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The larval development of the Asian clam, *Corbicula fluminea* in the hatchery

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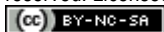
Abstract: The larval stage is given high priority in seed production, which is an essential aspect of any farming practice. Induced breeding was carried out by integrating temperature, salinity, and gametes suspension. Individuals from spawning broodstocks were sacrificed to observe larval development at an early stage. The released larvae (veliger) development was monitored until they settled on the substrate. As a result, three developmental stages have been identified: marsupial, planktotrophic, and benthic. The marsupial stage lasts until the pediveliger (D-shaped with velum and developed ciliated foot) is brooded in the innerdemibranches. The planktotrophic stage begins when the broodstocks release the first straight-hinged veliger (D-shape). Finally, the juveniles reach the benthic stage when they start to settle at the bottom of the culture chamber. Before metamorphosing into planktonic juveniles, the veligers were nurtured for about a month. Internal organs such as double-looped gills, digestive, mantle, foot, and prominent shell ridges developed and matured in planktonic juveniles. Therefore, the documentation of *C.fluminea* larval revealed significant development stages for seed production in the hatchery conditions.

INTRODUCTION

The *Corbicula* was captured and consumed as food in Asia, and it was considered one of the most economically important aquatic species (Korniushin & Glaubrecht, 2003; Wang, Zhang, Zhang, Li, & Xiao, 2014). Instead of *C.sandai* and *C.leana*, the *Corbicula fluminea* is the most prominent species of the genus *Corbicula* that lives in freshwater and is found throughout this region (Ramli, Ayyapan, Yusoff, Eh Rak, & Lee, 2020). Currently, the clam has been overfished, particularly in several countries such as China (Zhu *et al.,* 2018), Japan (Okawa, Kurita, Kanno, Koyama, & Onikura, 2016), and Malaysia (Yusof, Sow, Ramli, Rak, & Wei, 2020). This species was solely harvested from the natural habitat to cater to the market demands. For instance, the total annual catch was 12 MT in East Asian countries (Chen *et al.,* 2013), whereas the earlier record was 20 MT per annum. However, relying solely on wild stocks has led to drastic depreciation instead of water pollution and habitat destruction. There is no specific record of *C.fluminea* being captured in Malaysia, although their current presence on market shelves was gained speculatively from Thailand and Cambodia. The market price of clams has escalated dramatically due to

this status quo. This continuous occurrence has driven scientists to develop seeds to fulfil market demand through aquaculture.

Given these circumstances, the clam industry needs new sources in addition to the hatchery-produced seeds that look to be a feasible alternative. However, a lack of knowledge on larval production, growth, and development hindered the hatchery's capacity to produce this clam. The *Corbicula fluminea* is a well-known monoecious species that fertilises internally and broods its larvae in innerdemibranches, with the ability to produce over 10 million embryos. (Glaubrecht, Fehér, & Köhler, 2007; Okawa *et al.,* 2016). Previously, two techniques for producing clam seed were advocated: collecting from gravid broodstocks in natural habitats or using a hatchery system (Aji, 2011). Furthermore, the previous study emphasised the laboratory-bred, in which shelled larvae were acquired by dissecting wild clams in the laboratory and kept in laboratory conditions. (Nichols & Black, 1994). On the other hand, field-caught and continually grown in the hatchery is a typical method of obtaining shelled larvae.

Consequently, producing seeds could be a viable option for discovering a different source of *C. fluminea*. Attempts to artificially produce *C.fluminea* seeds in laboratory conditions have been made to date (King *et al.,* 1986). On the other hand, this study was performed to explore the early development of larvae in laboratory culture. Other freshwater bivalves, such as *Margaritifera spp.* have been extensively cultivated and reproduced in the laboratory and hatchery, but no *Corbicula* species has. (Hastie & Young, 2000; Preston *et al.,* 2007; Kovitvadhi *et al.,* 2008). In brief, external influences induced the fertilisation and spawning of hatchery-produced bivalves. As a result of the early study, three parameters were discovered to be significant: temperature, salinity, and feed supply. Therefore, this research discusses broodstock husbandry, induced breeding procedures, spawning, larval development of *C.fluminea* juveniles in a hatchery.

MATERIALS AND METHODS

Collection of the Corbicula fluminea.
The *C.fluminea* were obtained from the Pergau Lake, Jeli, Kelantan, Malaysia (5°37'13.4" N, 101°42'11.5" E). This lake is the largest source of *C.fluminea* for the local market, and a dredger (1.1m x 0.9m x 0.3m) was dragged from the boat to the permitted area (Rak *et al.*, 2021). Then, the clams with more than 20 mm were separated from the clutch and placed in a cold box to prevent stress or spontaneous spawning. Finally, they were transported to the Aquaculture Laboratory, Universiti Malaysia Kelantan, Jeli Campus. The clam's unwanted sizes (<10 mm) were released into the lake.

Broodstocks conditioning
The freshwater was gradually introduced into the box until achieving the equilibrium temperature. Then, 1000 broodstocks of *C.fluminea* were cleaned and placed in the recirculating aquaculture system (RAS) tank (700L) with supplemented sediment (fine sand), continuous, and aeration for eight weeks. The size is varied due to the sexual maturity of the clams being independent of the size. The broodstocks were fed by the diluted microalgae *Nannochloropsis sp.* (Reed Mariculture Inc, USA) and were continuously supplied at a density of 15×10^6 cellsmL^{-1} through a modified feeder (5 L). The microalgae volume was adjusted by observing the consumption through daily counts of the algal residual in the Neubauer chamber (Lima *et al.,* 2011). The dead clams were removed, the condition index (CI) was monitored, and the breeding induction was carried out when the CI value was at a range of 3.0-4.0 or above (Rahim, Idris, Kamal, Wong, & Arshad, 2012).

Breeding inductions
The *C.fluminea* (n=10) were randomly selected and sacrificed to obtain the gametes. Extracted gametes comprised of spermatozoa and ova. The gonads area was dissected under a microscope and ground using plastic micro mortar in the centrifuge tube (15 ml) with distilled water. The pulverised gonads are used for inseminating other broodstocks. Then, the gametes were diluted in the distilled water at 1×10^3 to 10^5 spermatozoa/ml. The concentration of the gametes was determined using a haematocytometer and observed under a light microscope (Leica, USA). Five hundred broodstocks were maintained in breeding tanks (10 L) with eight L adjusted water and without substrate (duplicate). Salinity adjustment to eight parts per million (ppm) using dechlorinated water or sodium chloride (NaCl), and the salinity was measured with a portable refractometer. The temperature was gradually increased from 27.0°C to 32.0°C, controlled by a water heater (DoPhin Heater 100W, China). The diluted microalgae

Nannochloropsis sp. was pipetted onto the broodstocks in the breeding tanks, stimulating them to extend their siphons. Then, the pulverised gonads were sieved using a 60 µM sieve, and 10 mL pipetted thoroughly to their extended siphons. The spawning induction was repeated every 48 h since the changes in turbidity of the water could not be the primary indicator of success (Baba *et al.,* 1999). The broodstocks spawning activities and responses were observed. However, the sperms or veliger releasing could not be seen through the naked-eyed. Every seven days after the induction process, the tank water was sequentially filtered using 150 µm, 120 µm, 100 µm, and 60 µm plankton net to catch the released veliger and juveniles. They were harvested five times after induction was carried out.

Spawning

The spawning activity of the *C.fluminea,* such as releasing sperms and eggs were not viable since the fertilisation occurred inside the parent body. Furthermore, this freshwater clam incubated its embryos inside the brood chambers in the gills for two weeks up to a month (Rajagopal *et al.,* 2000). The spawning of *C.fluminea* was defined when the broodstocks released the pediveliger (usually in marsupial) or early juvenile (Rajagopal *et al.,* 2000). A recent study scarcely found pediveliger while early juveniles were abundant in the breeding tank.

Larval rearing and development

Great care during dissection was taken to preserve the integrity of the mantle and visceral mass. The dissection was conducted under the dissecting microscope (Leica, USA). The live clam was gently forced apart by scalpel and holding while iridectomy scissors were used to cut the adductor muscle that holds the mantle lobes. Then, the left shell valve was removed, and the detached mantle lobe was lowered onto the visceral mass. The left mantle lobe was pulled back to examine the clam gills, exposing the gills and visceral mass. Embryo or larvae were observed *in situ* before both sides' gills were removed by incised using iridectomy scissors. After the gills were removed, a small incision was carried out on the exposed visceral mass. The covering epithelium layer was gently pulled back, which exposed the superficially located oogenic and spermatogenic follicles. Usually, the follicles were whitish and finely granular masses. The follicles were carefully removed from several locations on the visceral mass to avoid contamination and smeared on the glass slide. Then, smeared tissues were examined under light microscopes (Leica, USA). Further examination was carried out on the four removal gills. Typically, the embryos are incubated in the inner demibranch (second layer gills). Therefore, it was placed on a glass slide with a few drops of distilled water. The incubated embryos were gently teased and freed from the marsupial gill. The stages development of embryos was determined as follows: (a) no embryo present; (b) blastula; (c) gastrula; (d) trochophore; (e) veliger; (f) pediveliger; (g) early straight-hinged juvenile and (h) straight-hinged juvenile (Kraemer & Galloway, 1986). The removal of brooded veliger could not be survived in the conditioned water (culture water). As a result, the larval development examination was initiated after the early straight-hinged juvenile was discharged from the broodstocks. The released larvae were reared in the closed recirculating aquaculture system (RAS) supplemented with the substrate (125-250µm) and fed with the commercial microalgae, *Nanochloropsis sp.* at density 1-2 x 10^6 cells/ml. Images determined the development of larvae, captured using a compound camera microscope (Leica, USA). This study determined three stages of life: marsupial, planktotrophic, and benthic stages.

RESULTS

Spawning and Larval Development of Corbicula fluminea

Spawning (release gametes) events were intricated to determine since the occurrence is unnoticeable. However, the extracted gametes (sperms and eggs) from the mature broodstocks (Fig.1) were the sperms found in biflagellate. The oogenesis and spermatogenesis were found in all evaluated broodstocks. The sperms (S) were approximately in range 12-16 µm linear distance from end to end and biflagellate (Fig. 1). These male gametes can be found in all dissected broodstocks. Meanwhile, the blastulae abundantly found were removed from the innerdemibranches of the sacrificed broodstocks in the form of a gelatinous envelope (GE) and suspended in water (Fig. 2). The presence of blastulae reduced the sperm's existence in the sample. However, this observation was found not in all dissected broodstocks.
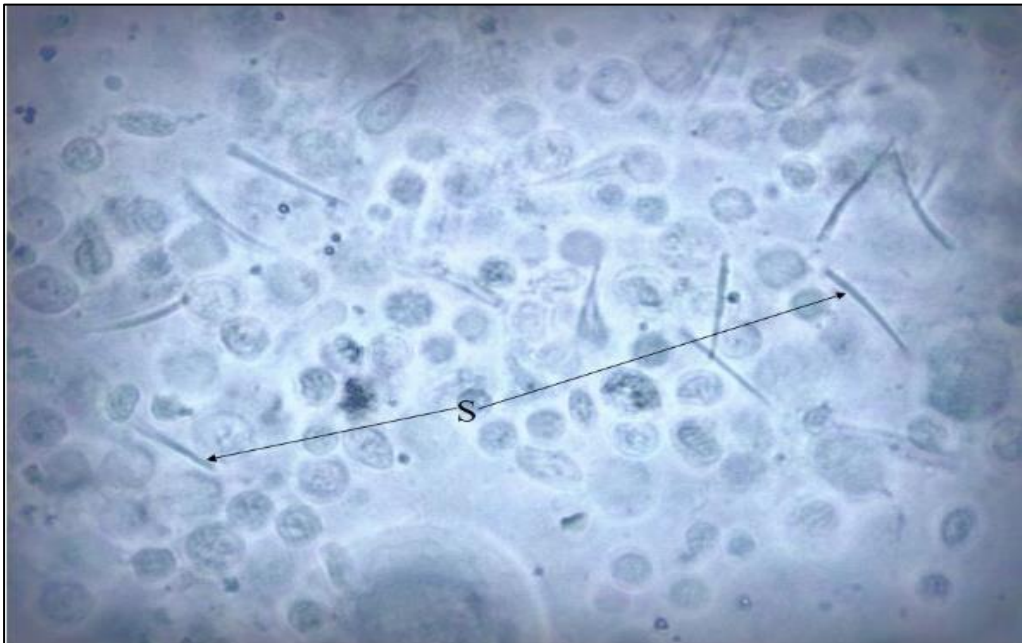
Figure 1. *Corbicula fluminea* sperms extracted from the gonad (400x magnification). S=Sperm

Figure 2. Gonad smear showing abundant blastulae (400x magnification). GE= Gelatinous envelope

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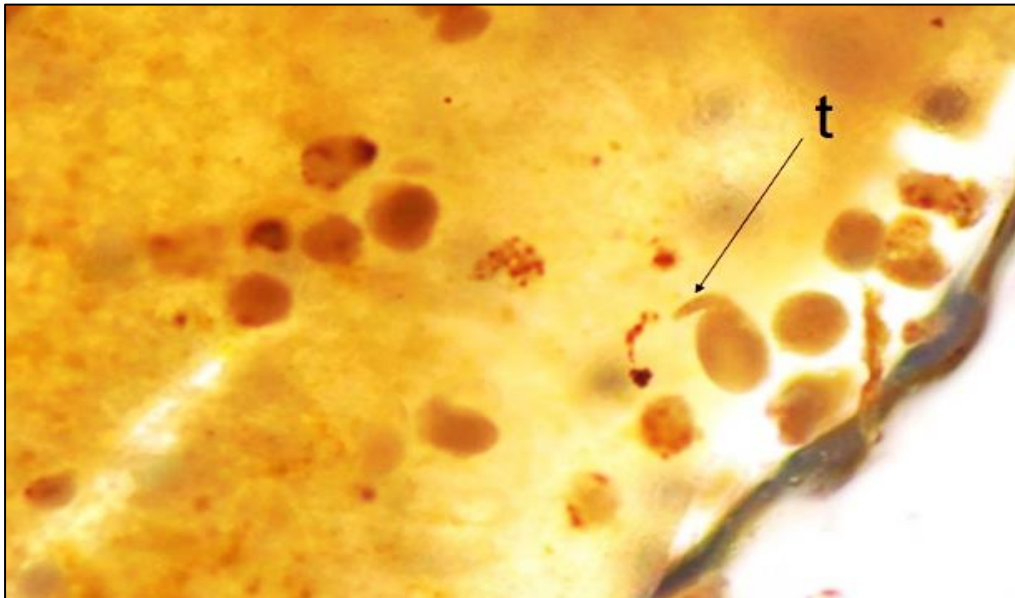
Figure3. Trochophore larva of *Corbicula fluminea* developed in innerdemibranch and apical tut (t) (400x magnification).

Early trochophore larvae developed after 14 h (Fig. 3). Cilia were not visible at 14 h on trochophores removed from parental gills, liberated from the gelatinous envelope, and suspended in water. However, particles moving in currents around the larvae were observed. After 17 h, tiny cilia covering the apical surface were visible, and by 18 h, the larvae's whole surface was covered with cilia. After that, trochophores were motionless while retained on the innerdemibranch. After 20 h, trochophores formed an apical ciliary tuft (t), which looked like a spike (Fig. 3).

Figure 4. The early straight-hinged (veliger) larva of *Corbicula fluminea* 36 h after spawning bearing a velum (V) (400x magnification).

Early straight-hinged larvae (veliger) were first observed at 42 h after spawning (Fig. 4). At this stage, the larvae are immotile, retained in the gills. In this study, the early straight-hinged larvae were flushed out from the gills of sacrificed broodstocks and suspended in the water. The velum of the D-shaped larvae was seen to be extended (Fig. 4). There was little movement detected, and the veliger could not move against the current. This veliger uses the cilia on the velum to trap microalgae cells. Inner organs were not visible at this planktotrophic stage, and the velum was expected to shed. However, all

veliger were not survived, suggesting that the planktotrophic is the immature larvae that withstand outside conditions.

Figure 5. Late straight-hinged juveniles (100 x magnification). C= cilia

Late straight-hinged juveniles were found as first released by the broodstocks after spawning (Fig. 5). At this stage, the late straight-hinged juveniles bearing a large ciliated foot (C) were found crawling at the bottom of the observation disc. The vela, which sheds during metamorphosis, is missing in late straight-hinged juveniles. Gills and other internal organs were observable at this stage. The broad ciliated foot is utilised to crawl towards the feed particles while anchoring against the water current. During the observation, the late straight-hinged juveniles are actively crawling.
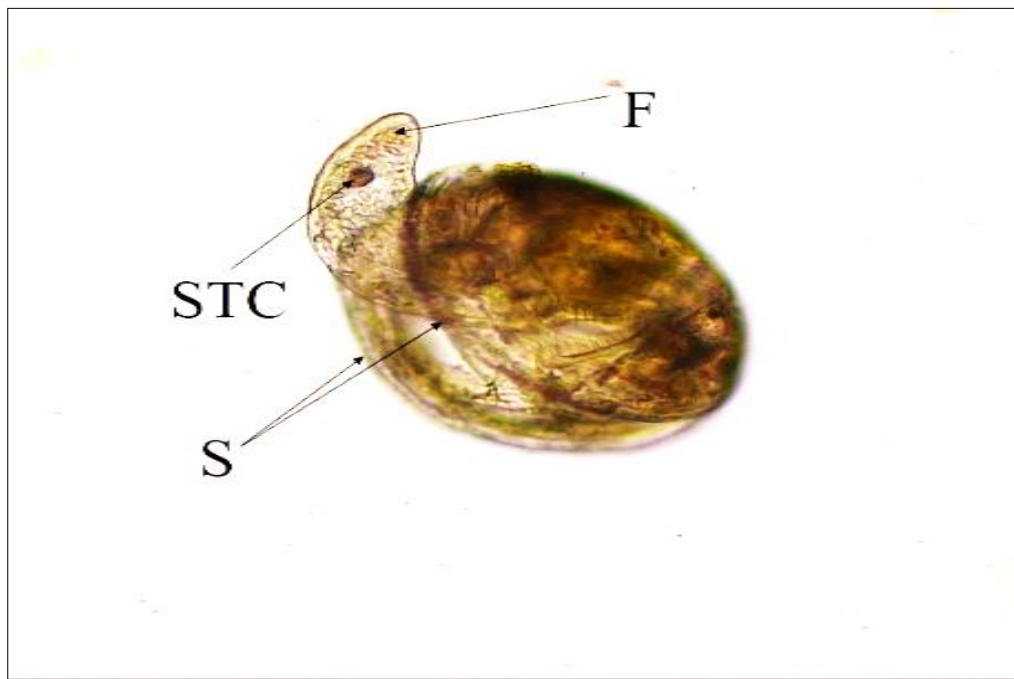
Figure 6. Late straight-hinged juveniles showing conspicuous statocyst (100x magnification). F=foot, STC=Statocyst, S=shells.

The juvenile of *C.fluminea* grown in the rearing chamber is shown in Fig. 6. The juveniles' movement is characterised by the opening of their feet (F) and shells (S). The statocyst (STC) is visible on the juvenile's foot. The STC aids the juveniles to stay oriented and balanced during swimming. The

STC acts as a sensory receptor, allowing the foot to approach the feed particles. At this point, microscopic villi can still be seen, and the ingestion feed mechanism is similar to that of late straight-hinged juveniles. The juvenile is sensitive to the stimulus. Due to the high-water current-like suction, the juveniles' shells closed for a while, and they drifted in the suction direction. This mechanism explains the easy dispersion in the natural water bodies.
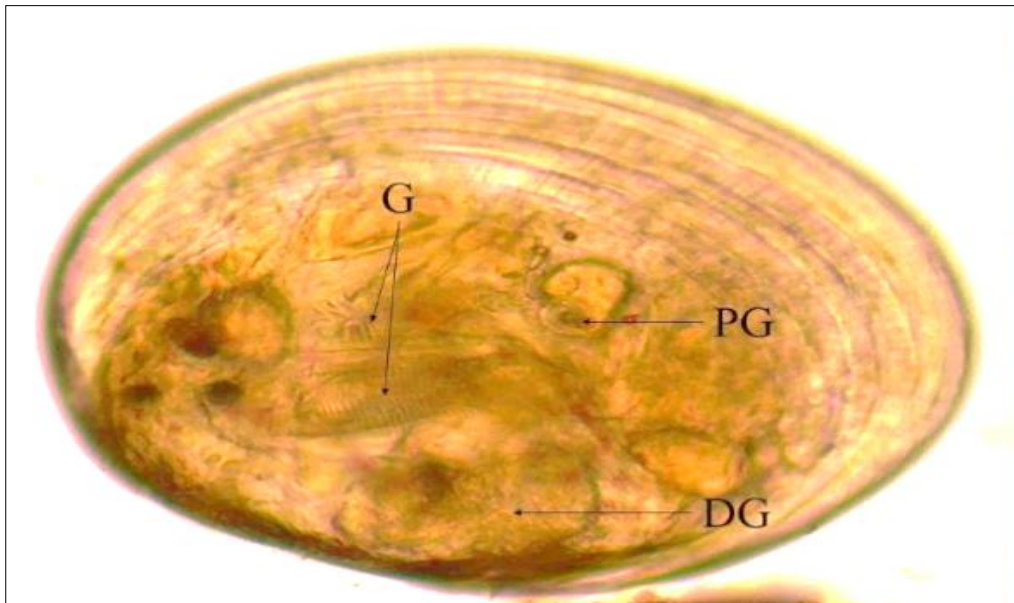
Figure 7. Late juvenile showing conspicuous double-looped gills, pedal ganglion, and digestive organs (40x magnification). G=Gills, PG=Pedal ganglion, DG=Digestive.

The late juvenile of *C.fluminea* grown approximately two months showed the shell ridges. This juvenile has well-developed a digestive system (DG) and respiration organ (G=gills). In Fig. 7, pedal ganglion (PG) could be seen under transparent shells responsible for the foot movement. Internal organs such as gills and digestive organs, and shell ridges become visible at this phase. Late juveniles are less active than earlier stages and rarely extend their foot to move. Late juveniles allow water to enter the mantle by opening the shell valves. The labial palps use this mechanism to catch and filter the feed particles for ingestion. The aperture frequently excretes pseudofaeces.
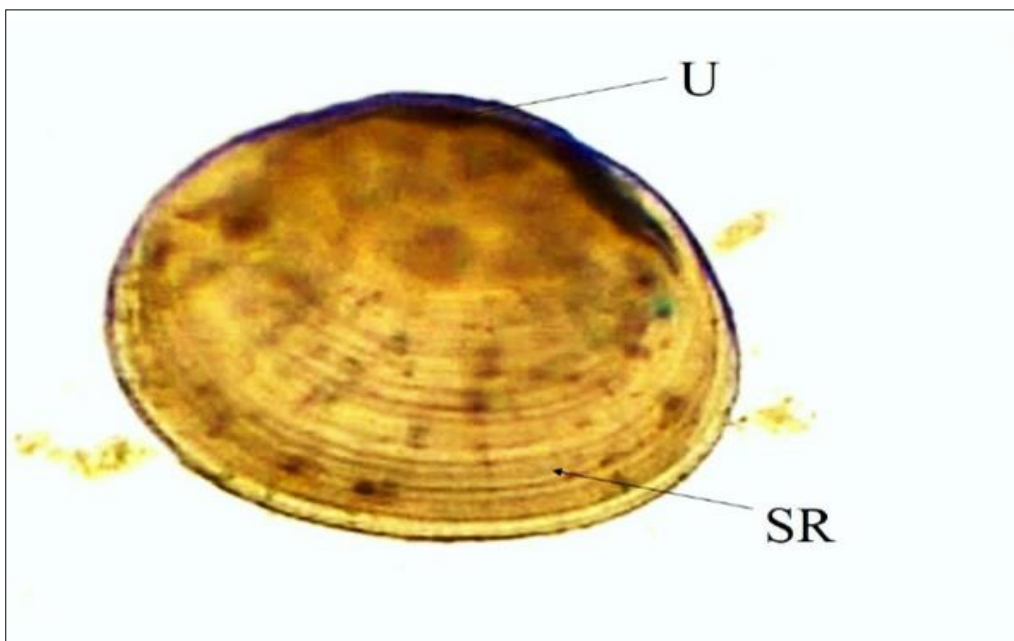
Figure 8. Umbonal juveniles of *Corbicula fluminea* (40x magnification).

The calcified shell of the juvenile is impervious to observe the organs inside. This umbonal juvenile embedded themselves in the sediment. Fig. 8 clearly shows the shell ridges (SR) and umbon (U) of the shell. At this stage, the juveniles rarely extend their foot to move. During the observation, the juvenile

remained still and closed the valves. The movement activity of the juveniles gradually decreased as their size approached one mm. The juveniles easily flow with the water current when there is no substrate. The juveniles died as a result of extensive valve closure.

DISCUSSION

Hatchery-produced seeds were successfully produced by inducing the rearing broodstocks by integrating temperature, salinity, and gamete suspension. This methodology was adopted from the natural conditions where the temperature and salinity cued the fertilisation and spawning of the *Corbicula* (Kimura, Soutome, & Sekiguchi, 2004; Rajagopal *et al.,* 2000)*.* In the development history of *ex-situ*-produced seeds, King *et al.* (1986) employed temperature and salinity as the breeding parameters for *C.fluminea* reproduced in laboratory conditions. These two natural physical parameters critically affect the aquatic animal. These parameters influence the ingestion rates, feeding, respiration, metabolic activity, growth, gametogenesis, and reproduction (Xiao *et al.,* 2014). A present study employing salinity in range 0-8 ppt which the *C.fluminea* is tolerated to that range. Other studies advocate the salinity tolerance for this freshwater species as high as 24 ppt (Aji, 2011.). Recent observation shows the *C.fluminea* remains cessation and lack activities as the increasing of salinities over the range. This indicates the salinity limiting the distribution of the *C.fluminea* in brackish areas. Similarly, the salinity was found to affect the physiological process of the estuarine species, such as survival, osmolarity of the hemolymph, tissue water content, and have other sublethal effects (Xiao *et al.,* 2014; Matsuda *et al.,* 2008). On the other hand, additional gamete suspension into the breeding tank escalated the fertilisation time in a recent study. The reproductive biology of *C.fluminea* classified them as hermaphrodites, with mature sperms found in any individual, as shown in Fig.1. Though, in a more recent finding, *C.fluminea* was identified as hermaphrodites where the androgenesis dominated the reproductive biology (Komaru, Yamada, & Houki, 2013). Therefore, results from these studies were varied due to different periods of examination like in a recent study, and all the sacrificed individuals contained matured sperms and eggs.

The previous study had integrated temperature, mechanical, and salinity shocks to stimulate the spawning of the *C.fluminea* in laboratory conditions (King *et al.,* 1986). Sperms and eggs of the *Corbicula* in different degrees of detail. For instance, the sperms of the *C.fluminea* were found comprised of the conical-headed with biflagellate (Gomes *et al.,* 2016; Lee, Siripattrawan, Foighil, & Ituarte, 2005). Meanwhile, in the earlier study, King *et al.* (1986) reported that the American *Corbicula* produced sperms with a spherical head diameter of two µm and bear a single flagellum around 15 µm in length. In a recent study, the sperms were biflagellate and conical-headed, where the length is estimated in the range of 15-18 µm. On the other hand, several reports on the size of *Corbicula* eggs vary from 20 µm to 280 µm (Kimura *et al.,* 2004; King *et al.,* 1986). Unfortunately, all the sacrificed clams (n=10) contained small numbers of rounded eggs during the experiment, estimated in the range of 95-120 µm. Kimura *et al.* (2004) described the eggs released by *C.japonica* was found in spherical with a diameter of 103 µm. During the examination, the gamete sizes and shape variation could be due to species, environmental factors, or different gametogenesis stages. On the other hand, energy supply during gametogenesis is also responsible for determining egg size. In this case, food availability significantly contributes to carbohydrates and lipid as energy sources during gametogenic development.

The fertilisation inside the broodstocks was intricating the observation in early cell division. In an attempt to observe the cell division, a recent finding found the early embryo encased with the gelatinous envelope (GE). It is abundantly extracted from the innerdemibranches of the broodstocks (Fig.2). According to previous work, the brooded embryos were estimated seven h after fertilisation (King *et al.,* 1986). The time sequence of development stages varied, overlapping consecutive stages among the evaluated individuals. Gonads' maturity and brooding period vary, possibly affecting the development stages of the embryo observed recently. Several studies described the embryo of *C.fluminea* developed into trochophore larvae which were estimated around 14 h after fertilisation occurred (King *et al.,* 1986; Kraemer & Galloway, 1986). In a recent study, the trochophore larvae were found 24 to 48 h after fertilisation, bearing a flagellum-like, namely apical tuft (t) projected from the gelatinous envelope (GE) (Fig. 3) which coinciding with the photomicrograph taken by King *et al.* (1986). This apical tuft (t) facilitates the larvae to swim and acts as a sensory function directing the larvae to feed. Further details, the apical tuft is composed of cilia that joint together and remain until reached the pediveliger stage (Britton & Morton, 1982). The apical tuft is significant to the species that released the planktonic larvae without brooding period, usually found in estuarine bivalves (Morton, 1982). Thus, for brooded species such as *C.fluminea,* the trochophore larvae grew inside as marsupial larvae.

During the examination of the broodstock innerdemibranches, the marsupial larvae were teased out from the brooding chambers, and various stages of development were found. For instance, the veliger (Fig. 4) was observed bearing a velum. At this stage, the veliger could not use the velum for swimming, and they were scarcely being found in the water. The premature larvae were observed recently, which indicates the aborted brood due to environmental stress. The newly released juveniles are considered in the planktotrophic stage. The juveniles bearing a ciliated foot and anatomically rounded shells allow them to be well adapted on substrate and water current (Fig. 5). The juvenile was actively moving at this stage, foraging the food using its foot. By locomotion, the juvenile elongates the foot while the cilia around the foot sense and attracts the food particles. Then, the foot is contracted and pulled the shells in the direction. During this stage, the juveniles grew significantly but had low survival during the first 20 d after the juveniles were detected. In a recent study, the poor rearing conditions such as pseudofeces particles attached to the juvenile were not easy to clean due to indistinguishable sizes.

As the juvenile grows, the dark spot on the body and foot, such as statocyst (STC), a bead-like structure, emerges (Fig.6). The statocyst consists of a small sac with sensory cilia that detects the movement of mineral mass (Mackie & Claudi, 2009). In this study, the juveniles move toward and capture the microalgae at their ciliated foot. Besides, the statocyst is significant for maintaining balance, correcting the orientation and sense. It is positioned in the distal semi or the proximal third of the foot. Statocysts are positioned differently as the foot lengthens and differentiates (King *et al.,* 1986). Meanwhile, during the late juvenile stage, internal organs such as gills (G), digestive organs (DG), and pedal ganglion (PG) are observed under the light microscope (Fig.7). The pedal ganglion is a chemoreceptive sense organ (osphradium) connected to the cerebropleural ganglia via nerve fibres that control the foot movement (Mackie & Claudi, 2009).

Furthermore, juveniles at this size could be seen by naked eyes, and they are moving in the water like scattered "tiny whitish dots". In a recent study, these juveniles were transferred into the sand substrates chamber for further growing. The juveniles grew better in substrate conditions compared with none. However, monitoring the growth is complicated due to indistinct sizes between the juvenile and sands grains. The internal organ of the juveniles could not be seen due to the calcified shells impeding the observation while distinct umbonal could be observed (Fig. 8). On the other hand, clear ridges on the shells were observed, and they were inactive once they were settled in the benthic area. Hence, the growth of the juveniles was found at slow rates for growing and settled calcified juveniles (Fig. 8). The time estimation of the sequence of the developmental stages is based on the first observation of each developmental stage. However, there were consecutive stages that overlapped. Developmental time may vary due to water temperature, feed availability, and time-released from the brooder.

CONCLUSION

In conclusion, the present finding discovered the marsupial, planktotrophic and benthic stages of *C.fluminea* seeds produced in captive rearing. The broodstocks were not synchronised spawned, although they were induced. Hence, the recent study would be a reference for propagating this freshwater bivalve

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