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Variations in age and size at sexual maturity of female green mud crab (*Scylla paramamosain*) under different captive growout conditions

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Abstract
Sexual maturity of female green mud crab (*Scylla paramamosain*) under different grow out protocol was assessed by evaluating the composition of different gonad development stages in respect to age and size. Female crabs attained sexual maturity following five gonad development stages of proliferation, previtellogenesis, primary vitellogenesis, secondary vitellogenesis and tertiary vitellogenesis. First sexual maturity initiated at 110.40, 112.60 and 116.80 g of body weight and 7.10, 7.30 and 7.90 cm of carapace width under indoor compartment, outdoor boxes and outdoor tanks, respectively. After settlement as crablet, the first sexual maturity appeared at 5 months of age under outdoor tanks and 5.5 months for outdoor boxes and indoor compartment. The median maturity age (MA50) was 6.5 months for outdoor tanks and 7 months for other two protocols. Earliest commencement of sexual maturity and production of larger sized highest number of gravid females in outdoor tank culture system suggested as suitable over other two protocols.

Keywords: age and size, female, sexual maturity, mud crab

1. Introduction
Mud crab is one of the most valuable crustacean species next to shrimp, and mainly exploited from wild sources by artisanal fishermen [1]. Thriving international demand and high market price led to over exploitation of all size groups of crab in South-East Asian countries [2-6] and hindering regular recruitment of spawner in the natural stocks [7].
Understanding the reproductive biology is the basic tool for effective management strategy and planning in protecting the prospective breeders, which is done for ensuring the regular recruitment in wild stock of commercially exploited species like mud crab [8, 9]. Clear concept of age, size and morphological features at first sexual maturity and gonad maturation stages is pre-requisite for successful copulation (mating), breeding and establishing of hatchery management protocols. Reproductive biological features like age and size at sexual maturity of many species have been derived from the study on gonad maturation stages [10] through histological observations. Studies on gonad development of *Scylla serrata* [11-13], *S. paramamosain* [14, 15], *S. olivacea* [16, 17] have been conducted on wild sourced samples with unknown age. That might contain various age groups of population, originating from different parents or had more than a single species. Mud crab produced from hatchery with known uniform age group might able to provide accurate information regarding growth, sexual maturity and reproductive capability. In addition, a captive broodstock might open other areas of future research on nutritional requirements and genetic selection for resistance to disease [18]. But, information on biological study and sexual maturity of the same age group of mud crab seems fragmentary. Meanwhile, growth and reproductive development of crustaceans are reportedly influenced by heredity, geographical location, culture environment, nutrition and by external environmental factors [17, 23, 28-30]. The mud crab genus *Scylla* consists with four different species [25]. Among the four species, *Scylla paramamosain* seems widely distributed in south-east Asian countries. Breeding of mud crab solely dependent on naturally collected broods and scarcity of suitable broodstocks very often preventing normal hatchery operations [13, 21] and seed production research. In this circumstances, development of broodstock under captive condition deserves priority. Therefore, this study aimed to observe, whether the different growout system in captive condition affect the sexual maturation or not with a typical species the green mud crab, *Scylla paramamosain.*

2. Materials and Methods

2.1 Study site

The study was conducted at Centre for Marine and Coastal Studies (CEMACS) under Universiti Sains Malaysia (USM), Penang, Malaysia during the period of 2012 to 2015. The study site situated at North-East part of Penang Island beside the Penang National Park and located under 5° 28' 2.3664" N and 100° 12' 2.8728" E in Global Positioning System (GPS).

2.2 Source of experimental animals

Sampled animals were managed by breeding the natural base female (P_0) and subsequent rearing the larvae in hatchery condition. For the first two months, crablets were reared in communal basis until they reached the juvenile stage. Then the female juveniles were assigned in different grow out protocols (Treatments) such as, T1: crabs reared in outdoor fibre glass tank bottom; T2: crabs grown individually in outdoor floating plastic boxes; and T3: crabs grown under indoor plastic drawers/compartment. All the crabs were fed with trash feed collected from the trawl net @3-5% body weight twice in a day.

2.3 Collection of animals (crabs) and measurement

From 4 months of age onwards, 5 female crabs from each grow out protocols were randomly collected at 15 days intervals, anesthetized by means of cold shock (-20ºC for about 30 minutes), measured (TW; total weight and CW; carapace width) and recorded for each crabs. The allometric growth organs of the female (shape and color of the abdominal flap as well as presence or absence of setae at the edge of abdominal flap and on the walking legs) were monitored following standard methods [14, 19].

2.4 Dissection of crabs, collection and preservation of gonad/ovary samples

The anesthetized crabs were taken out from the freezer, dissected the anterolateral and frontal spines. The upper shell (carapace) was gently removed, thin membranes over digestive organ was carefully cleaned which revealed the ovary/gonad. Photos of gonad morphological features were taken with a digital camera (Panasonic, DMC-FH25). The gonad was gently removed, weighed followed by drying with bloting papers and the thickness of the ovary was measured. Three sub-samples from anterior, middle and posterior part of the ovary were taken for each crab. Collected samples were separately preserved in Eppendorf tube filled with Davidson's fixatives for 24 hours, then transferred to 50% ethanol until histological process [14, 16].

2.5 Calculation of Gonad Somatic Index (GSI)

Gonad Somatic Index (GSI) for each female crab was calculated using the formula:

$$\text{Gonad Somatic Index (GSI)} = \frac{\text{Weight of gonad or ovary}}{\text{Total weight (TW) of crab}} \times 100 \quad \textbf{(1)}$$

2.6 Histological slide preparation and analysis of gonad development stages

Histological slide of gonad tissues were prepared through routine histological procedures of dehydration in ascending order of ethanol starting from 70% and ended at 100% at 10% intervals, xylene treatments, embedded in paraffin wax blocks, sectioning at 6 µm, placed onto a glass slide and stained with Haematoxylen-Eosin [20]. The stained tissues were covered with a cover slip and observed under a light stereo microscope. Images of the cells of gonad development stages and diameter of the cells were taken by using a digital computer with an image capture analysis system (Image Cell B, Stereomicroscope, Camera-X cam-α, Olympus SZX91CAS, Japan). Histological classification of gonad development stages was performed according to Islam *et al.* and Ikhwanuddin *et al.* [14, 17].

3. Results

3.1 Histological features and classification of gonad development stages

Based on the appearance of the most advanced oocytes from histological observation, in all the growout protocols, female green mud crab accomplished five distinct gonad development stages (Plate 1 and Table 1) under captive growout protocols.

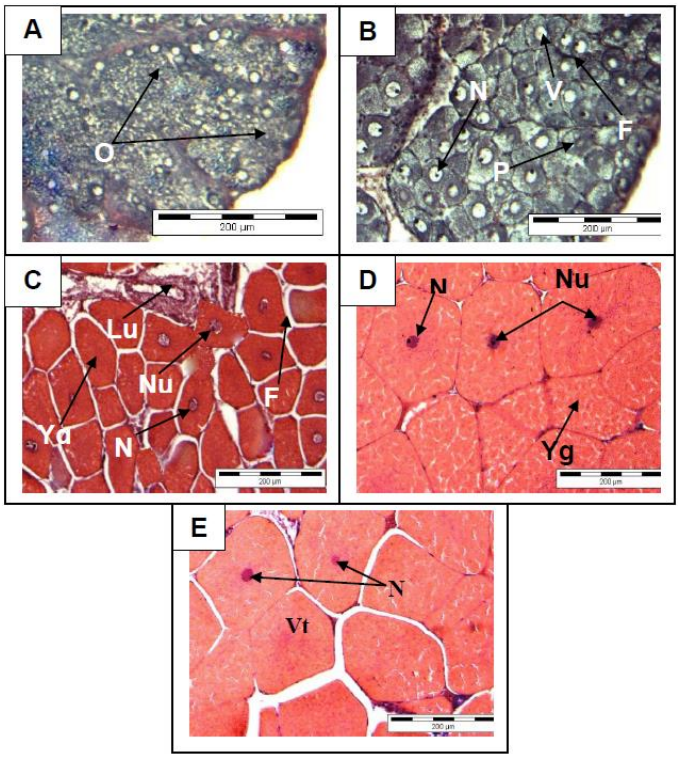

Plate 1: Histological features of different gonad developmental stages in female mud crab under captive conditions; A: Proliferation, B: Pre-vitellogenesis, C: Primary vitellogenesis, D: Secondary vitellogenesis, E; Tertiary vitellogenesis (mature); *Note: [O= Oogonium, F= Follicles, Lu= Lumen, N= Nucleus, Nu= Nucleolus, P= primary oocytes, Yg= Yolk globules, V= Vacuolated globules, Vt= Vitellus]

The proliferation (Stage-1; plate 1 A), pre-vitellogenesis (Stage-2; plate 1 B), primary vitellogenesis (Satge-3; plate 1 C), secondary vitellogenesis (Stage-4; plate 1D) and tertiary vitellogenesis (Stage-5; plate 1 E) had the oocyte diameter ranged from 12.92 - 35.20 µm, 35.5 - 65.95 µm, 66.87 - 124.0 µm, 138.16 - 206.57 µm and 153.26 - 240.16 µm, respectively (Plate 1 and Table 1). Highest proportion (46.15%) of secondary vitellogenesis stage (stage-4) was achieved from outdoor growout system followed by indoor compartment (30.77%) and outdoor floating boxes (23.08%). Similarly, the proportion of tertiary vitellogenesis stage (stage-5) was also highest (40%) under outdoor growout protocol followed by both indoor compartment and outdoor floating boxes (30%) (Table 1). The proliferation stage is

characterized by the presence of round shaped clustered oogonia in the ovary lobe and cytoplasm barely visible (Plate 1 A and Table 1). The pre-vitellogenesis stage is categorised by the staged oocytes with vacuolated globules, follicle cells and cytoplasm, but the nucleus is rarely visible (Plate 1 B and Table 1). Primary vitellogenesis stage is easily recognised from the formation of yellowish color yolk globules from cell periphery. All essential cell elements like nucleus, nucleolus and follicle cells are present, but the vacuolated globules has disappeared (Plate 1 C and Table 1). The secondary vitellogenesis stage is recognized from the spreading of yolk globules to the entire cytoplasm with the presence of a nucleus, nucleolus and follicle cells within the oocyte (Plate 1 D and Table 1). The tertiary vitellogenesis stage contains identical individual oocytes with prominent yolk globules fused to each other, the nucleus was occasionally visible and nucleolus positioned at the periphery of the nucleus (Plate 1 E and Table 1).

Table 1: Histological characteristics of the ovary development stages of female green mud crab grown under different captive growout protocols

Stages	Histological features	Composition of stages (%)		
		T1: outdoor tank	T2: outdoor boxes	T3: indoor compartment
Proliferation	Cluster of oogonia in ovarian lobe; oogonium globular shape, diameter 12.92 – 35.20 µm; cytoplasm barely visible	33.33	37.04	29.63
Pre-vitellogenesis	Formation of oocytes; oocytes staged or overlapped; both vacuolated globules, follicle cells and cytoplasm are visible; nucleus rarely visible; oocyte diameter 35.5 – 65.95 µm	33.33	33.33	33.33
Primary vitellogenesis	Formation of yolk globules from cell periphery; nucleus, nucleolus, follicle cells visible; oocyte diameter 66.87 – 124.0 µm	36.36	36.36	27.28
Secondary vitellogenesis	Yolk globules spread to the entire cytoplasm; nucleus, nucleolus, follicle cells visible; oocyte diameter 138.16 – 206.57 µm	46.15	23.08	30.77
Tertiary vitellogenesis	Individual oocytes; yolk globules prominent and fused; nucleus occasionally visible; oocyte diameter 153.26 – 240.16 µm	40.00	30.00	30.00

3.2 Anatomical features of gonad morphology at different development stages and size at sexual maturity

Likelihood the histological features, the morphological development of gonad also occurred with five discrete stages for all growout protocols (Plate 2 and Table 2).

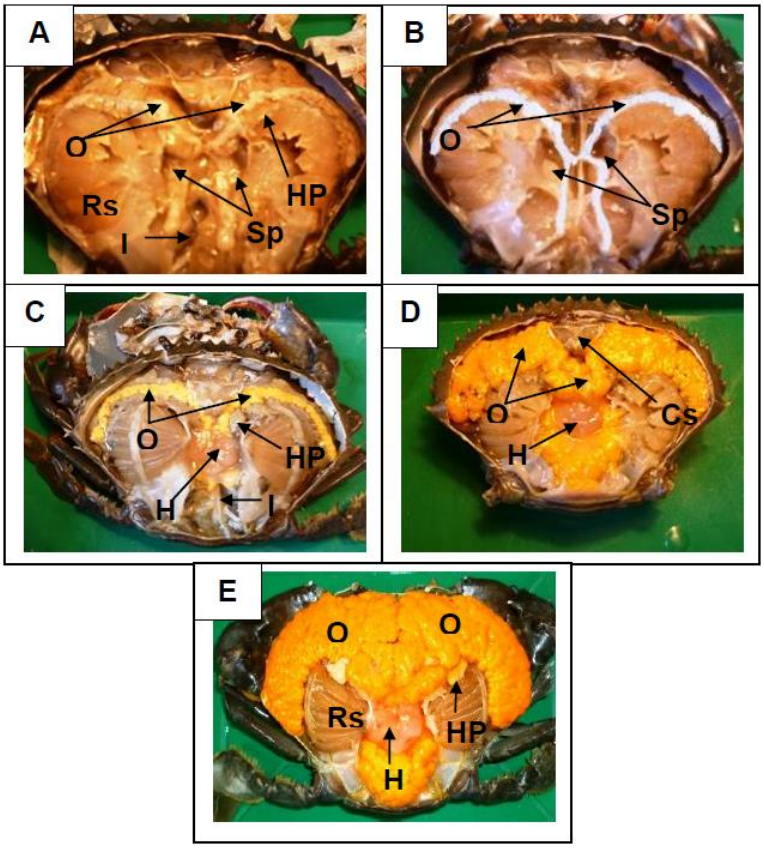

Plate 2: Anatomical view of different gonad developmental stages of female mud crab under captive condition; A: Proliferation, B: Pre-vitellogenesis, C: Primary vitellogenesis, D: Secondary vitellogenesis, E: Tertiary vitellogenesis (mature); *Note: [O= Ovary, HP= Hepatopancreas, I= Intestine, H= Heart, Sp= Spermatotheca, Cs= Cardiac stomach, Rs= Respiratory organ]

Initiation of sexual maturity was first observed in crabs at 110.40, 112.60 and 116.80 g of body weight (TW) and 7.10, 7.30 and 7.90 cm of carapace width (CW) under indoor compartment, outdoor boxes and outdoor tank culture

protocols, respectively (Table 2). Size of crab did not differ significantly at first sexual maturation among difference grow out protocols.

In stage-1 (immature stage), the gonad appeared with a thread of translucent, watery tissues and very hard to distinguish from the hepatopancreas (Plate 2 A). The ovary had the thickness of approximately <2 mm and occupied about <1% of body cavity (Table 2). For stage-2 (developing stage), the gonad appeared as two lines over the hepatopancreas and easily separated from the digestive gland with the prominent size and creamy to whitish in color. The ovary thickness was 2-3 mm and filled approximately 1-2.5% of body cavity. The spermatheca is visible after removal of the heart (Plate 2 B and Table 2).

Table 2: Morphological features of different gonad development stages and size distribution of female crabs under different growout systems (BW= body weight, CW= carapace width)

Stages	Morphological features	Crab size	Grow out protocols		
			T1: outdoor tank	T2: outdoor boxes	T3: indoor compartment
1. Immature stage (Plate 2 A)	Thread tissue, ribbon like structure over hepatopancreas; hard to distinguish; watery to translucent color; thickness <2 mm, occupy <1% of body cavity	BW (g)	116.80±8.50[a]	112.60±9.70[a]	110.40±12.40[a]
		CW (cm)	7.90±0.57[a]	7.30±0.53[a]	7.10±0.54[a]
2. Developing stage (Plate 2 B)	Creamy to off-white color; ovary thickness 2-3 mm; occupy 1-2.5% of body cavity; spermatheca visible after removal of heart	BW (g)	135.50±11.40[a]	128.40±8.70[a]	119.50±8.90[a]
		CW (cm)	8.90±1.05[a]	8.60±1.00[a]	8.50±0.90[a]
3. Early maturing stage (Plate 2 C)	Pale or light yellow in color; ovary thickness 3-8 mm; occupy 10-25% of body cavity.	BW (g)	185.40±18.30[a]	166.60±14.50[ba]	148.40±12.70[cb]
		CW (cm)	9.80±.0.90[a]	9.20±0.60[a]	9.00±0.80[a]
4. Late maturing stage (Plate 2 D)	Deep yellow to yellow-orange in color; ovary thickness 8-12 mm; occupy 25-75% of body cavity.	BW (g)	208.60±16.80[a]	182.90±14.90[ba]	176.20±13.60[cb]
		CW (cm)	10.40±0.80	9.50±0.50	9.40±0.60
5. Mature stage (Plate 2 E)	Individual eggs are noticeable; deep yellow to yellow-orange in color; ovary thickness 12-20 mm; occupy >80% of body cavity	BW (g)	234.00±14.50[a]	204.40±11.60[b]	198.40±10.40[cb]
		CW (cm)	10.80±1.10[a]	9.70±0.80[a]	9.60±0.70

The initiation of the vitellus was the main feature of the gonad in the early development stage (stage-3), the vitellogenesis started from the cell periphery and made the gonad yellowish in color (Plate 2 C). Pale or light yellow gonad occupied 10-25% of the body cavity with the thickness of 3-8 mm (Table 2). In late maturing stage (stage-4), the gonad lobules has been developed notably in the upper hepatopancreas and in sterno carapace (Plate 2 D). The gonad thickness was 8-12 mm and approximately 25-75% of the body cavity was filled with deep yellow ovary (Table 2). At the mature stage (stage-5) the gonad elongated to utmost and homogeneously shed the digestive gland and cardiac stomach (Plate 2 E). More than 80% of the body cavity were filled with deep yellow to yellow-orange gonad. The gonad had the thickness of 12-20 mm (Table 2) and individual eggs were visible (Plate 2 E).

3.3 Gonad somatic index (GSI) in respect to different maturation stages

Irrespective of grow out protocols, GSI seemed very low at maturing stages (Stage-1 and Stage-2) while started to increase as yolk formation began (Stage-3). Highest mean GSI values of over 10% were found at advanced stage (stage-5) (Table 3).

Table 3: Classification of GSI (Gonad Somatic Index) and frequency of GSI under different gonad development stages

Category of GSI (%)	Sample frequency (%)				
	Stage-1	Stage-2	Stage-3	Stage-4	Stage-5
< 1	81.48	33.33	0.00	0.00	0.00
1-5	18.52	66.67	72.73	0.00	0.00
5.1-10	0.00	0.00	27.27	69.23	60.00
> 10	0.00	0.00	0.00	30.77	40.00
No. of samples	27	9	11	13	10
Proportion (%)	38.57	12.86	15.71	18.57	14.29
GSI (%) Mean±SD	0.67±0.55	1.26±0.44	3.10±1.45	7.82±2.35	10.35±4.86

Among the sampled crabs, the majority (38.57%) of the females were in the immature stage (stage-1), while lowest was (12.86%) for the pre-vitellogenesis stage (stage-2) (Table 3) indicated shorter existance of the stage.

3.4 Age at first sexual maturity under different growout protocols

Occurrance of first sexual maturation (stage-1) of female *S. paramamosain* was noticed among only 20% of the samples at the age of 5 months for outdoor tank (T1), while, it was 5.5 months for both outdoor boxes and indoor compartments (Fig. 1.

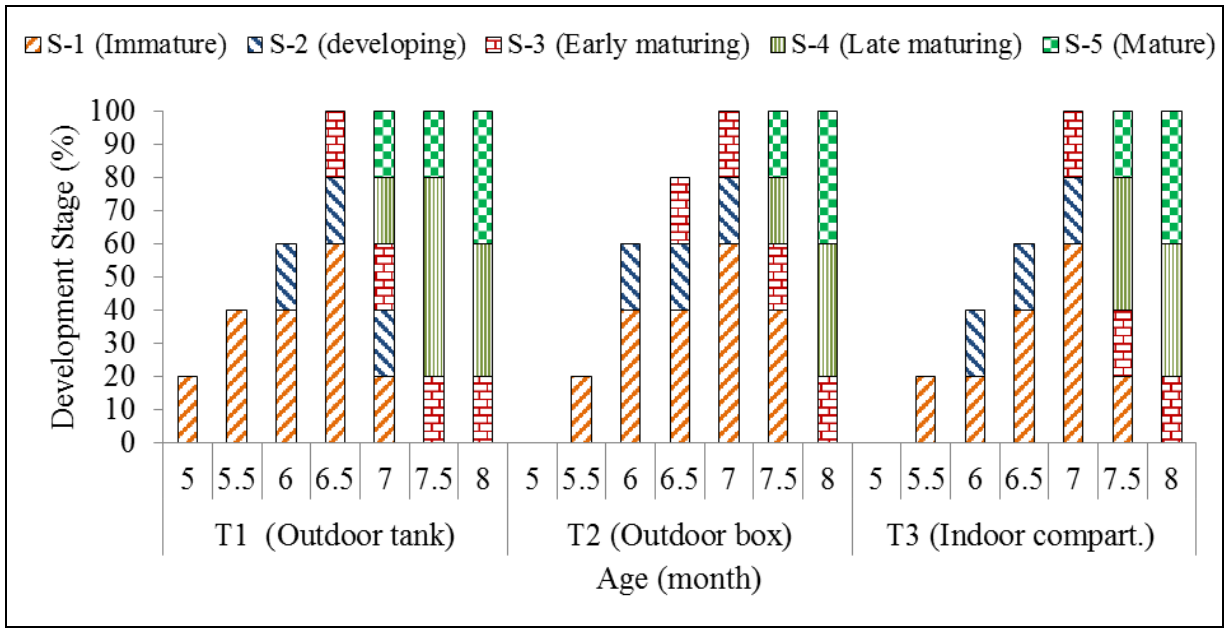

Fig 1: Composition (%) of different ovary maturity stages in relation to age of the female green mud crab grown under different captive growout protocols

The proportion gradually increased as the age increased and immature female was found up to 7 months of age for outdoor tank and 7.5 months of age for both outdoor boxes and indoor compartments (Fig. 1). In outdoor tanks, more than 50% of the sample was found to be maturing at the age of 6.5 months indicated median sexual maturity age (MA_{50}), whereas for both outdoor boxes and indoor compartments the median sexual maturation age (MA_{50}) was 7 months (Fig. 1). Both first maturity and median maturity level commenced earlier in the outdoor tank culture system compared to the outdoor boxes and indoor compartment systems.

4. Discussion

Virtually, all the essential organs of an animal were developed during gamete formation and embryogenesis, but some of the organs remained in rudimentary forms and developed slowly with progress in age as well as with size increment. In the case of female green mud crab (*S. paramamosain*), sexual organs were not traced with naked eyes before 5 months of age (Fig. 1). Based on the histological observation, five gonad maturation stages (Stage-1 to Stage-5) for female *S. paramamosain* (Plate 1) were strongly compatible with the morphological progress of the gonad (Plate 2). The oocyte diameter increased (Plate 1 and Table 1) with the progress of maturation stages which led to an increase in gonad volume (Plate 2) and gonad somatic index (GSI) (Table 3). The common histological characteristics regarding oogenesis formation and gonad maturation phases observed in this study coincided with the finding of wild *S. serrata* [12, 13] and wild *S. paramamosain* [21, 14], but differed with previously reported four stages for *S. olivacea* [17]. In another study, previtellogenic stage that restrains the primary oocytes and the vitellogenic stage that allows the oocytes to be larger with the appearance of yolk globules in the cytoplasm, has been considered as two major gonad development stages in brachyuran crabs [22].

This study observed the first appearance (immature) of gonad at 5 months and 5.5 months of age and fully mature female was observed at 7 and 7.5 months of age. Maturation was asynchronised with 50% of maturation at the age of 6.5 and 7 months under different culture conditions (Fig. 1). Regardless of heredity, this type of maturation might be associated with nutritional factors and obesity. High protein and less fibrous food enhance the obesity and thus advances puberty in females, but delays for the male [23]. However, findings on maturity age of this study is supported by previous authors [6, 7, 24].

This study observed highest proportion (38.57%) of gonads for the immature stage (stage-1) and lowest proportion (12.86%) for the maturing stage (stage-2) (Table 2) indicating short existence of stage-2. This observation varied with the observation on wild *S. paramamosain* [14] and for wild *S. olivacea* [17]. They observed lowest proportion for the mature stage, perhaps due to their sampling from mangrove swamps and escaping of mature females due to migration for mating and spawning [6, 21, 25, 26].

From this study, female crabs started maturing at different sizes (Table 2) and ages (Fig. 1) when reared under different protocols. The trend showed that the maturation in the outdoor tank (T1) seemed faster. It has been reported that, the reproductive development might be associated with age and size [27], nutrition and obesity [23]. Reproductive development thus greatly influenced by photoperiod [28]; photoperiod and temperature [29]. In crustaceans, it might be regulated by nutrition, temperature and salinity [17, 30, 31]. In this study, the crabs reared under outdoor condition were cultured in communal basis and sheltered with seaweed, thus received enough sunlight and in addition, nutrient from the tank bottom, shelter (seaweed) and through cannibalizing on other crabs might have triggered the earliest onset of sexual maturation and provided highest proportion of gravid crabs (stage-5).

5. Conclusion

Suitable environmental conditions required for growth, sexual maturity and reproductive development of marine crustacean. The crab samples used in this study were exclusively reared in captive conditions under different protocols. A portion of female crabs showed faster sexual maturity, whereas, the majority began maturing uniformly and the last portion showed slow maturation. Sexual maturity was faster and number of gravid broods was higher under outdoor growout

system. The protocols used for broodstock development might be a baseline guide to the hatchery managers for rearing and managing the broodstock in captive condition.

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