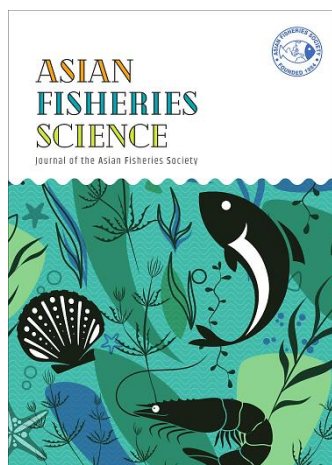




Gonadal Development of Tree-Climbing Mangrove Crab *Episesarma mederi* (H. Milne Edwards, 1853) from Capiz, Philippines

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Abstract

Episesarma mederi (H. Milne Edwards, 1853) belongs to the dominant crab groups in estuarine and mangrove areas that play vital roles in the nutrient cycling and substrate biochemistry of tropical ecosystems. It is a traditional coastal resource in Asia, but its status remains unassessed and unmonitored. The foremost constraint in managing this crab species is the lack of knowledge on its reproductive biology. Hence, this study analysed the gonadal maturation stages of *E. mederi* from President Roxas, Capiz, Philippines. Morphometric analysis and sexual determination were performed. Staging of gonad development was established by complementing morphological features (i.e. colour, volume) with histological analysis (i.e. cell type and size). A total of 448 crabs (264 females, 184 males) were collected. Results showed that males were larger and heavier than females. The smallest recorded sexually mature individuals had a carapace width of 28.6 mm in females and 26.0 mm in males. Ovarian histology showed five stages of development: immature, developing/redeveloping, maturing, mature (two substages – early mature and late mature), and spent. In males, only two stages of gonad development were identifiable: immature and mature. The gonadal morphology and histology suggest that *E. mederi* has a continuous reproductive strategy. This is supported by the presence of post-spawning ovigerous females with redeveloping ovaries and the occurrence of mature males throughout the study period. The present study provides preliminary information on the reproductive aspects of *E. mederi* that may be essential for its sustainable utilisation.

Keywords: histology, oocyte, reproductive biology, spermatocyte, sesarmid

Introduction

The mangrove crab *Episesarma mederi* (H. Milne Edwards, 1853) is classified as a burrow-dwelling and facultative tree-climbing crab species since it recurrently migrates between burrows nearby mangrove roots and trees (Sivasothi, 2000; Fratini et al., 2005; Lee et al., 2015). It belongs to Family Sesarmidae, which is among the dominant brachyurans in tropical and subtropical habitats with several endemic genera in the Indo-West Pacific (Schubart et al., 2006). During water level rise at high tides, *E. mederi* crabs climb up trees up to 6 m and remain motionless on treetops to evade predators (Ng and Sivasothi, 2001). Their burrowing activities consequently alter the topography and biochemistry of the substrate that facilitates nutrient cycling

(Kristensen, 2008). As primary consumers of mangrove litter, these crabs increase organic carbon turnover rate by shredding the leaves, which in turn hastens the consumption of estuarine detritivores (Robertson, 1986; Hogarth, 2015). In Asian countries, *Episesarma* spp. have traditional food value, but their collection remains unregulated similar to other small-scale fisheries of crabs (Ng, 1998; Ng and Sivasothi, 2001; Maynard and Oxenford, 2014).

Given the significant ecological and socioeconomic roles of *E. mederi*, investigating the reproductive biology of this mangrove crab species is a preliminary step to understand its population dynamics and establish a management plan (Nicolau et al., 2012). Studies on the taxonomy (Tan and Ng, 1994; Lee et al., 2015), niche preferences (Sivasothi, 2000), and

population genetics (Supmee et al., 2012) of *Episesarma* spp. have been reported. There has been no study reported on the reproductive biology of *E. mederi*, which also holds true for its genus. Even reproductive studies on Family Sesarmidae crabs are relatively few (Kyomo, 1986; Flores et al., 2002; Lima et al., 2006; Silva et al., 2007; Ribeiro et al., 2012; Ribeiro and Bezerra, 2014), particularly on histological and morphological aspects of gonad development (Santos et al., 2009).

Gonadal development and sexual maturity in crabs are often determined using macroscopic and secondary morphological features, whereas microscopic characterisation is rarely used (de Souza and Silva, 2009; Nicolau et al., 2012). However, several studies have shown that physiological (i.e. gametogenic development) and morphological sexual maturity in some crab species are asynchronous, implying that a single technique is not always sufficient in such assessments (Castiglioni and Negreiros-Fransozo, 2006; Benetti et al., 2007).

Thus, this paper focuses on the morphology and histology of the female and male gonads of *E. mederi* to characterise the stages of its gonadal development and provide information in determining sexual maturity.

Materials and Methods

Sampling

The *E. mederi* used in this study were gathered from a mangrove area in President Roxas, Capiz, Philippines (11° 26' 34" N, 122° 55' 23" E) from February 2015 to January 2016 (Fig. 1). Species identification was verified using the key of Lee et al. (2015) based on the morphological characteristics (e.g. structures, colourations) of *Episesarma* spp. The most distinct features of *E. mederi* are its chelae, which have purple to violet (top half) and reddish (bottom half) colouration on the palm with whitish granules on the outer surface and white pincer tips. A minimum of 30 crabs was randomly handpicked during the first week of the months within the 1-year sampling period. The crabs were collected at night in the muddy substrate surrounding their burrows during low tide to account for the dominant nocturnal activity of sesarmids (Kyomo, 1986). Mangrove trees such as *Avicennia* spp. and *Nypa fruticans* were abundant at the sampling site, and few coconut trees were also present.

Collected crab samples were placed in perforated polystyrene boxes layered with wires to prevent overcrowding. Crabs were covered with a cloth moistened with water from the collection site during the 5-h transport to the microtechnique laboratory of the Institute of Marine Fisheries and Oceanology, University of the Philippines Visayas, Miagao, Iloilo for dissection and histological preparation.

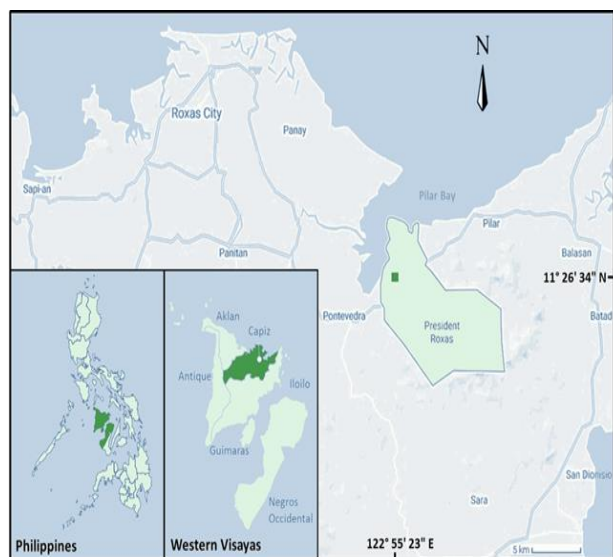


Fig. 1. Map of the sampling site for studies on gonadal development of *Episesarma mederi* in President Roxas, Capiz, Philippines.

Morphometrics and sexing

All crab samples were measured and sexed. Crabs were cold-anaesthetised at 4 °C for about 15 min. Carapace width (CW) and carapace length (CL) were measured to the nearest 0.1 mm using a vernier calliper. The distance between the tips of the epibranchial spines was measured as CW while the distance between the posterior edges of the eye orbits and the carapace was measured as CL (Fig. 2a).

Body weights (BW) were measured using a Shimadzu ELB2000 (Kyoto, Japan) digital electronic balance with sensitivity of 0.01 g. Sexing of the crab samples was based on abdominal shape – narrow and triangular in males while broad and rounded in females. Males also have more prominent round projections (tubercles) in the dactylus (Lee et al., 2015) as shown in Figure 2b. Female crabs carrying eggs were recorded as ovigerous (Fig. 3).

Morphological description and histological analysis of the gonads

Gonad developmental stages of *E. mederi* were determined through morphological and histological examination of its ovarian and testicular tissue samples. Only anaesthetised live crabs were dissected since tissue deterioration was observed in dead specimens. The carapace was cut along the periphery using dissecting scissors. The thin black membrane covering the gonads was then carefully removed using forceps to expose the gonads. The gonads were located dorsal to the hepatopancreas. For each sex, at least 10 specimens that exhibited the same distinct gonad colour and size were tentatively grouped as prospect gonadal stages for histological verification.

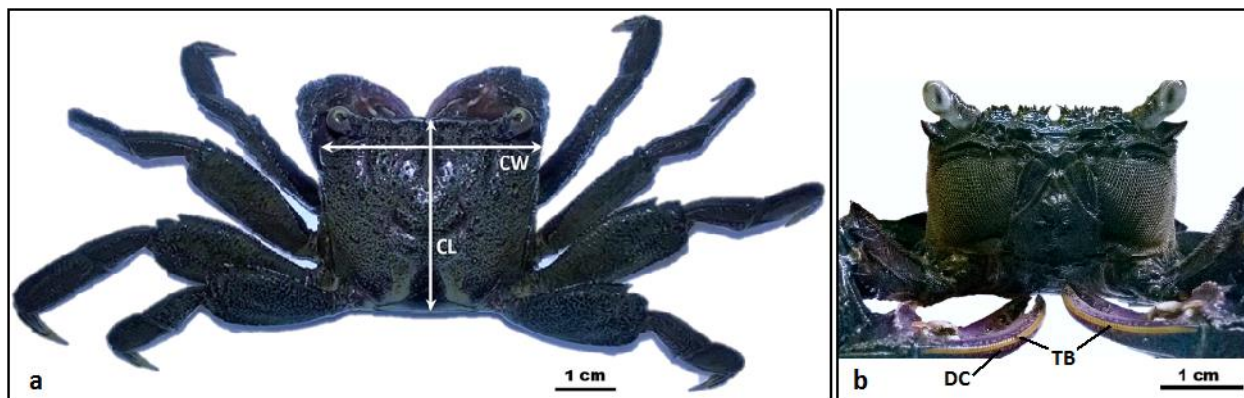


Fig. 2. Distinct physical features of *Episesarma mederi*. (a) Dorsal view showing its pointed hook-like leg tips, widely separated eyes, and squarish carapace with illustration of the carapace width (CW) and carapace length (CL) measurements; (b) Front view highlighting the location of the tubercles (TB) in each dactylus (DC).

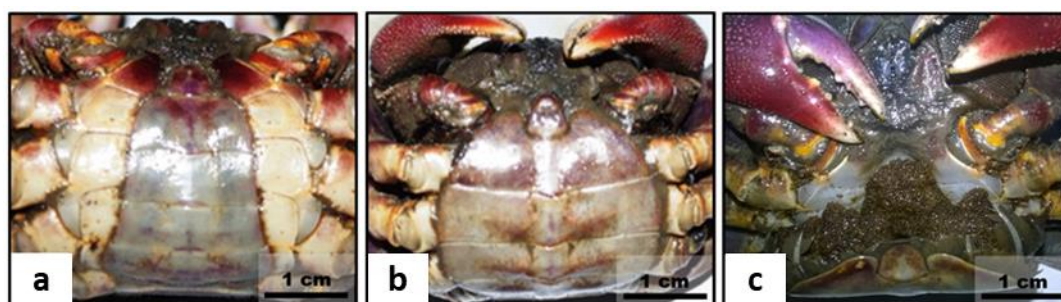


Fig. 3. Sex differentiation of *Episesarma mederi*. (a) Male with narrow, triangular abdomen; (b) Female with broad, rounded abdomen; (c) Ovigerous female with eggs attached to the hairs underneath its abdomen.

Extracted gonads were fixed in Bouin's solution for 1 h and then stored in 70 % ethyl alcohol. Standard histological processing was performed based on Humason (1972). Embedded gonad tissues were transversely sectioned (5 μ m) using a microtome and stained with haematoxylin and eosin.

Histological sections of the gonads were examined to determine the appearance of germ cells at each developmental stage. Three representative samples were selected per stage, and 30 cells of every germinal cell type present were analysed. The long, short, and nucleus diameters of the germinal cells were measured using Olympus SZ61 (Tokyo, Japan) image analyser. The ovarian developmental stages were established based on the predominant female germ cells, arrangement of follicle cells, size and dye affinity of oocytes, and changes in the nucleus diameter. The testicular stages were based on the occurrence of each male germ cell and their staining reaction. The staging was compared with related literature (e.g. Santos et al., 2009; Nicolau et al., 2012; Liu et al., 2014).

Statistical analysis

Data on the morphological characteristics including BW, CL, CW, and CL-CW ratio of *E. mederi* were presented as mean and standard deviation (SD). Prior to analysis, diagnostic tests were performed to

determine the distribution and homogeneity of variance for each group by using the Shapiro-Wilk test and Levene's test, respectively. Both tests revealed that the data is suitable to be analysed using parametric tests t-test and ANOVA. Two-sample t-test was used to analyse the sexual differences relative to these morphological parameters. Differences in the measurements of these parameters relative to different stages of maturation of female and male *E. mederi* were analysed using the one-way ANOVA and two-sample t-test, respectively. Post-hoc test using the Tukey's adjustment and Tukey-Kramer adjustment, whichever was appropriate depending on the homogeneity of variance, was used to detect significant differences following the ANOVA. One-way ANOVA and post-hoc tests were also employed to describe and compare the measurements of germinal cells in different stages of development of female *E. mederi*. The level of significance was set at 0.05 threshold. All data analysis was performed using STATA 12.

Results

Body weight, size, and sex

A total of 448 *E. mederi* were collected over the 12-month study period. The samples, comprised of 264 (58.93 %) females and 184 (41.07 %) males, had a sex ratio of 1:0.70 (F:M). Sexual differences in terms of

body weight (BW) and size were analysed (Table 1). Significant differences were found in the BW, CL, and CW between females and males ($P < 0.05$). Male crabs were significantly heavier and larger than female crabs.

Table 1. Body weight (BW), carapace length (CL), carapace width (CW) of female and male *Episesarma mederi*.

	Female (n = 264)	Male (n = 184)
BW(g)	35.9 ± 10.6 ^a	50.1 ± 18.3 ^b
CL(mm)	33.6 ± 3.7 ^a	35.9 ± 5.5 ^b
CW(mm)	35.6 ± 3.7 ^a	37.9 ± 4.3 ^b

Means (± SD) with different superscript letters within the same row indicate a significant difference ($P < 0.05$).

Characterisation and staging of female gonads

The reproductive organs of female *E. mederi* were composed of symmetrically distributed pair of ovaries joined by a transversal bridge, gonoducts, and the extensions of the latter that function for storing spermatozoa – the spermatheca. These organs formed an H-shaped structure in the cephalothorax cavity.

The development of ovarian maturation in *E. mederi* was classified into five stages by complementing the observations between morphological features (i.e. colour, volume of ovaries) and histological analysis based on the presence of the most advanced oocytes as well as the average germ cell diameters of the female specimens (Tables 2–3; Fig. 4).

Table 2. Morphological and histological description of the female reproductive system in *Episesarma mederi*.

Stage	Macroscopic description	Microscopic description
I. Immature	Thin ovaries; translucent to white	Oogonia (~12 µm) and previtellogenic oocytes (~46 µm) found in germinative and maturation zones, respectively; follicle cells irregularly distributed throughout the ovaries
II. Developing/ Redeveloping	Slender and slightly larger ovaries; off-white to yellow	Fewer oogonia and previtellogenic oocytes, some of which differentiated into endogenous vitellogenic oocytes (~62 µm), especially in redeveloping ovaries; follicle cells surround the oocytes
III. Maturing	Anterior horns of ovaries cover almost half of the hepatopancreas; orange	Endogenous oocytes located in the inner region surrounded by the exogenous oocytes (~101 µm); nucleus was large with darkly stained nucleolus; follicle cells form one layer around the oocytes
IV. Mature		
IV-A. Early mature	Ovaries greatly increase in size but not enough to cover the entire hepatopancreas; brown	Nearly mature oocytes (~162 µm) with large yolk globules in the cytoplasm; nucleus decreased in size with less visible nucleolus and follicle cells
IV-B. Late mature	Ovaries reach their maximum size, covering almost the entire hepatopancreas; dark brown	Fully mature oocytes (~230 µm) with larger yolk globules; nucleus and follicle cells were hardly recognisable
V. Spent	Shrunken and flaccid ovaries; light brown	All cell types present in disarray as new germ cells developed and some mature oocytes were reabsorbed

Table 3. Average cell and nucleus diameters of female *Episesarma mederi* germinal cells.

	Oogonia	Oocyte stage				
		Previtellogenic	Endogenous vitellogenic	Exogenous vitellogenic	Nearly mature	Mature
Cell diameter (µm)	11.9 ± 0.2 ^a	45.7 ± 0.6 ^b	61.5 ± 1.0 ^c	101.0 ± 1.1 ^d	162.0 ± 2.4 ^e	230.0 ± 2.7 ^f
Nucleus diameter (µm)		20.5 ± 4.4 ^a	20.9 ± 3.5 ^a	26.1 ± 5.5 ^b	20.4 ± 4.5 ^a	

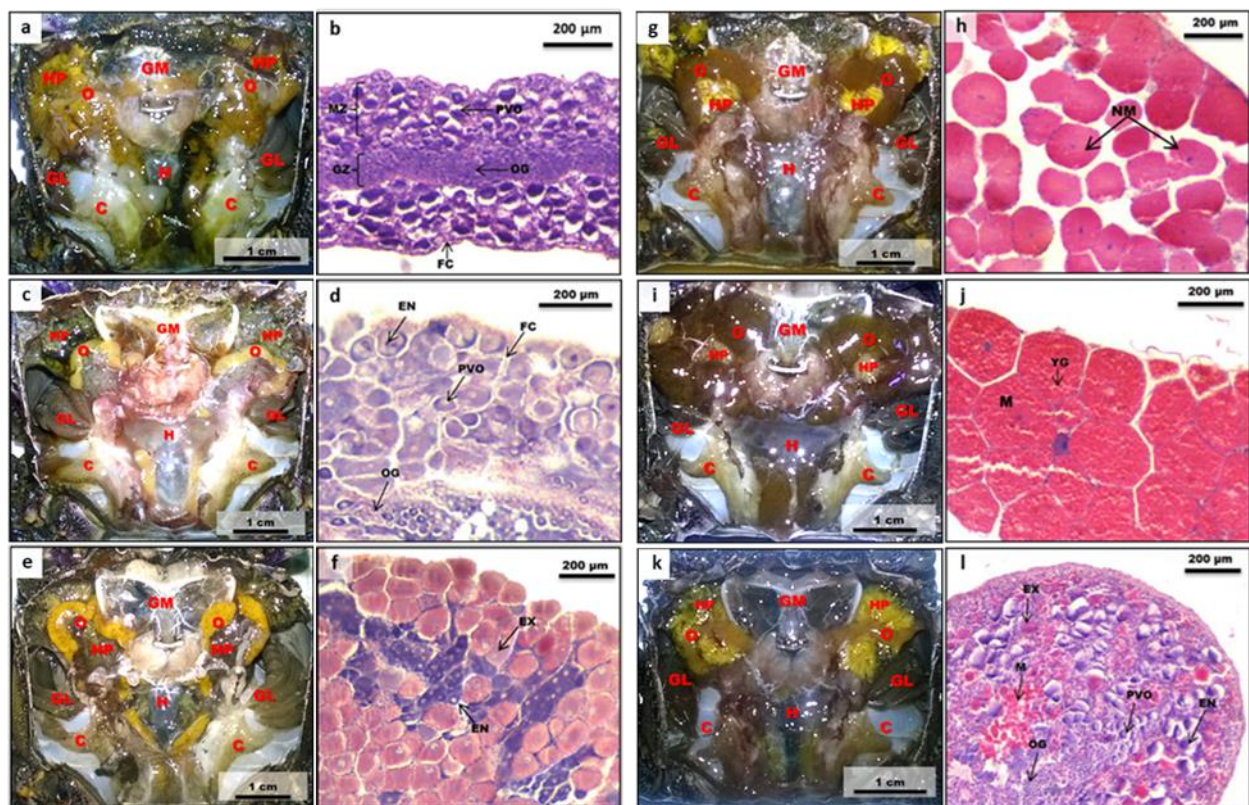


Fig. 4. Macroscopic and microscopic ovarian developmental stages of *Episesarma mederi*. In the immature stage (a, b), the ovaries were translucent to white with a layer of oogonia (OG) in the inner germinative zone (GZ), previtellogenic oocytes (PVO) in the outer maturation zone (MZ), and follicle cells (FC) were randomly distributed. In the developing/redeveloping stage (c, d), the ovaries were off-white to yellow with few OG and PVO but with differentiated endogenous vitellogenic oocytes (EN). In the maturing stage (e, f), the ovaries were orange and mainly composed of endogenous and exogenous vitellogenic oocytes (EX). In the early mature stage (g, h), the ovaries were brown and dominated by nearly mature oocytes (NM). (E). In the late mature stage (i, j), the ovaries were dark brown and made up of mostly mature oocytes (M) with large yolk globules (YG). In the spent stage (k, l), the ovaries were light brown with all cell types in disarray as gradual release and replacement of oocytes occur. GM – gastric mill, HP – hepatopancreas, O – ovary, H – heart, GL – gills, and C – cartilage.

As the ovaries develop, the mean values of oogonia and oocyte diameters were found to be significantly different ($P < 0.05$) among each other. There was an increase in the average diameter of the germ cells as they progressed from the immature to the mature stage, which was confirmed via post-hoc test. The exogenous vitellogenic oocytes in the maturing ovary (Stage 3) were found to have a significantly bigger nucleus than the rest.

Stage I (Immature)

In this stage, the ovaries were translucent to white, very thin, and flaccid in appearance extending up to the first abdominal segment in reference to the segments found on the ventral side of the crab. Two distinct regions can be observed under the microscope: the germinative zone and the maturation zone. The former was the inner region composed of oogonia (~12 μm) with scattered follicle cells. The latter was found in the outer region dominated by the more mature previtellogenic oocytes (~46 μm) with disorganised follicle cells. The oogonia, small spherical germ cells with minute amounts of cytoplasm and nucleus, were hardly recognisable. The

previtellogenic cells with irregular shape had more cytoplasm and conspicuous nuclei compared to that of the oogonia. Follicles cells were flattened in shape with barely distinguishable cytoplasm and nucleus. All cell types at this stage had a basophilic cytoplasm and were bluish.

Stage II (Developing/Redeveloping)

The ovaries became off-white to yellow and thicker compared to the previous stage. The gonads extended up to the second abdominal segment. Previtellogenic cells were found in between the inner region composed of few oogonia and the periphery of the ovarian lobe where endogenous oocytes were found. Endogenous vitellogenic oocytes (62 μm) were larger than previtellogenic oocytes. They had a large nucleus with a darkly stained nucleolus and cytoplasm that was slightly acidophilic and light pink. Follicle cells were randomly distributed around the oocytes. This stage also included the redeveloping females that had either fully released (i.e. extruded to the abdomen for incubation) or reabsorbed their mature oocytes at Stage V and had already undergone complete reorganisation of the ovary.

Stage III (Maturing)

The ovaries were orange and larger than the previous stage. The anterior horns of the ovary covered almost half of the hepatopancreas. The newly matured exogenous vitellogenic oocytes dominated the ovaries, and the germinative zone disappeared. A new batch of endogenous vitellogenic oocytes matured in the inner region, which had the size and appearance typical for its germ cell stage. Exogenous vitellogenic oocytes were larger (~101 µm). These cells had a larger nucleus (~26 µm) with a darkly stained nucleolus and a highly acidophilic cytoplasm. The cytoplasm appeared granulated because of the accumulation of small yolk globules. The follicle cells were flattened in shape and arranged in one layer around the oocytes.

Stage IV (Mature)

This stage was divided into two substages.

Stage IV-A (Early mature)

In this substage, the ovaries turned brown and noticeably increased in size. The anterior horns of the ovaries were larger but not enough to cover the entire hepatopancreas. The oocytes were bigger (~162 µm) and medium-sized yolk globules accumulated in the cytoplasm, making them acidophilic. The nucleus became smaller (~20 µm) compared to the previous stage and was darkly stained. The nucleolus was no longer visible. Only a few follicle cells in a thin layer were found to surround the oocytes.

Stage IV-B (Late mature)

The ovaries were dark brown and had reached their largest volume. The anterior horns of the ovaries occupied most of the thoracic cavity, almost covering the entire hepatopancreas. The majority of the oocytes were fully mature and at their maximum size (~230 µm). Large yolk globules occupied the entire cytoplasm, making them strongly acidophilic exhibiting a dark pink colouration. Follicle cells were still arranged as very thin layers around the oocytes.

Stage V (Spent)

The ovaries turned light brown and reduced in size similar to that of the developing (Stage II) but were more flaccid in appearance. The change in colour and size of the ovaries can be attributed to the gradual release of the mature oocytes during spawning. Generally, all types of ovarian germ cell were present and distributed in disarray as new ones replaced the released mature oocytes. Oogonia first reappeared and formed several germinative zones that were scattered over the entire ovarian lobe together with postovulatory follicle cells. The presence of mature oocytes that were not yet extruded at this stage suggests that the mature oocytes were only partially spawned, and several batches of eggs can be laid by

E. mederi. Some of these non-released oocytes underwent atresia and were reabsorbed.

Characterisation and staging of male gonads

The reproductive system of the male *E. mederi* was composed of a paired testis bridged by a commissure, vas deferens, and ejaculatory ducts, which were located in the cephalothoracic area near the hepatopancreas. The location and H-shaped orientation of the organs resembled that of the female counterpart. The tubular testes were located at the posterior portion of the hepatopancreas and were hardly visible. The vas deferens passed over the intestine and connected to the ejaculatory duct of the gonopod.

Two verifiable stages of male gonad maturation were identified: Immature (Stage I) and Mature (Stage II). The preliminary identification of the stages of the male gonads was based on the macroscopic observation of the size and colour of the testes and vas deferens. The staging was further confirmed by the microscopic analysis of the gonads by assessing the prevalence of each germ cell type (Table 4; Fig. 5)

Stage I (Immature)

The immature testes were translucent white and difficult to locate compared to the distinct and whitish vas deferens. Histological analysis revealed that spermatogonia and spermatocytes dominated the gonads, although few other advanced germ cells can be observed in some samples. Spermatogonia were larger compared to the spermatocytes and were distributed in a germinative zone surrounded by the more advanced germ cells. Spermatogonia appeared lightly stained and had large nuclei that were clear due to the peripheral distribution of heterochromatin. The primary and secondary spermatocytes were indistinguishable. These cells had smaller nuclei and darker basophilic staining than the spermatogonia.

Stage II (Mature)

In this stage, the testes turned creamy white, and the vas deferens became more prominent in colour and size. The germ cells were mainly in the later stages of spermatogenesis. Spermatozoa were the dominant cell types, followed by spermatids that tend to compress in testicular cysts. Few spermatogonia and spermatocytes can also be observed in such clusters while undergoing gametogenic development. Spermatids were smaller than spermatocytes and more darkly stained. During spermiogenesis, spermatids differentiated into spermatozoa that were smaller and slightly ovoid in appearance. Males at this gonad stage were observed to be present during each monthly sampling.

Table 4. Morphological and histological description of the male reproductive system in *Episesarma mederi*.

Stage	Macroscopic description	Microscopic description
I. Immature	Thin and inconspicuous testes, vas deferens slightly coiled; translucent white	All germ cell types can be found but dominated by spermatogonia and spermatocytes that were distributed in distinct germinative and maturation zones, respectively
II. Mature	Thicker testes, vas deferens highly coiled; creamy white	All germ cell types can be found, but the testes were mainly composed of spermatozoa and several spermatid groups in testicular cysts

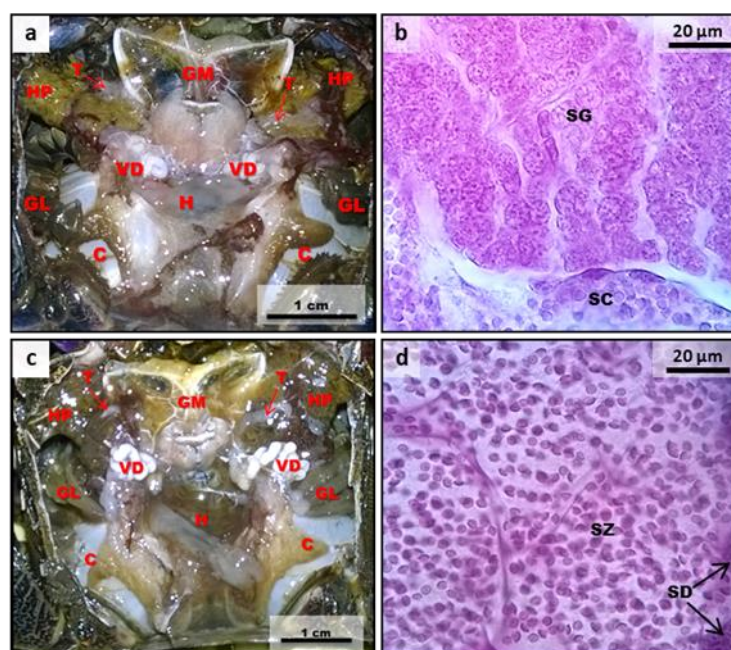


Fig. 5. Macroscopic and microscopic testicular developmental stages of *Episesarma mederi*. In the immature stage (a, b) the testes were translucent white with prevalent centralised spermatogonia (SG) in germinative zones surrounded by grouped spermatocytes (SC). In the mature stage (c, d) the testes became more visible with their creamy white colour, and the vas deferens turned brighter and larger; spermatozoa (SZ) dominated the testes with some spermatids (SD) distributed in testicular cysts and few spermatocytes and spermatogonia. GM – gastric mill, HP – hepatopancreas, T – testes, VD – vas deferens, H – heart, GL – gills, and C – carapace.

Size distribution and morphometric measurements at different gonadal stages

The size of female crabs ranged from 25.6–48.1 mm CW. The smallest sexually mature female based on its gonad morphology and physiology had 28.6 mm CW, whereas the smallest ovigerous female collected had 29.0 mm CW. Table 5 shows that CW was significantly different in female *E. mederi* at different stages of maturation ($P < 0.05$). A general trend of increment can be observed in the BW of female crabs from immature stage to spent stage. This was further analysed using the post-hoc test. A significant increase in the BW of females in the immature stage to females in the spent stage was also shown with a

mean increase of 8.6 ± 0.4 g (mean $\pm$ SD). Furthermore, a pattern of increase in the CW can be observed from immature to maturing stage that was confirmed in the post-hoc analysis, showing a mean increase of 4.6 ± 1.3 mm (mean $\pm$ SD).

Male crabs ranged from 26.0–47.0 mm CW. The smallest sexually mature male based on the same morphological and physiological criteria had 26.0 mm CW. BW and CL parameters were significantly different in male *E. mederi* between the two stages of maturation ($P < 0.05$) as seen in Table 6. The BW of mature males significantly increased by 17.5 ± 5.7 g (mean $\pm$ SD) compared to immature males. The increase in the CW of 5.7 ± 1.0 mm (mean $\pm$ SD) was also significant between the two stages.

Table 5. Body weight (BW), carapace length (CL), and carapace width (CW) of female *Episesarma mederi* at different stages of ovarian maturation (n = 232).

	Stages of ovarian maturation				
	Immature	Developing/ Redeveloping	Maturing	Mature	Spent
BW(g)	31.1 ± 11.8 ^a	34.3 ± 8.5 ^{ab}	37.4 ± 11.9 ^{ab}	37.1 ± 10.2 ^{ab}	39.7 ± 12.2 ^b
CL(mm)	32.2 ± 4.4 ^a	32.8 ± 3.6 ^a	35.1 ± 3.5 ^b	34.0 ± 3.4 ^a	33.8 ± 4.4 ^a
CW(mm)	32.5 ± 5.1 ^a	34.7 ± 3.4 ^{ab}	37.1 ± 3.8 ^c	36.0 ± 3.2 ^{bc}	36.5 ± 3.7 ^{bc}

Means(± SD) with at least one same superscript letter within the same row are not significantly different ($P < 0.05$).

Table 6. Body weight (BW), carapace length (CL), and carapace width (CW) of male *Episesarma mederi* at different stages of testicular maturation (n = 162).

	Stages of testicular maturation	
	Immature	Mature
BW(g)	33.9 ± 12.6 ^a	51.4 ± 18.3 ^b
CL(mm)	33.0 ± 7.9 ^a	35.9 ± 5.8 ^a
CW(mm)	34.2 ± 4.2 ^a	38.0 ± 4.3 ^b

Means(± SD) with the same superscript letter within the same row are not significantly different ($P < 0.05$).

Discussion

Body weight, size, and sex

In this study, *E. mederi* males were significantly larger and heavier than females. This specific trend of sexual dimorphism has also been observed in other sesarmids (Leme, 2002; Silva et al., 2007; Ribeiro et al., 2012), mangrove crabs (Benetti et al., 2007), and brachyurans in general (Castiglioni et al., 2011). Female crabs have lesser somatic growth than males because they allocate greater energy toward reproduction (i.e. gonad development) since oocytes are more energy demanding to produce compared to spermatocytes (Silva et al., 2007). Moreover, females must spend additional energy to incubate the eggs, whereas males can mainly focus on somatic growth to increase mating potential (Bezerra and Matthews-Cascon, 2007; Castiglioni et al., 2011).

The sex ratio 1:0.70 showed more females than males in the studied *E. mederi* population in President Roxas, Capiz, Philippines. This deviation from the evolutionarily stable proportion of 1:1 is normally observed in crustaceans (Silva et al., 2007). It is typically attributed to the varying natural population parameters (e.g. growth, mortality) between sexes, as well as sampling strategies (Ribeiro et al., 2012). Larger members of the population under *Episesarma* spp. tend to climb trees that synchronise with tidal movement (Sivasothi, 2000). This isophasic or mobile behaviour could have affected the observed sex ratio. Since the larger males have the tendency to stay in the treetops, they were probably less accessible on

their common fishing grounds, which also served as the collection zone of this study

Characterisation and staging of female gonads

Macroscopic appearance (i.e. colour, size) of the *E. mederi* ovaries in this study showed differentiation as development progressed from initial to advanced stages. The change from lighter coloured ovaries in earlier stages of development to darker ovaries in the advanced stages of *E. mederi* has been similarly reported in other brachyuran species, such as the red-clawed mangrove tree crab *Goniopsis cruentata* (Latreille, 1803) (de Souza and Silva, 2009), fiddler crab *Uca (Minuca) rapax* (Smith, 1870) (Castiglioni et al., 2007), mangrove crabs *Scylla serrata* (Forskål, 1775) (Quinitio et al., 2007) and *Scylla paramamosain* Estampador, 1950 (Silva et al., 2012), marine crabs *Portunus sanguinolentus* (Herbst, 1783) (Sukumaran and Neekalantan, 1998) and *Portunus pelagicus* (Linnaeus, 1758) (Liu et al., 2014), and red frog crab *Ranina ranina* (Linnaeus, 1758) (Minagawa et al., 1993; Baylon and Tito, 2012). This colour variation of the ovaries is associated with the accumulation of yolk globules in the cytoplasm of the oocytes during vitellogenesis, wherein high amounts of the yolk are concentrated in the middle of the oocyte and are typical for arthropods (Quinitio et al., 2007). The lipoproteins in the yolk globules contain carotenoids (Liu et al., 2014), which include b-carotene, astaxanthin, and other unidentified carotenoids that cause their yellow or orange colouration (Adiyodi and Subramoniam, 1983; Lee and Puppione, 1988).

Carotenoid pigments were reported to be important in the protection of embryo against solar radiation (Adiyodi and Subramoniam, 1983).

Description and establishment of the five ovarian development stages in *E. mederi* was mainly based on the histological analyses of the mangrove tree crab *Aratus pisonii* (H. Milne Edwards, 1837) (Nicolau et al., 2012), the sesarmid *Armases rubripes* (Rathbun, 1897) (Santos et al., 2009), and on the blue swimming crab, *P. pelagicus* (Liu et al., 2014). Developmental stages of other related crab species were also compared with the present observation on *E. mederi*. These data were then complemented with the measurements of the germ cells in the gonads to establish the maturation stages.

The immature stage (Stage I) in *E. mederi* had a germinative zone composed of oogonia and a maturation zone comprised of previtellogenic oocytes. This finding is similar to what have been reported in other crab species such as *A. pisonii* (Nicolau et al., 2012), *S. serrata* (Quinitio et al., 2007), *Scylla olivacea* (Herbst, 1796) (Islam et al., 2010), and *P. pelagicus* (Liu et al., 2014). In other studies, these previtellogenic oocytes were referred to as primary oocytes (Quinitio et al., 2007) or 'oocytes in early development' (Nicolau et al., 2012) for distinction purposes.

The developing/redeveloping stage (Stage II) was characterised by fewer oogonia but with numerous oocytes in previtellogenic and endogenous vitellogenic stages. The previtellogenic oocytes had large nuclei, implying that the germ cells in this stage were actively undergoing vitellogenesis. This led to the differentiation of previtellogenic oocytes into endogenous vitellogenic oocytes, which had a distinct colourless eosinophilic component in their cytoplasm. This component contains proteins and lipids that are used as basic structural materials in the synthesis of more tissues (Adiyodi and Subramoniam, 1983). This differentiation into endogenous vitellogenic oocytes started at the periphery of the ovarian lobe. The presence of oogonia, previtellogenic oocytes, and endogenous vitellogenic oocytes in Stage II is in agreement with the staging of Liu et al. (2014) on the portunid *P. pelagicus* and Santos et al. (2009) on the sesarmid *A. rubripes*.

It is important to take note that several Stage II females in this study were found to be ovigerous, which corresponds to the redeveloping post-spawned females. Pinheiro and Fransozo (2002) also reported the occurrence of ovigerous females with developing gonad stage in the speckled swimming crab *Arenaeus cribrarius* (Lamarck, 1818). Interestingly, they found that the ovigerous females predominantly possessed developing gonads (77.6 %) instead of mature gonads (22.4 %). In the said study, no ovigerous female possessed an immature ovary, which was logical since redeveloping post-spawned females were

already sexually mature. Moreover, all ovigerous females identified in this present study had a rounded abdomen, which supports the justification that this morphological modification is essential for egg incubation and can be an indicator of sexual maturity in these crab species (de Souza and Silva, 2009).

The redeveloping post-spawned *E. mederi* females were placed together with the developing juvenile females in Stage II of this study because no distinct histological differences were observed between the two subgroups. Moreover, the areas previously occupied by the released and/or resorbed mature oocytes during spawning were replaced by previtellogenic and endogenous oocytes, as well as few newly formed oogonia. This finding is similar to that of post-spawning females of the mangrove tree crab *A. pisonii* (Nicolau et al., 2012).

The maturing stage (Stage III) was microscopically characterised by the dominance of both endogenous and exogenous vitellogenic oocytes. This finding is comparable to that of the early vitellogenic stage of de Souza and Silva (2009) on the mangrove tree crab *G. cruentata*. They reported that vitellogenesis of the oocyte is divided into two successive phases: (a) primary vitellogenesis, which is characterised by endogenous protein yolk accumulation in the cytoplasm of the oocyte, and (b) secondary vitellogenesis, which is characterised by cells that incorporate extracellular protein substances in their cytoplasm through pinocytosis. This is comparable to the vitellogenesis substage under Stage II of *A. rubripes* (Santos et al., 2009) and Stage III of *P. pelagicus* (Liu et al., 2014).

The mature stage (Stage IV) in this study was divided into two substages: early mature (Stage IV-A) and late mature (Stage IV-B). The early mature stage was characterised by a light brown ovary that was predominantly composed of nearly mature oocytes (~162 μ m) with smaller yolk globules in their cytoplasm. On the other hand, the late mature stage had dark brown ovaries containing mature oocytes with larger and more acidophilic cytoplasmic yolk globules (Nicolau et al., 2012). This proposed staging conforms to the previous studies on the sesarmid *A. rubripes* (Santos et al., 2009), mangrove tree crab species *G. cruentata* (de Souza and Silva, 2009), and *A. pisonii* (Nicolau et al., 2012), which used a single stage for the mature ovarian phase dominated by late-stage vitellogenic and fully mature oocytes. However, the substaging was deemed necessary in this paper to account for the previously mentioned distinct macroscopic and microscopic differences on the ovaries at this stage. These substages are equivalent to the separate late maturing and fully maturing ovarian stages of *S. serrata* (Quinitio et al., 2007) and the Stage IV and Stage V ovarian stages of *P. pelagicus* (Liu et al., 2014).

The spent stage (Stage V) was characterised by ovaries composed of oocytes at various stages of development. The presence of few fully mature oocytes together with cells that were in the early stages of oogenesis indicates that some mature oocytes had already been released for spawning. While spawning was taking place, the ovary was also simultaneously undergoing redevelopment. Proof of this is the presence of numerous oogonia undergoing mitosis. The histological characteristic of the ovaries at this stage is similar to the spent stage identified by Quintio et al. (2007) on the mangrove crab *S. serrata*, and the post-spawning (Stage IV) identified by de Souza and Silva (2009) on the red-clawed mangrove tree crab *G. cruentata*.

Characterisation and staging of male gonads

The male reproductive stages in *E. mederi* were established by complementing macroscopic and histological characteristics of the testes. The immature (Stage I) testes were initially translucent, which suggests the absence of spermatogenesis. The vas deferens, on the other hand, possessed a distinct whitish colouration indicating the considerable presence of seminal fluid needed in the encapsulation of spermatozoa into a spermatophore. During the immature to mature stages of development, the colour of the testes and vas deferens of *E. mederi* ranged from light to dark (i.e. translucent to milky white). This distinctive change signifies the increase of various glycosaminoglycans (GAGs) necessary in the maturation of male gametes in the testes and the processing of spermatozoa into spermatophores in the vas deferens (Santos et al., 2009; Nicolau et al., 2012; Ganapiriyar et al., 2017).

Histological analysis revealed variations in the staging of testicular maturation across literature due to the diversity of reproductive patterns among crustaceans. It was reported that it was impossible to establish clear maturation stages based on testicular histology in the swimming crabs *Callinectes ornatus* Ordway, 1863 (Nascimento and Zara, 2013) and *C. danae* (Zara et al., 2012) since spermatogenesis occurs continuously all year round (Santos et al., 2009). Thus, the presence of the maturity markers – spermatogonia (Stage I), spermatocytes (Stage II), and spermatids and spermatozoa (Stage III), have been occurring simultaneously. While in other species such as *P. sanguinolentus* and *P. pelagicus* (Sukumaran and Neekalantan, 1998), *A. rubripes* (Lima and Oshiro, 2006), and *S. olivacea* (Islam and Kurokura, 2012), three stages were recognised that included transitional stage between the immature and mature stages. However, in this present study on *E. mederi*, only two stages for males were identifiable: immature (Stage I) and mature (Stage II). This finding agrees with another study of the sesarmid *A. rubripes* (Santos et al., 2009) and the males *R. ranina* (Baylon

and Tito, 2012) wherein two developmental stages (i.e. immature, mature) were reported. Since a defined intermediate stage was not observable in *E. mederi*, it can be hypothesised that the chromatin condensation during spermatocyte and nuclei formation in spermatids is transpiring quickly (Stewart et al., 2010).

Size distribution and morphometric measurements at different gonadal stages

There were differences in the morphometric parameters on the females (Table 4) and males (Table 5) of *E. mederi* in this study. These variations became significantly different as individuals entered the maturing and/or mature stages. Individually, females seemed to reach sexual maturity at a larger size (28.6 mm CW) than males (26.0 mm CW) based on their smallest sexually mature representatives. Female crabs are said to attain sexual maturity later than males because they must first develop larger abdomens to provide greater support and ensure moisture for their eggs during incubation (Masunari and Dubiaski-Silva, 1998). However, if we compare the mean morphometric values of immature and mature stages of both sexes, males were in fact heavier and larger than females. This observation coincides with the computed CW50 (the size at which 50 % of the individuals became sexually mature) for this *E. mederi* population, which is 32.3 mm for females and 37.5 mm for males (Leonida et al., unpublished data). Likewise, smaller size of sexual maturity in females was reported in the sesarmid mangrove crab *Sesarma rectum* Randall, 1840 (Ribeiro et al., 2013). This trend can be attributed to the greater energy expenditure of females toward reproduction compared to somatic growth (Silva et al., 2007; Ribeiro et al., 2012). Differences in size at first sexual maturity between sexes may also be due to the dependency of growth rates on seasons, as well as on geographical locations and sampling strategies (Sara et al., 2002; Ribeiro et al., 2012).

Reproductive pattern and strategies

The presence of the spermatheca in some female decapods allows the storage of spermatozoa that can be used for several spawnings (Adiyodi and Subramoniam, 1983; de Souza and Silva, 2009). The results of this study suggest that *E. mederi* females were also capable of multiple ovipositions or egg-laying. This inference is supported by the occurrence of post-spawning ovigerous females with a Stage II ovarian development that gradually transitioned from the gametogenic reorganisation in Stage V. In fact, the caged experiment of Kyomo (1986) documented at least three spawning events of the sesarmid *Sesarma intermedia* after a single mating, with a 30-d incubation and 7-d oviposition interval per egg mass. This continuous reproduction is apparently possible due to the regular supply of spermatozoa that fertilise the oocytes. Multiple ovipositions increase

reproductive success (Kyomo, 1986), and continuous reproduction strategy is common among tropical crustaceans due to the low-temperature variability in the tropics (Pinheiro and Fransozo, 2002). Other studies also reported the continuous presence of ovigerous females throughout the year in the sesarmids *A. rubripes* (Lima et al., 2006) and *S. rectum* (Silva et al., 2007).

In this study, the presence of mature *E. mederi* males during each monthly sampling further affirms a continuous reproductive strategy in this species. This agrees with the constant gamete production observed by Santos et al. (2009) in the males of the sesarmid *A. rubripes*. In the mangrove tree crab *A. pisonii*, male gametes were also continuously produced, which also suggests that these crabs are capable of year-round reproduction (Nicolau et al., 2012).

Conclusion

The complementary morphometric and histological analyses performed on *E. mederi* indicated a synchronised morphological and gametogenic onset of sexual maturity in this species. Five ovarian stages and two testicular stages were identified. Males were generally larger and heavier since females allocate greater energy toward reproduction. The smallest recorded sexually mature individuals had 28.6 mm CW for females and 26.0 mm CW in males. The gonadal development of *E. mederi* suggests a continuous reproductive strategy, which is supported by the presence of post-spawning ovigerous females with redeveloping ovaries and year-round occurrence of mature males. This is the first study conducted on the reproductive aspects of this tree-climbing mangrove crab species. The preliminary information from this investigation is a significant contribution to the reproductive biology of *E. mederi* in the Philippines and will be essential for future management and population dynamics studies of this species.

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