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Gamete characteristics and early development of the hooded oyster *Saccostrea cucullata* (Born, 1778)

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ABSTRACT

The hooded oyster, *Saccostrea cucullata* (Born, 1778), is an important aquaculture species on the coast of Thailand. Its aquaculture is mainly based on wild seed collection, the availability of which is becoming unstable due to environmental changes and pollution. To support aquaculture of this species, seed production in the hatchery is therefore required. Unfortunately, information on seed production is not available in this species. Therefore, the aim of this work was to study gametes and early stage larval development in order to improve current knowledge of fertilization and larval development in this species. Spermatozoa and oocytes were collected from mature oysters by a strip spawning method and characterized under a light microscope and a transmission electron microscope (TEM). *In vitro* fertilization was performed in 30 ppt filtrate seawater to observe developmental stages until the D-larvae. Results showed that oocytes, when fertilized with spermatozoa, underwent meiosis I (15 min) and meiosis II (30 min), indicated by the presence of the first and second polar bodies, respectively. The first cleavage, holoblastic unequal type, occurred 50 min after fertilization. A polar lobe was observed during the first cleavage and fused with one of the two cells to form one small and one large macromere. The larvae then continuously developed to trochophore larvae over 8 h. D-larvae were observed 24 h after fertilization. Results obtained from this work improve current knowledge of gametes and early developmental biology of the larvae in this species. This knowledge is important for developing hatchery methods for *S. cucullata*.

1. Introduction

In oysters, larval development at early stages has been studied in many species of the genus *Crassostrea* (Galtsoff, 1964; Wallace et al., 2008; Nor Idayu et al., 2015; Ver, 1986; Yurchenko et al., 2018) and the genus *Saccostrea* (Kakoi et al., 2008; Kalyanasundaram and Ramamoorthi, 1987; Nowland et al., 2018). Unfertilized primary oocytes released from gonads are typically arrested at the first prophase of meiosis. Oocytes are induced to complete meiotic cell division once fertilized with spermatozoan to achieve one mature oocyte and polar body. Pronuclei fusion of the mature oocyte and spermatozoan then forms a nucleus leading to development of a zygote (Kakoi et al., 2008). The zygote then develops into an embryo followed by a larval development.

Oysters and other members of Mollusca possess spiralian embryogenesis, a common character of which is unequal holoblastic cleavage (Rabinowitz and Lambert, 2010). This suggests a shared evolutionary origin and has been used as an important character for taxonomic classification of Animalia.

Knowledge of oyster embryo and larval development is essential for development of species specific production methods. In the past, oyster culture around the world has been based on wild seed collection, however wild seed availability can fluctuate and be insufficient for aquaculture because of increasing levels of water pollution (Chueachat et al., 2018). To overcome this problem, hatchery systems were successfully developed and are used in many temperate countries around the world, including France (Buestel et al., 2009), Brazil (Singaraja, 1980), Australia (Nowland et al., 2018), Cuba (Vega et al., 2014), and Vietnam (Nowland et al., 2020). Currently, oyster production is mostly or entirely hatchery based in many areas. The Pacific oyster, *Crassostrea gigas*, (Mondol et al., 2015) and the American oyster, *Crassostrea virginica*, (Wallace et al., 2008) are among the most successful species grown in

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hatchery systems.

The hooded oyster, *Saccostrea cucullata* (Born, 1778), is an economically important species in tropical region such as Thailand, Malaysia, Japan, Sri Lanka, Indonesia, the Philippines, and India (Szuster et al., 2008; Nowland et al., 2020; FAO, 2019). This species has potential to be used for hatchery based farming in these regions and allow for economic development (Nowland et al., 2020). Developmental stages of this species were previously reported by Kalyanasundaram and Ramamoorthi (1987) and Bussarawit and Cedhagen (2012). However, only some larval developmental stages were described. Information such as cellular events during fertilization, completion of meiosis I and II, and the ultrastructure of gametes is still limited in this species. Additionally, hatchery production techniques have not been successfully developed for this species. To support hatchery production of *S. cucullata*, knowledge of gamete characteristics and larval development is required.

In Thailand, three oyster species are cultured including *Crassostrea belcheri*, *Crassostrea iredalei*, and *Saccostrea cucullata*. All of these cultures are currently based on wild seed collection (Chalermwat et al., 2003; Szuster et al., 2008). The availability of wild seed is becoming less reliable and insufficient for aquaculture because of environmental changes and water pollution (Chueachat et al., 2018). To support oyster aquaculture, nursery and grow-out techniques are developed for *Crassostrea belcheri* (Tanyaros et al., 2008) and *Crassostrea iredalei* (Chueachat et al., 2018). However, none of those techniques and hatchery methods have been successfully established for the hooded oyster. Therefore, the aim of the present work was to study developmental stages and gamete characteristics of this species to enable future development of hatchery methods.

2. Materials and methods

2.1. Oyster

The hooded oyster were collected from an oyster farm in Chonburi Province during the spawning season in April-June 2019. Adult oysters with shell length of 5−7 cm were chosen and brought back to the Aquaculture Laboratory, Faculty of Science, Burapha University, Chonburi. Oysters were cleaned and acclimatized for one week in 30 ppt aerated filtered (0.22 μm) seawater with a density of one individual/l. They were fed daily with 1 % v/v of a mixture of *Nannochloropsis* sp. and *Chaetoceros* sp. at a density of approximately 2×10^7 cells/mL during acclimatization. These oysters were used for gamete collection and fertilization. The experimental protocol was in accordance with animal ethics standards and was approved by the Burapha University Institution Animal Care and Use Committee (23/2561).

2.2. Gamete collection

To collect gametes, shells of reproductively mature oysters were opened. Sex was identified by sampling a small amount of gametes from gonads and observing under a light microscope. Five males and five females were used for gamete collection. Sperm and oocytes were collected by the stripping method and pooled separately for each gender in 30 ppt filtered seawater. Sperm was filtered using three layers of nylon mesh with pore sizes of 120, 30 and 10 μm to remove gonad tissue. Oocytes were filtered using four layers of using nylon mesh with pore sizes of 300, 120, 68 and 30 μm. Oocytes that remained on the nylon mesh with pore size of 30 μm were used in this experiment. Spermatozoa and oocytes were counted and used for *in vitro* fertilization. Another group of unfertilized gametes were also observed separately under a microscope at 24 h.

2.3. Transmission electron microscopy

The ultrastructure of spermatozoa and oocytes of the hooded oyster were characterized. Spermatozoa and oocytes were separately fixed in 2.5 % glutaraldehyde in 0.1 M PBS (pH 7.2) for 4 h and post-fixed at 4 °C in 1 % osmium tetraoxide in 0.1 M PBS (pH 7.2) for 2 h. Samples were dehydrated in a series of ethanol washes increasing from 70 % to absolute ethanol. Samples were then infiltrated with propylene oxide (PO) and embedded in pure araldite resin. Ultrathin sections of 90 nm were cut using a Hestion ERM 3000 microtome (Hestion, Australia) and stained with uranyl acetate in 70 % methanol followed by 0.1 % lead citrate and examined with a Tecnai 20 transmission electron microscope (Philips, USA).

2.4. In vitro fertilization and larval development observation

In vitro fertilization was modified from Song et al. (2009) and Kingtong et al. (2013). Briefly, 80,000 oocytes were incubated in a glass container containing 200 mL of 30 ppt seawater for 30 min. Spermatozoa were then introduced to fertilize the oocytes in the container with the ratio of approximately 400 sperm/oocyte. Time lapse between gamete collection and fertilization was 30 min. Water temperature, salinity and pH were 25 °C, 30 ppt and 7.8–8.0, respectively. Oocytes were immediately observed under a light microscope to study the fertilization process and developmental stages from cleavage to D-larvae. Three replicates were performed. Samples of embryos and larvae in early stages were fixed in Davison's fixative for nucleus staining.

2.5. Nuclear staining

Hoechst 33342 (Thermo Fisher Scientific, USA) fluorescent dye was used to stain nuclei of oocytes in order to track the nucleus of the 1st polar body and the 2nd polar body during meiosis I and meiosis II, respectively. It was also used to track nucleus at early cleavage stages. Staining steps were performed according to the manufacturer's protocol. Briefly, the glutaraldehyde-fixed oyster oocytes and embryos were washed once with phosphate buffered saline (PBS) and then stained with Hoechst 33342 at a final concentration of 1 μg/mL for 5 min. The stained oocytes and embryos were washed twice with PBS before imaging. Hoechst 33342 fluorescent photographs were taken by a digital camera attached to an inverted fluorescence microscope (Eclipse Ti-S Inverted Research Microscope, Nikon), using an ultraviolet filter. The images with scale bars were processed by NIS-Elements Imaging Software (Nikon).

3. Results and discussion

3.1. Gamete characteristics

3.1.1. Spermatozoa

Spermatozoa of the hooded oyster started to move quickly using flagella once released to seawater. Spermatozoa nuclei were observed as small dots under a light microscope (Fig. 1A). Ultrastructure results showed that spermatozoa of the hooded oyster consisted of an acrosome complex, a round nucleus, a midpiece anchored with four spherical mitochondria, and a flagellum (Fig. 1B). The acrosome complex was composed of an acrosomal vesicle anteriorly and a subacrosomal space filled with an axial rod in the middle. The nucleus was barrel-shaped, containing invaginated subacrosomal space anteriorly. Four spherical mitochondria were found at the midpiece. The mitochondria localized at the proximal base of the flagellum (Fig. 1B, upper inlet). The flagellum was composed of a 9 + 2 microtubule arrangement (Fig. 1B, lower inlet). Spermatozoa of the hooded oyster closely resemble those of other oysters such as *Saccostrea commercialis* (Healy and Lester, 1991), *Saccostrea mordax* (Yurchenko, 2012), *Crassostrea gigas* (Gwo et al., 1996; Yurchenko, 2012), and *Crassostrea angulata* (Sousa and Oluveira, 1994). Nucleus length and width of spermatozoa were 1.29 ± 0.15 μm and 1.81 ± 0.14 μm (n = 40), respectively. The length/width ratio was 0.72 ± 0.08, which is higher than the length/width ratio of the genus *Crassostrea*

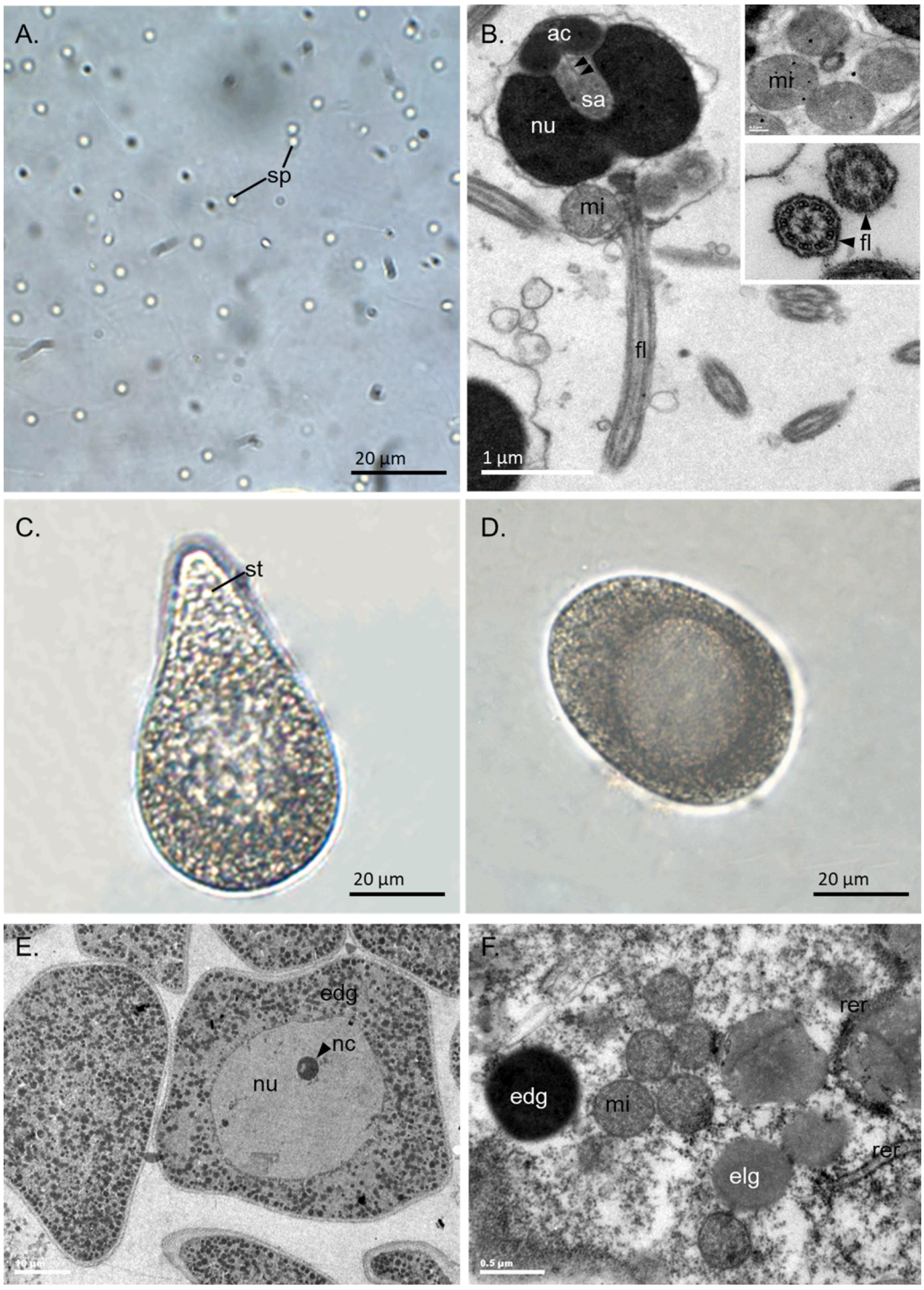

Fig. 1. Gamete characteristics of *S. cuccullata*. Sperm are observed under a light microscope (A). Ultrastructure of sperm shows details of nucleus, midpiece, acrosome, and flagellum (B). Oocyte appear in both pear-shape (C) and round-shape (D). Ultrastructure of oocytes (E) shows euchromatin nucleus with a nucleolus. Electron dense granules (edg), electron translucent granules (elg), rough endoplasmic reticulum (rer), and mitochondria (mi) are plentiful in ooplasm (F). (ac, acrosome; ax, axial body; fl, flagellum; nu, nucleus; nc, nucleolus; st, stalk).

ranging from 0.54–0.62 (Gwo et al., 1996; Sousa and Oluveira, 1994; Yurchenko, 2012), and consistent with reports of other *Saccostrea* species ranging from 0.69–0.72 (Healy and Lester, 1991; Yurchenko, 2012). Therefore, the length/width ratio of spermatozoa nucleus may be useful for morphological classification at the genus level.

Spermatozoa of the hooded oyster are able to swim in seawater for a long period of time, at least 24 h. This is similar to the Pacific oyster in which spermatozoa can swim at least 20 h (Suquet et al., 2010b). Spermatozoa moving periods of oysters are much longer when compared to other aquasperm types that have external fertilization such as fish (Dreanno et al., 1999; Alavi et al., 2010 ; Suquet et al., 2010a). This could be explained by the difference in mitochondria size and volume of spermatozoa. Mitochondrial volume has been shown to determine flagellar beat frequency (Cardullo and Baltz, 1991) and correlate with ATP content and sperm swimming velocities in mammals (Gu et al., 2019). Although mitochondria volume was not measured in this studied, the result showed that the four mitochondria of the hooded oyster were obviously large which indicates the ability to produce large amount of ATP content to supply long term flagellum movement. This characteristic is important for sessile animals such as oysters to accomplish their external fertilization in open sea habitats.

3.1.2. *Oocytes*

In this work oocytes were collected during the spawning season in

order to ensure their reproductive maturity. The results showed that oocytes of the hooded oyster, once stripped from the gonad, were found in both pear- and round-shapes (Fig. 1C–D). The pear-shaped oocytes changed to round-shaped oocytes after incubation in seawater for 30 min. Oocyte diameter was measured only in the round-shaped form. The average size of the oocyte in the hooded oyster was 48.27 ± 3.55 μm with a maximum of 55.59 μm (n = 30). Mature oocyte diameter of the hooded oyster is in the same range as reported for other species within Ostreidae (Kakoi et al., 2008; Eckelbarger and Davis, 1996; Masaru et al., 2017; Brousseau, 1995; Lango-Reynoso, et al., 2000; Lim et al., 2003; Leverett and Thain, 2013).

Ultrastructure results showed that oocytes of the hooded oyster were composed of a large nucleus and that the nucleolus was clearly visible within the nucleus (Fig. 1E). Ooplasm was filled with many yolk granules and lipid droplets, which were observed as electron dense granules and electron translucent granules, respectively (Fig. 1F). Yolk granules and lipid droplets are important for nutrient storage. Mitochondria and rough endoplasmic reticulum (RER) were also abundant in the ooplasm (Fig. 1F). These findings are similar to the oocytes of the Eastern oyster *Crassostrea virginica* (Eckelbarger and Davis, 1996), indicating the production of oocyte yolk bodies during oogenesis.

3.2. Fertilization and meiosis of the oocyte

The embryonic development in the hooded oyster was observed (Fig. 2). After introducing spermatozoa to oocytes, the primary oocyte started to undergo the first meiotic cell division, indicated by the presence of the 1st polar body within 15 min (Fig. 2A1 and B1). The second meiotic cell division occurred at 30 min, which could be observed by the presence of the 2nd polar body (Fig. 2A2 and B2), forming a zygote. It is not easy to distinguish the 1st polar body and the 2nd polar body under a light microscope (Fig. 2A1−2). However, this could be confirmed by staining the nucleus using Hoechst 33342 fluorescent dye (Fig. 2B1−2). Fluorescent nucleus staining also aided observation of chromosomal and nuclear movement during cell division. The primary oocytes remained unchanged if not fertilized with spermatozoa. This is consistent with a previous report that bivalve oocytes were arrested at prophase I (Deguchi and Osanai, 1994). Spermatozoa is required to induce meiotic cell division of the oocytes.

No fertilization membranes were observed in this species. We hypothesized that the lack of fertilization membrane of fertilized oocytes is probably a common character among the members of Mollusca. This is supported by a report of the gastropod *Nassarius reticulatus* in which cortical granules could not be observed. In this case, the vitelline layer would not be able to transform into a thick fertilization membrane (Schmekel and Pioroni, 1975). This is a possible reason why polyspermy occurs during fertilization in oyster (Stephano and Gould, 1988), and it is therefore important to optimize the ratio of spermatozoa and oocyte for artificial fertilization (Song et al., 2009).

3.3. Early larval development of the hooded oyster

The first cleavage division was complete at 50 min after fertilization. The zygote started to undergo mitotic cell division, indicated by chromosomal condensation and movement (Fig. 2B3). During the first cleavage, the polar lobe started to form (Fig. 2A4−5, B4−5). These included small lobes close to the polar body and one large polar lobe at the opposite side. Nuclei were found only in the two small lobes. Cytoplasmic content in the polar lobe then fused with one of the small lobes to become a large cell and vegetal pole (Fig. 2 A6, B6). Two blastomere cells were obtained at the end of the first unequal holoblastic cleavage, including one large and one small cell (Fig. 2A7 and B7).

The current study shows the existence of polar lobe formation during the first cleavage in *S. cuccullata*. In Mollusca, spiralian embryos can be divided into two groups: those with and those without polar lobe formation (Dorresteijn, 1990). Polar lobe formation has been reported in the Pacific oyster *Crassostrea gigas* (Deno, 1998; Longo et al., 1993), the American oyster *Crassostrea virginica* (Masaru et al., 2017), and common slipper shell *Crepidula fornicate* (Henry et al., 2006). Whereas, embryos without polar lobe formation have been reported in the zebra mussel *Dreissena polymorpha* (Luetjens and Dorresteijn, 1998) and clam *Corbicula sandai* (Obata et al., 2006). The formation of the polar lobe depends on microtubule organization and orientation during cell division (Longo et al., 1993; Masaru et al., 2017). Existing evidence indicates that polar lobe formation appears to be diverse in Mollusca, not particularly restricted to specific taxa. However, result from the current work in light of previous reports suggest that formation of the polar lobe is a common character of oysters in family Ostreidea.

The second cleavage division occurred 60–65 min after fertilization (Fig. 3B). Four blastomeres were obtained, including three small blastomeres and one large blastomere. In this study, the evidence showed that the first two cell divisions synchronously divide the embryo into four macromeres, similar to the American oyster *Crassostrea virginica* (Masaru et al., 2017). However, cell divisions after the four blastomere stage were not synchronous. The larvae then developed to further stages (Fig. 3C and D). At the end of cleavage, one large blastomere was still dominant at the vegetal pole, whereas small blastomeres were located at the animal pole (Fig. 3D).

The blastula stage was observed around 4 h (Fig. 3E). At the end of the blastula stage, a pseudo-blastopore was observed at the vegetal pole

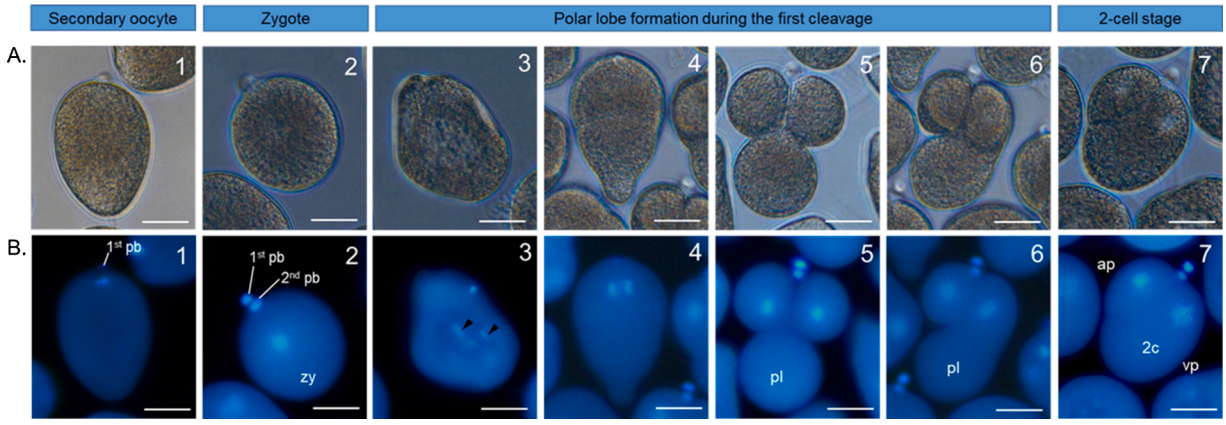

Fig. 2. Fertilization and the first cleavage in *S. cuccullata*. Cellular events under a light microscope (A) were confirmed by nuclear staining using Hoechst 33342 fluorescent dye (B). After sperm introduction, secondary oocyte (1) and zygote (2) occurs within 15 and 30 min, respectively. The zygote undergoes the first cleavage division *via* polar lobe formation (3-6). The polar lobe fuses into one of small cells to become a large cell at the vegetal pole (7). (pb, polar body; zy, zygote; 2c, two-cells stage; pl, polar lobe; ap, animal pole; vp, vegetal pole; arrow head, condensed chromosomes; scale bar = 20 μm).

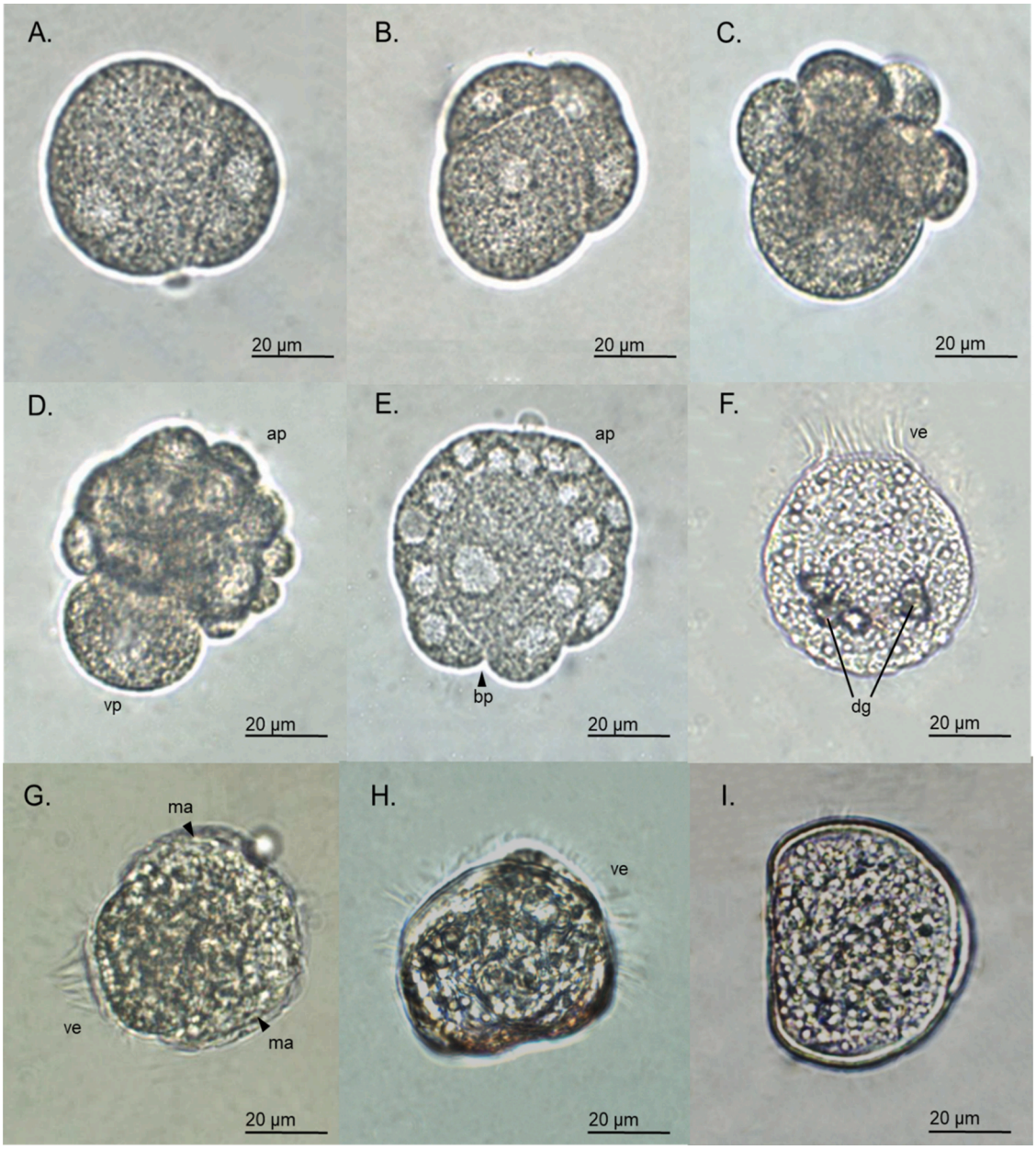

Fig. 3. Early development of *S. cuccullata*. The embryo develops from a zygote to stages of 2-cell (A) 4-cell (B), 8-cell (C) and later stage of cleavage (D). Pseudo-blastopore is observed at the end of blastula (E). Digestive organ appears in trochophore larva (F). Mantles starts to form and produce shells at the end of trochophore larva (G). Intestine appears, shells develop (H) and become D-larvae (I). (ap, animal pole; bp, pseudo-blastopore; dg, digestive glands; mt, mantle; ve, velum; vp, vegetal pole).

indicating the beginning of the gastrula stage of larval development. (Fig. 3E). At this stage, one large blastomere at the vegetal pole invaginated into the embryo resulting from the expansion of small blastomeres from the animal pole covering the entire embryo.

Prototroch, the ciliated ring characteristic of trochophore larvae, was observed around 6 h after fertilization (Fig. 3F). At this stage, internal organs, such as digestive glands, developed inside the larvae. At the end of trochophore larvae, mantles started to form on both sides of the larvae (Fig. 3G). The mantles play important roles in shell calcification. The larvae metamorphosed from trochophore to veliger larvae in which shells were produced along both sides of the mantle. A digestive tract was clearly observed in veliger larvae 20 h after fertilization (Fig. 3H). In this stage, shells were dominant and covered soft tissue of the larvae. The shells developed into a D-shaped structure in veliger stage so called D-larvae (Fig. 3I) 24 h after fertilization. Timing of early developmental stages vary among oysters in the family Ostreidae. However, most of species reach the D-larvae at about the same time. In the genus *Saccostrea*, D-larvae were reported from 18−25 h after fertilization (Kakoi et al., 2008, Nowland et al., 2008; Kalyanasundaram and Ramamoorthi, 1987), whereas greater variation is reported in the genus *Crassostrea*, ranging from 16−52 h (Galtsoff, 1964; Wallace et al., 2008; Idayu et al., 2015; Ver, 1986).

The internal organs, such as digestive glands, were observed in trochophore larvae and the intestine was clearly visible 20 h after fertilization. We hypothesize that larvae in this stage have feeding capability, however, the significance of food to larvae at this stage was not investigated in this work.

4. Conclusion

This is the first report of gamete characteristics and early larval development in the hooded oyster, *Saccostrea cuccullata*. Spermatozoa of the hooded oyster are composed of an acrosome complex, a barrel-shaped nucleus, a midpiece anchored with four spherical mitochondria, and a flagellum which is similar to other oyster species. The length/width ratio of the nucleus was 0.72 ± 0.08, which may be a useful characteristic for taxonomic classification of oysters at the genus *Saccostrea*. Oocyte diameter in this species was 48.27 ± 3.55 μm, and yolk granules and lipid droplets are abundant in the ooplasm of the oocytes. In the *in vitro* fertilization, the first and second meiotic cell division of oocytes were indicated by formation of the first and second polar bodies and occurred 15 and 30 min after introducing spermatozoa to oocytes, respectively. This experiment provides evidence of the existence of polar lobe in this species. This polar lobe fused with one of the two cells to form one small and one large blastomere. Generally, the developmental stages of larvae in this species are similar to other oyster species in genus *Saccostrea* and *Crassostrea*. The results obtained from this work improve current knowledge of gamete characteristics and early developmental biology of the larvae. *In vitro* fertilization technique obtained from this study can be applied to set up a hatchery system for this species.

Declaration of Competing Interest

The authors report no declarations of interest.

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