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Morphometric Measurements, Gonopod and Gonopores Appearances in Early Sexual Crablet Stage of *Scylla paramamosain* (Estampador, 1949)

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ABSTRACT

This study was aimed to describe the morphological appearances of gonopod and gonopores along with the carapace sizes at early crablet stage of mud crab, *Scylla paramamosain.* Changes in abdominal shape were recorded from instar stage 1 (Crablet 1 or C1) of the crabs for sexual differentiation. In addition, comprehensive measurements of their sizes, appearances and description of forked pleopod and both gonopod and gonopores were also recorded. Experiments were carried out using an optical microscope to facilitate the observation of the crablets' abdomen along with a digital camera which connected to a laptop to capture the sample images. In this present study, the difference of abdominal flap shape began to appear more clearly at C5 stage using an optical microscope with 8-20x magnifications. Female's abdominal flap in C5 stages was assumed wider than the male's abdominal flap and the notch on the side was not as clear as in male. The difference of abdominal flap shape between male and female could be recognized with the naked eye when the carapace width reached ±2 cm (±C9 stage). The existence of gonopod in the Carapace Width (CW) range of 05.01–10.00 mm were still difficult to be detected and the existence of gonopod in this range was assumed still a small bulge at the base inside abdomen flap and as well as for forked pleopod. The gonopod and forked pleopod were detected more clearly in CW >10 mm using an optical microscope with 10-20x magnifications. The data from this study can be used to compare male and female of *S. paramamosain* in the crablet stage, which are valuable during the early stocking of the juvenile crab in the aquaculture facilities or crab ponds and for its selection breeding program or monosex culture development.

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Authors' Contribution
MNS and MI designed experiment. MNS performed experiments, collected and analyzed data and wrote the first draft of the manuscript. CZC-I, AA-S, MS and ABA-M helped in the preparation of the experiment facilities. MI and MNA helped in the first draft of the manuscript and edited it.

Key words
Abdominal shape, Aquaculture, Carapace width, Crab morphology, Sex differentiation

INTRODUCTION

Brachyuran crabs are known to have around 7000 species (De Grave *et al*., 2009; Ng *et al*., 2017), and the family Portunidae is important for its economical value and contribution to the crab fisheries of the world (Abol-Munafi and Azra, 2018; Gunarto *et al*., 2018; Azra *et al*., 2019). Some species such as the blue swimming crab, *Portunus pelagicus,* and the mud crab, *Scylla* spp., are well-known in international markets and usually marketed live or soft-shell product (Paterson and Mann, 2011; Azra and Ikhwanuddin, 2015, 2016; Tavares *et al*., 2018). The mud crabs, *Scylla* spp. are a group of four commercially important Portunid species that are found in intertidal and subtidal sheltered soft-sediment habitats, particularly mangroves, throughout the Indo-Pacific region (Keenan *et al*., 1998; Le Vay *et al*., 2008). The taxonomy of the mud crabs, genus *Scylla* has been clarified using allozyme electrophoresis, DNA sequencing and morphometrics to identify four *Scylla* species from crabs collected throughout their distribution from Red Sea to the Indo-Pacific Ocean (Keenan *et al*., 1998; Shelley and Lovatelli, 2011). The revision of the genus *Scylla* into four species provides a basis for the development of a better understanding of their ecology, population biology

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and the sustainable management of fisheries, as well as the selection of species for aquaculture (Keenan *et al*., 1998; Imai *et al*., 2004; Rouf *et al*., 2016).

Most malacostracan Crustacea are gonochoristic, but males and females are not distinguishable at hatching and sexual characteristics develop with each successive molt (Hasegawa *et al*., 1993). The similarities between male and female juveniles of Brachyura are very similar, including in their abdomen sizes at the first post-assessment stage (Shinozaki-Mendes and Lessa, 2019). In mud crabs, *S. serrata*, the differential shape of the male and female abdomen enabled the sexing of crabs above 3 cm carapace width on casual inspection, while the sex of crabs below this size can be examined at 30× by means of a binocular microscope (Heasman, 1980) which may differ between mud crab species. The differences between the sexes of mud crab become more apparent as the crabs mature based on their abdominal flap, where the female has much broader abdominal flap than that of males (Ikhwanuddin *et al*., 2011).

Although there are some studies that found the genetic method of sexing several species of crustacean at early development stages, such as *Macrobrachium rosenbergii* (Ventura *et al*., 2011) and in the mud crab itself (Shi *et al*., 2018), the method requires special equipment and skills with a high cost. In general, sexing of a species is based on the observation of their morphology. Particularly in the mud crab, abdominal flap or cheliped size was used to differentiate male and female. But this morphological observation takes a long time for the difference to be seen by the naked eye.

Thus, this study tried to demonstrate for the first time that the mud crab crablet stage in males and females can be morphologically distinguished, additionally showing how the appearance of their gonopods, forked pleopods and gonopores manifest in the early crablet stage.

MATERIALS AND METHODS

Crab sources

Juvenile crabs at C1 stage were produced at the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu. In brief, the matured broodstock were sampled in Setiu Wetland, coastal water of Terengganu, Peninsular Malaysia. They were maintained in a 100 L fibreglass tanks at 1 crab per tank or maintained communally (2 ind/m^2) in a 7.5 m^2 (2.5x3 m) of square fibreglass tank and occasionally fed with Scadfish, *Decapterus* spp. one or two times daily as much as 3% of biomass until they spawned. Spawned crabs were transfer to the 500 L fibreglass tanks until they hatched. After the eggs hatched completely, aeration in the incubation tank was turned off for several minutes and larvae that clustered and actively swam on the surface were collected by gentle scooping. Larval crabs were cultured in larval rearing tanks until they reached the megalopa stage. The megalopas then reared individually in plastic cups with a bottom diameter of 6-9 cm to produce of the crablets as samples in this study. Larval rearing and culture methods referred to the previous published methods (Shelley and Lovatelli, 2011; Azra and Ikhwanuddin, 2015; Syafaat *et al*., 2019).

Sex differentiation in the crablet stages of mud crab

To ensure the supply of particular crablet stage, some crablets were maintained individually in plastic cups with 6-9 cm of bottom diameter and placed into a rectangular fiber glass tank (0.7×1.17 m and 1×2 m). Some crablets are maintained communally in a rectangular fiber glass tank (1×3 m) or maintained individually in plastic cups to obtain sufficient numbers of crablets during this experiment. Crablets were fed daily with shrimp pellet, blended fish flesh or adult frozen *Artemia* once a day. Crablet carapace size was measured using digital calipers with an accuracy of 0.1 mm. Sex differentiation observation was carried out starting from stage C1 and so on until the difference between male and female morphologically developed and the observation focused only on abdomen flap shape (Fig. 1). Abdominal observation was carried out in each crablet stage either in live samples or old carapace (exuviae) from the previous molting. If there was already difference in the abdominal flap shape (to predict male and female) at a certain stage visually, then a number of size ratios were made on the abdominal flap and further analyzed to determine whether there was a quantitative difference at that stage. Lines within the abdominal flap were measured using the software of GIMP-GNU Image Manipulation Program (the line size produced from this program were not a factual size but only an approach size that made to facilitate making the ratios). Thirty (30) samples, 15 males and 15 females, were used in this quantitative test.

Observation of gonopod, gonopore and forked pleopod in the crablet stages

Gonopod, gonopores and forked pleopod were observed in the crablet stages with carapace sizes ranging from 5–35 mm. Moreover, 10 crablets were taken for each abdomen that resembled male and female then maintained until difference of their abdominal shapes appeared more clearly between them (include gonopod and gonopores checking) to confirm visual prediction done earlier on. In this study, optical microscope (Nikon® optical microscope type SMZ645) was used to facilitate the observation of the crablets abdomen and Dinoeye® camera type AM-423X were used to capture the sample images under the microscope.

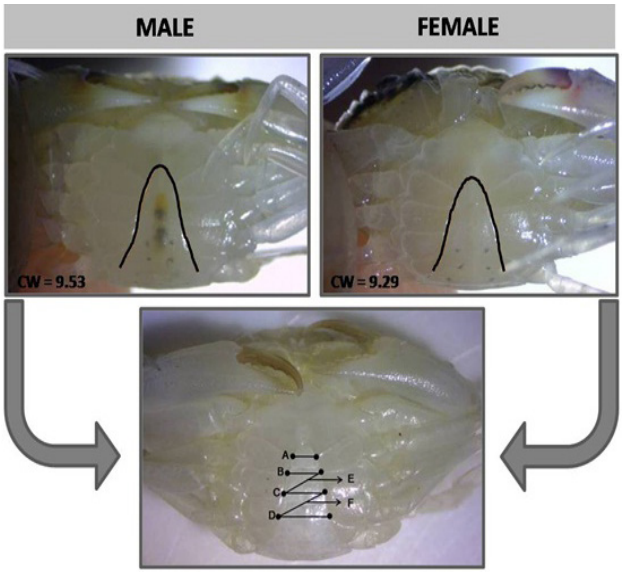


Fig. 1. Estimation of male and female based on abdominal flap shape in C5 stages and the six lines on abdominal flap that used to make a ratio for t-test purpose.

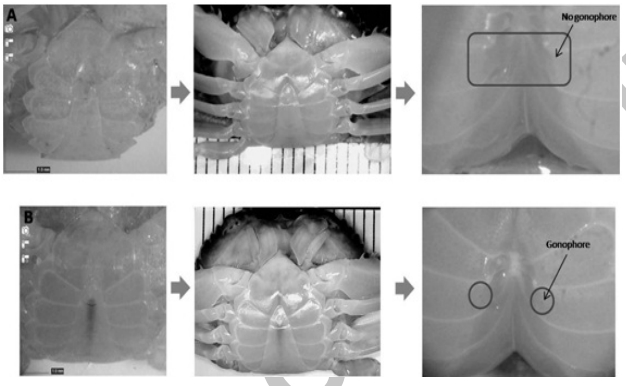


Fig. 2. Verification of sex prediction. (A) Male; (B) Female.

Table I. T test value of the six ratios that made based on six lines in abdominal flap of C5 stage of *S. paramamosain*.

Parameter	Average ratio		P value	Statistic test
	Male	Female		
B/C	0.83±0.02	0.82±0.03	0.1278	T-test
C/D	0.85±0.02	0.82±0.02	0.0075	T-test
E/C	1.02±0.02	1.01±0.02	0.5454	T-test
F/D	1.00±0.01	0.99±0.02	0.1697	MW-U test*
E/F	0.86±0.02	0.84±0.02	0.0051	T-test
(E+F)/D	1.87±0.03	1.82±0.04	0.0104	MW-U test

*MW-U test, Mann-Whitney-U test.

Data analysis

The independent *T*-test was used to analyze differentiation of the six ratios based on six lines in abdominal flap of male and female in C5 stage. Data were presented as mean ± standard deviation at a significance level of $P<0.05$. Microsoft Excel was used to create a graphic, while R-program version 3.5.1 was used for all of the other data analyzes.

RESULTS

Morphological sexing

Sexing based on abdominal flap differences in the early crablet stages (C1-C2) was not recognizable yet because their abdominal flap were very similar (Table II). Abdominal flap differences between crablets began to be detected in C3-C4 stages but the difference was not too clear. In this present study, sexing of the crablet started at the C5 stage because the difference in abdominal flap began to appear more clearly using an optical microscop (magnification of 8-20x). Female's abdominal flap in C5 stages were wider than male's abdominal flap and the notch on the side was not as clear as in male. The difference of abdominal flap between male and female in the crablet stage of *S. paramamosain* would be seen more clearly when the carapace width reached ±2 cm (±C9 stage). In this size, gonopod and gonopores (include forked pleopod in female) were also detected clearly (Figs. 5 and 6). Furthermore, sex predictions done in C5 stages were 90% corret where 9 of the 10 samples tested were correct both for male and female crablets (Figs. 2, 3 and 4). Furthermore, from the six ratios based on six lines in abdominal flap of C5 (B/C, C/D, E/C, F/D, E/F and (E+F)/D) (Fig. 1), three of the ratios were significantly different ($P<0.05$) i.e., C/D, E/F and (E+F)/D (Table I).

Gonopod, gonopore and forked pleopod in crablet stages

In this experiment, a total of 105 crablets 41 males and 63 females were used. The CW ranged around 5–35 mm and were divided into 6 size ranges i.e., 5-10 mm, 10-15 mm, 15-20 mm, 20-25 mm, 25-30 mm, and 30-35 mm. The shortest CW used for male crablet observation was 5.6 mm and the longest was 31.5 mm, while the shortest and the longest CW for female were 5.6 mm and 33.4 mm, respectively. The results showed that the gonopod were still difficult to be detected in the CW range 05.01 – 10.00 mm. The gonopod in this range was still a small bulge at the base inside abdomen flap (Table III). The gonopod was detected more clearly in CW >10 mm (Fig. 5). Furthermore, the presence of gonopore and forked pleopod could be detected in the CW range 5.01 - 7.50 mm but their appearance was not so clear (Table IV). In

this range, although the gonopores could be detected but it was still difficult to determine and the presence of forked pleopod was assumed to be a small bulge at the base inside abdomen flap (Fig. 6).

Table II. Crablet's abdominal flap observed from C1 to C7 crablet stages of *S. paramamosain*.

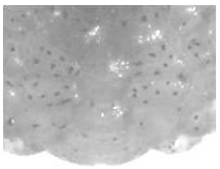
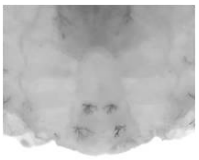
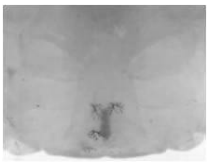
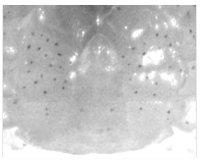
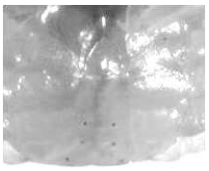
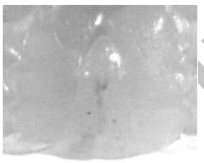
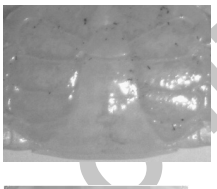
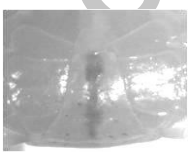
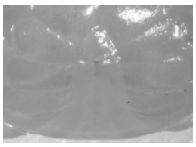
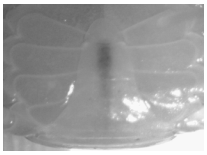
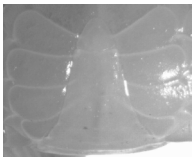
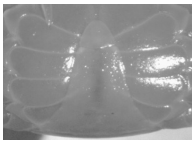
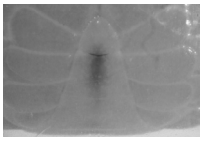
Crablet stage (mean CW±SD)	Abdomen shape	
	Males	Females
C1 (3.40±0.22 mm)		
C2 (4.56±0.21 mm)		
C3 (5.80±0.33 mm)		
C4 (7.43±0.58 mm)		
C5 (9.10±0.68 mm)		
C6 (11.18±0.76 mm)		
C7 (14.30±0.72 mm)		

Table III. Size range distributions for observation of the appearance of gonopod in male crablets of *S. paramamosain*.

Size range (mm)	Frequency	Percentage (%)	Appearance frequency	Appearance percentage (%)
05.01 – 07.50	2	4.76	1	50
07.51 – 10.00	2	7.14	1	50
10.01 – 12.50	2	4.76	2	100
12.51 – 15.00	11	26.19	11	100
15.01 – 17.50	13	30.95	13	100
17.51 – 20.00	8	19.05	8	100
20.01 – 22.50	1	2.38	1	100
22.50 – 25.00	1	2.38	1	100
25.01 – 27.50	0	0	0	-
27.51 – 30.00	0	0	0	-
30.01 – 32.50	1	2.38	1	100
32.51 – 35.00	0	0	0	-
Total	41	100	39	

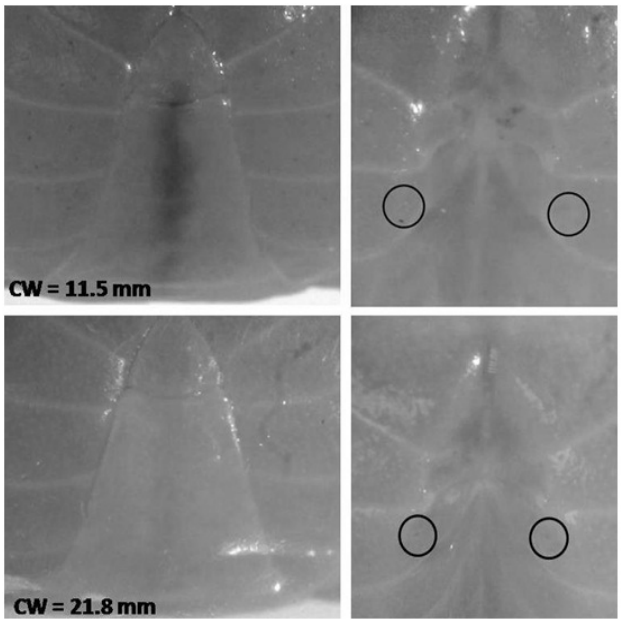


Fig. 3. Abdominal flap shape and the appearance of gonopores (circle) in different carapace width of female crablet of *S. paramamosain*.

Table IV. Size range distributions for observation of the appearance of gonopore and forked pleopod in female crablets of *S. paramamosain.*

Size range (mm)	Frequency	Percentage (%)	Appearance frequency of gonopore	Appearance Percentage of gonopore (%)	Appearance frequency of forked pleopod	Appearance Percentage of forked pleopod (%)
05.01 – 07.50	3	4.76	2	66.67	2	66.67
07.51 – 10.00	3	3.17	3	100	3	100
10.01 – 12.50	5	7.94	5	100	5	100
12.51 – 15.00	13	20.63	13	100	13	100
15.01 – 17.50	9	14.29	9	100	9	100
17.51 – 20.00	10	15.87	10	100	10	100
20.01 – 22.50	12	19.05	12	100	12	100
22.50 – 25.00	1	1.59	1	100	1	100
25.01 – 27.50	3	4.76	3	100	3	100
27.51 – 30.00	1	1.59	1	100	1	100
30.01 – 32.50	2	3.17	2	100	2	100
32.51 – 35.00	2	3.17	2	100	2	100
Total	64	100	63		63	

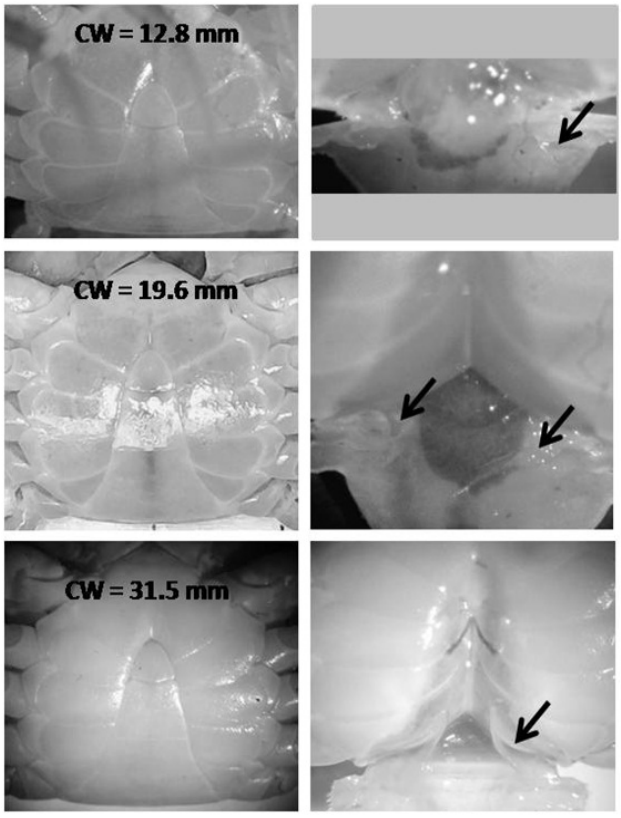


Fig. 4. Abdominal flap shape and the appearance of gonopod (arrow) in different carapace width of male crablet of *S. paramamosain.*

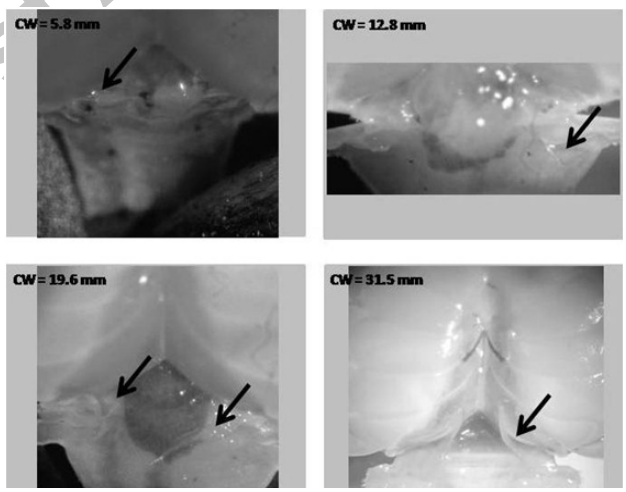


Fig. 5. The appearance of gonopod inside abdomen flap of crablet with different carapace width of *S. paramamosain*

DISCUSSION

In this present study, the morphological differences between males and females of *S. paramamosain* crablet through the naked eye have begun to appear at a CW of ±2 cm (±C9 stage) but further observations using a microscope (magnification of 8-20x) showed that the difference could be detected visually in CW of ±1 cm (±C5 stage). Although the difference of the abdomen flap shape on the C5 stage was still vaguely visible, but testing of several size ratios (Fig. 1) that made on the abdominal flap showed that there

was a quantitative difference ($P<0.05$) between the two forms of abdominal flap (Table I). Furthermore, subsequent maintenance of C5 until it reached the C7-C8 stage to prove the correctness of the sex prediction showed that there was a consistency of the same abdominal shape by 90% and this result was proven by examining the presence of gonopore and gonopod in female and male respectively on C7-C8. In *Callinectes danae* (Crustacea: Brachyura), based on ontogenetic trajectories for males and females show that they have similar origins and follow different directions over the instars (Shinozaki-Mendes and Lessa, 2019), where its sexual dimorphism becomes apparent from the fourth juvenile stage onwards (Bolla *et al*., 2014). In *Eriocheir japonicus,* sexual differentiation first occurs at the megalopa stage in two different orientations of the gonoducts between males and females (Lee *et al*., 1993). The rudiments of the gonopores (i.e., the endings of the paired gonoducts at the thoracic stemite) in both sexes appear at the first crab stage. From the third crab stage the gonopores in both sexes become concave (Lee *et al*., 1993). From this stage the gender can be determined visibly by the presence of first pleopods in males (i.e., the first gonopods) under dissecting microscopy and up to the fifth crab stage, no androgenic gland appears along the gonoducts of the male crabs (Lee *et al*., 1993).

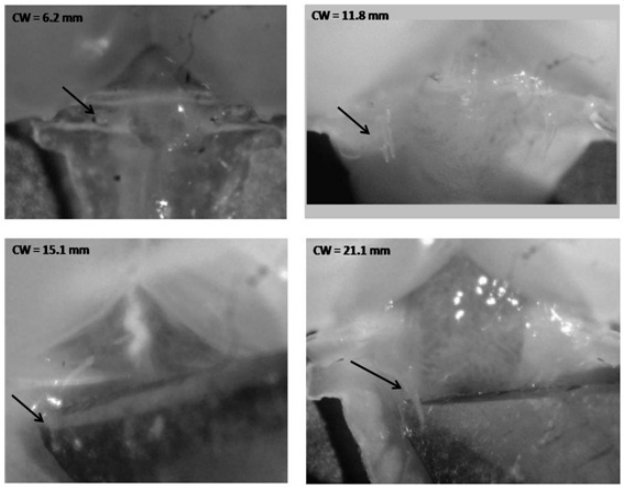


Fig. 6. The appearance of forked pleopod inside abdomen flap of *S. paramamosain* crablet with different size of carapace width (CW).

Study on the crabs *Callinectes sapidus* and *Rhithropanopeus harrisii* showed sex differentiation can be determined on the basis of the appearance of the gonopores in the females and the appearance of the first pleopods in males at the second crab stage for the primordial gonopores first appear at this stage, and at the next stage the gonoducts extend to the stemites where the primordial gonopores are situated (Hong, 2004). In this present study, the presence of the gonopore had been detected vaguely at the C3 and C4 stage but its determination was still rather difficult because the hole was small and it located on an uneven surface so the observation needs to be done carefully. Further, the presence of gonopods and forked pleopods in male and female respectively also could be detected at the C3-C4 stage but its presence was recognized still as a small lump at the bottom side of inside the abdomen flap. Furthermore, the examination of the gonopore and gonopod inside the abdomen flap at C1 and C2 was still difficult because the abdominal flap was still soft and may require a special tool to monitor it at that stage.

By knowing the existence of gonopod, gonopores and forked pleopod of *S. paramamosain* crablet at a particular stage and size with a specific form of the abdomen flap, we can distinguish male and female based on their different form of the abdomen flap. Females were recognized by the presence of 4 pairs of biramous pleopods (forked pleopod) and of oviduct depression on the sternites of the 6th thoracic segment, where males were recognized by the presence of copulatory pleopods (gonopod) and the absence of oviduct depressions (Heasman, 1980). One of the advantages that can be obtained from knowing the existence of primary sex characteristics (gonopores and gonopod) in the crablet stage is that it can be used to determine the right time to make a sex reversal treatment. Previous study showed that to achieve successful masculinization in crabs, animals used should be not later than the third to fifth crab stages (Hong, 2004). Lee *et al*. (1993) reported that androgenic gland transplantation in the females of *E. japonicus* at the third crab stage, masculinization was found at the following stages with the appearance of the male copulatory appendages and the degeneration of the third to fifth pleopods.

The ability to distinguish sex of mud crab in the crablet stage could help stimulate the monosex seed market which is scarce, while simultaneously triggering the development of the mud crab hatchery industry. In mud crab culture, monosex culture of all male shows higher specific growth rates (SGRs) compared to all female (Khatun *et al*.,2009) while culture trials of mono-sex culture (all-male and all-female) has yielded significantly higher production and survival compared to that of mixed culture (Venugopal *et al*., 2012). Availability of monosex seeds is essential to support monosex mud crab farming. In the selective breeding program, the ability to distinguish sex during the crablet stages allows for improved handling of prospective broodstock earlier, e.g. allow us to select the samples and provide special treatment based on sex earlier.

CONCLUSION

The morphological differences between males and females based on abdominal flap shape of *S. paramamosain* crablet could be detected at C5 stage (using an optical microscope with magnification of 8-20x). Furthermore, the presence of gonopores, gonopod and forked pleopod could be detected at the C3-C4 stage but their presence was still vaguely. This result was expected to be useful in selection program, sex reversal and monosex culture development of mud crab in the future.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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