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Characterization of the Embryonic and Larval Development of the Mangrove Clam *Anodontia Edentula* (Family: *Lucinidae*)

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Abstract: **The different stages in the development of the mangrove clam *Anodontia edentula* from fertilization up to metamorphosis was described in terms of timing, immediate morphology, and growth including the rearing conditions that lead to the successful completion of cultures. *Anodontia edentula* exhibited the typical bivalve life cycle starting with the trocophore larvae which further developed into the veliger and pediveliger stages before undergoing metamorphosis to form spats. It was established that the larval rearing of this species in the lab is difficult. Most trial cultures stayed for about 5-7 days reaching only the veliger stage. After employing relevant strategies, the cultures were stretched up to 22 days until metamorphosis. This study was able to update literature on the early life history of *A. edentula*. It explored effective management of bivalve larvae cultures for experimental purposes. This study also has direct implications in bivalve aquaculture and restoration Biology. To be able to artificially propagate this species both for food and for the conservation and rehabilitation of mangrove stands, knowledge of its developmental stages as well as the conditions for efficient survival is imperative.**

Keywords: **mangrove clam, *Anodontia edentula*, development, life cycle, aquasilviculture.**

I. INTRODUCTION

As the human population expands, so is the consumption of marine bivalves. These usually resulted to overharvesting of wildstocks and the eventual necessity for artificial culture. The mangrove clam *Anodontia edentula* is never an exemption. Like other bivalves, it also has a potential economic importance in the seafood industry. Mangrove clams are burrowers in the intertidal and subtidal zones. They are known to harbor sulphur-oxidizing bacteria in their gills from which they derive most of their nutrition. *A. edentula* are strategically situated in sulfide-rich anoxic substrates but also gain access to oxygenated seawater through a ventilation burrow or tube. By locating the opening of this burrow, collectors can detect the presence of a buried clam and harvest it non-destructively with a blade or bare hands. In contrast, the indiscriminate tilling of wide mangrove areas can damage mangrove plants. Collectors complained of decreasing clam sizes and numbers (Primavera et.al., 2002).

Several studies (Lebata, 2001; Primavera et.al., 2002; Brissac et.al., 2011; Natan, 2009; Argañosa and Geduspan, 2013; Tanduyan, 2014) have been done on the biology of this species including its role in mangrove ecosystems, social and economic importance. There is no published research so far on *A. edentula's* early life history from fertilization to metamorphosis. In the case of *A. edentula*, the ultimate goal of studying its early life history is twofold. In the face of the pressure of exploitation and destruction of mangrove areas, there is a necessity to establish artificial culture techniques for systematic repopulation and rehabilitation of these ecosystems by restoration biologists. The morphological features of each larval stage must be established to be able to identify this species in plankton samples. Secondly, knowledge on embryonic and larval development as well as the optimal conditions for rearing determines the feasibility of a large scale aquaculture of the animal for food. It is on this light that this study was conducted.

II. MATERIALS AND METHODS

This section describes the most efficient strategies and methods employed that lead to reliable results. It is important to note that several trial and error runs in a discontinuous series of experiments spanning for about 2 years were made to ensure the survival of experimental animals especially the larval stages. Without such perseverance, the experimental cultures could not have reached the metamorphosis stage. This experience implies the difficulty that can be met by researchers who wants to venture into *A. edentula* larval rearing if they are not informed by this study. Nevertheless, the scheme described below was able to produce good results in various trials.

Preparation of Spawners:

Adult clams ranging from 30-50 mm long were bought from local collectors in Guimaras, Iloilo, Philippines. In the lab, they were placed in aerated 30-liter aquaria containing aged seawater at 1 clam/5 liters density. They were fed *ad libitum* of cultured microalgae mix which consists of *Chaetoceros calcitrans, Isochrysis galbana*, and *Nannochloropsis spp.* These clams were considered to be mature and ripe and were cultured for 3 days under laboratory conditions before they were induced to spawn. Some clams were sacrificed to produce gonadal extracts that were used to induce spawning. About 20 grams of gonad material is macerated using a blender in 500ml water. The suspension was filtered using a 180µm sieve and stored in a freezer.

Obtaining Gametes and Fertilization:

Clams taken from the wild were assumed to be reproductively mature. Each clam was placed in 350ml plastic bowls for easy observation during the spawning process and to prevent mixing of gametes. The gonadal extract was raised to approximately 30-35 ^{0}C by submerging the container bottle in lukewarm water before they are injected at 0.5ml in the mantle cavity of each clam. The spawning bowls were observed for spawning activity. Generally, they spawn within a period of 1 hour after injection. A somewhat fine, milky suspension and a coarse clumpy suspension would indicate sperm and eggs released respectively. Measurements were done using a stage micrometer calibrated on the computer screen for measuring captured pictures of gametes and larvae. Sperm and egg densities were also determined using a hemacytometer for the latter and Sedgwick counting chamber for the counting of eggs. All released eggs are mixed in a 10-liter aquarium and maintained at a density of 80,000 cells/L. Sperm densities may vary but it was not considered a significant factor to maintain it to a specific value since they are very small and will unlikely crowd the culture vessel. Sperm suspension from various spawning bowls was also mixed and about 500ml is poured into the aquarium and mixed thoroughly with the eggs using a stirring rod. Time was noted as the start of fertilization.

Monitoring of Development:

Sampling for the monitoring of embryonic development is done by mounting 0.5 ml of developing embryo and larval suspension in a depression slide and observed under LPO. Documentation of the embryonic and larval stages of *A. edentula* larvae was done using a video microscope attached to a PCI TV card in a desktop computer. Video clips and snapshots were taken at significant time intervals based on the observable morphologic changes on the computer screen. Starting with the trocophore larvae, measurement of larval length and width was regularly done using a stage micrometer calibrated on the computer screen. Larval stages were fed with a mixture of *Chaetoceros calcitrans* (50,000 cells/ml) and *Isochrysis galbana* (33,000 cells/ml). Larval rearing usually lasted for about 19- 21 days. A circular vinyl disc previously conditioned in seawater tanks was fitted at the base of each culture aquarium. This disc served as the settlement substrate when the pediveliger stage was reached.

III. RESULTS

Relevant Issues Observed and Resolved:

Spawners cultured in the lab prefer *ad libitum* feeding with mixed microalgal species. The spawners seemed to be stressed and their spawning becomes unpredictable if they are feed with only one type of microalgae at a specific density as recommended by some literature. Serotonin was initially used to induce spawning but gonadal extract was also tested and was found to be equally effective. Raising the temperature of the gonad extract a few degrees higher than the culture environment was observed to make spawning faster. After spawning, literature suggests that eggs are washed and filtered to remove debris or lower bacterial and protozoan load. It was however observed that the viability of the eggs is affected as the fertilization rate is lowered and some eggs are physically damaged. The success of the experimental runs depends on the number of successfully developing eggs thus it was maintained that they are no longer physically agitated after

spawning. Spraying gonadal extracts to the spawning bowls can also induce spawning but it was decided to directly inject it into the mantle cavity. During embryonic development, it is best that the culture container is not aerated as this can cause deformities in the developing embryos. Aeration can only be introduced after a significant amount of motile larvae are produced. This is also the time where microalgal food can be introduced. Larvae prefer relatively small-sized algae. In this study, *C. calcitrans* is preferred over *I. galbana*.

One of the difficulties encountered in rearing larvae was protozoan infestation and bacterial contamination. All seawater was initially UV-treated to kill potential pathogens but significant mortalities from poor water quality remained. These organisms may be introduced as early as fertilization probably from spawners and microalgal cultures. Constant washing and changing water was done every 24 hours but with little improvements. The use of antibiotics streptomycin and oxytetracycline was effective in decreasing the mortalities. It was however felt that antibiotic use must be avoided. Eventually it was hypothesized that new untreated seawater is better than UV-treated seawater because it also contains good bacteria that maintains the health of the seawater. These bacteria are killed by UV light. Using fresh seawater improved the condition of larval culture containers but there are times where inconsistencies are observed. Aging the seawater for 2 weeks was found to produce consistent results. It is hard to hypothesize at this point as to how aging seawater maintained the health of the culture containers. Interventions were also done on the spawners and the microalgal cultures. Before microalgae is fed to the culture, temperature is lowered to 15-20 degrees Celsius in a chiller for about 3-5 hours. This reduced the appearance of ciliates in the culture containers. The spawners were also scrubbed clean prior to placing them in spawning bowls.

Embryonic Development:

Gonadal extract was able to induce spawning in 13 clams out of the 15 clams injected. Male clams spawn faster than females taking only about 10 to 15 minutes, whereas female clams took about 30 minutes to 1 hour to release eggs. Spermatozoa of *A. edentula* have a hooked shaped head with lengths approximated to be between 5 and 10 µm. Their flagella cannot be seen clearly as they are motile and exhibit erratic movements. Eggs are completely spherical with diameters ranging from 85 to 90 µm. Fertilization is never a problem since almost 98% of the eggs were fertilized. The remaining unfertilized eggs were allowed to remain in the culture container given that it is difficult to separate them from the fertilized ones.

The appearance of a thick fertilization membrane was captured only within a minute after sperm was added into the egg suspension. After another 12 minutes, almost 50 percent of the fertilized eggs released the first polar body which appeared as a protrusion at the egg's animal pole. This timing is an approximate average value as we look at the entire culture as a whole and not the individual fertilization event for every gamete. Table I below shows the timing of the different embryonic stages after fertilization. Data here was transformed to increments of whole numbers.

TABLE I. Approximate Mean developmental timing of the embryonic stages of *Anodontia edentula* after mixing gametes and under laboratory conditions

Stage	Time
Fertilization Membrane	1.2 min
Polar Body	13.3 min
First Cleavage	41.0 min
Second Cleavage	1.19 hrs
Third Cleavage	2.08 hrs
Fourth Cleavage	3.61 hrs
Blastula	14 hrs
Gastrula/ Motile Blastula	22 hrs

The first cleavage (see Fig. 1) occurred 38 to 45 minutes after fertilization. The second and third cleavages occurred about 1 to 2 hours after. Fourth cleavage occurred 3.6 hours after fertilization. The fifth cleavage was no longer timed because it is already hard to distinguish it as a solid or hollow ball of cells since the latter can only be seen clearly if the microscope contrast is moved several times in the microscope. Thus, the observation can only be focused to the blastula stage. The blastula and gastrula stages were reached about 14 and 22 hours respectively.

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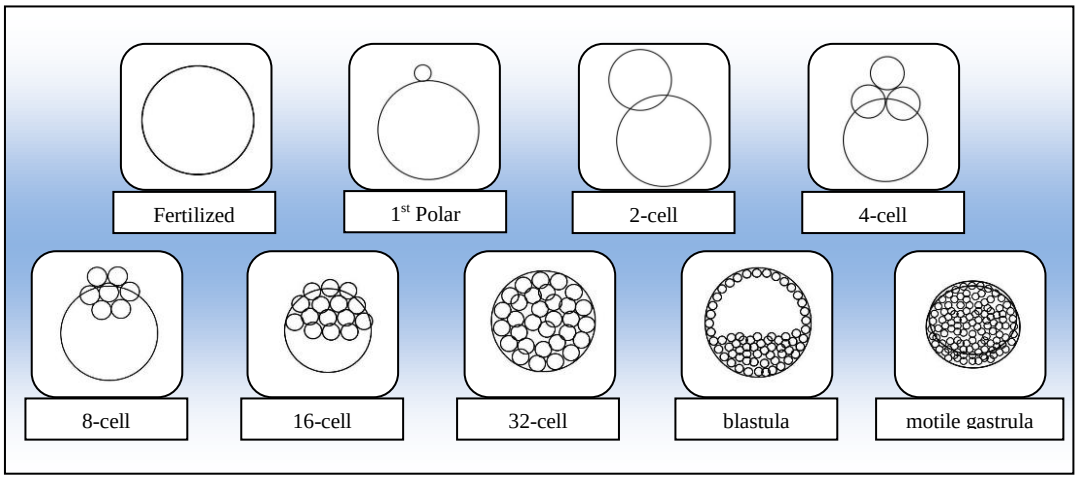

Figure 1. Observed Embryonic Stages of *Anodontia edentula*

Larval Development:

The larval stages shown in Fig. 2 started with the trocophore larva which was observed approximately 26 to 28 hours after fertilization. Straight- hinged D- larvae were obtained 12 hours after the trocophore with mean length and width of 149 x 114 µm. The hinge line is from 95 to 110 µm in length. During this time, the larvae start to feed using a ciliated velum. The D larvae lasted for about seven (7) days. The development of the umbo (note the protrusion at the hinge) was apparent after 10 days with mean size of 180 x 147 µm. The eyespot which represents the internal organs is prominent at this stage up to the remainder of the larval period. The foot was observed after 16 days indicating that the pediveliger stage was reached. There is a considerable slowing down of movement during this period. The larvae still use its velum for swimming and feeding but the foot allowed the larvae to crawl on the floor of the culture container and depression slide.

TABLE II. Timing of the larval stages of *Anodontia edentula* after fertilization

Stage	***Time***
Trocophore	26 hrs
Straight-hinged Veliger	38 hrs
Umboned Veliger	10 days
Pediveliger	16 days
Early Juvenile	22 days

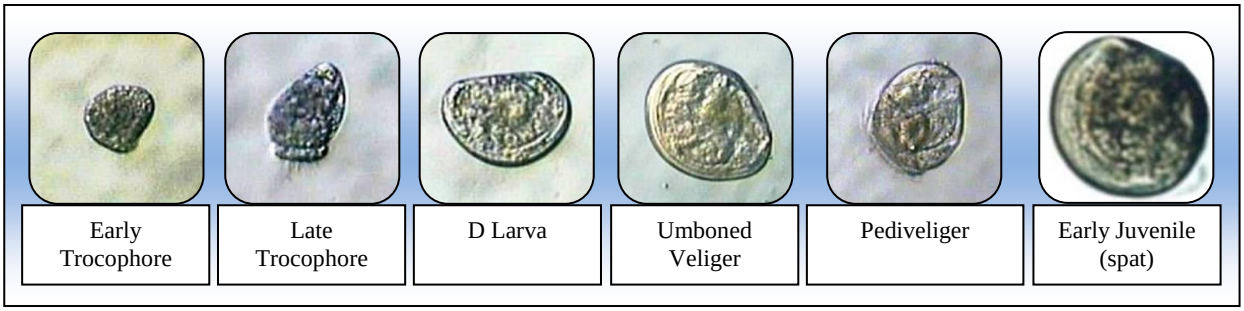

Figure 2. Observed Larval Stages of *Anodontia edentula*

Metamorphosis occurred after 19 to 22 days. Mortality was almost 99% indicating failure, at least numerically. This is however normal like in their natural habitats. The early juvenile (spat) above measures 248 x 270 µm. The cultures were terminated after the spats died the next day. Perhaps the seawater cannot support the mineral requirements needed by the spats. The biofilms which may be essential to the survival of the spats in the settlement discs may also have collapsed after they are transferred to the culture containers. It is advised that immediately after metamorphosis, the settled juveniles in the settlement discs are transferred back into the flow-through system where the discs were previously conditioned.

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Table III below represents the different morphometric measurements in µm of the different stages. Eggs measured approximately 85 to 90 µm, whereas sperm is around 5 to 6 µm. Take note that during embryonic development there is no observable increase in size. The developing embryo is still almost the size of the ovum. A significant increase can be observed after the larval stages are reached when the larvae starts feeding (Fig. 3 and Fig. 4).

TABLE III. Relative Mean Measurements of the Larval Stages of *Anodontia edentula*

Stage	*Mean (µm) ± SD*
Egg	86 ± 4.2
Spermatozoa	5.0 ± 0.7
Trocophore	L:140; W:86 ± 3.5 x 2.2
Straight-hinged Veliger	L:149; W:114 ± 7.4 x 2.2
Umboned Veliger	L:180; W:147 ± 6.1 x 8.4
Pediveliger	L:187; W:163 ± 9.7 x 12.0
Early Juvenile	L:248; W:237 ± 17.6 x 5.8

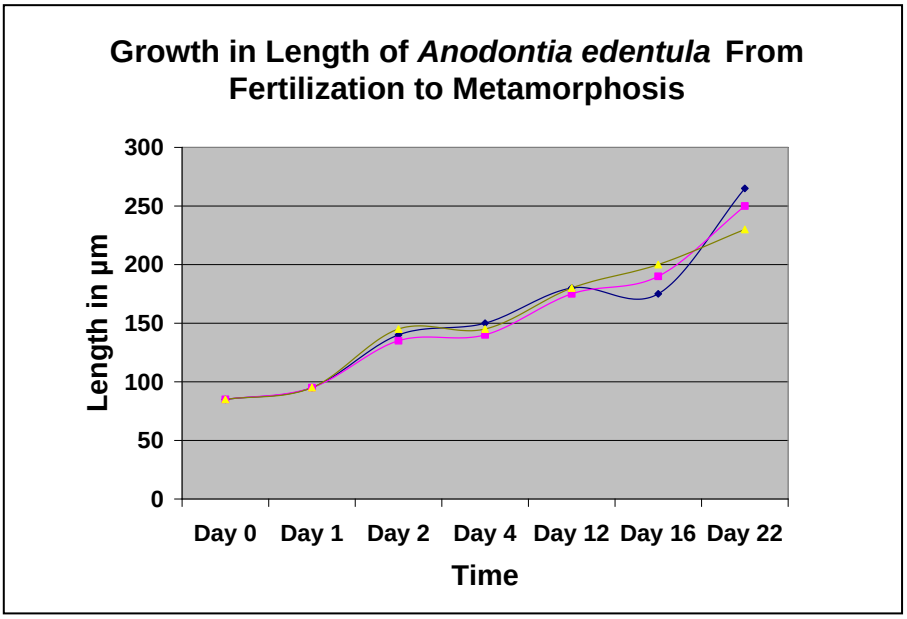

Figure 3. Growth Curve (in length) in 3 Runs

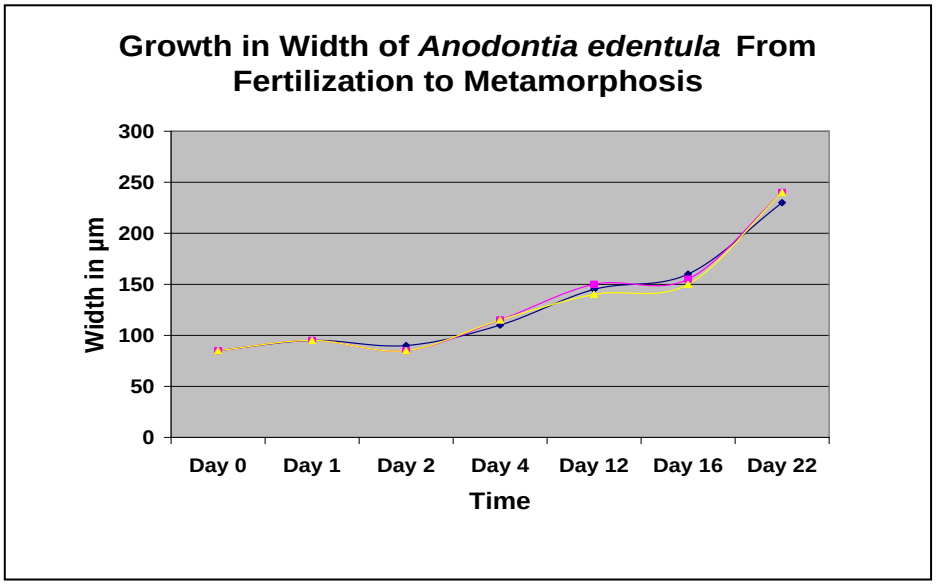

Figure 4. Growth Curve (in width) in 3 Runs

IV. DISCUSSION

Although, some literature (eg. FAO, 2004) implicated the use of serotonin and its probable adverse effects on gamete viability, no such effects were observed in *A. edentula*. Almost 98% of the eggs were fertilized and undergo successful embryonic development. Sexual stimulation using gonad extract was also tested. This is accomplished by introducing extract of ripe gonad either into the medium where the broodstock were immersed or directly into the gonads. The present of gamete in the water obviously provides a stimulus that triggers a spawning response in *A. edentula.* According to Heslinga (1990) ripe gonads contain pheromones that induce other clams to spawn. When clams release sperm or eggs, others are likely to follow to spawn which is called epidermic spawning. A simple squirt of gonads extracted from a sacrificial clam into the incurrent siphon of sexually mature clam is adequate to induce spawning. In this study, it was injected directly into the mantle cavity. Raising the temperature of the extract a few degrees higher than the spawning bowls was observed to make spawning faster.

The morphological features of *Anodontia ed*entula eggs and sperm are similar to all other bivalves in general. The spiralian pattern of cleavage is also typical of what has been observed in other bivalve species (Kin, Kakoi & Wada, 2009). The timing of developmental stages during embryogenesis may vary between various species but all-in-all complete the transition from fertilized egg to trocophore larva occur within 18–24 h after fertilization. This relatively rapid rate of embryogenesis is a characteristic of most tropical oviparous oysters (Kakoi et al., 2008). In contrast, temperate species generally require 32–48 h to reach D-stage (Loosanoff & Davis, 1963; Dinamani, 1973). Since electron microscopy was not used, this study is only limited to characteristic changes in shape and obvious features. Specific details on embryogenesis, gastrulation, and eventual shell formation is hard to observe. There is an observable change in shape during the trochophore stage of *A. edentula*, suggesting it is a common phenomenon in the *Lucinidae*. Doroudi & Southgate (2003) observed that *Pinctada margaritifera* trochophores extend along their longitudinal axis, resulting in the anterior region becoming broader than the posterior region. There is a similar transformation from ovoid to conical in *A. edentula*. Early shell material compresses the trochophore laterally, giving rise to a distinctive heart shape for the few hours preceding D-stage.

A larval period of 18–24 days is common in many oviparous bivalve species, regardless of latitudinal distribution (Southgate & Lee, 1998; Kakoi et al., 2008). The pediveliger stage is quite remarkable as it has the ability to crawl using its foot or swim. During this stage, it is hypothesized that it is growing and storing energy in its biomass in preparation for settlement or metamorphosis stage. Perhaps the crawling behavior is already a form of assessing the substrate for future benthic existence. Why do the larvae swim so long; or short? Several hypotheses can be formulated to answer this question. Species with feeding larvae usually produce smaller and more numerous eggs than similar species with nonfeeding larvae. Feeding larvae are also usually in the plankton for a longer time than nonfeeding larvae and thus offer greater potential for dispersal. Hypotheses on feeding versus nonfeeding development concern a shifting balance between hazards of a longer planktonic stage on the one hand and the advantages of either growth or dispersal on the other. In a different perspective, the long planktonic periods associated with feeding larval stages are attributed entirely to the time required for growth, and the enhanced dispersal associated with feeding larval stages is ignored. The presence or absence of a suitable substrate for settlement and metamorphoses may also play a role. Whether these arguments are correct or not, it must be expected that larvae grown in laboratory conditions must have a relatively shorter period of larval duration since all necessary requirements are optimized for growth. For *A. edentula*, several aspects of nutrition and larval feeding must be further investigated to test whether the duration of larval stages is indeed environmentally dependent.

The settlement discs offered to the larvae were previously soaked in running seawater to accumulate natural diatoms and biofilms. This method was previously tested by Moss and Tong (1991) for enhancing larval settlement of Abalone *Haliotis iris* on artificial surfaces. Settlement was probably successful with these preconditioned discs. The high percentage of mortality may be attributed to overcrowding during metamorphosis since the surface area of the beaker floor is too small to accommodate the remaining pediveligers (> 8 larvae/ml) in a 1-liter container. Bacterial proliferation follows due to abundance of decaying organic matter which almost ruined the cultures before this study is completed. Also, the spats may be requiring adequate amounts of a limiting nutrient such as calcium to build more shell or sulfur to feed their associated symbiotic bacteria.

V. CONCLUSIONS AND RECOMMENDATIONS

Anodontia edentula is hard to culture in the lab especially to those with little knowledge on shellfish larval rearing. There is a need to make the laboratory conditions especially the nutrient content of the water as similar as those found in the mangroves. Contamination by predatory ciliates and opportunistic bacteria must also be controlled. It is therefore necessary to increase the survival rate of spats after metamorphosis if this species is to be considered for large scale aquaculture or for restocking rehabilitated mangrove stands. This study was able to provide some solutions to some of these issues. Moreover, the growing popularity of aquasilviculture utilizing mangrove areas can really consider the production of this clam in polyculture with other commodities such as fish and crustaceans.

Anodontia edentula exhibited the typical bivalve early life history. Embryonic stages were completed in about 24 hours while larval stages stayed for 22 days. It is recommended that electron microscopy is used in the characterization of embryonic and larval morphology to be able to cope up with technology use and for reliable comparison with the findings of other researchers on other bivalve species. Also, larval rearing in their natural habitat must also be studied. It is not yet known whether the duration of the larval development in the lab is the same as in the wild. This will provide information whether the environment controls how long the larvae stay on the water before settling for benthic existence.

The techniques employed in this study should not only be limited to *A. edentula*. There are a lot of economically important clams in the Philippines that are not yet studied in terms of their potential for artificial culture. More studies are expected in the near future using the findings of this study as basis for developing more efficient strategies.

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