Spawning induction and embryonic development of the clam

Ameghinomya antiqua (King, 1832)

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ABSTRACT. Artificial cultivation increases clams’ availability and is an alternative to the extraction from natural banks. The culture of clams requires species-specific research in the different growth stages, and studies on the effects and interactions of culture parameters are essential to obtain and control the proper development of larvae. This paper aims to compare methods to induce spawning, describe the embryonic development, and compare the effect of different culture densities on the yield of "D" larvae of the "taca" clam Ameghinomya antiqua. Breeders were collected on the southwest coast of Quinchao Island, Chiloé, Chile. Spawning induction assays were performed comparing different combinations of biological and physical factors. Experiments on the effect of embryonic density in the obtention of "D" larvae were performed, and the embryonic development was described at 11 ± 1°C. The spawning inductions were successfully achieved with the addition of food combined with temperature changes, resulting in the liberation of oocytes with a jelly coat with a diameter of 140 µm. Trochophore larvae were observed at 40 h post-fertilization. The percentage of embryos developed showed significant differences when testing cultures with densities of 20, 40, and 60 embryos mL⁻¹. Experiments with 20 embryos mL⁻¹ density were the ones that obtained a greater number of developed embryos (50%). These results suggest spawning induction with the addition of food and temperature changes with a density of 20 embryos mL⁻¹. This paper describes the embryonic development and technology development for spawning induction for the first time.

Keywords: Ameghinomya antiqua; clam culture; jelly coat; small-scale aquaculture; southern Pacific

INTRODUCTION

In Chilean clam fisheries, Ameghinomya antiqua (King, 1832) represents 72.87% of the national landing (SERNAPESCA 2021). The A. antiqua fishery has shown a sharp decline over time (Jerez et al. 2006, González & Barahona 2007, Jerez & Figueroa 2008) with 43,761 t in 1988 and 11,288 t in 2020 (SERNAPESCA 2022). Authorized artisanal divers carry out clam extraction from the natural banks.

The Undersecretary of Fisheries (SUBPESCA by its spanish acronym) has implemented measures for the recovery of natural banks and the development and diversification of aquaculture activities in management areas (AMERB) and small-scale aquaculture (APE) with native species (Uriarte 2008). A. antiqua has been one of the five selected species to enhance research in the cultivation of native resources.

The Veneriidae, A. antiqua (=Venus antiqua), has separate sexes with two spawning periods in an annual
cycle (Zagal & Hermosilla 2007) with maximum maturity in austral spring and summer (September and February) (Lozada & Bustos 1984). The minimum legal extraction size is 5.5 cm (Jaramillo et al. 2003, SERNAPESCA 2020). Because of the actual status of the fishery, a two-year ban is in effect in the main extraction area, which accounts for 70.96% of the landing (SUBPESCA 2021).

Studies on the cultivation of A. antiqua addressed the development of technology for the mass production of spat and their subsequent fattening in the environment (Bustos & Olavarría 2000, González & Barahona 2007). Currently, there are no commercial cultured clams in Chile; only experimental and pilot cultures with fattening in the natural environment have been carried out (FAO 2016).

The induction to spawning is key in the cultivation of bivalves. Physical stimuli are the most used, among them temperature variation (thermal shock) and biological stimulation using gonadal extracts (Reverol et al. 2004, Helm et al. 2006, Barría et al. 2021). The thermal shock is the less stressful method and induces a successful gametes expulsion (Bustos & Olavarría 2000, Helm et al. 2006, Ng et al. 2009, Rengel et al. 2009, Aranda-Burgos et al. 2014, Castillo-Durán et al. 2015).


The culture density of embryos has been studied in the clam Mercenaria mercenaria, showing that at high embryo densities, the development only reaches the morula stage, and abnormal larvae are obtained (Loosanoff & Davis 1963). Helm et al. (2006) mentioned that for many of the commonly cultured oviparous oysters and clams, embryo culture density could be as high as 50 to 80 mL⁻¹, but 20 mL⁻¹ is considered more suitable. For A. antiqua, Bustos & Olavarría (2000) used an embryo culture density of 40 and 60 mL⁻¹. The pectinid Chlamys asperrima embryos can be incubated at densities up to 100 mL⁻¹ with an effective yield of D larvae at the end of incubation (O’Connor & Heasman 1995).

Identifying the optimal ranges of factors such as density, temperature, and feeding for each species is critical for successful and efficient production (Loosanoff & Davis 1963, Gruffydd & Beaumont 1970, Liu et al. 2010). On the other hand, studying the effects and interactions between physical, chemical, and biological parameters is essential to obtain and control adequate larval development (Reverol et al. 1998, Castagna 2001).

Based on the above background, this study seeks to describe the embryonic development and obtain the technology for the larval culture of the clam A. antiqua, considering methodologies for the induction to spawn and the effect of the embryonic density on the D larvae production.

**MATERIALS AND METHODS**

**Spawning induction of Ameghinomya antiqua**

Breeders (n = 211; mean length = 45 ± 4.2 mm) were collected during austral spring (September-October) from the southwest coast of Quinchao Island (42°29’S, 73°30’W), Chiloé, Chile.

In the Copulhue SpA hatchery, the clams’ maturity was evaluated in a sample before induction by macroscopic analysis of the gonad. Clams were randomly selected, and three clams per sex were used for gonad analysis according to Helm et al. (2006) methodology. An optical microscope (AMSCOPE 40C-200x camera for photographic recording) was used to observe spermatozoa mobility (400x) and the presence of mature oocytes (100x).

Seven methods were sequentially tested for spawning induction using 30 breeders per treatment with three replicates for each one: 1) biological: spermatozoa obtained by gonad stripping were diluted in 500 mL of filtered seawater and added to the trays with the breeders; 2) gradual temperature increase: seawater temperature was increased by 2°C every 30 min from 9 to 23°C using a heater controlled by a thermostat; 3) sudden temperature change: clams were kept in a 9°C tank for 30 min and then changed to a 19°C tank, this sudden change was repeated four times (Bustos & Olavarría 2000); 4) gradual temperature increase plus biological induction: stripped male gonad diluted in 250 mL of filtered seawater was added to the trays. The temperature was increased by 2°C every 30 min until it reached 23°C when an extra 250 mL of diluted spermatozoa were added; 5) dry periods and sudden temperature changes: breeders were kept out of water for 30 min, then immersed at 9°C water and subsequently in 19°C water. This cycle was repeated three times; 6) salinity changes, dry periods, and sudden
Table 1. Assays for the spawning induction of *Ameghinomya antiqua* with biological and physical factors.

<table>
<thead>
<tr>
<th>Induction methods</th>
<th>Description</th>
<th>Aeration</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Biological</td>
<td>500 mL of seawater with spermatozoa at 12°C.</td>
<td>Yes</td>
</tr>
<tr>
<td>(2) Gradual temperature increase</td>
<td>Gradual increase from 9 to 23°C.</td>
<td>Yes</td>
</tr>
<tr>
<td>(3) Sudden temperature change</td>
<td>Sudden change from 9 to 19°C in four cycles.</td>
<td>No</td>
</tr>
<tr>
<td>(4) Gradual temperature increases plus biological induction</td>
<td>250 mL of sperm maceration + gradual increase from 11 to 23°C + 250 mL of sperm maceration</td>
<td>Yes</td>
</tr>
<tr>
<td>(5) Dry periods and sudden temperature changes</td>
<td>Sudden changes of T° from 9 to 19°C + 30 min dry periods (breeders) between seawater changes.</td>
<td>No</td>
</tr>
<tr>
<td>(6) Salinity change, dry periods, and sudden temperature change plus biological induction</td>
<td>One hour in fresh water at 9°C + sudden changes in T° from 9 to 19°C + dry breeders between seawater changes + 250 mL of sperm macerate</td>
<td>Yes, during the time with sperm</td>
</tr>
<tr>
<td>(7) Induction with the addition of food and temperature increase</td>
<td>3 L of microalgae + heater for 12 h up to 18-20°C + without heater with 3 L microalgae for 1 h + cleaning with fresh water + breeders at 11°C with 3 L of microalgae.</td>
<td>Yes, during the time with heater</td>
</tr>
</tbody>
</table>

Effect of embryonic density for obtaining D larvae

After fertilization, the embryos were sieved and placed in a 1 L test tube with seawater filtered at 1 μm. Homogeneous samples of 1 mL were counted in a Sedgwick Rafter chamber under an optical microscope (Amscope).

The experimental densities were selected based on the densities used by Bustos & Olavarría (2020) in *A. antiqua* and those suggested in the literature for oysters and clams (Helm et al. 2006). Three treatments were used in the embryonic densities experiment: 20, 40, and 60 embryos mL⁻¹, each with three replicates. The embryos were placed in plastic containers (HDPE) with 10 L of filtered seawater at 14°C, and aeration stones (13×25 mm) were used with low pressure to avoid water stirring.

Then, after 60 h, the homogeneous samples were taken in 1 L. Samples of 1 mL were taken, placed in a Sedgwick Rafter chamber, and observed under an optical microscope. D larvae and embryos were counted, and their length and height were measured. The D larvae obtained were determined as a percentage of the initial number of embryos in the sample to evaluate the effect of different densities.

Data analysis

A Shapiro-Wilk test was performed to assess normality and a Levene test to check homoscedasticity. The effect of embryonic density on the percentage of embryos developed and D larvae obtained was analyzed with an ANOVA, and the differences between the means with Tukey *a posteriori* test. The same test was applied to the D larval length and height. A *P* < 0.05 was used as a significance criterion for all tests. Data were analyzed using Minitab 18 statistical software.
RESULTS

Spawning induction of *Ameghinomya antiqua*

Table 2 shows the results of the induction experiments. In treatment (1) with biological induction (addition of spermatozoa), 90% of the individuals opened their valves with siphons fully extended, and, in some cases, an active displacement was registered. In addition, the expulsion of pseudofeces was observed.

In the induction with the gradual temperature increase method (2), when rising the temperature, the clams closed the valves for the first 10 to 15 min, then reopened them and extended their siphons. In treatment (3) with sudden temperature changes, the breeders remained with their valves closed throughout the experiment.

On the other hand, when the combination of gradual temperature increases and biological induction with stripped male gonad was tested (treatment 4), the clams remained with their siphons extended during the whole period; additionally, pseudofeces were expelled, and the animals moved actively. In the treatment with dry periods and sudden temperature changes (5), the clams remained closed for the first 25 min. Then, in the last 5 min of the cycle, some individuals began to extend their siphons, which was interrupted when the next cycle started. In the last cycle, 95% of the breeders remained with their valves closed for 30 min.

In the treatment (6) with salinity changes, dry periods, and sudden temperature changes plus biological induction, the clams remained with closed valves during the experiment. The food and temperature variation method (7) produced an abundant spawning (Fig. 1) with a success rate of 99% of the induced breeders. Prior to spawning, the individuals presented fully extended siphons without movement nor pseudofeces expulsion. The clams that initiated the expulsion of their gametes generated coordination with the other specimens that began to spawn. That is, an individual expelled his gametes for a short time and then stopped emitting, and another individual began the partial expulsion of his gametes; after the second individual stopped emitting, the first one spawned again, and so on.

Males emitted their gametes with a continuous flow, forming a white ribbon suspended in the water column. The females expelled the gametes in a continuous grainy white flow that sank to the bottom. Once the gametes were expelled, the water turned completely milky. Hydrated oocytes with a jelly coat had a diameter of 140 ± 50 μm (Fig. 2) and without the jelly coat 90 ± 20 μm. Spermatozoa had a sickle-shaped form, high motility, and a length of 5 ± 0.5 μm.

Embryonic development

The post-fertilization time and size for each development stage is summarized in Table 3. Successful fertilization was indicated by the presence of the fertilization membrane (Fig. 3a) at 20 min after adding the sperm to the water with oocytes. In oocytes with fertilization membrane, the jelly coat is not visible anymore. The first polar corpuscle was observed at 45 min post-fertilization (pf). At 2 h and 15 min pf, the first mitotic cleavage was observed, dividing the egg unevenly into two cells (Fig. 3b).

The second division was seen at 3 h and 15 min pf, forming a tetrad with a macromer and three micromeres (Fig. 3c). After 4 h and 15 min, the third cleavage occurred, forming eight cells, maintaining a macromer and several irregular micromeres (Fig. 3d). The morula was observed at 6 h and 15 min pf (Fig. 3e), with no sign of a macromer. At 12 h pf, the blastula with the blastopore generated by cellular invagination was formed (Fig. 3f), and after 15 h pf, the rotary blastula was observed (Fig. 3g) with slight movements and spun on its axis (Fig. 3h). After 16 h pf, the gastrula (Fig. 3i) stage began, with cilia on its surface, allowing a rotary displacement in different directions. Finally, at 40 h pf, the trophophore larval phase was reached (Fig. 3j). This larva had an oval shape, was covered by cilia, and had a long apical flagellum that allowed high mobility and displacement with spins around its axis.

Effect of embryonic density for obtaining D larvae

A one-way ANOVA showed no significant differences between the three density embryo treatments (F = 0.22; P > 0.80). In the treatment with a density of 20 embryos mL⁻¹, 47.2 ± 6.7% (average ± standard error) of the embryos developed to D larvae. In the treatment with 40 embryos mL⁻¹, a mean value of 48.6 ± 7.2%, and finally, in the treatment with the highest density (60 embryos mL⁻¹), a mean of 43.3 ± 3.1% of embryos turned to D larvae. Figure 4 shows the proportion of D larvae generated at the end of the experiment at a temperature of 14°C.

The mean lengths of the D larvae obtained in the different embryo densities experiments were 121 ± 5 μm for 20 embryos mL⁻¹, 121.6 ± 8.5 μm for 40 embryos mL⁻¹, and 122.6 ± 4.2 μm for 60 embryos mL⁻¹. A one-way ANOVA showed no significant differences between the mean length of D larvae in the three density treatments (F = 0.71; P > 0.49). The height of D larvae also does not show significant differences between treatments (F = 0.65; P > 0.52).
Table 2. Results of spawning induction experiments in *Ameghinomya antiqua*.

<table>
<thead>
<tr>
<th>Types of induction</th>
<th>Observation</th>
<th>Observation time (h)</th>
<th>Spawning</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Biological</td>
<td>Extended siphons and expulsion of pseudofeces</td>
<td>3</td>
<td>No spawning</td>
</tr>
<tr>
<td>(2) Gradual temperature increase</td>
<td>Extended siphons</td>
<td>3</td>
<td>No spawning</td>
</tr>
<tr>
<td>(3) Sudden temperature change</td>
<td>Closed valves</td>
<td>6</td>
<td>No spawning</td>
</tr>
<tr>
<td>(4) Gradual temperature increase plus biological induction</td>
<td>Extended siphons and expulsion of pseudofeces</td>
<td>3</td>
<td>No spawning</td>
</tr>
<tr>
<td>(5) Dry periods and sudden temperature changes</td>
<td>Closed valves</td>
<td>8</td>
<td>No spawning</td>
</tr>
<tr>
<td>(6) Salinity change, dry periods, and sudden temperature change plus biological induction</td>
<td>Closed valves</td>
<td>13</td>
<td>No spawning</td>
</tr>
<tr>
<td>(7) Induction with the addition of food and temperature increase</td>
<td>Extended siphons, without displacement and expulsion of pseudofeces</td>
<td>35</td>
<td>Abundant spawning</td>
</tr>
</tbody>
</table>

**Figure 1.** Spawning of male *Ameghinomya antiqua*.

**DISCUSSION**

Among the methodologies described in the literature to achieve the expulsion of gametes, the most used for clams is the thermal shock with a high percentage of spawning (Bustos & Olavarría 2000, Castagna 2001, Helm et al. 2006, Hadley & Whetstone 2007, Da Costa et al. 2008, Contreras-Guzmán et al. 2014). This methodology has been used with previous conditioning in *Mercenaria mercenaria*, *Ruditapes philippinarum*, *Chione cancellata*, *Ameghinomya antiqua*, *Chionista fluctifraga*, and *Pholas orientalis* with temperature differences between 8-9°C and induction periods of 2 to 3 h (Bustos & Olavarría 2000, Castagna 2001, Ng et al. 2009, Rengel et al. 2009, Aranda-Burgos et al. 2014, Castillo-Durán et al. 2015). The gradual temperature increase has been successful for *Katelysia rhytiphora* and *Mulinia edulis* (Nell et al. 1994, Oliva et al. 2013). In *Tawera elliptica* and *Tivela mactroides* the best results have been achieved with biological induction with gonad extract (Reverol et al. 2004, Barría et al. 2021) and in *Venerupis pullastra* with desiccation (Cerviño 2011). On the other hand, other authors have used a mix of thermal shock, food and gonadal extract, and a combination of thermal shock and desiccation (Hur et al. 2005, Cerviño 2011, Aranda-Burgos et al. 2014, Contreras-Guzmán et al. 2014). They achieved spawning in *Gari solida*, *R. philippinarum*, *Venerupis pullastra*, *Cyclina sinensis*, and *Metrix lusoria*. However, methodology did in this study not have positive results in this study. The breeders of *A. antiqua* had abundant spawning after the stimulus caused by varying the temperature and adding food, a method not previously used in this species. In these experiments,
Table 3. Characterization of the embryonic development of taca clam (*Ameghinomya antiqua*) at 11 ± 1°C.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Post-fertilization time</th>
<th>Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertilization membrane</td>
<td>20 min</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>Formation of the first polar corpuscle</td>
<td>45 min</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>First cleavage</td>
<td>2 h 15 min</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>Second cleavage</td>
<td>3 h 15 min</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>Third cleavage</td>
<td>4 h 15 min</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>Morula</td>
<td>6 h 15 min</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>Blastula</td>
<td>12 h</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>Rotary blastula</td>
<td>15 h</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>Gastrula</td>
<td>16 h</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>Trochophore</td>
<td>40 h</td>
<td>90 ± 10</td>
</tr>
</tbody>
</table>

Figure 3. Embryonic development of *Ameghinomya antiqua* at 100x. a) Oocyte with fertilization membrane, b) first polar corpuscle, c) the first cleavage: two cells, d) the second cleavage: four cells, e) third cleavage: eight cells, f) morula, g) blastula, h) rotary blastula, i) gastrula, j) trochophore larvae. PC: polar corpuscle, B: blastopore, F: flagellum. Scale bar = 30 μm.

the clams were induced to spawn in spring without previous conditioning, and 50% of the embryos developed into D larvae in contrast to Bustos & Olavarría (2000), who obtained an 80% with clams conditioned for 45-60 days. In both experiments, the proportion of sperm/oocyte used was 5:1.

The breeders were not stimulated by inducing spawning with the thermal shock method. These results differed from those of Bustos & Olavarría (2000), who achieved successful spawning with previously conditioned breeders. This difference may be because spawning is dependent on endogenous and exogenous factors (Martínez-Guzmán 2008), such as the initial condition of the breeders (Reverol et al. 2004) and temperature (Vélez et al. 1993). Therefore, Martínez-Guzmán (2008) suggests previous conditioning of the breeders for successful spawning. Many bivalves from cold and warm water habitats require between 4 to 8 weeks of conditioning to reach spawning maturity and disengage from the reproductive periods of the species (Helm et al. 2006). In this study, the breeders were not conditioned, and successful and abundant spawning was achieved. Having two periods of annual spawning, *A. antiqua* may not be conditioned and can be induced to spawn with the gradual increase in temperature and food, thus avoiding the costly hatchery conditioning. Among the exogenous factors, temperature and food availability are key variables in the reproductive cycle of marine bivalves (Freites et al. 2014); in this sense, the coupled effect of these two variables on *A. antiqua* mature breeders led to successful spawning.

The expulsion of gametes in both sexes of *A. antiqua* agreed with the description of Bustos & Olavarría (2000) and Cerviño (2011), and the sex ratio was close to 1:1 as described by Prida et al. (2018). However, the oocytes presented a jelly coat that had not
been described for the species in previous studies (Olavarría et al. 1996, Bustos & Olavarría 2000). The oocytes with the jelly coat have a diameter of 140 ± 50 μm, and without the jelly coat, 90 ± 20 μm, values that are greater than those indicated by Bustos & Olavarría (2000) of 60-65 μm. Within the family Veneridae, species without gelatinous cover have similar oocytes diameters (Table 4). The gelatinous-coated species are *M. mercenaria* (Loosanoff & Davis 1963) and *T. elliptica* (Barría et al. 2021) (Table 4), with diameters similar to those obtained in this study.

The jelly coat is an extracellular structure with different functions surrounding oocytes and embryos (Barría et al. 2021). In female gametes of certain species, it prevents the penetration of more sperm (Cerviño 2011), thus avoiding polyspermy (Gosling 2015).

The embryonic development of *A. antiqua* follows the pattern described for bivalve mussels, where the first, second, and third division stages are achieved, followed by the state of morula, blastula, rotating blastula, gastrula, and trochophore (Reverol et al. 2004, Hur et al. 2005, Da Costa et al. 2008, Aranda-Burgos et al. 2014, Contreras-Guzmán et al. 2014, Barría et al. 2021). Nevertheless, in *A. antiqua*, the jelly coat disappears after the oocyte is fertilized, unlike *T. elliptica* (Barría et al. 2021), where this layer disappears in the stage of gastrula-trochophore, and *G. solida*, the jelly coat remains during the complete embryonic cycle (Contreras-Guzmán et al. 2014).

The trochophore larval stage in the present study was reached at 40 h pf with a length of 90 ±10 μm, whereas Bustos & Olavarría (2000) describe that the trochophore larva is observed at 16 h pf with a length of 70 μm at a temperature of 16°C. The development times are similar to those described for *Semele solida* cultured at 22°C and *M. edulis* at 9.8 ± 1.5°C, which reached the trochophore larval stage at 48 and 34 h, respectively (Cisneros & Bautista 2006, Barría et al. 2017). *T. elliptica*, which also has a jelly coat, reaches trochophore larva in a shorter time than *A. antiqua* (Table 4, Barría et al. 2021). However, the length of the larvae is very similar in both species, cultivated under the same temperature conditions. In the case of *T. elliptica*, the experiments were performed at 10.7 ± 0.9°C and in the present study at 11 ± 1°C. In species such as *G. solida*, the trochophore larvae were obtained within 20 to 23 h (Contreras-Guzmán et al. 2014), within 13-14 h in *V. pullastra* (Cerviño 2011), 13 h in *Ruditapes decussatus* (Aranda-Burgos et al. 2014), around 10 h in *R. philippinarum*, *C. sinensis* and *M. lusoria*, and 7 h in *T. mactroides* and *Gafrarium tumidum* (Reverol et al. 2004, Jagadis 2011) (Table 4). The time to reach the stage of trochophore larvae of *A. antiqua* in this study is longer than in the previous ones on the same species or other clams. The temperature at which embryonic development experiments are carried out is relevant (Loosanoff & Davis 1963, Clotteau & Dubé 1993, Castagna 2001, Hadley & Whetstone 2007), and the species mentioned were cultured in temperatures above 19°C. Therefore this could influence the shorter time of embryonic development up to trochophore larva of those species. When performing embryonic and larval development in the case of *M. edulis*, regardless of the season of the year, the time of appearance of the stages decreases at a higher temperature (Barría et al. 2017).

D larvae were obtained in all experimental densities (Fig. 4). According to Helm et al. (2006), the recommen-
Figure 4. Percentage of D larvae obtained at different embryonic densities (20, 40, and 60 embryos mL\(^{-1}\)) and mean larval length ± standard error.

ded densities are 20-40 embryos mL\(^{-1}\) for \textit{R. philippinarum} and 15-25 embryos mL\(^{-1}\) for \textit{M. mercenaria} and \textit{M. arenaria}. In the case of \textit{M. mercenaria}, high densities were tested and 3000 embryos mL\(^{-1}\) did not present developed embryos. However, at 250 embryos mL\(^{-1}\), the complete embryonic cycle was obtained (Loosanoff & Davis 1963). Although there were no differences between the treatments for obtaining D larvae in this study, it is necessary to test higher embryonic densities to obtain a greater number of D Larvae. According to the results, future research should consider the effect of temperature on the embryonic development of \textit{A. antiqua}. In addition, a higher proportion of sperm/oocyte in experiments with higher embryonic densities should be tested to improve the production of D larvae.

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