppt during the study. Water pH was quite stable at 7.6, while dissolved oxygen ranged 5.9 to 6.50 mg/L for all treatments. Ammonia level at week 2 was between 0.083 to 0.167 ppm and decreased to 0 ppm for all treatments, while nitrite was not detected throughout the experimental period. Nitrate levels were between 0 and 6.67 ppm at week 2 and between 5 and 6.67 ppm at week 3, but later decreased to between 0 and 3.33 ppm at the end of study period for all the treatments.

Figure 1: *Hippocampus barbouri* juvenile with gas bubble (red circle) at abdomen when cultured at 31°C.

Table 4: Survival of *H. barbouri* juveniles cultured at 25, 28 and 31°C throughout the experimental period.

Week	Temperature (°C)		
	25	28	31
	Survival (%)		
0	100±0.00	100±0.00	100±0.00
2	90±10.00	96.67±5.77	96.67±5.77
4	90±10.00	83.33±5.77	86.67±5.77

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Table 5: Water quality parameters measured for experiment on temperature throughout the study period for the experiment on temperature.

Water parameter	Temperature (°C)	Week			
		1	2	3	4
Salinity (ppt)	25	30	30	32	31
	28	30	30	30.67	31
	31	30	30	31.67	31
pH	25	7.6	7.6	7.6	7.6
	28	7.6	7.6	7.6	7.6
	31	7.6	7.6	7.6	7.6
DO (mg/L)	25	6.4	6.5	6.1	6.1
	28	6.4	6.5	6.0	5.9
	31	6.4	6.5	5.9	5.9
Ammonia (ppm)	25	0	0.167	0	0
	28	0	0.167	0	0
	31	0	0.083	0	0
Nitrite (ppm)	25	0	0	0	0
	28	0	0	0	0
	31	0	0	0	0
Nitrate (ppm)	25	0	6.67	6.67	1.67
	28	0	0	5	0
	31	0	1.67	5	3.33

Discussion

In this study, finding showed that stocking density did not affect the growth of *H. barbouri* juveniles. It may be that the stocking densities applied in this study has not reached it maximum limit, hence no significance differences between treatments. Similarly, stocking density did not affect the growth of *H. whitei* (Wong and Benzie, 2003). Study by Martinez-Cardenas and Purser (2012) on 3 weeks old *H. abdominalis* juveniles showed that different stocking density tested did not affect growth or survival of *H. abdominalis* juveniles.

However, study by Lin *et al.* (2010) on *H. erectus* juveniles, reported that as stocking density increased, the weight and standard length decreased. Usually, when stocking density increased, growth would decrease due to competition for space and food. Another study on *H. erectus* juveniles suggested that stocking density affected the growth of even after the age of 25 days (Zhang *et al*., 2010). Study on *H. abdominalis* showed that significantly better height and weight after 2 months (Woods, 2003). Lin *et al.* (2009) reported that sub adult *H. erectus* at the highest stocking density of 1.5 juv/L showed the lowest height and weight increments. Wrestling and grasping were normal behaviors of seahorses when cultured in tank. They tend to play and gather at the same area. It is believed that the expenditure of energy for these behaviors may affect the growth and survival of seahorse juveniles (Woods, 2003).

In the present study, stocking density of 10 juv/tank showed the highest survival of 90 % as compared to the other two stocking densities. *Hippocampus erectus* juveniles showed highest survival at stocking density of 1.5 juv/L as compared to other densities (Lin *et al.*, 2010). Culture of early stage seahorses at high stocking density could have an advantage as seahorses do not cannibalize (Bergert and Wainwright, 1997). However, Martinez and Purser (2012) stated that it may or may not be conducive for growth and survival.

In the present study, mortalities occurred at week 6 on 54 DAB juveniles; however this condition stabilized after week 9 when juveniles reached 75 DAB. As for water quality parameters, the readings for DO, pH, ammonia, nitrite and nitrate are within the acceptable limit as described by Boyd (1979), Forteath *et al.* (1993) and Erlania *et al.* (2010). Therefore, the mortality in this experiment may not be due to water quality.

Lin *et al*. (2010) and Woods (2003) suggested the culture of *H. erectus* and *H. abdominalis* at stocking density of 1 or less than 1 juv/L. Further studies should be carried out to determine the optimum stocking density to avoid high mortality during the culture of seahorse.

Temperature is one of the main factors that greatly affect the growth and survival of seahorse (James and Woods, 2001; Wong and Benzie, 2003). Tropical and subtropical seahorse juveniles are usually raised between 23 and 28°C whereas temperate species between 13 and 24°C (Olivotto *et al*., 2011). Optimum temperatures for the culture of juveniles and adult seahorses for most of the species were still unknown. *Hippocampus barbouri* is a tropical species, found at shallow seagrass bed up to 10 m depth (Lourie *et al*., 2004). According to Ambas (2009), *H. barbouri* juveniles, at early life stage are unable to withstand temperature higher than 31°C. Total mortality occurred even when cultured at short study period of 7 days.

In the current study, growth of seahorse juveniles at 25 and 28°C is better as compared to higher temperature of 31°C. This finding is similar to that reported by Ambas (2009), whereby *H. barbouri* juveniles grow better at 26°C. Wong and Benzie (2003) reported that sub adult of *H. whitei* increased in length with the increase of temperature. This suggested that effect of temperature on growth may be species-specific or depends on life stages. The optimum temperature for the survival of *H. kuda* juveniles is between 24 and 28°C (Lin *et al*., 2006). Survival of *H. barbouri* juveniles is highest at 26°C and the lowest at 32°C (Ambas, 2009). Better growth and survival of *H. erectus* are at temperature range of 26 to 28°C (Lin *et al.*, 2010). In the present study, *H. barbouri* juveniles showed more than 80% survival when cultured between 25 and 31°C, with the highest survival of 90±10.00% at 25°C. These findings

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implied that *H. barbouri* juveniles can withstand temperature between 25 and 31°C.

A notable observation on *H. barbouri* juveniles cultured at higher temperature of 31 °C is in its tendency to develop gas bubble disease (GBD). *Hippocampus barbouri* juveniles with GBD will usually float at the water surface, and this will disrupted their feeding ability, resulted in starvation, finally mortality. Similar observation was reported by Lin *et al.* (2010), whereby *H. erectus* juveniles cultured at lower temperature of 26°C showed less occurrence of GBD. Therefore further investigation should be carried out to determine if temperature influence the occurrence of GBD in tropical seahorses. Du *et al.* (2004) mentioned that diet affects GBD rate in *H. kuda* juveniles. *Artemia* nauplii are the most suitable initial food for larvae of aquarium fishes (Rakowicz, 1972; Carlberg and Van Olst, 1976). In seahorse aquaculture, *Artemia nauplii* are often used for the feeding of newborn and juveniles. As *Artemia* nauplii are photo positive and usually gather at water column near to water surface, causing seahorse juveniles to ingest air, therefore may be one of the causes for the mortality. Further study is needed to confirm on this.

Study on *H. trimaculatus* juveniles showed that water temperature affects the feeding behavior (Sheng *et al*., 2006). In the present study, although *H. barbouri* juveniles feed less at low temperature of 25 and 28°C, they grow much better compared to those in 31°C. Ambas (2009) stated that temperature would affect metabolism rate of organisms and dissolved oxygen level that may led to mortality at larvae stage. Optimum growth of seahorse juveniles could be obtained with optimum cultured temperature. Energy gained from food at non-optimal temperature would be utilized for physiological processes particularly for environmental adaptation. Wong and Benzie (2003) reported that seahorses showed higher metabolic rate when cultured at temperature higher than 26°C.

In the present study, water quality parameters such as salinity, pH and nitrite were quite stable with very low fluctuation. Dissolved oxygen (DO) readings are above 5 mg/L for all treatments throughout the experimental period. According to Boyd (1979), DO above 5 mg/L was sufficient to support the normal living of aquatic animals. Hence, DO level was less likely contributed to the mortality of *H. barbouri* juveniles in this study. Optimum ammonia level recommended for the culture of seahorse should be less than 0.1 ppm (Boyd, 1979). A total of 4 mortalities in week 2 may be due to the increase of ammonia level between 0.083 and 0.167 ppm. High ammonia at the first 2 weeks of culture maybe is due to the used of new biomaterial in the filtration, thus the inefficient breakdown ammonia. After week 2, ammonia level decreased to 0 ppm and this level remained until the end of the experimental period. Acceptable limit of nitrate for marine organisms should be less than 20 ppm (Forteath *et al*., 1993; Erlania *et al.*, 2010). In the present study, the highest nitrate level was at 6.67 ppm during week 2 and 3, and then gradually decreased until the end of experimental period.

Therefore, based on the findings of this study, it can be concluded that *H. barbouri* juveniles can be cultured at stocking density of 0.5 juv/L and temperature ranged of 25 to 28°C for best survival.

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