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Captive Breeding, Rearing and Closing of Reproductive Cycle of the Three Spot Seahorse, *Hippocampus trimaculatus* (Leach, 1814)

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Abstract

Seahorse aquaculture can provide a platform to reduce pressure on its wild populations and to meet the demand for global trade. However, techniques for breeding seahorses are yet to established for every species and information is limited especially for species dwelling in Malaysian waters. *Hippocampus trimaculatus* is among the heavily exploited species and red listed as 'Vulnerable' under IUCN [1]. This study was conducted for one and a half years at the Fisheries Research Institute, Pulau Pinang, Malaysia. The goal of this study was to develop a simple yet practical husbandry technique for *H. trimaculatus*. The study described protocols applied to the establishment of brooders, from the newborn to adulthood, and ultimately to the closure of the reproductive cycle. The seahorses were fed with a live diet *ad libitum* daily. Juveniles attained a total length of 50mm within a rearing period of 90 days. At 7 months old, the seahorses had reached their matured sizes of 111mm and 106mm for males and females respectively. After a year in captivity, we achieved the closure of the reproductive cycle for the captive-born *H. trimaculatus*. The results showed that the captive breeding of *H. trimaculatus* is feasible in Malaysia. These findings will be useful in promoting this species for commercial seahorse culturing and conservation purposes.

Keywords: *Hippocampus trimaculatus*; Seahorse; Captive breeding; Husbandry; Reproductive cycle closure.

1. Introduction

Seahorses of genus *Hippocampus* can be found in all tropical, subtropical and temperature waters worldwide [2-4]. However, they are considered vulnerable to loss of their natural habitats and global exploitation either for traditional Chinese medicine, curios or aquarium trades [2, 3, 5, 6]. Captive-bred seahorses are essential for establishment of seahorse aquaculture and for conservation goals, in order to reduce the pressure on wild populations and provide a consistent supply of seahorses [2, 4, 5]. Establishment of technology for mass production will help in achieving these objectives. These reasons are the major factors that inspire researchers around the world to study seahorses [7].

At least ten species of seahorses, of which seven are heavily exploited, are found in Malaysian waters [8]. The Fisheries Research Institute (FRI), Pulau Pinang has been conducting breeding and rearing of two of these native species i.e. *Hippocampus kuda* [9] and *H. barbouri* [10]. Another potential candidate for seahorse culture is *Hippocampus trimaculatus*, commonly known as three-spot seahorse. It is one of the most highly traded species [7] and has been subjected to a "Significant Trade Review" through CITES [3]. This species is cited as vulnerable on the Red List of Threatened Species [1] and is of great conservation concern.

To date, no captive bred production of *H. trimaculatus* has been conducted and reported in Malaysia, although it has been done in other countries [11-13]. Moreover, the growing demand for information on seahorses may prompt each nation to perform research on species occurring in its national waters [7]. As seahorses are species and venue specific, previous studies on this species in other countries or studies of other seahorses might not apply to *H. trimaculatus* in Malaysia. The present study is focussed on establishing the breeding and rearing technology of *H. trimaculatus* under captive conditions. This paper provides general information on culturing this species until it reaches its adulthood and completes its reproductive cycle.

2. Materials and Methods

2.1. The Culture Protocols

This study was conducted from March 2018 to August 2019 in an indoor nursery of the Fisheries Research Institute (FRI), Pulau Pinang, Malaysia. In March 2018, nineteen (19) sub-adults *H. trimaculatus*, 13 males and 6 females, were obtained from a local aquarium shop. They were transferred and kept in a single 200L (90cm L x

45cm W x 50cm H) rectangular nursery tank, following the method suggested by Mohamad Saupi and Muhammad Fadzil [9], until they reached their adult sizes. Later, they were capable of choosing their own partners. Once they had established their own partners, the adults were transferred to their own brooder tanks (half the length of the nursery tank). The minimum height of 50cm was considered necessary for the successful completion of courtship and transfer of eggs in this species.

Five pairs of potential parent seahorses were selected for this study. Initial body height and weight measurements were obtained for the adults. Out of the five pairs, only three pairs successfully mated and gave birth. Once the males became pregnant, the females were transferred out of the brooder tanks, leaving the males isolated until the newborns were released. The newborns were then transferred to their own tanks and the females were rejoined with their chosen partners. All newborns were counted and reared in 200L rearing tanks, which were of the same type, size and conditions as the nursery tank. A limit of 100 individuals per tank was arranged.

All tanks used in this study were supplied with filtered seawater sourced from a nearby fishermen's port. The tanks were provided with mild aeration using small aeration tubes (5mm in diameter), placed at the bottom of the tanks, at a rate of 5-10 bubbles per second. No air stones were used in the tanks to minimize the issue of air bubble ingestion, commonly known as 'gas bubble' disease. For maintenance of good water conditions, tanks were cleaned daily, before the morning feeding, by siphoning out leftover food and faeces, and replenished with up to 20% of fresh filtered seawater. All tanks were kept bare, except for the aeration tubes and holdfasts.

The juveniles were provided with holdfasts, starting from day 10, following the method by Mohamad and Yap [14]. Polyethylene lines of 1mm thickness and 30cm in length were unwound and tied onto a weight and placed at the bottom of the tanks. The lines were replaced with cable ties (size 3mm in diameter) after day 60. As the seahorses grew bigger and reached adult sizes, plastic chains, size 5 mm in diameter, tied to sinkers served as the holdfasts.

2.2. The Water Conditions

Throughout the study, routine measurement and checking of some water quality parameters were performed periodically. Temperature was maintained, using as submersible thermostat heater, at 28 $\pm$ 2^{o}C, salinity at 30-33ppt, pH at 7.8-8.0, dissolved oxygen at 4.6-5.1mg/l, calcium at 370-390mg/l, and ammonia (NH_3), nitrate (NO_3) and phosphate (PO_4) at less than 0.05mg/l. These parameters were within the optimum range for marine aquarium water quality as described by Tullock [15]. For the rearing tanks, a photoperiod of 10h light: 14h dark, supplied by fluorescent lights, was set from 8am to 6pm, following the method by Lipton and Thangaraj [11]. The lamps were placed approximately 15-20cm above the water surface, providing the light intensity of 1000-1500lx. For the other tanks (brooder and nursery), only natural light regime was provided.

2.3. The Seahorse Diets

The parents and adult *H. trimaculatus* were fed twice daily (9am and 3pm) with live, wild caught coastal mysid shrimps (5-10mm) to satiation. For juvenile seahorses, *ad libitum* feeding was practiced two times a day, using a standardized feeding regime, i.e. first week, second week, first month, and second month. Starting from birth until 1 week old, the newborns were weaned onto live rotifers (*Brachionus* spp.). Then, the juveniles were fed with live *Artemia* enriched with fish oil. Beginning of day 30, 3 to 7 days old *Artemia* nourished with rice flour, were offered to the seahorses. After 60 days, live mysid shrimps, together with the adult *Artemia* (>7 days old), were introduced as the diet for *H. trimaculatus*. After reaching 3 months old, all seahorses were fed mostly with live mysid shrimps until the end of the study.

2.4. Data Collection and Analysis

Day-to-day monitoring and routine data sampling were carried out throughout the study. Mortality, either due to starvation or diseases, was continuously monitored. Behaviour of seahorses was noted qualitatively. Total length and wet weight of seahorse juveniles were measured every 10 days for birth until day 90. Length was measured according to Lourie, *et al.* [16] as a distance from the tip of the coronet to the tip of the uncurled tail. Weight was taken using microbalance to the nearest 0.001g after blot drying using paper towel. Specific growth rate (SGR) was calculated as SGR = 100 x (ln W_f - ln W_i) / t, where W_f and W_i are the final and initial body weights (g) and t is time (days).

Three batches of newborns from three different parents, born within the same week, were used for growth experiment. From each batch, 10 individuals were randomly sampled for body length and weight measurement. Seahorses were measured alive and returned to the tanks. After 90 days, the routine measurement was stopped and all surviving seahorses were counted, combined and raised in the same nursery tanks until they reached their adulthood. Percentage survival of the seahorses was measured as the remaining number at a specified age divided by the initial number at birth, and multiplied by 100. Then, only data for age at maturity, sex ratio and number of newborns produced (F2 generation) were recorded. The final count was done after the captive-bred seahorse released its first newborns and completed the reproductive cycle. All statistical tests were conducted using the Statistical Program for Social Sciences (SPSS) software version 16. Means are given with SD unless otherwise indicated.

3. Results

Seahorse *H. trimaculatus* started to exhibit courtship behaviour after three month's rearing in the nursery tank. The behaviour included changes in body colour, swimming as a pair in the water column, and drifting vertically snout-to-snout towards the water surface (Figure 1). While mating, the female seahorse transferred her eggs, using its ovipositor, into the fully developed male's brood pouch. This action took only a second and was almost unnoticeable. The mating process was considered complete once the couple began to swim separately and the body colouration returned to their original dull colours. Male *H. trimaculatus* incubated the fertilized eggs between 10 to 12 days before giving birth to newborns. Spawning normally occurred in the morning and occasionally in late afternoon.

Figure-1. Adult seahorses showing their courtship behavior

Table-1. Size of parent seahorses after giving their first births

Parent	Total length (mm)		Wet weight (g)	
	Male	Female	Male	Female
1	124	122	8.62	8.48
2	128	117	8.84	7.57
3	120	119	7.36	7.07
Average	124.00 ± 3.27	119.33 ± 2.05	8.27 ± 0.65	7.71 ± 0.58

Table-2. Details of newborns released from each parent.

Parent	Batch	Date of Birth	No. of newborns
1	101	24/06/18	382
	102*	04/07/18	344
	103	16/07/18	164
	104	28/07/18	264
	105	27/01/19	177
	106	17/02/19	433
	107	01/03/19	503
		Average	323.86 ± 118.62
2	201	30/06/18	200
	202*	10/07/18	287
	203	24/07/18	371
	204	03/08/18	252
	205	23/08/18	239
		Average	269.80 ± 57.75
3	301*	05/07/18	175
	302	23/07/18	188
	303	02/08/18	184

	304	22/08/18	130
	305	21/09/18	156
	306	21/02/19	217
		Average	175.00 + 27.08
		Total Average	274.47 + 103.26

Note: *batches used for growth experiment.

After spawning, the parents were observed to almost immediately perform the courtship rituals for the next mating. The average length-weight for male and female parent seahorses when they released their first newborns were 124.00 + 3.27mm; 8.27 + 0.65g and 119.33 + 2.05mm; 7.71 + 0.58g respectively (Table 1). The males were bigger than females for all parents.

From June 2018 to March 2019, 18 batches of juveniles were released from three male parents, with an average of 6 batches per parent (Table 2). The spawning happened from June to September 2018, and it continued from January to March 2019. The average brood size of *H. trimaculatus* was 274.47 + 103.26, ranging from 130 to 508 individuals.

Newborns *H. trimaculatus* were small 'replicas' of typical adult seahorses. Shortly after birth, they began to swim freely, with the help of gentle water currents. They then congregated near the water surface in search of live food. The newborns were pelagic and became active swimmers after a few days. Their pelagic life lasted for two weeks. After that they started to attach themselves to the holdfasts provided. Then, they became almost passive, spent most of their time resting at the holdfasts and only moved to pursue live prey. By the third week, all juveniles had settled themselves to the bottom of the tank. Most of them started to accept the given diet.

Three batches of newborns from three different parents (batch 1 from parent 1, batch 2 from parent 2 and batch 3 from parent 3) were used for growth experiment. Growth of juvenile seahorses for 90 days is depicted in Figure 2. At the time of birth, newborns of *H. trimaculatus* were 6-8mm in length. Thirty days later, the juveniles of batch 1, 2 and 3 attained a length of 16.80 + 1.55mm, 17.33 + 1.58mm, and 17.70 + 2.91mm respectively, as shown on Figure 1. From 30 to 60 days, the length of seahorses continued to increase quickly. At this age, the juveniles had doubled their previous sizes. The growth was 36.33 + 4.16mm, 33.38 + 3.74mm and 36.50 + 3.03mm for batch 1, 2 and 3 respectively. However, the gender of seahorses could not be concluded as the brood pouch had yet to be developed. The first noticeable male was detected on day 90 after birth. Development of brood pouch in males could be observed during this stage when the males measured more than 50mm in length. Thus, the other seahorses of similar sizes to males, but without the pouches, were assumed to be females. The average lengths for all batches on day 90 were 50.00 + 5.66mm, 49.25 + 12.91mm and 46.30 + 5.98mm respectively.

Growth rates of the juveniles were high during the first three months of study, as supported by the data on weight and length (Table 3). Seahorses weight at the start of the study was 0.003 + 0.001g for all batches. Specific Growth Rates (SGR) and average increment shows only small differences between batches, with the SGR values of 8.40, 8.20 and 8.13 and daily increment (mm) of 0.48, 0.48 and 0.45 for batch 1, 2 and 3 respectively.

Figure-2. Growth in length (mm) of three batches of *H. trimaculatus* during 90 days

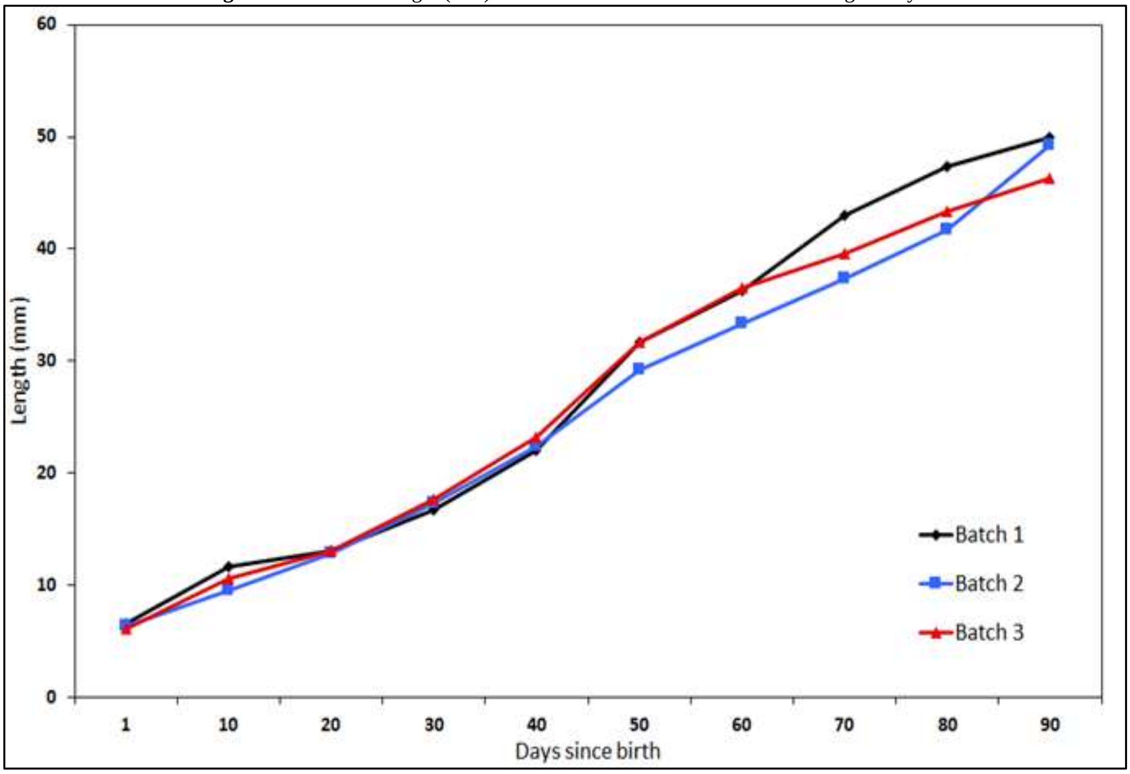

Table-3. Growth and survival of *H. trimaculatus* in captivity from birth until 90 days

Batch	Initial length (mm)	End length (mm)	Initial weight (g)	End weight (g)	Average Increase (mm/day)	SGR (%)	Survival (%)
1	6.60±0.97	50.00±5.66	0.003±0.001	5.971±0.636	0.48	8.40	33.14
2	6.40±0.52	49.25±12.91	0.003±0.001	4.631±1.107	0.48	8.20	39.72
3	6.20±0.42	46.30±5.98	0.003±0.001	4.377±1.169	0.45	8.13	34.29

Survival rate for each batch was calculated from the number reaching 90 days out of the total number of newborns. Mortality was high, up to 50%, during the first 10 days after birth. Starvation was the main reason for mortality of newborns during the first week, while mortality after that was caused mainly by ingestion of air bubbles. Mortality was low after the juveniles began feeding on live *Artemia*. Survival of juveniles, after 90 days of rearing, for batch 1, 2 and 3 was 33.14%, 39.72% and 34.29% respectively.

Juvenile *H. trimaculatus* were mostly black or dark in colour. The seahorses reaching sexual maturity were recognizable by their colour changes and ritual movement exhibited early in the morning. Changes in body colour from darker to brighter, to almost white, were obvious particularly during courtship (Figure 1). At sexual maturity they were 7 months of age and with the total length of 111.00 ± 6.10mm for males and 106.17 ± 6.94mm for females. The male to female ratio was approximately 1:1.

Mating of F2 generation seahorses was detected from the age of 7 months onwards. However, no egg transfer was observed during these mating periods. The reproductive cycle of F1 generation was finally completed on day 395. The number of newborns released by the first pregnant male was 114 individuals.

4. Discussion

Throughout this study the mechanism for captive-bred *H. trimaculatus* to successfully breed were monitored. The sizes of brooders, 124.00mm for males and 119.33mm for females, were comparable to other studies (119.9 ± 15.3mm as stated by Murugan, *et al.* [12]. Lourie, *et al.* [16] and Yip, *et al.* [17] also reported *H. trimaculatus* to reach sexual maturity size at 12cm. The observed brood size (274.47 ± 103.26 individuals), however, was not in accordance with previous studies on *H. trimaculatus* by Lipton and Thangaraj [11] and Murugan, *et al.* [12], which reported 389 ± 56.11 and 457-533 newborns respectively. Nonetheless, the gestation period of 10-12 days is shorter compared to 12-16 days reported by Lipton and Thangaraj [11] and Lourie, *et al.* [16].

Among the goals of this study was documenting the rearing performance (growth and survival) of juvenile *H. trimaculatus*. In this study, the average daily growth increment in 90 days old *H. trimaculatus* was 0.45-0.48mm. Murugan, *et al.* [12], also observed a comparable growth rate of 0.5mm/day from birth to 90 days.

Seahorse breeders are often challenged by the food requirements of young seahorses. Suitable diet and quality of feed are the crucial factors for achieving optimal growth, survival and reproductive performance of seahorses ([12, 18]. Feeding during the first week after birth was a critical period in seahorse rearing. During this period, proper size and type of diet was essential to avoid mass mortalities. In this study, in the absence of suitable diet, death of newborns was observed beginning from day two after birth. High mortality of nearly 50% continued within the first two weeks after birth. Most of the mortality cases were due to starvation.

Starvation related mortality could be due to the initial diet. Newborn *H. trimaculatus* preferred to feed on copepods rather than other live prey, such as rotifers, during the first week of rearing [12, 13]. Wild juveniles are also reported to prefer copepods [17]. As observed by Sheng et al. (2006), feeding on copepods resulted in improved survival and growth. However, in this study, the newborns were solely fed with rotifers during the first week of rearing. Copepods were not available throughout the study. For unknown reasons, some of the newborns rejected the rotifers provided as food. Thus, it seems likely that rotifers are not the preferred diet for newborns *H. trimaculatus*.

Another cause of starvation was the ‘gas bubble’ disease or air bubble ingestion. It was found to be one of the major causes of seahorse mortality [2, 12, 19]. Newborns *H. trimaculatus* were pelagic and Lin, *et al.* [19] reported that while feeding near the surface, they might unintentionally have gulped the air excessively. The trapped air inside their bodies will inflate their swim bladders, make them unable to move down the water column and causes them to float passively at the water surface. Once floating, they are incapable of catching food, quickly leading to starvation and death. This situation resulted in low survival rates during the first two weeks of rearing.

The highest mortality of *H. trimaculatus* appears to be confined to the first two weeks after birth, a pattern similar to that observed in some other seahorse species reared in Malaysia [9, 10, 14]. Mortality at later stages was less once the preferred diet was available.

Varied diet seems to be important to juveniles. Thus, it is crucial to change the diet as the seahorse grows [4]. Although an *Artemia* diet was successful, it should not be the sole feed in rearing seahorses. The juveniles preferred to feed on mysid shrimps and a shift in feed preference to mysid shrimps was observed the moment they were introduced to the seahorses on day 45. Murugan, *et al.* [12], reported that the juveniles preferred larger size prey as they were growing in size. Mysid shrimp was also the preferred food of adult *H. comes*, another common Indo Pacific seahorse, when compared to *Artemia* and other live diets [18]. They also reported that mysid shrimp successfully gave higher brood size, shortened parturition period, higher parturition occurrence and thus reproductive performance was significantly improved. This might be the reason for the higher brood size of 274 individuals on average achieved in this study.

Although the early mortality was high, the protocols established in this study resulted in a survival rate of nearly 40% after 90 days of rearing. This agrees with the study by Job, *et al.* [5] on another common Indo-Pacific seahorse, *H. comes*, supporting the good husbandry practices adopted in this study. It is, however, considered low compared to works by Murugan, *et al.* [12], who managed to achieve a survival rate of 65%.

The seahorses reached full maturity after 7 months in captivity. Then after a year, we achieved the closure of the reproductive cycle for the captive-born *H. trimaculatus*. The brood size of F2 generation of 114 individuals was higher than studies by Murugan, *et al.* [12]. They reported an average brood size of 65 individuals. In comparison, this study has produced improved results in closing of the reproductive cycle. It is hoped that this might lessen reliability on wild brooders.

In total, 18 batches of newborns were observed from June 2018 to March 2019, with the peak months for spawning taking place in July and August. Thus, it is assumed that *H. trimaculatus* might display a seasonal breeding period. This is, however, not in accordance with majority of Indo Pacific seahorses that breed all year round, as described in Lourie, *et al.* [16]. At the present time, no conclusive explanation can be proposed for these observations.

5. Conclusions

A captive breeding program can be of significant value in supplying commercial stocks of seahorses, especially when wild population numbers are falling. In the case of *H. trimaculatus*, it is quite feasible to culture this seahorse in captivity. The successful closure of the reproductive cycle during this experimental breeding will facilitate the development of seahorse aquaculture as a viable alternative to wild caught seahorses and an important conservation initiative. Although the optimal culture conditions have yet to be achieved, the present study provides the basic information for future work that could be conducted with regards to seahorse management plans. These plans could include developing a husbandry manual for culturing seahorses and establishing captive breeding programs. It is hoped that the results of this study could be used for mass scale captive breeding of seahorses in Malaysia with the aim of conservation and expanding this important export market.

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