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ORIGINAL ARTICLE

Broodstock conditioning, spawning, embryonic and early larval development of a novel baby clam species for aquaculture in the southeast Pacific: *Tawera elliptica* (Lamarck, 1818)

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Abstract

Tawera elliptica is a small size (baby) clam with an export fishery in southern Chile. The species is dioecious with external fertilization and without sexual dimorphism. The aim of this work was to optimize broodstock conditioning, induce spawning and describe embryonic development until the early larval stage in a hatchery. Broodstock were conditioned at 3 treatments (with/without sand; water circulation) for 48 days. The clams were fed with a mixed diet of macroalgae, with a daily ration of 250,000 cells/ml. Broodstock response was quantified using condition index and gonad development stages (histological techniques). For spawning induction, physical and biological methods were tested. The best response was achieved in males with physical induction. Histological analysis showed that the water circulation system treatment exhibited the highest percentage of maximum maturity. A fertilization ratio of 500:1 sperm:oocyte was used. The incubation was carried out in 3-L containers with filtered seawater at 10.7 ± 0.9°C without aeration. Observations, measurements and the duration of each embryonic and larval stage were recorded. The oocyte has a prominent gelatinous layer (= jelly coat) that disappears between the gastrula and trochophore stages. The status of the D-larval stage was reached at 107 h post fertilization with a mean valve length of 91.4 ± 8.9 µm. This is the first record of the complete embryonic development of this baby clam until the early larval stage and the conditioning in a water circulation system for 48 days are suitable for the species.

KEYWORDS
gelatinous layer, juliana clam, laboratory conditioning, small scale aquaculture, Veneridae

1 | INTRODUCTION

Tawera elliptica (Lamarck, 1818; MolluscaBase, 2020) is a Veneridae clam, commonly known as juliana clam, inhabits sandy bottoms (Osorio, 2002) and is distributed from Valparaíso (32°S; Osorio et al., 1979; Soot-Ryen, 1959) on the Pacific coast to Mar del Plata on the Atlantic coast (38°S; Carcelles, 1950).

The juliana clam has a maximum length of 40 mm, it is a baby clam, and exports are mainly destined for European markets (Vega et al., 2016). The commercial extraction of the juliana clam began in 2000 (Sánchez et al., 2007). Currently, the fishery is regulated through a management plan and a legal minimum size of 25 mm for the species (SSP, Res. Ex. 1825, 2014; Figueroa-Fábrega et al., 2018; Vega et al., 2016).

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The landing reached a maximum of 9608 t in 2010, registering a subsequent fall with no recovery to date, with 3334 t landed in 2018 (Sernapesca, 2019). Currently, there are several exhausted juliana clam banks, so artisanal fishermen have exploited new banks in the inland sea of Chiloé (Sánchez et al., 2007; Vega et al., 2016). The development of culture at a commercial level for this and other species of molluscs may be an alternative to traditional extraction fishing from natural banks and, on the other hand, would strengthen small-scale aquaculture (SSA) in Chile (Abarca et al., 2012; Oliva et al., 2014). The SSAs play an important role in the diversification of aquaculture, being part of a sustainable development strategy and generating a significant number of jobs, especially in rural areas (Wurmann-Gotfrit, 2008). For the development of SSA, it is essential to have seeds from the wild or hatcheries (De la Fuente et al., 2015). Vega et al. (2016) working on the principal natural banks in the inner sea of Chiloé Island did not register the presence of recruits under 10 mm; therefore, the gathering of seed from the wild is not a viable alternative and it is necessary to develop the technology to produce seeds in hatcheries.

At a global level, efforts are being made in the cultivation of molluscs to meet the world demand for seafood (da Costa et al., 2008; Helm et al., 2004; Lovatelli et al., 2008). In order to be successful in cultivating marine species, it is necessary to know the optimal environmental conditions for each species, so knowledge of the initial stages of development is crucial (Castillo-Durán et al., 2013; Da Costa et al., 2008; Helm et al., 2004; Reverol et al., 1998). The study of reproductive cycles is an essential part in the knowledge of the biology of a species, and the determination of this cycle provides valuable information for fisheries management and the development of technologies for the cultivation of each species (da Costa et al., 2012a; Gosling, 2003; Martínez-Guzmán, 2008).

Tawera elliptica is a dioecious species, without sexual dimorphism and with external fertilization (Lozada et al., 1991). It presents a continuous asynchronous or mixed reproductive cycle, without an evident period of gonadal rest (Jerez et al., 1999; Lozada et al., 1991; Sánchez et al., 2007). Successive samplings in different banks have shown continuous gametogenic activity throughout the year (Jerez et al., 1999; Lozada et al., 1991; Sánchez et al., 2007); however, the evacuation of gametes would occur in specific periods (April–May, July–September and November–January). In its southernmost distribution (>54°S), the gonads are mature throughout the year and a main spawning is recorded during November (austral spring) in the Ushuaia area in Argentina (Lomovasky et al., 2005; Morriconi et al., 2007).

When spawning periods in the natural environment are restricted to a short period of time, it is recommended to condition the broodstock to have larvae for a longer time range for productive crops. Conditioning is understood as the set of treatments applied to the breeders to achieve sexual maturity outside their natural cycle (da Costa et al., 2008; Helm et al., 2004; Utting & Millican, 1998) and involves the maintenance of stable temperature, salinity, feeding and photoperiod conditions in order to enhance or accelerate gametogenic development (da Costa et al., 2012b; Helm et al., 2004). The first spawning in a laboratory was carried out by Trigo (2013), which conditioned breeders for 18 days. However, a low number of embryos were obtained, and they did not reach the stage of type D veliger.

To achieve the first stages for the culture of *T. elliptica*, the following objectives have been set: (a) to determine the effect of different conditioning treatments in adult specimens of *T. elliptica* and (b) to describe the embryonic and early larval development of this species.

2 | MATERIALS AND METHODS

2.1 | Experimental design and sample collection

A total of 3500 *T. elliptica* adults (30.99 ± 2.06 mm) were obtained from a natural bank in Isla Alao, Chiloé Archipelago (42°35′S 73°17′W), using Hookah diving, and transported to the Copulhue SpA hatchery on the Island of Quinchao (42°28′S, 73°31′W) for conditioning. The transfer of the individuals to the hatchery did not exceed 24 h and they were kept in expanded polystyrene (EPS) containers at low temperature (<10°C) until their arrival at the laboratory.

The breeders were conditioned for 48 days between 14 August and 1 October 2017. The treatments to be tested were (a) no sand watertight system (NSWS), (b) sand watertight system (SWS) and (c) sand and water circulation system (SWCS). Each treatment had three replicates with 300 breeders evenly distributed in plastic trays of 59 × 38 × 20 cm. The experimental density was in the range of observed densities in natural banks in Chiloé Archipelago (Jerez et al., 2000; Vega et al., 2016).

In the SWS and SWCS treatments, 5 cm of fine sand was used as substrate. For the NSWS and SWS systems, the seawater was changed every 24 h and maintained with smooth and constant aeration. In the SWCS treatment, the tray with the broodstock was located on top of a 1000-L tank, and an air-lift system was installed to circulate the water. Five per cent of the water in each SWCS replica was changed daily. The different treatments and replicates were fed with 60% *Isochrysis galbana* (var Tahiti), 20% *Phaedactylum tricornutum* and 20% *Nannocloropsis* sp., maintaining a ratio of 250,000 cells/ml supplied once a day. The water temperature in the trays and tanks was recorded daily.

Every 15 days, 10 specimens were randomly selected from each replica and treatment. Five clams were used to evaluate the wet weight and the dry weight of the tissue, as well as to calculate the condition index (CI). Another 5 clams were used for the histological analysis.

In each sampling, the breeders were previously marked with graphite pencil on one of their valves for identification and subsequent monitoring. Measurements were made with a calliper with a precision of 0.1 mm. Prior to the condition index analysis, the clams (sand treatment) were kept in a container with clean seawater to remove all traces of sand to avoid weight reading (Hawkins & Rowell, 1987).

The condition index (CI) was calculated in a sample of 30 specimens, before starting the experiment. The relations of Rainer and Mann (1992) and Matozzo et al. (2012) were used based on tissue dry weight, shell dry weight and shell cavity volume. For this, the soft tissue (meat) was extracted by cutting the musculature in the area of insertion to the valves, and the tissue was left in glass containers to be then gently pressed on paper to remove the excess moisture. An analytical balance with a precision of 0.01 mg (DNA model GF-400) was used to weigh the tissue and valves individually. The soft tissue samples were dried in a Memmert oven (UM-500) at 60°C until constant weight was achieved. The volume of the valve cavity was obtained by positioning both (right and left) on a surface that kept them stable and subsequently adding distilled water into each of them, separately, using a 5-mL graduated syringe (until the water reached the edge). Using the same (dry) syringe, all the distilled water contained in them was withdrawn, the graduation achieved was observed, and the specific volume of each shell (left, right and total volume) was noted. The CIs were calculated according to the following equations:

$$CI_{vol} = (\text{Dry meat weight} \times 100/\text{Shell cavity volume}) \quad (1)$$

$$CI_{shell} = (\text{Dry meat weight} \times 100/\text{Dry shell weight}) \quad (2)$$

The soft tissue (edible meat) yield was evaluated according to the formula used by Mohite et al. (2008):

$$MY = (\text{Wet soft tissue weight} \times 100/\text{Total weight with shell}) \quad (3)$$

For the histological analysis, samples were taken on day 0, 37 and 53, and the clams were fixed in Davidson's for 24 h. The fixative was then removed and transferred to another container with 70% alcohol for 24 h. Subsequently, the second 70% alcohol change was made for a minimum period of 12 h. The samples were processed for routine histology (Humason, 1962) by being dehydrated using an ascending series of alcohols, then rinsed in butanol and included in histological paraffin and finally cut with a microtome. They were stained in 5-µm-thick sections with Harris haematoxylin and eosin (Howard et al., 2004). Slides with individualized reproductive tissue samples were analysed using a Zeiss Axiostar Plus microscope. For the study of the gametogenic cycle, the scale proposed by Brown et al. (1999) was used, which divides the gametogenic cycle into 5 stages (initial maturity IM, advanced maturity AM, maximum maturity MM, initial evacuation IE, total evacuation TE).

2.2 | Spawning, fertilization and embryonic development

For spawning induction, two methods were used (a) physical induction (PI), which consisted of keeping the clams with filtered seawater at 10 µm at a temperature of 15°C for 1 h and then leaving them without water for another hour, repeating this 2–3 times, and (b) biological induction (BI), by adding male gametes to the breeders. Each experiment had constant aeration and food ad libitum. For the physical induction (PI) method, 45 specimens were used per treatment for the case of SWS and SWCS, while only 15 specimens were used for the NSWS treatment because spontaneous spawning was recorded in 2 of their replicates. For biological induction (BI), male gametes obtained through the stripping technique (Helm et al., 2004) of 6 mature specimens were used. Forty-five clams were used per treatment, and sperm was added to each container. The temperature of the seawater was also maintained at 15°C.

Once the gametes were released, the clams were sexed and placed separately in 1-L containers. One millilitre of sample was taken from the female container and the oocytes were counted with a Sedgewick-rafter chamber, subsequently being kept in 1-µm filtered seawater at for hydration until the moment of fertilization. Similarly, a sample was taken from the male container using a Pasteur pipette to count the sperm in a Neubauer chamber and checking that the sperm showed type 3 motility (active movement of the flagellum and effective forward movement; Dupré & Joo, 2006). Fertilization was carried out in 3-L containers of seawater filtered at 1 µm, at room temperature (10.7 ± 0.9°C) and without aeration. Hydrated oocytes were fertilized with a ratio of 500:1 sperm/oocyte. Fertilization and embryonic development were observed with light microscopy, equipped with a graduated eyepiece and an AMSCOPE 40C-200X camera for photographic recording. To establish the percentage of each stage of development, a 1 ml aliquot was extracted and observed in a Sedgewick-rafter chamber. Monitoring was carried out every 30 min in the first 4 h, then every 2 h until the gastrula stage, and finally every 5 h until reaching the stage of the type D veliger larva. The period between stages of development was set when 50% of the sample reached an established phase.

2.3 | Statistical analysis

The wet weight, dry weight and condition index data for each sampling of the 3 conditioning treatments were evaluated to test whether they met the normality assumptions using a Shapiro–Wilks test. For the homogeneity of variances, a Levene test was used. The final conditioning survival data and condition indices were transformed to the arcsine value of the root. To compare the temperatures between the treatments, and the volume of the right and left shell cavity, a Student test was performed, and the homogeneity of the variances was first verified with a previous *F* test. A one-way analysis of variance was applied to the wet weight and dry weight values obtained from each conditioning treatment. If significant differences were obtained, a Tukey HSD test was applied afterwards. In case of heterogeneous variances, a Kruskal–Wallis non-parametric test was performed, and a subsequent Mann–Whitney *U* test was applied for differences between two independent samples. A significance level of $p < 0.05$ was used. STATISTICA 8 (Statsoft) was used for statistical analysis.

2.4 | Ethics statement

The Comité Institucional de Bioética para la Investigación con Animales (CIBICA) from the Universidad de Valparaíso, Chile, approved the experimental protocols (Acta 058-2015) for the Juliana clam, *Tawera gayi*, and informed this decision to the Comisión Nacional de Ciencia y Tecnología (Conicyt).

3 | RESULTS

3.1 | Initial conditions

The initial morphometric parameters for 30 breeders are shown in Table 1. The initial average length of the specimens used for the conditioning experience was 30.99 ± 2.06 mm and their average total weight (with valves) was 9.91 ± 1.61 g. The average fresh (wet) tissue weight was 1.86 ± 0.34 g, with an average meat yield of 19.12%. The weight of the valves in the analysed sample amounted to an average value of 6.57 g, which corresponds to 66% of the total weight. The meat yield of adult specimens of *T. elliptica* ranged from 24.6% to 15.1%.

The female gonad of *T. elliptica* is characterized, macroscopically, by presenting a milky and whitish colour texture, while the male gonad is distinguished by a grainy and beige colour, characteristics that were corroborated by smear and subsequent observation by light microscope.

3.2 | Wet and dry weight of *Tawera elliptica* during conditioning

Table 2 shows the values of the wet weight and average dry weight ±standard deviation (SD) for each of the treatments during the study period. In all the treatments, a decrease in weight is evident between the start and the first test (18 days) of conditioning. The no sand treatment (NSWS; Table 2) is the only one that showed a decrease in the average value of dry weight in two consecutive samplings, also considering the adhesion of microalgae in the valves, which became a permanent dark layer on the shells of clams after 1 week of conditioning.

The SWCS treatment resulted in the highest average weight increase and on days 32 and 48 exceeded the initial value of both wet and dry weight (Table 2). An analysis of variance (Kruskal–Wallis) applied to the values of wet weight after 32 days, detected significant differences between the treatments ($H = 11.92$; $p = 0.026$). The least effective treatment regarding wet weight was NSWS, showing significant differences ($p < 0.05$) from SWS and SWCS treatments on day 32 and from SWCS on day 48 (Table 2). Like the results of the wet weight, all treatments showed a decrease in the dry weight between the beginning of the study and the first sampling (Table 2). Regarding the weight of the dry tissue in the three samplings carried out (days 18, 32 and 48), the SWCS treatment leads to significantly higher values with respect to the wet and dry weights, mainly in comparison with the NSWS treatment.

The SWS and SWCS treatments recovered from the initial weight loss, with a greater increase in weight for SWCS towards the end of the study (Table 2). SWCS showed significant differences in the first sampling in the evaluation of the dry weight of the tissue ($F = 3.24$; $p = 0.049$). At the end of the experiment (48 days), significant differences were detected between SWCS and NSWS treatments ($F = 7.03$; $p < 0.01$).

The average temperature for the NSWS and SWS treatments in trays was 10.77 ± 2.14°C and 11.01 ± 1.85°C for the SWCS treatment. A *t*-test, after an *F* test for variance of two samples, found no significant differences in the temperature of both groups ($t = 0.58$; $p = 0.55$).

Spontaneous spawning was recorded on day 18 of conditioning in replicate 3 of the SWCS treatment, evidenced by the presence of foam in the water and oocyte clouds. Partial spawning was also recorded, associated with gradual and sudden increases in temperatures in the NSWS treatment on days 35 and 37.

3.3 | Condition index

The initial condition index (CI) was 8.22 ± 1.11 for the volumetric index (CI_{vol}; equation 1) and 5.27 ± 0.93 for the gravimetric index (CI_{shell}; equation 2). As recorded with the wet weight and dry weight data, in the first CI_{vol} check, a decrease in values was

TABLE 1 Initial morphometric and gravimetric parameters for the broodstock sample ($n = 30$) of *Tawera elliptica*

	Average	SD	Maximum value	Minimum value	CV (%)
Shell length (mm)	30.99	2.06	34.69	26.93	6.64
Shell height (mm)	25.26	1.78	28.59	21.76	7.04
Shell width (mm)	16.01	1.04	18.02	13.45	6.52
Total weight (g)	9.91	1.61	13.70	6.00	16.24
Meat yield (%)	19.12	2.42	24.66	15.15	12.66
Wet soft tissue weight (g)	1.86	0.34	2.78	1.34	18.09
Shell weight (g)	6.57	1.25	9.13	3.81	19.08
Dry soft tissue weight (g)	0.68	0.10	0.96	0.52	14.83
Internal shell cavity (ml)	4.17	0.71	5.20	2.70	17.00

Abbreviation: CV, coefficient of variation.

TABLE 2 Average values and standard error of wet and dry weight, and condition indices (CI_{vol} and CI_{shell}) for conditioning treatments of *Tawera elliptica*: (a) no sand watertight system (NSWS), (b) sand watertight system (SWS) and (c) sand and water circulation system (SWCS)

		Condition treatments				
Condition/date	**Days**	**UWS**	**SWS**	**SWCS**	***F* value**	***p***
Wet weight (g)						
14-Aug-17	1	1.86 ± 0.34	1.86 ± 0.34	1.86 ± 0.34	–	–
01-Sep-17	18	1.60 ± 0.29 (a)	1.52 ± 0.30 (a)	1.68 ± 0.35 (a)	0.82	0.4479
15-Sep-17	32	1.42 ± 0.33 (a)	1.79 ± 0.21 (b)	1.90 ± 0.43 (b)	*H* = 11.92 (*)	0.0261
01-Oct-17	48	1.59 ± 0.23 (a)	1.86 ± 0.35 (ab)	2.02 ± 0.43 (b)	5.89	0.0056
Dry weight (g)						
14-Aug-17	1	0.34 ± 0.07	0.34 ± 0.07	0.34 ± 0.07	–	–
1-Sep-17	18	0.30 ± 0.06 (a)	0.26 ± 0.06 (a)	0.32 ± 0.07 (b)	3.24	0.0493
15-Sep-17	32	0.30 ± 0.08 (a)	0.37 ± 0.06 (ab)	0.40 ± 0.10 (b)	6.09	0.0048
1-Oct-17	48	0.28 ± 0.04 (a)	0.32 ± 0.07 (ab)	0.38 ± 0.09 (b)	7.03	0.0023
Index 1 (CI_{Vol})						
14-Aug-17	1	8.22 ± 1.11	8.22 ± 1.11	8.22 ± 1.11	–	–
01-Sep-17	18	6.40 ± 0.74 (a)	5.81 ± 0.71 (a)	7.57 ± 1.51 (b)	*H* = 15.92 (*)	0.0003
15-Sep-17	32	5.41 ± 1.10 (ab)	6.21 ± 1.22 (a)	7.06 ± 0.98 (b)	8.38	0.0009
01-Oct-17	48	7.30 ± 1.47 (a)	6.99 ± 1.14 (a)	9.13 ± 1.55 (b)	9.57	0.0004
Index 2 (CI_{Shell})						
14-Aug-17	1	5.27 ± 0.93	5.27 ± 0.93	5.27 ± 0.93	–	–
1-Sep-17	18	4.48 ± 0.92 (a)	4.13 ± 0.86 (a)	5.38 ± 0.98 (b)	7.37	0.0018
15-Sep-17	32	5.20 ± 1.30 (ab)	5.64 ± 0.83 (a)	6.32 ± 1.34 (b)	3.45	0.0410
1-Oct-17	48	4.40 ± 1.10 (a)	4.57 ± 1.21 (a)	5.32 ± 1.07 (a)	2.84	0.0700

Note: The letters in brackets indicate homogeneous groups according to the Tukey HSD test. (*) = Analysis of k samples with the Kruskal–Wallis test and subsequent paired analysis of Mann–Whitney.

recorded for all treatments. The SWCS treatment remained close to the initial CI (7.57 ± 1.51) and was significantly higher than the other two treatments (H = 15.92; p < 0.01). The initial value of the CI_{vol} (Table 2) was only exceeded in the last test of the SWCS treatment, with a value that was considerably higher than the rest of the treatments (F = 9.57; p < 0.01). Regarding CI_{shell}, the SWCS treatment is the only one that exceeded the initial condition index in all samples (Table 2). The SWCS treatment exceeded the initial values with respect to the wet and dry weights and the two condition indices evaluated.

3.4 | Histological analysis on conditioning treatments

At the beginning of the study, 22.2% of the males were in a state of advanced maturity, 61.1% with maximum maturity, 11.1% with partial evacuation and 5.6% with total evacuation. Females presented 50% of advanced maturity, 41.7% of maximum maturity and 8.3% with partial evacuation.

Figure 1 illustrates the state of gonadal maturity according to the five states proposed by Brown et al. (1999). In Figure 1a, the interacinar space and decreased connective tissue show little development of spermatids, and in Figure 1b, an increase in acini and a consequent decrease in the interaction space can be observed. In the maximum maturity (MM) stage, the interaction space is reduced, and the interior of the acini shows sperm covering its lumen (Figure 1c). In the partial evacuation stage (Figure 1d), the acini have irregular shapes with decreased sperm. Finally, in Figure 1e, a disorganized gonad is shown with undefined interaction spaces and with few remaining sperm.

Figure 1f shows ovogonies and previtellogenic oocytes. Oval oogonia can be seen, and the previtellogenic oocytes show a peduncle that determines their pyriform appearance. The advanced stage of maturity (Figure 1g) shows a reduced interaction space with respect to the previous stage and ovogonies and previtellogenic oocytes in various maturation stages. The maximum maturity in females (Figure 1h) exhibits a very reduced interacini space. The interior of the acini is full of vitellogenic oocytes. Subsequently (Figure 1d), in the presence of partial spawning, the acini are disorganized and with an irregular perimeter. As in males, in some sectors there are still complete acini and others are moderately empty. In the total evacuation stage (TE), the acini are more disorganized, with an irregular perimeter and a small size (Figure 1j).

The frequencies of the reproductive states for males and females in the initial condition and for days 32 and 48 of conditioning for

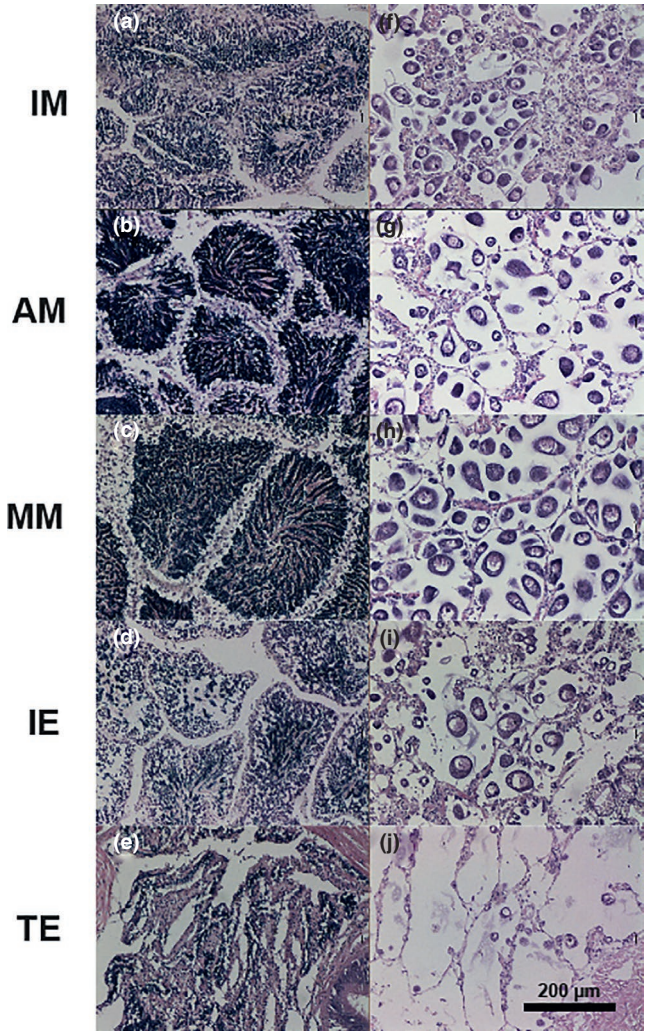

FIGURE 1 Photomicrographs of the gonadal stages for males (left) and females (right) of *Tawera elliptica*. AM, advanced maturity; IE, initial evacuation; IM, initial maturity; MM, maximum maturity; TE, total evacuation

the 3 treatments are presented in Figure 2. The NSWS treatment, at the end of the study, registered a high percentage of specimens with partial and even total evacuation of gametes, while the SWS treatment did not show many differences with respect to the initial condition except on day 32, where it increased to 20% of specimens with partial evacuation, maintaining the maximum maturity fraction at 42.8% on day 48 (Figure 2). Females from the NSWS treatment showed the highest percentage of partial evacuation compared with the other treatments at the end of the period (12%; Figure 2). The SWS treatment showed a decrease in maximum maturity and a 2% increase in the partially evacuated state.

In the SWCS treatment, the maximum maturity fraction in females changed from 41.7% to 87.5% after 48 days of conditioning. In both cases (males and females), a decrease in maximum maturity was observed on day 32, with a rapid recovery 16 days later, with both sexes having a presence of over 70% of gametes at the stage of maximum maturity (Figure 2).

Table 3 shows the survival recorded at the end of the conditioning period. Survival was observed to be high during the conditioning period. At the end of the experiment, survival was 98.6% ± 0.38 in the SWCS treatment, 98.0% ± 0.33 in SWS and 97.11% ± 1.64 in NSWS. An analysis of variance did not detect significant differences between treatments ($F = 1.61$, $p = 0.27$).

3.5 | Spawning and embryonic development

The response to spawning induction was low in both males and females for the 3 groups of conditioned breeders and in both induction treatments (PI and BI). The highest response was achieved with physical induction in the group of males conditioned in NSWS (20%; Table 3). Females showed a low response to the physical inductor, the highest response being 6.7% in the SWCS treatment. Biological induction had a very low response in both males and females (<3%). Both PI and BI generated evacuation of gametes in both sexes for SWCS conditioned breeders (Table 3).

The males were the first to spawn, regardless of the method used. Males expel their gametes with a continuous flow forming a ribbon (Figure 3a), while females expel it in the form of short pulses, expelling groups of oocytes (Figure 3b,c) which then decant at the bottom of the container. Oocytes were observed with a prominent gelatinous layer, hydrating for 30 minutes after being evacuated, with a diameter (including the gelatinous layer) of 92.00 ± 7.21 µm in unhydrated oocytes and 166.54 ± 9.95 µm in fully hydrated oocytes (Figure 3d). The thickness of the gelatinous coating was 39.95 µm in hydrated oocytes. The sperm had motility from its emission to approximately 2 h after being evacuated, and the sperm heads had a length of 5.83 ± 0.72 µm.

It was also observed that prior to the emission of gametes, in both sexes, the siphons presented a vertical position, opening the valves and exposing the mantle (Figure 3b). The angular orientation of the siphons was in favour of the current generated by the air diffuser stone. The female used in this experiment evacuated 574,500 oocytes. Fertilization was verified only until the appearance of the polar corpuscle 15 min after the introduction of the sperm (Figure 4a; Table 4). A total of 405,000 embryos were counted; the gelatinous layer was shown with a large amount of sperm on its perimeter. At 50 min post fertilization (pf), the first mitotic division was observed, segmenting the egg into 2 cells (Figure 4b; Table 4). The second division that gave rise to 4 cells was observed at 2 h and 10 min pf, forming a tetrad with a macromer and 3 µm (Figure 4c; Table 4). After 5 h, the third division occurred, forming 8 cells which conserved the macromer observed in the previous division (Figure 4d). The morula appeared at 10 h and 50 min pf (Figure 4e; Table 4) and the blastula at 16 h; in the latter, the blastopore was clearly visualized (Figure 4f; Table 4). At 26 h pf, the embryo reached the gastrula stage, which had cilia throughout its surface that allowed it to rotate within the gelatinous layer (Figure 4g; Table 4). After 56 h and 50 min, the trochophore larva stage was reached, which had a bell shape surrounded by cilia and

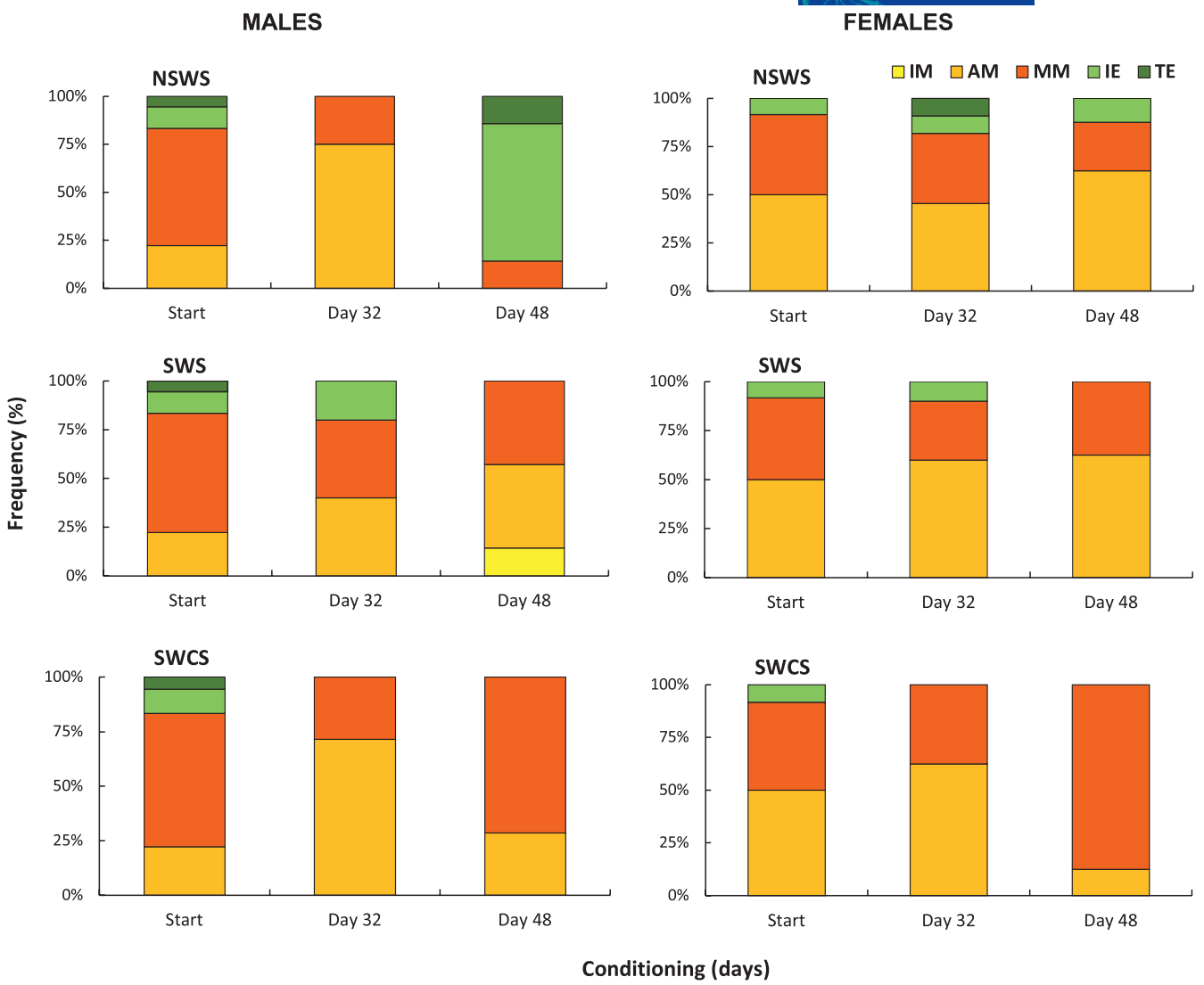

FIGURE 2 Frequency of gonadal stages for males and females of *Tawera elliptica* (AM, advanced maturity; IE, initial evacuation; IM, initial maturity; MM, maximum maturity; TE, total evacuation) in treatments no sand watertight system (NSWS), sand watertight system (SWS) and sand and water circulation system (SWCS)

TABLE 3 Spawning response (%) of males and females of *Tawera elliptica* clams under biological (BI) and physical (PI) induction for each conditioning treatment

			Spawning response (%)							
	N		Male		Female		No response		Survival (%)	
Treatment	BI	PI	BI	PI	BI	PI	BI	PI	Mean	SD
NSWS	45	15	0.0	20.0	0.0	0.0	100.0	80.0	97.1	1.64
SWS	45	45	0.0	8.9	2.2	4.4	95.6	86.7	98.0	0.33
SWCS	45	45	2.2	11.1	2.2	6.7	95.6	82.2	98.6	0.38

with a short apical flagellum (Figure 4h; Table 4). The gelatinous layer disappears between these last two phases, its rupture and the free and linear swimming of the trochophore larva can be observed. The early veliger larva was achieved at 87 h and 20 min, where the initial shell formation was visualized (Figure 4i; Table 4). Finally, at 107 h post fertilization, the stage of the veliger type D larva was achieved, reaching a valve length of 91.43 ± 8.99 µm (Figure 4j; Table 4).

4 | DISCUSSION

This work shows that it is possible to condition broodstock of the juliana clam in a hatchery with a high survival rate (>96%). Under the tested conditions, broodstock with maximum maturity could be obtained in a period of 48 days. Clams did not present external sexual dimorphism and their gametic condition could only be evaluated by observing the gonad.

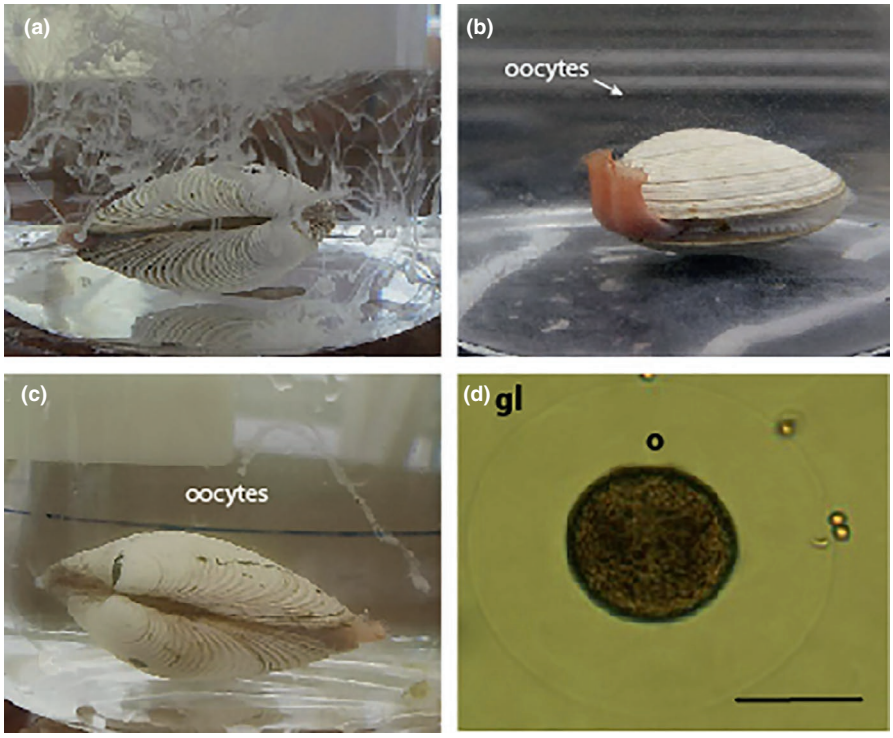

FIGURE 3 Spawning of *Tawera elliptica* under laboratory conditions (a) male expelling sperm, (b) female with vertical position of both siphons prior to gamete expulsion, (c) female expelling oocytes, (d) oocyte with its gelatinous layer, scale bar = 50 µm, gl, gelatinous layer; o, oocyte

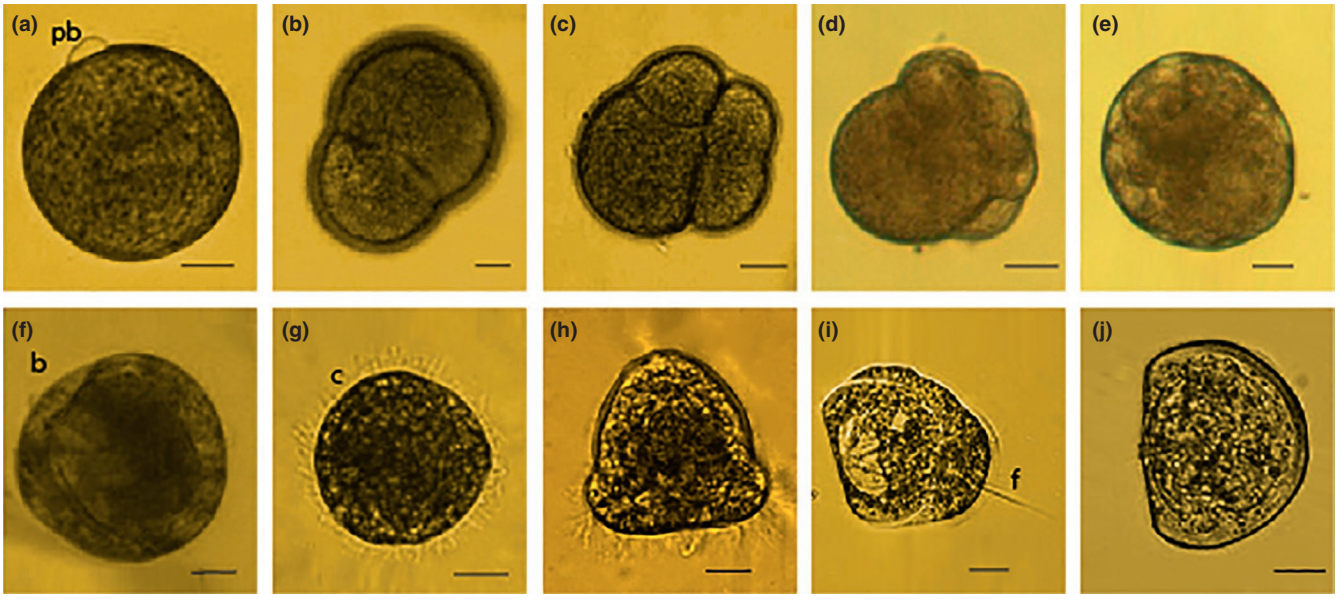

FIGURE 4 Embryonic and early larval development in *Tawera elliptica*. (a) Fertilized egg with polar body, (b) first segmentation (2 cells), (c) second segmentation (4 cells), (d) third segmentation (8 cells), (e) morula, (f) blastula, (g) gastrula, (h) late trochophore, (i) early veliger larva, (j) straight-hinged veliger; b, blastopore; c, cilia; f, flagella; pb, polar body. Scale bar = 20 µm

Several authors have shown that this species has a continuous gametogenic activity throughout the year and that the evacuation would occur mainly between the months of April to May, July to September and November to January (Jerez et al., 1999; Lozada et al., 1991; Sánchez et al., 2007). Morriconi et al. (2007) describe that this species in its southernmost distribution (sub-Antarctic waters) presents a massive spawning episode between November and December and small pulses of evacuation of gametes during the rest of the year. The specimens for this study were extracted at the beginning of August 2017, a date that can be considered as advanced or maximum maturity. The histological analysis carried out at the beginning of this study corroborates this. The 22.2% of males were in advanced maturity state and 61.1% in maximum maturity while the 50% of the females were in advanced maturity and 41.7% in maximum maturity. On the other hand, the collection period of the breeders to be conditioned is relevant since it directly influences the time

TABLE 4 Post-fertilization times from unfertilized oocytes to D-shaped larvae for *Tawera elliptica* at 10.7 ± 0.9°C

Development stage	Time post fertilization (min-h)	Size (µm)
Unfertilized egg	0	66.92 ± 4.7
First polar body	15 min	70.0
2-cell	50 min	76.67 ± 5.8
4-cell	2 h 10 min	76.67 ± 5.2
8-cell	5 h	77.50 ± 4.6
Morula	10 h 50 min	72.00 ± 4.5
Blastula	16 h	70.0
Gastrula	26 h	70.0
Trochophore	56 h 50 min	77.50 ± 7.1
Early D-shaped	87 h 20 min	83.75 ± 5.2
D-shaped larvae	107 h	91.43 ± 8.9

necessary to reach the desirable spawning condition. Matias et al. (2009) presented differences in the conditioning time of *Ruditapes decussatus* depending on the date of collection associated with the differences in the initial gonadal development of breeders. About 14 weeks of conditioning has been described *for R. decussatus* with breeders with gonads at rest, and only 6–7 weeks with breeders with advanced gonadal development (Abbas et al., 2018; da Costa et al., 2020). This is consistent with the conditioning time required for the species *T. elliptica* observed in this study, where in 48 days (about 7 weeks), it was possible to obtain mature individuals for induction, with 70% of breeders at maximum maturity stage for females and males in the SWCS treatment.

The weight loss in both wet weight and dry weight (Table 2) recorded during conditioning in all treatments between the start and first sampling after 18 days may be due to the fact that the specimens were in a state of maximum maturity and that under the stress conditions generated by (a) extraction from their place of origin, (b) transfer and (c) installation under laboratory conditions, partially released their gametes (Abbas et al., 2018; da Costa et al., 2020; Navneel & Azam, 2013).

It should be noted that SWCS treatment has the highest thermal stability, since the temperature is best preserved in a large volume. On the other hand, the availability of food is constant, unlike the other systems where the feeding is by pulses. Stability in these two variables may explain the best conditioning result to this experimental density.

From the point of view of the variation in weight, the SWCS conditioning treatment presents the best results in recovering from the weight loss observed in the intermediate samplings and in exceeding the initial value.

The condition index (CI) is useful to evaluate the nutritional status of bivalves, their commercial quality, as well as knowing their reproduction cycle in general (Baghurst & Mitchell, 2002; Castillo-Durán et al., 2013; Mason & Nell, 1995; Rainer & Mann, 1992).

The condition indices based on the dry weight of the valves (CI_{Shell}) and the volumes of the valve cavity (CI_{vol}) have limitations. The indices associated with the weight of the valve do not take into account the possible changes caused by the thickness depending on the age and growth of the specimens, as in the case of oysters (Rainer & Mann, 1992).

The condition indices, like the wet and dry weight values, show a decrease between the start of the conditioning study and the first sampling, which may be related with a stress response associated with gamete releases after the start of the study. The CI_{vol} presented a more robust result with both the wet weight and the dry weight, mainly for the SWCS treatment. This is consistent with the relationship between increases in CI and gonadal development in clams (Matias et al., 2009).

The survival of the *T. elliptica* specimens exceeded 97% during the 48 days of conditioning. Similarly, Trigo (2013) obtained a survival rate of 99% in conditioning adult *T. elliptica*, also under laboratory conditions. These results confirm that this clam adapts to laboratory conditions, achieving maximum maturity and evacuating its gametes.

The gonadal characterization in *T. elliptica* presented a difference in texture and coloration between the sexes not described for this species. The females presented a milky texture and a whitish colour, while the males presented a more granular and beige gonad. This slight difference in coloration is like the one recently described for the *Ameghinomya antiqua* clam (Prida et al., 2018), where the females presented a whitish gonad and the males more orange gonads. The embryonic and early larval development of *Tawera elliptica* follows the pattern described for bivalve molluscs, with the stages of the first, second and third division, morula, blastula, gastrula, trochophore larva and type D veliger larva (Contreras et al., 2014; da Costa et al., 2008; Gros et al., 1997, 1999; Raleigh & Keegan, 2007). However, *T. elliptica* shows differences in the way fertilization is carried out, because it occurs at the bottom of the container with agglutinations of oocytes and sperm and not necessarily in the water column. The total size of the oocyte (including the gelatinous covering) in *T. elliptica* (166.54 ± 9.95 µm) is similar to that described for *Gari solida* (174 ± 10 µm; Contreras et al., 2014), *Mercenaria mercenaria* (163–170 µm; Loosanoff & Davis, 1950) and *S. plana* (140–150 µm; Frenkiel & Mouëza, 1979; Raleigh & Keegan, 2007) and smaller than *Lucinoma aequizonata* (Gros et al., 1999) and *Codakia orbicularis* (Gros et al., 1997), which have a total diameter of 500 and 350 µm respectively (Table 5). Trigo (2013) records a total diameter of 108 µm for the juliana clam, a lower value for a hydrated oocyte than the one described in this study, being close to a newly evacuated oocyte without hydration (92 ± 5.88 µm). This size difference is probably related to the presence of hypotonated oocytes due to their immaturity.

The fertilization bioassay carried out by Trigo (2013) in *T. elliptica* was at a sperm: oocyte ratio of 10:1, a value lower than the one used in this experiment, where the ratio was 500:1. However, the number of embryos obtained in this study (405,000) was higher than the one obtained by Trigo (2013), who mentions that she only obtained a "low number" of embryos, so she could not obtain the type D veliger stage. In turn, Le et al. (2016) established a sperm:

TABLE 5 Thickness of the gelatinous layer in different clams and observations regarding their early embryonic and larval cycle

Species	Temperature (°C)	Oocyte diameter (µm)	Thickness of jelly coat (µm)	Oocyte diameter without jelly coat (µm)	Time from pf to D-larvae (h)	D-larvae length (µm)	Jelly coat permanence	References
Veneridae								
Venus striatula	–	nd	nd	nd	48	90	Trochophore	Ansell (1961)
Mercenaria mercenaria	22	163–170	50	70–73	44–48	122	Blastula-trochophore	Loosanoff and Davis (1950)
Tawera elliptica	nd	108	30	nd	nd	nd	nd	Trigo (2013)
Tawera elliptica	10.7 ± 0.9	166.5 ± 9.9	39.25	66.9 ± 10	107	91.4 ± 8.9	Gastrula-Trochophore	Present study
Ruditapes decussatus	20 ± 1	–	2	67.4 ± 3.6	26	89.9 ± 6.5	–	Aranda-Burgos et al. (2014)
Tivela mactriodes	25 ± 1	–	–	60.7 ± 0.9	14.5	92.8	–	Reverol et al. (1998)
Semelidae								
Scrobicularia plana	20–22	148.3 ± 8.4	42	64.0 ± 1.4	36–38	94.6	Complete	Raleigh and Keegan (2007)
Scrobicularia plana	16–18	140–150	37	75–80	60	nd	Complete	Frenkiel and Mouëza (1979)
Lucinidae								
Lucinoma aequizonata	10	500	150	200	12 (days)	240	Complete	Gros et al. (1999)
Codakia orbicularis	25–27	350	120	nd	48	160–170	Complete	Gros et al. (1997)
Psammobiidae								
Gari solida	15 ± 0.8	174 ± 10	55	65 ± 3	43–47	96 ± 5	Complete	Contreras et al. (2014)

Abbreviation: Nd, no data.

oocyte ratio equal to a range of 50–500:1 for optimal fertilization in *Panopea zelandica*, a value similar to the one used in this study, validating the good result in the percentage of embryos obtained in this clam versus the 10:1 ratio (sperm: oocyte) used by Trigo (2013). The first specimens to spawn were males, as mentioned by Trigo (2013); however, a longer period of motility was recorded in the sperm, which lasted up to 2 h.

The gelatinous layer present in female gametes in a variety of taxa is defined as a thick sticky gelatinous structure that surrounds or coats oocytes in some species. These layers are extracellular structures derived from the membranes that surround the oocytes and embryos (Shu et al., 2015). These layers present in oocytes from many molluscs include protein-rich layers in their innermost portion (close to the egg/embryo) and can vary as fine or thick gelatinous structures of oligosaccharides. They fulfil multiple functions during the early embryonic stages (Shu et al., 2015), since they mediate fertilization through the recognition of sperm, the initiation of the acrosomal reaction, protection of the embryo, adaptation to desiccation and changes in osmotic pressure in the environment and finally the blockage of polyspermy (Frenkiel & Mouëza, 1979; Gros et al., 1997; Wong & Wessel, 2006). This gelatinous layer would have a similar function to that proposed by Herrler and Beier (2000) for the zona pellucida that induces the oocyte–sperm interaction, the acrosomal reaction and prevention of polyspermy in mammals. The presence of this structure can be found in several families of bivalves, including Articidae (Lutz et al., 1982), Lucinidae (Gros et al., 1997), Semelidae (Frenkiel & Mouëza, 1979; Hughes, 1971) and Veneridae (Ansell, 1961; Loosanoff & Davis, 1950).

The post-fertilization time to reach the stage of type D veliger larvae in the juliana clam was 107 h, which is higher than other bivalve species with the presence of a gelatinous layer, except in the species *Lucinoma aequizonata* (Gros et al., 1999) where the post-fertilization time was 12 days at a temperature of 10°C and where, as in *T. elliptica*, a prominent gelatinous covering is observed (Table 5). This duration varies in other bivalve species, in *Codakia orbicularis;* for example, it is 48 h (Gros et al., 1997); in *Gari solida*, it is 43–47 h (Contreras et al., 2014), 48 h in *Venus striatula* (Ansell, 1961), 44–48 h in *Mercenaria mercenaria* (Loosanoff & Davis, 1950), 60 h (Frenkiel & Mouëza, 1979) and 36–38 h in *Scrobicularia plana* (Raleigh & Keegan, 2007; Table 5). The development time of the veliger larvae in those species is less than the time recorded for the development of the same early larval phase in this study. The temperature during embryonic development is relevant because the incubation temperature was above 15 °C in many of the aforementioned species, which could have influenced the shorter embryonic and early larval development time.

The gelatinous layer has been recorded with different duration times in the embryonic and early larval development of clams. In *Tawera elliptica*, the gelatinous layer is lost between the gastrula phase and the trochophore larva (26–57 h), similar to what is described for the *Mercenaria mercenaria* clam (Loosanoff & Davis, 1950) and *Venus striatula* (Ansell, 1961), where the Gelatinous structure is lost between the blastula phase and the trochophore larva. In other species such as *Codakia orbicularis* (Gros et al., 1997), *Scrobicularia plana* (Frenkiel & Mouëza, 1979; Raleigh & Keegan, 2007) and *Lucinoma aequizonata* (Gros et al., 1999), the gelatinous layer is maintained during full embryonic development and even the embryos rotate continuously within the perivithelial space that has grown until the appearance of type D veliger larvae (Table 5).

Under hatchery conditions, *T. elliptica* is a feasible species for pilot production. It should be noted that the spawned specimen generated 0.574×10^6 oocytes. For other commercial clams such as *Gari solida*, *Ameghynomia antiqua* and *Protothaca thaca*, potential fertility values of 11×10^6, 3.1×10^6 and about 4.4×10^6 of oocytes, respectively, have been described (Lozada & Bustos, 1984; Romero et al., 2011) for sizes over 55 mm of valve length. Fertility in *T. elliptica* should be verified in the future, since it shows that for the sizes used in the fishery (>31 mm), fertility in this species would be low due to its smaller size. Strong catches for these small clams will produce problems in the recovery of natural banks (Vega et al., 2016), so studies focusing on aquaculture of this species would diminish the fishing pressure.

This work is the first study on laboratory conditioning of specimens, induction of spawning and description of early embryonic and larval development in *Tawera elliptica*.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

DO and LRD gathered the funding; DO, LRD, and AA developed the concept of the paper; CB, DO, LRD and AA wrote the paper, CB, APC, DCo and PC performed the experiments; DCa analyzed the histological samples and all authors contributed to manuscript revision.

DATA AVAILABILITY STATEMENT

The data that support this study are available from the corresponding author.

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