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Artificial Insemination and Early Embryonic Development of the Mangrove Crab *Perisesarma bidens* (De Haan) (Crustacea: Brachyura)

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Md. Moniruzzaman Sarker, Md. Sirajul Islam, and Tsuyoshi Uehara (2009) Artificial insemination and early embryonic development of the mangrove crab *Perisesarma bidens* (De Haan) (Crustacea: Brachyura). *Zoological Studies* **48**(5): 607-618. The aim of this study was to explore a technique of artificial insemination and document the early embryonic development of the mangrove sesarmid crab *Perisesarma bidens* (De Haan). Observations were made on fertilization of the eggs of this crab by artificial insemination up to hatching under laboratory conditions. The female extruded the eggs into the abdominal cavity 24-48 h after copulation. Unfertilized eggs were collected from the pleopods of the female immediately after laying and were stored in 80% filtered sea water (FSW). Sperm were removed from the spermatheca of the same female and diluted in 80% FSW. The unfertilized eggs and sperm were shaken well in a glass beaker for artificial insemination. The eggs were rinsed 3-5 times with 80% FSW after 5 min of mixing of sperm with ova. Eggs were incubated in 100 ml flat cylindrical culture bottles containing 70 ml of 80% FSW at 25°C in a water bath. The fertilization membrane was observed 3-5 min after insemination. The other 2 outer layers of fertilized eggs were also observed 8-10 min after insemination. The internal yolk of the egg is the major source of nutrition for developing embryos. An average of 65% of fertilized eggs hatched as 1st zoeae after 17 d of incubation. It is therefore feasible to produce embryos by artificial insemination during the peak breeding season from May to Sept. This is the first report of early embryonic development from fertilization to larval hatching of *P. bidens* by artificial insemination. http://zoolstud.sinica.edu.tw/Journals/48.5/607.pdf

Key words: *Perisesarma bidens*, Artificial insemination, Cleavage, Embryonic development, Zoeae.

There are few studies on the reproduction and embryology of brachyuran crabs (Garcia-Guerrero and Hendrickx 2004), whereas other groups of decapod crustaceans have received much attention, and their embryology is better documented. Studies on the embryology of brachyuran crabs are recent and have included only a few species from different habitats, such as the hair crab *Erimacrus isenbeckii* (Atelecyclidae) by Nagao et al. (1999); the estuarine or mud crabs *Chasmagnathus granulata* and *Cytograpsus angulatus* (Varunidae) by Bas and Spivak (2000); the fiddler crab or mangrove ghost crab *Uca lacteal* (Ocididae) by Yamaguchi (2001); the mangrove crab *Ucides cordatus* (Ocypodidae) by Pinheiro and Hattori (2003); *Goniopsis pulchra* (Grapsidae) and *Aratus pisonii* (Sesarmidae) by Garcia-Guerrero and Hendrickx (2004); the green shore crab *Carcinus maenas* (Portunidae) by Chung and Webster (2004); the intertidal crabs *Hemigrapsus edwardsii* and *H. crenulatus* (Grapsidae) by Taylor and Seneviratna (2005) and Seneviratna and Taylor (2006); the blue crab *Callinectes sapidus* by Walker et al. (2006); and the blue king crab *Paralithodes platypus* by Stevens (2006).

On the other hand, studies on artificial

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insemination and early embryonic development of a dominant mangrove crab *Perisesarma bidens*, as well as most other crabs are almost completely lacking to date. Until now, only Lee and Yamazaki (1989) provided some information on artificial insemination of the freshwater Chinese crab, *Eriocheir sinensis*. The semiterrestrial sesarmine crab *P. bidens* (De Haan) is widely distributed in Indo-West Pacific mangrove regions, being known from the Bay of Bengal to the Andaman Is., Sri Lanka, India, the Malay Archipelago, the Philippines, Hong Kong, Taiwan, Korea, and Tokyo Bay to Kyushu and the Ryukyu Is. of Japan (Sakai 1976, Dai and Yang 1991, Islam and Shokita 2002). The complete larval development of *P. bidens* has been described in detail (Islam and Shokita 2000), although they did not investigate the early embryonic development nor the artificial insemination of this species. The embryology of brachyuran crabs has been examined haphazardly throughout the world by a few researchers, but no standard exists for defining the developmental stages (Stevens 2006).

Whether fertilization in crabs occurs internally or externally is controversial, and the possibility of artificial insemination also remains in doubt. However, Lee and Yamazaki (1990) observed the presence of a valve-like tissue in the reproductive organ of crabs. Eggs that are released from the ovaries are all unfertilized which is of great importance for obtaining unfertilized mature eggs for artificial insemination. This finding inspired us to study artificial insemination and early embryonic development of *P. bidens* which is of great importance to crab fisheries for artificial seed production, the biological balance in mangrove ecosystems, and conservation of species diversity and its management.

MATERIALS AND METHODS

Collection of animals and rearing

Crabs in nature exhibiting courtship behaviors were selected as being reproductively active, and such active male and female crabs were captured by hand from the Manko mangrove swamp, Okinawa I., Japan at low tide during their spawning season from May to Sept. in both 2005 and 2006. Collected crabs were immediately transported to the laboratory and reared as pairs (1 male + 1 female) in plastic containers (30 x 20 x 20 cm) filled with 80% filtered seawater (FSW) maintained with constant gentle aeration, and provided with some rough beach stones as shelter. A 12 h dark: 12 h light photoperiod was maintained. The water was changed daily until spawning or oviposition and brown leaves of *Kandelia ovovata* were offered as food. The temperature of the rearing water was maintained at 25°C.

Mating and copulation

Mature crab pairs were selected following Sarker and Tsuchiya (2007), with carapace length (CL)/carapace width (CW) ranging 15.5-24.8/17.1-26.6 mm for males, and 11.7-20.7/12.6-22.0 mm for females. Mating behavior was observed in a plastic container (30 x 20 x 20 cm) at noon every day in the laboratory. When copulation occurred, the duration of copulation and the stances adopted by the male and female during copulation were recorded.

Artificial insemination

Immediately after copulation, the male and female were separated, and the female was checked at 2-3 h intervals until it began laying eggs. Unfertilized eggs were collected from the pleopods of the abdomen immediately after laying and were kept in 80% FSW. At the same time, sperm were removed from the spermatheca of the same female. The collected sperm were diluted in 80% FSW for insemination.

Unfertilized eggs and sperm were combined and shaken well in a 250 ml glass beaker containing 80% FSW for artificial insemination. Sperm were allowed to remain attached to the eggs for 3-5 min; excess sperm were removed by 3 consecutive washes with 80% FSW. These eggs were transferred to 100 ml flat-bottom cylindrical culture bottles containing 70 ml of 80% FSW at 25°C in temperature-controlled water baths (Thermo Minder SJ-10R, TAITEC Co., Ltd., Saitama, Japan). The maximum density of zygotes in each bottle was 6/ml. The water in each bottle was changed daily until hatching.

Incubation period

In this study, incubation refers to the time (in days) from the extrusion of eggs to the hatching of larvae.

Video observation and photography

Cleavage and development

After insemination, eggs were taken from the culture bottle and continuously examined until cleavage began and then at 2-6 h intervals until hatching using a light microscope (Nikon M-B, 291, Japan) equipped with a digital camera. The developmental stages of the embryos are reported in days after microscopic analysis with this scheme. For video observations, 10 eggs were placed on a glass slide, washed with seawater, and surrounded by a Vaseline ring. A cover slip (22 mm square) was pressed onto the Vaseline ring until contact was made with the eggs. The first 10 eggs encountered were classified as "fertilized" if they had reached the 2-4 cell stage of development. Measurements of living embryos and distinct developmental stages, and larval release from the eggs were monitored using a video camera on a TV monitor connected to a time-lapse video set to record for 48 h for each 120 VHS tape with an ocular micrometer. Changes in embryonic development were monitored and recorded using a microscope. The room was kept at 25°C, and a 12 h dark: 12 h light photoperiod was maintained. Some unfertilized eggs were kept in 80% FSW as controls to compare with the inseminated eggs. After 14 d of incubation, developing embryos were dissected from the egg membrane, and organs which could be discerned were described.

Hatching and release of larvae

Before hatching, many chromatophores were visible in developing embryos. The egg-case was transparent, and pigment cells were easily distinguishable under a light microscope. As hatching approached, the developing embryos frequently moved their abdomen and appendages. The eggs were somewhat oval-shaped and were tightly squeezed in the egg membrane. At this time, the embryos were not freely moving inside the egg. All processes and breakage of the egg membranes were observed under the light microscope with the video camera.

Hatching occurred within 17 d of incubation. From newly hatched larvae, the most photopositive zoeae were mass-reared under laboratory conditions using 20% FSW at 25°C in gently aerated 10 L plastic bowls (5 bowls, each with about 200 larvae). Larval release of selected cultured eggs was videotaped at close range and real-time speed to precisely record the event. Zoeae were fed daily with newly hatched nauplii of *Artemia* sp. (at about 12-15 nauplii/ml).

RESULTS

Copulation and egg-laying

Copulation was initiated by the active behavior of males. Males approached females using various types of precopulatory activities. Soon after a male had grasped and mounted a female, copulation began. Copulation continued for 0.25-4 h. The females extruded unfertilized, mature eggs 24-48 h after copulation. The eggs were immediately attached to the non-plumose setae of the endopodites of the female abdomen through the stalk or funiculus 5 min after laying and then were encased in an investment coat.

Sperm and their motility

Normal spermatozoa, collected from the spermatheca of a female which had copulated, appeared uniformly spherical in shape, measuring 15-20 μm in diameter, with several slender radiating nuclear arms and a prominent acrosome overlying the nuclear mass (Figs. 1A, B). Semen that was examined after collection from the spermatheca contained non-motile spermatozoa. When mixed with whole eggs, sperm motility was restored. The site of motility of the sperm was specifically in the polar body region.

Unfertilized eggs

Mature unfertilized eggs were obtained from the pleopods of the female. Newly released mature eggs were spherical and centrolecithal, and were evenly filled with yolk components; they had an average diameter of 313.05 ± 8.41 μm (Table 1). There was only 1 layer of egg membrane closely attached to the egg body. The thin distinct periplasm was homogenous throughout the egg. Neither the micropyle nor signs of egg polarity were observed immediately after egg-laying (Fig. 2A).

Eggs after insemination

Fertilization

Fertilized eggs were macrolecithal, centrolecithal, and sphericale, and had a uniform dark olive color, without evidence of development. No polar bodies were observed in eggs examined immediately after having been laid. The fertilization membrane was first observed after 3 min of insemination, and the fertilization cone or ring-like structure was seen 5 min after insemination. These had been reabsorbed by 30 min after incubation. The 1st and 2nd polar body-like structures ware extruded from the zygote 10-15 and 20-25 min after insemination, respectively (Figs. 1C, D, Table 1). At that time, the yolk components were yellowish. Three distinct egg membranes were observed after 25-30 min of insemination (Fig. 1E).

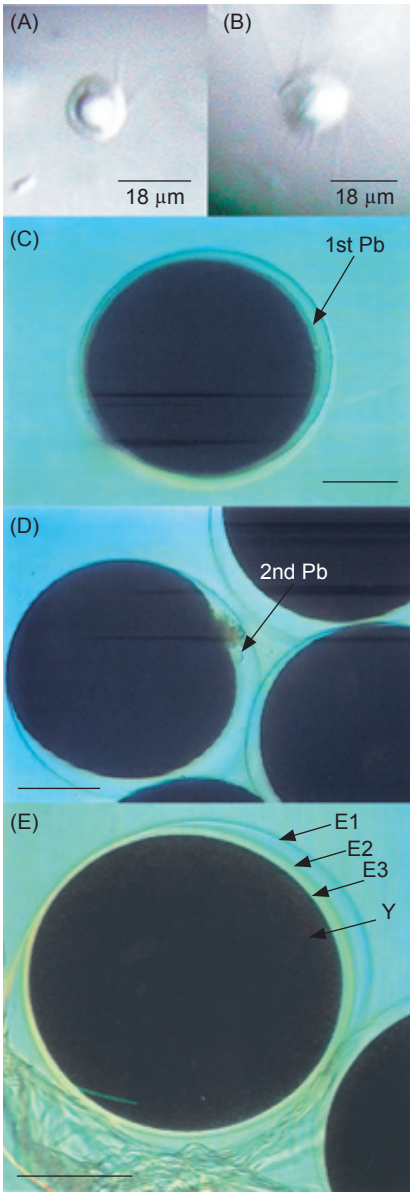

Fig. 1. (A, B) Sperm of *Perisesarma bidens*; (C) formation of the 1st polar body; (D) formation of the 2nd polar body; (E) 3 distinct layers of the fertilized egg. Pb, polar body; Y, yolk; E1, E2, and E3, 1st, 2nd, and 3rd layers of the fertilized egg, respectively.

Embryogenesis

Developmental stages, the average diameter (long axis), and incubation periods of artificially inseminated eggs are presented in table 1. Embryonic development of *P. bidens* was completed within 17 d of incubation. The chronology of embryological events at a constant room temperature of 25°C are shown in figures 2 and 3.

Cleavage

The cleavage was equal and holoblastic and was characterized by the early occurrence of a cleavage cavity.

Two-cell stage

The 1st cell division occurred within 23-24 h after insemination. At the 2 cell stage, a wide cavity or cleavage furrow had formed between the blastomeres (Figs. 2D, E). The 1st division was typical and passed through the animal vegetal pole. About 75% of eggs reached the 2 cell stage of development.

Four-cell stage

Two-cell embryos underwent the 2nd equal division of cleavage within the next 2 h of incubation, and reached the 4 cell stage producing 4 large, equal blastomeres (Fig. 2F).

Eight-cell stage

At 27-29 h of incubation, 4 cell developing embryos underwent the 3rd cleavage event and reached the 8 cell stage (Fig. 2G).

Sixteen-cell stage

By the 4th cleavage at 31-32 h of incubation, the developing 8 celled embryos reached the 16 cell stage (Fig. 2H).

Thirty-two-cell stage

The 16 cell developing embryos usually underwent the 5th cleavage within 33-35 h of incubation (Fig. 2I). Due to the large density of blastomeres, the latter stages of cleavage and blastomeres could not clearly be detected.

Blastula

At about 44-45 h after insemination, when approximately 128 blastomeres were present, the developing embryos reached the blastula stage (Figs. 2J-L). The surface of each blastomere was so granular that the internal features could not be detected using only a microscope and without applying any special technique for counting the blastomeres.

Gastrula

Two days after insemination and incubation, the developing blastula reached the early gastrula stage. The phase between blastula and gastrulation was not clearly marked but was a gradual transformation. Gastrulation took place by epiboly (Figs. 2M, N).

Tissue cap

Evidence of tissue formation was observed after 3 d of incubation (Fig. 2O). There was a cluster of presumptive primordial cells beginning to form as a patch located in the ventral position.

Table 1. Chronology of development of *Perisesarma bidens* at 25°C by artificial insemination under laboratory conditions

Time after insemination	Size in diameter (μm) (mean ± SD, *n* = 10)	Remarks and stages of development
0	313.05 ± 8.41	Insemination
3-5 min	313.15 ± 8.91	Elevation of fertilization membrane
10-15 min	313.76 ± 9.98	1st polar body
30-45 min	313.80 ± 9.81	2nd polar body
23-24 h	314.11 ± 8.57	2 cell stage
25-26 h	314.38 ± 6.97	4 cell stage
27-28.5 h	314.42 ± 9.92	8 cell stage
31-32 h	314.69 ± 5.40	16 cell stage
33-35 h	315.18 ± 4.83	32 cell stage
37-38 h	315.54 ± 9.21	64 cell stage
44-45 h	316.36 ± 7.42	Blastula stage
2 d	327.15 ± 8.91	Gastrula stage
3 d	336.76 ± 9.98	First traces of whitish portion of tissue cap
4 d	338.80 ± 9.81	Globular tissue into the white portion, tissue cap, organogenesis
5 d	341.11 ± 8.57	Yolk-free portion increases
6 d	343.38 ± 6.97	First evidence of ocular and thoracic-abdominal organs
7 d	345.21 ± 9.92	Evidence of eyes observed
8 d	348.09 ± 5.40	Eyes increase in size
9 d	351.18 ± 4.83	Heart first observed with slow jerking movements
10 d	354.24 ± 9.21	Yolk components arranged into 4 lobes
11 d	356.36 ± 7.42	Heart beating rapidly
12 d	361.90 ± 8.39	Black chromatophores, distinct eyes
13 d	373.03 ± 7.34	Ocular lobes in their final position, appendages larger and incompletely segmented
14 d	384.55 ± 9.07	Yolk components arranged into 2 lobes
15 d	388.48 ± 9.43	Orange chromatophore and yolk complete
16 d	410.17 ± 6.55	Orange and black chromatophores more distinct
17 d	987.98 ± 2.65	Hatching of zoeae

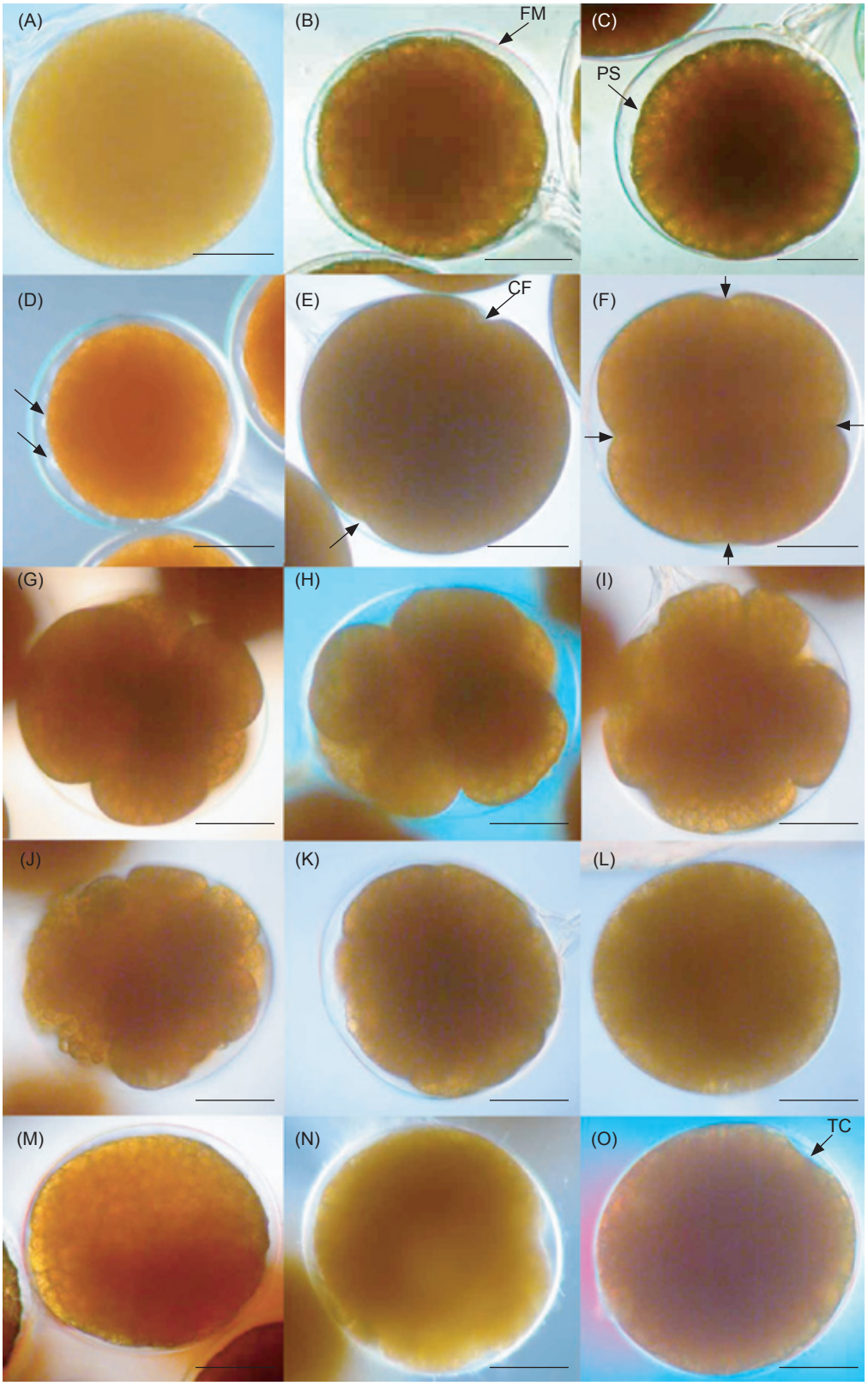

Fig. 2. Stages of development in embryos of *Perisesarma bidens* (De Haan). (A) Unfertilized egg; (B-D) fertilized eggs before cleavage; (E) 2 cell stage; (F) 4 cell stage; (G) 8 cell stage; (H) 16 cell stage; (I) 32 cell stage; (J-L) blastula; (M, N) gastrula; (O) tissue cap. FM, fertilization membrane; PS, perivitelline space; CF, cleavage furrow; TC, tissue cup. Scale bars = 100 μm.

Developing embryos

Four days of incubation

Yolk droplets were somewhat larger and had a lighter-yellowish color (Fig. 3A). The tissue cap grew larger.

Five days of incubation

Yolk droplets were larger and had a more-distinct yellowish color. The yolk-free portion had increased in size. No specific organs were observed (Fig. 3B).

Six days of incubation

A further increase in the yolk-free portion was observed. Evidence of tissue formation was more distinct and clearer in the yolk-free portion (Fig. 3C).

Seven days of incubation

The 1st evidence of organs was visible in the ventrolateral position of the yolk-free portion. The cluster of primordial cells had differentiated into major embryo structures in the ventral region of the developing egg. Ocular and cephalic appendages, and thoracic-abdominal portions of papillae were observed at this time (Fig. 3D).

Eight days of incubation

Crescent-shaped eyes first became visible (Fig. 3E). Utilization of the yolk by the developing embryos was notable, exposing the cephalothorax.

Nine days of incubation

The developing eyes were larger in size (Fig. 3F). No evidence of a heart or heartbeat was observed at this stage.

Ten days of incubation

A heart-like structure was first observed. The heartbeat was slow at about 60-78 beats per minute (bpm). The eyes were more or less complete in size (Fig. 3G).

Eleven days of incubation

Yolk components were arranged in 4 lobes. Further development of the heart and heartbeat were observed. The heart was beating at 100-110 bpm. Eye development was incomplete (Fig. 3H).

Twelve days of incubation

The eyes were more visible (Fig. 3I). Further reduction of the yolk granules was observed. The heartbeat was faster than the day before.

Thirteen days of incubation

The yolk components were arranged in 2 lobes (Fig. 3J). Chromatophores had appeared on the abdomen. The eyes had grown and were triangular.

Fourteen days of incubation

Embryos occupied 1/2 of the volume of the egg in the ventral position. The shape of the heart was complete, and the heart was beating faster (120-130 bpm). The eyes were larger, the cornea was forming, and eye pigmentation was more intense. The antennae and mandible were comparatively more developed (Fig. 3K).

Fifteen days of incubation

About 3/4 of the yolk granules had been consumed, and the remaining yolk was divided into 3 patches. Embryos had grown considerably and now occupied the entire egg volume except for the reserve yolk. The heart was in a dorsal position. Ocular lobes were in their final position, and the eyes were larger and more complex. All appendages were larger, incompletely segmented, and developing setae. The 1st evidence of the rostrum was visible (Fig. 3L).

Sixteen days of incubation

All structures of the embryos were distinct, and yolk droplets were still stored dorsal to the cephalothorax, which was entirely visible. The eyes were completely differentiated into the cornea and retina. Abdominal metamerization was complete, and each segment bore chromatophores. The heart had grown larger, and the rostrum was more distinct (Fig. 3M).

Seventeen days of incubation

Just before hatching, the embryo occupied

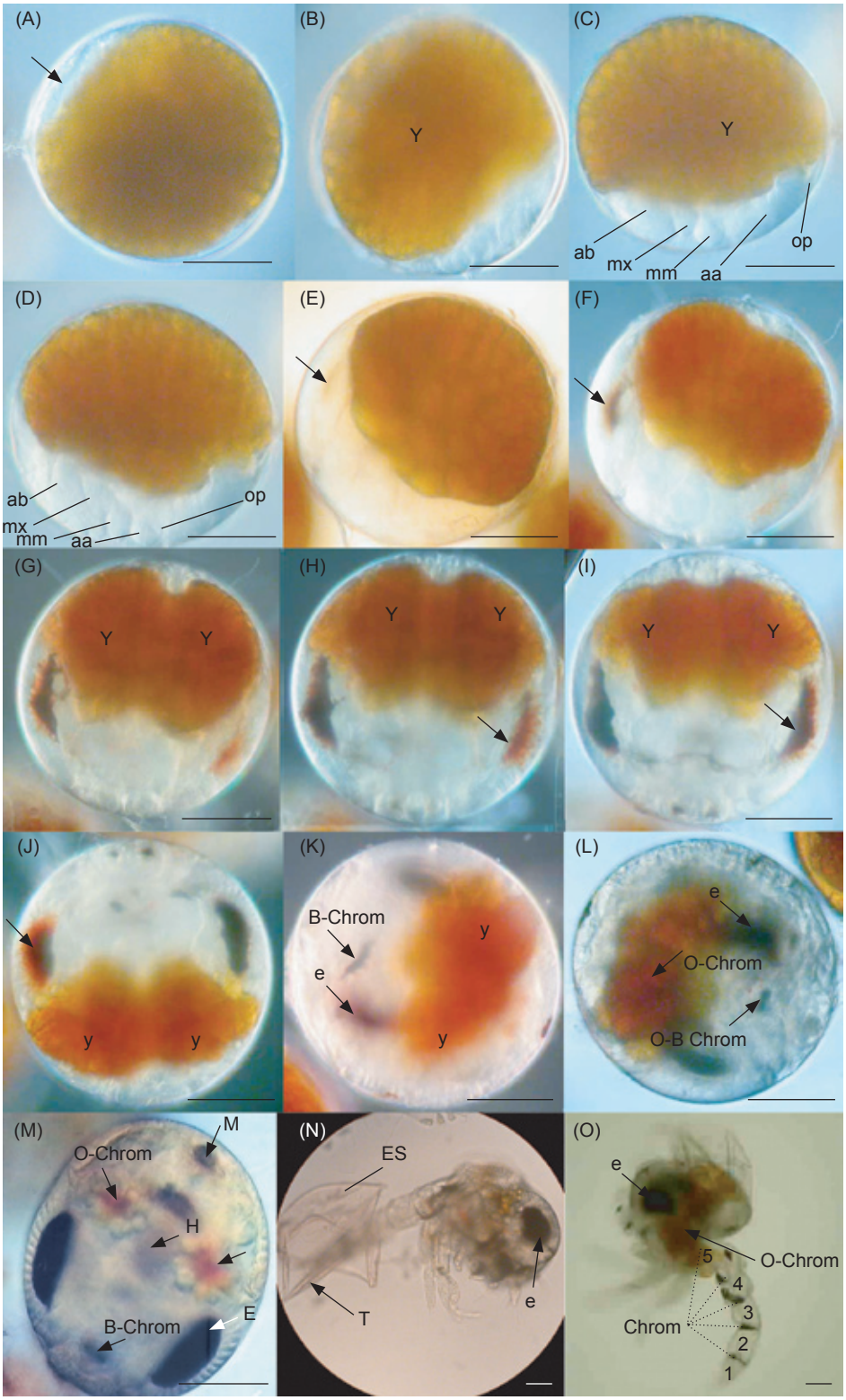

Fig. 3. Stages of development in embryos of *Perisesarma bidens* (De Haan). (A) 4 d embryo; (B) 5 d embryo; (C) 6 d embryo; (D) 7 d embryo; (E) 8 d embryo; (F) 9 d embryo; (G) 10 d embryo; (H) 11 d embryo; (I) 12 d embryo; (J) 13 d embryo; (K) 14 d embryo; (L) 15 d embryo; (M) 16 d embryo; (N) hatching embryo on 17th day; (O) 1st zoea hatching. ab, thoracic-abdominal; mm, maxillule-maxilla; mx, maxillipeds; aa, buds of antennule-antenna; op, optical; B-Chrom, black chromatophore; O-Chrom, range chromatophore; O-B Chrom, orange-black chromatophore; T, telson; e, eye; ES, egg shell; Y, yolk free into the eggs; y, reserve yolk in the developing zoeae; Arrow head indicate eye. Scale bars = 100 μm.

almost all of the available egg volume. The yolk was almost completely depleted, with only traces remaining in the cephalothorax cavity. The carapace, maxillipeds, and telson were well differentiated. There were many chromatophores, mostly black, in the embryo. The egg membrane was transparent, so the pigment cells could easily be distinguished under a stereomicroscope. As hatching approached, the embryo frequently moved its abdomen and appendages in the egg membrane. The heartbeat was faster than the previous stage at 110-140 bpm (Fig. 3N).

Hatching

The internal movements of the larvae caused the egg to rotate 180°, allowing the telson and rostral spine to face towards the funiculus. During the hours prior to hatching, the egg dilated as a result of water uptake and reached its maximum size. Larval release was completed within 5-40 min during the night, although some larvae were also released early in the morning as zoeae-1. About 65% of the larvae hatched as zoeae-1 after 17 d of incubation (Fig. 3O).

Pigmentation

The location of the chromatophores was discernible (Figs. 3K-M, O). The eyes were dark reddish-brown. A pair of yellow chromatophores was located on abdominal segment 5 near its point of attachment with the telson. A single yellow chromatophore was present on abdominal segments 2 and 3. On the 1st abdominal segment, a yellow chromatophore was situated anteriorly and medially (Fig. 3O). A large yellow chromatophore was found above the heart and stomach, while a red chromatophore appeared near the base of each mandible.

DISCUSSION

Mating and copulation

Our laboratory experiments showed that the *P. bidens* female normally copulated with males before oviposition. This, in fact, is in agreement with the findings of (Fukui 1993). The duration of copulation was very long and varied. Mating and copulation under laboratory conditions lasted for about 1-6 h. The reproductive ecology of *Sesarma* sp. under laboratory conditions was described by Zimmerman and Felder (1991), but they did not report copulation or its duration.

Egg-laying

The adult female retains the spermatophores acquired from the male during copulation in its own spermatheca via the gonopores and extrudes unfertilized eggs into the abdominal setae at 24-46 h after copulation. The time of egg-laying after copulation varies with species. Yamaguchi (2001) reported a similar trend in *Uca lacta* with the time between copulation and egg-laying ranging 1-3 d. That study was done in a natural habitat where it was difficult to record the precise time and duration of copulation. But Lee and Yamazaki (1989) observed egg-laying of *Eriocheir sinensis* in the laboratory 10 h after copulation. The present study indicated that up to 10 min after laying, newly released eggs were unfertilized. Determination of the precise egg-laying time after copulation is essential for successful artificial insemination.

Sperm morphology

The lack of literature on sperm structure within the grapsid limits comparisons. Bhavanishankar and Subramoniam (1997) reported that normal spermatozoa released from the spermatheca and from spermatophores appeared uniformly spherical, measuring 3-4 μm in diameter, with several nuclear arms and a prominent acrosome overlying the nuclear mass. This shape is similar to the sperm of *P. bidens* described in the present study but the size greatly differs. They also reported that the highest numbers of normal sperm were collected from the spermatheca, which is an essential component of successful insemination and fertilization.

Fertilization

Oocytes of some decapod crustaceans are fertilized when they pass through the seminal receptacles where sperm are stored (Hartnoll 1968). In most decapod crustaceans, fertilized eggs extruded from the gonophores attach to the ovigerous hairs within the incubation chamber of the female (Saigusa et al. 2002). Males of the spiny king crab, *Paralithodes brevipes*, mate with females and fertilize the eggs externally within the brood chamber formed by a flap under her body (Sato et al. 2005). In brachyuran crabs, females incubate their eggs in the body cavity from

spawning to hatching.

The female releases sperm into the abdominal cavity where they attach to the eggs and fertilization occurs. Therefore the fertilization pattern of this crab can be regarded as incomplete internal fertilization. In the present study, we observed unfertilized eggs being released by the female. Sperm were also collected by dissection out of the spermatheca of the same females that were the targets of our study on artificial insemination of this crab. The eggs of *E. sinensis* were unfertilized immediately after laying (Lee and Yamazaki 1990). Lee and Yamazaki (1989) also reported that they collected eggs of *E. sisnensis* directly from the ovary. The rate of fertilization success in the present study as determined by the cleavage rate was 95%, and about 65% of fertilized eggs hatched as zoeae. On the other hand, the rate of fertilization success of the Chinese freshwater crab *E. sinensis* was recorded as 100%, while only 20% hatched as zoeae at 20°C (Lee and Yamazaki 1989) by artificial insemination.

Early embryonic and larval development

Taylor and Seneviratna (2005) reported the cleavage, blastulation, gastrulation, and formation of eyespots and heart of the intertidal crabs *Hemigrapsus edwardsii* and *H. crenulatus* at 15°C in 100% seawater. All crabs had longer developmental periods compared to *P. bidens* which may have been due to its incubation at a lower temperature. However, our data closely fit those of blue crab early embryonic development as described by Walker et al. (2006) at 28°C. Durations of developmental stages to the 1st and 2nd polar bodies and the 1st cleavage were approximately 10-15 min, 30-45 min, and 23-24 h, respectively, which were similar to the findings of Lee and Yamazaki (1989) for *E. sinensis*.

Most authors recognized 5 or fewer embryonic stages (Henmi 1989) and as many as 15 embryonic stages before hatching (Fukui 1988, Yamaguchi 2001). Stevens (2006) first used both visual and cluster analysis of morphometric measurements of crab embryology. In the present study, we followed the general terminology of fundamental embryology up to cleavage, and then defined the continuous daily progress of developing embryos. Undivided (just-spawned) eggs were identified as stage 1, which is similar to the findings of Kobayashi and Matsuura (1996), but they recognized 2 and 4 cell embryos each as separate stages while 2-32 cell embryos were identified as a single stage in the present study. Yamaguchi (2001) recognized undivided eggs to 32 cell embryos as a single stage, which also greatly differs from the present study. Undivided (just-spawned or -fertilized) eggs characteristically differ from cleaved eggs and were distinguished as stage 1, which is more appropriate in the embryology of brachyuran crabs. The incubation period depends on the species and temperature. At low temperatures, the incubation period is prolonged, while it is shorter at higher temperatures (Yamaguchi 2001). The embryonic development of *P. bidens* is similar and matches the general embryonic pattern observed in many brachyuran crabs, including those of much-larger tropical and subtropical species (Garcia-Guerrero and Hendrickx 2004) and of cold water species

Table 2. Comparison of embryonic development of 4 species of mangrove crabs including *Perisesarma bidens*. Sources: 1, Garcia-Guerrero and Hendrickx (2004); 2, present study; 3, Yamaguchi (2001); 4, Taylor and Leelapiyanart (2001); 5, Taylor and Leelapiyanart (2001)

Species	Embryonic stages	Incubation period (d)	Diameter of embryos (mm)	Incubation temp. (°C)	Reference
Grapsidae					
Sesarma retiulatum	10	17.3	0.34 - 0.42	28	1
Goniopsis pulchra	9 periods	15	0.60 - 0.64	26 - 28	2
Clyclograpsus lavauxi	5	56	0.25 - 0.30	15	5
Sesarmidae					
Aratus pisonii	8 periods	14	0.57 - 0.62	26 - 28	2
Perisesarma bidens	16	17	0.31 - 0.41	25	3
Ocypodidae					
Uca lacteal	15	15.4	0.24 - 0.32	28	4

that experience very slow embryonic development (Nagao et al. 1999). The total incubation period was 17 d for *P. bidens* in the present study compared to 14 d for *Aratus pisonii* and 15 d for *Goniopsis pulshra* as observed by Garcia-Guerrero and Hendrickx (2004). Walker et al. (2006) described 12 stages of development in *Callinectes sapidus* at 28°C, and the incubation period was 12 d. Taylor and Seneviratna (2005) described the ontogeny of salinity tolerance and hyper-osmoregulation in the intertidal crabs *H. edwardsii* and *H. crenulatus*. They described 5 stages of development up to hatching at 15°C.

In the present experiment the largest diameter of undivided (just-fertilized and uncleaved) eggs of *P. bidens* was 0.31 mm, which increased to 0.41 mm before hatching. Yamaguchi (2001) reported that the largest diameter of undivided eggs of *Uca lectea* was 0.24 mm, which increased to 0.32 mm in stages 14 and 15. Garcia-Guerrero and Hendrickx (2004) found that the egg diameter of *Aratus pisonii* was 0.57-0.62 mm, and that of *Goniopsis pulchra* was 0.60-0.63 mm. Comparative data of embryonic stages, egg diameters, and incubation periods and temperatures in 5 species of mangrove brachyuran crabs are shown in table 2. Eggs (from fertilization to embryos) of *P. bidens* were always spherical, which is the most common shape for brachyuran eggs (Nagao et al. 1999, Yamaguchi 2001, Pinheiro and Hattori 2003, Garcia-Guerrero and Hendrickx 2004).

Hatching

In many crustacean embryos, hatching occurs after a long period of incubation. For example, in *Sesarma haematocheir*, embryos are incubated by females for about 1 mo after fertilization (Saigusa 1980). Larval release by *Sesarma intermedia* on the Hataka I. seashore is not strongly related to tidal movement, but rather to the sunset (Kyomo 1986). This suggests that light is a major factor inducing the release of larvae. Release of larvae occurs under well-defined conditions of light, tide, and several other exogenous and endogenous factors. In all cases, all females carrying mature eggs wait until sunset or dusk before proceeding to the seashore. Furthermore, results show that larval release is not a sporadic event, but that it takes place within a specific time interval. Similar observations were reported by other authors, e.g., Saigusa and Hidaka (1978) on semilunar rhythms in *Ses. intermedia*. Larval release at night by the fiddler crab was observed by De Coursey (1981). She suggested that nocturnal larval release is advantageous for both adults and larvae to avoid predators. Though *P. bidens* is not nocturnal, in most cases in the present experiment, larval release occurred in the evening and early in the morning.

CONCLUSIONS

The present work is the first report on the successful artificial insemination of *P. bidens*. Our results strongly suggest that *P. bidens* fertilization is of the moderately internal type that occurs in the abdominal cavity or flaps. Females store sperm in the spermatheca, and their release was induced by internal stimuli after egg extrusion into the pleopods of the female. The collection of reproductively active pairs of crabs from their natural habitat in order to collect gametes for artificial insemination is very time-consuming and season-dependant. Thus the findings of the present study add a new avenue for managing mangrove ecosystems and biodiversity conservation using artificial insemination of this species. The technique is simple and can be used during the peak breeding season for large-scale larval production at minimal costs. However, more research is necessary to explore the induction of mating so that gametes can easily be collected for artificial insemination in order to facilitate large-scale seed production in crab farming. Further study is also needed to observe the survivability of larvae produced by artificial insemination up to their mature stages.

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