Reproductive performance of the fluted clams (*Tridacna squamosa*) through different serotonin hormone doses for spawning induction

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Abstract. Exploring the reproductive performance one of the giant clam species being cultured in Indonesia. The fluted clams (Tridacna squamosa) are a keystone species for Indonesia's marineculture and restocking program, categorized as a protected commodities by Convention on International Trade in Endangered Species (CITES) due to anthropogenic pressure on wild population. To assess and improve their reproductive performance, induction of different serotonin was applied consisting of control (0 mM), T1 (1.1 mM), T2 (1.4 mM), and T3 (1.7 mM). New significant insights emerge, results indicate response's time of pre-spawning T1 and T2 were approximately 45 s, while T3 was 60 s, respectively T2 is highest spawning success at 91.67%, contrasting with 50% by T3, also spawning latency varied, with the quickest gamete release (3 min) by T2, on the contrary T3 (4 min). Observed eggs released at approximately 24 min (T2), 84 min (T1) with an egg diameter of 128.72±1.56 µm yet absent in T3. Polynomial regression of fecundity from differential dosing approach attained an R^2 = 0.3885, highlights an optimal 1.36 mM dosage for maximizing spawning performance, indicating T2 as an adequate dose with moderate egg abnormalities 8.7% and. FR, HR, and SR ranged from to 70-80% respectively.

1 Introduction

The largest group of bivalves, known as giant clams, belongs to the *Tridacnid* family, consisting of 2 genera (*Tridacna* and *Hippopus*) and 11 species [17]. These molluscs inhabit coral reef ecosystems in the Indo-Pacific region, engage in symbiosis with photosynthetic dinoflagellates (*Symbiodinium spp.*), and are renowned for their beautiful mantles. This symbiotic relationship leads to hypertrophy in the mantle muscles, forming a gathering place for dinoflagellates that produce colours, known as the Zooxanthella Tubular System (ZTS). The resulting colours and patterns serve as an attraction for aquarium enthusiasts, increasing the demand for marine ornamental clam commodities in the market, which, unfortunately, contributes to the decline in their natural populations due to overfishing [15-18]. This market demand, coupled with activities such as tourism, coastal urbanization, and water quality

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degradation, poses anthropogenic pressures on the sustainability, population, and habitats of clams in their natural environment. These indications and factors are key reasons for the looming threat of extinction of various clam species in coastal ecosystems [20].

In response to the alarming conditions of giant clam populations in 1983, the Convention on International Trade in Endangered Species (CITES) categorized all clam species as protected commodities, regulating and restricting all activities related to their exploitation and utilization from nature (Appendix II [1]. Safeguarding giant clam populations, the Indonesian government issued a regulation by the Ministry of Environment and Forestry in 2018, specifying that the species *Hippopus hippopus* is classified as a protected wildlife. Further measures for protection, rehabilitation, and conservation were outlined in the 2007 government regulations. However, domestic clam cultivation activities are governed by the Ministry of Forestry Permit Letters. The International Union for Conservation of Nature (IUCN) has also classified certain clam species, such as *T. gigas*, *T. derasa*, *T. roswateri*, and *T. mbalavuana* as vulnerable, while *T. crocea*, *T. maxima*, and *T. squamosa* are categorized as least concerning because of insufficient data [27].

Fluted clam (*T. squamosa*) is a clam species found in Indonesia and is prevalent in various tropical waters, particularly in the Indo-Pacific region. These clams inhabit coral and sandy substrates up to a depth of 20 m [25]. Morphologically, clams share common characteristics, including a hinge, byssal opening, umbo, folds, fluted shell, mantle, an incurrent siphon, and excurrent siphons. *T. squamosa* exhibits a distinct morphology in its fluted shell, featuring raised scales as an adaptive defence mechanism against predators. *T. squamosa* plays a crucial role in the productivity of coral reef areas. The presence of giant clams significantly influences the richness and diversity of fish and other organisms in degraded coral reef regions, with clam siphon tissues, feces, and gametes serving as food sources for predators and detritivores [25].

Over the past four decades, increasing attention has been paid to clam cultivation for both the ornamental fish export market and consumption. Giant clam cultivation is categorized as challenging with a very low survival rate. The primary obstacle in clam cultivation is the stimulation of spawning in the broodstock. Artificial induction methods carry risks that may result in the death of the broodstock due to errors in hormone induction. The second challenge involves obtaining sufficient eggs, requiring mature and large gonad specimens, considering that clams are simultaneous protandrous hermaphrodite gonochoristic animals. The third and most formidable obstacle is the low survival rate, as larvae must complete onto genic cycles and metamorphosis [11, 12, 15, 18].

Serotonin (5-hydroxytryptamine; 5-HT) serves various functions in the vertebrate brain, including emotional control, endocrine responses, stress coping, and aggression. Synthesized from the amino acid tryptophan, with the assistance of tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase (DDC), serotonin functions as a neurotransmitter that plays a role in reproduction in bivalve molluscs. Serotonin has been reported to induce oocyte maturation, as evidenced by germinal vesicle breakdown (GVBD), release of eggs from ovarian tissues, sperm motility stimulation, and parturition [23]. Giant clams are characterized as simultaneous protandrous hermaphrodite gonochoristic organisms, which implies that they released sperm first, followed by eggs as an individual [13]. The primary function of serotonin during spawning is oocyte maturation. Serotonin receptors induced by estradiol-17b (E2) are distributed in the oocyte membrane and increase during oocyte development, leading to increased sensitivity to the serotonin involved in oocyte maturation [26]. However, utilization of serotonin induction for giant clam spawning lacks global standard dosage used, with varying dosages reported in different journals ranging from 0.8 mM to 2.0 mM [21, 19, 4, 5]. As there is no global standard for dosage used, this study aimed to evaluate the reproductive performance of *T. squamosa* through different doses of serotonin

induction to gain a benchmark for global standards in the use of serotonin hormones for spawning induction.

2 Material and methods

2.1 Broodstock collection

T. squamosa broodstock were F_2 cultured with an aged of ≥9 years, a mean (±SE) shell length and height measuring 24.7±0.5 x 18.3±0.4 cm and weighing 3.17±0.18 Kg. Broodstock was sourced from the ocean nursery facility of the PT. Dinar Darum Lestari. Twelve *T. squamosa* broodstock individuals were collected and distributed across four treatments, each with three replicates. The broodstock was acclimated using drip acclimation. After acclimatization, they were placed in a prepared broodstock tank.

2.2 Spawning induction

Broodstock spawning involves serotonin induction via intra-gonadal injection at predetermined treatment dosages of 0 mM, 1.1 mM, 1.4 mM, and 1.7 mM. The intra-gonadal injection technique outlined the injection point and the organs to be monitored during the procedure, with the injection point on the anterior part of the clam located beneath the excurrent siphon. Following induction, the response of the broodstock was observed, recording both the reactions and time required for gamete release [16, 21]. Broodstock exhibiting pre-spawning contraction behaviours were transferred to a bucket filled with filtered seawater for gamete release and observation, where they released sperm first as the sperm was collected, the opacity of the bucket became opaque, and the broodstock was transferred to a new bucket, which was repeated until it was observed with backwashing behavior. Following this behavior, they released eggs. Therefore, careful observation is crucial to prevent self-fertilization by released sperm. The collected sperm was treated with streptomycin to ensure quality and prevent contamination. The eggs obtained were quantified and examined for their condition, followed by fertilization with the collected sperm after observation.

3 Results

The induction of various doses of serotonin resulted in diverse spawning responses, with spawning performance recorded based on time (minutes) for each treatment. The control treatment did not exhibit a pre-spawning contraction response, which is the closing and opening of the valve due to contraction of the adductor muscle of the clams (**Table 1**).

Table 1. The spawning response of *T. squamosa* under different doses of serotonin hormone induction treatment

Treatment	Response (minutes)	Spawning Percentage	Sperm Release (minutes)	Eggs Release (minutes)
Control	-	-	-	-
1.1 mM	00:48 ± 00:11	83.34%	03:41 ± 01:11	84:31 ± 64:38
1.4 mM	00:43 ± 00:15	91.67%	03:21 ± 00:29	24:38 ± 02:58
1.7 mM	01.01 ± 00:12	50%	03:58 ± 00:52	-

The initial spawning response in the serotonin hormone induction treatments, 1.1 mM, and 1.4 mM occurred within 45 seconds, demonstrating a faster reaction compared to the response in 1.7 mM, which took around 60 seconds. This early response involved clam contractions directing water flow towards the excurrent siphon, and after some time, the clams released gametes. The spawning success rate was the highest at 91.67% (1.4 mM) and the lowest at 50% (1.7 mM). The first gamete release occurred at 3 min and 21 s (1.4 mM), whereas the longest duration was approximately 4 min (1.7 mM). Following sperm release, the clam broodstock performed a backwash on its excurrent siphon to clean the residual sperm and prepare for egg release. The average time for egg release was 24 minutes and 38 seconds for 1.4 mM, and the longest was 84 minutes and 31 seconds for 1.1 mM, while 1.7 mM did not produce eggs, potentially indicating negative feedback at higher doses.

The fecundity value was determined based on the number of eggs obtained, and these values were incorporated into a scatter plot graph to examine the correlation between serotonin hormone dosage and the resulting egg quantity, along with its regression coefficient (**Fig. 1**).

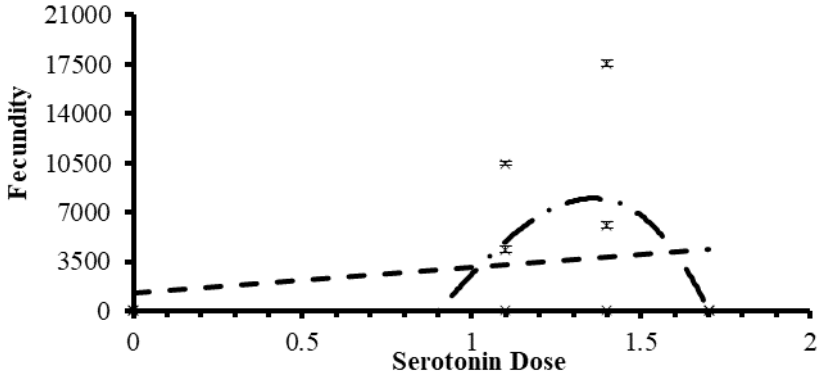

Fig. 1. The influence of various doses of serotonin hormone induction on the fecundity *T. squamosa* broodstock.

The fecundity values exhibited an increase corresponding to the serotonin dosage, as indicated by the linear regression formula $y = 1821.1x + 1291.6$. However, the coefficient of determination for the linear regression was $R^2 = 0.0468$, suggesting a weak correlation between dosage enhancement and fecundity. To further assess the relationship, a polynomial regression analysis was conducted, revealing the polynomial regression formula $y = -37615x^3 + 97846x^2 - 57630x - 7_{E-09}$ with an R^2 of 0.3885, indicating a moderate correlation. The moderate coefficient of determination implies that the dosages employed in this study approach the assumption that an optimal dosage exists for fecundity results. The peak dose of serotonin hormone based on the fecundity formula is 1,36 mM and the resulting highest number of eggs based on the formula is 7980 eggs.

The diameter of the eggs represents the central axis in measuring the length of an egg, assessed either through scales or software during observations using a microscope. The distribution of the egg's central axis plays a crucial role in determining its development, as well as the gonad readiness (**Fig. 2**).

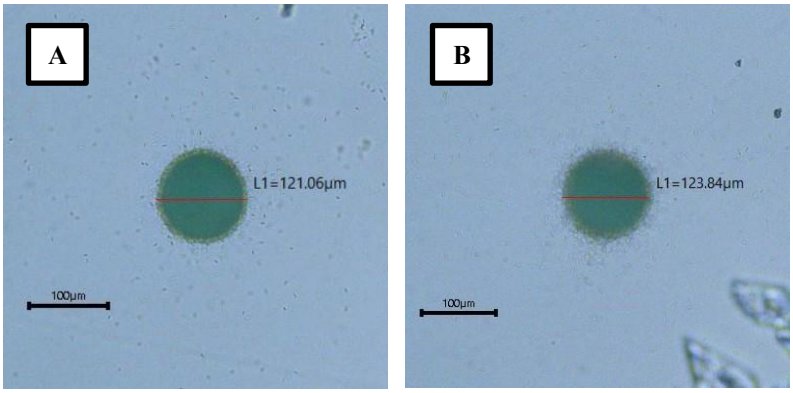

Fig. 2. *T. squamosa* egg diameter was conducted using the *IndomicroView* software. (A) Represents the image of egg diameter at a magnification of 40x under the microscope, measuring 121.06 µm in diameter. (B) Depicts the image of egg diameter at a magnification of 40x under the microscope, with a diameter of 123.84 µm.

The obtained egg diameter through sampling measures 128.72±1.56 µm, with the smallest recorded diameter being 100.22 µm and the largest diameter reaching 147.13 µm. These egg diameter values were compared with data from published journals to establish correlations between egg diameter and spawning induction outcomes.

The percentage of egg abnormalities in the spawning induction treatment with different serotonin dosages showed no significant difference (**Fig. 3**).

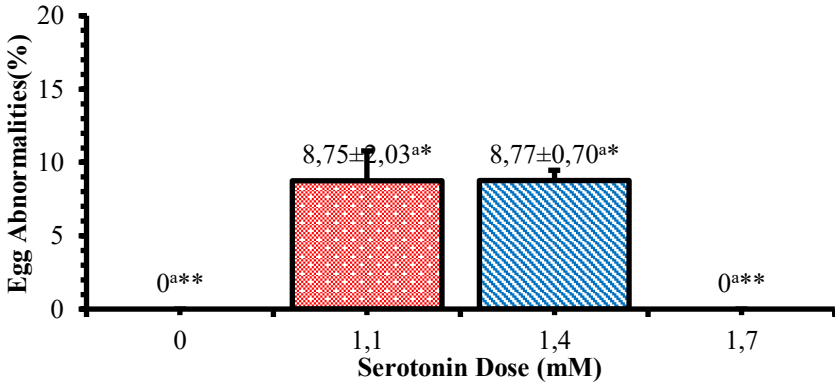

Fig. 3. The abnormality percentage of *T. squamosa* egg morphology. Note: (*) indicates repetition with no egg release, (**) signifies that all repetitions did not produce eggs.

The level of egg abnormalities due to various doses of serotonin did not exhibit a statistically significant impact (P>0.1). The observed abnormalities, with an average value of 8.7%, manifested as external deformities and yellowish discoloration of the egg, potentially affecting fertilization (**Fig. 4**).

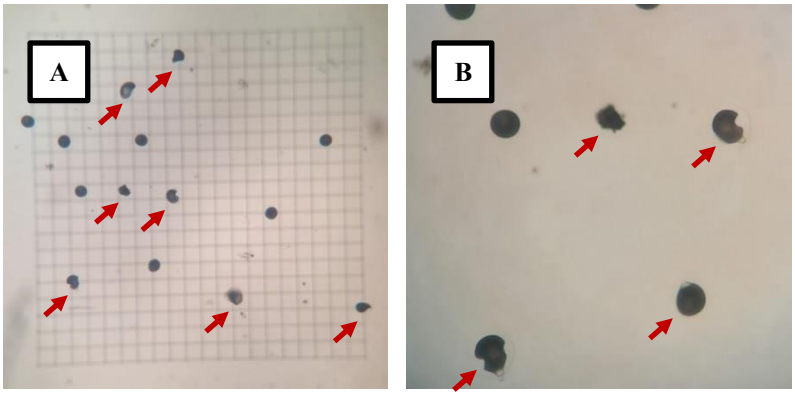

Fig. 4. Abnormal *T. squamosa* eggs. (A) Defective eggs at a magnification of 10x under the microscope (→). (B) Defective eggs at a magnification of 40x (→).

The number of eggs exhibiting embryogenesis 20 min post-fertilization in the induction treatments using different doses of serotonin hormone showed a significant difference compared to the control, except for 1.7 mM (**Fig. 5**). Various doses of serotonin did not significantly affect the fertilization rate (P>0.1). However, there was a significant difference compared with the control. The hatching rate of eggs, based on the number of swimming trochophores 24 h post-fertilization, indicated the influence of serotonin hormone dosage (**Fig. 6**).

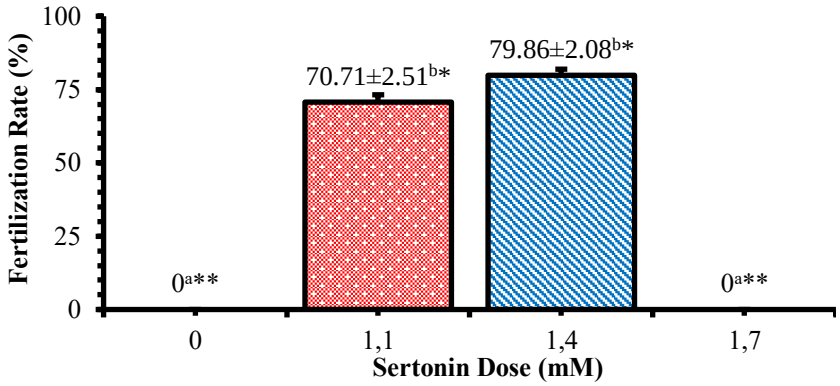

Fig. 5. The fertilization rate in *T. squamosa* eggs, spawning under different doses of serotonin induction. Note: (*) indicates repetition with no egg release, (**) signifies that all repetitions did not produce eggs.

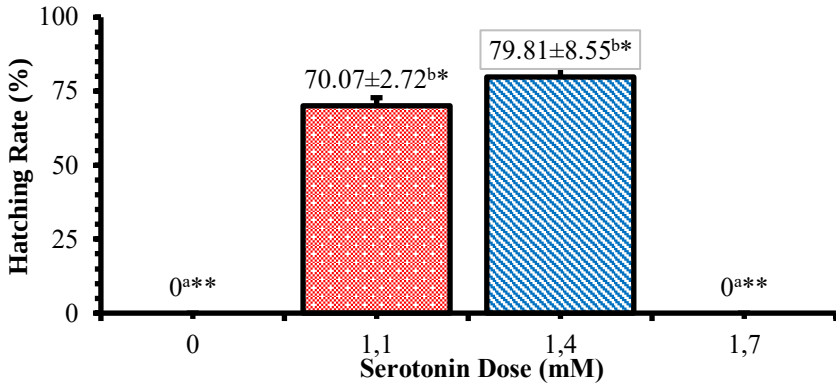

Fig. 6. The hatching rate in *T. squamosa* eggs, spawning under different doses of serotonin induction. Note: (*) indicates repetition with no egg release, (**) signifies that all repetitions did not produce eggs.

The serotonin dosage in the spawning of scaly clams significantly influenced the quantity of veligers obtained from sampling at the age of 7 days (**Fig. 7**). The survival rate of larvae under the application of serotonin doses of T1 and T2 did not show a significant difference (P>0.1), but it significantly differed from that of the control and T3 (P<0.1).

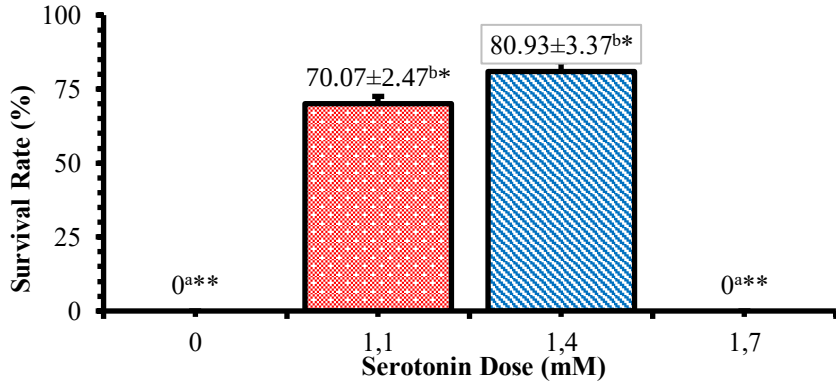

Fig. 7. The survival rate in *T. squamosa* eggs, spawning under different doses of serotonin induction. Note: (*) indicates repetition with no egg release, (**) signifies that all repetitions did not produce eggs.

4 Discussion

The spawning hormone induction response using different serotonin doses exhibited the fastest results in treatments T1 and T2, occurring at 48 s and 43 s, respectively, compared to T3 (61 s), while the control did not spawn. The hormonal induction response manifested as the initial spawning behavior of the giant clam, involving contraction of the posterior abductor muscle, leading to the opening and closing of the clam shell. This response spanned 1–3 min [8]. The contraction response directed water flow to the excurrent siphons, with the generated current aimed at expanding the area for gamete release. However, this contraction response does not always lead to successful clam spawning. Serotonin treatment resulted in the highest spawning percentage at 91.67% (T2) and the lowest at 50% (T3). Successful spawning was observed when the clam released its gametes, primarily sperm, owing to the simultaneous protandrous hermaphrodite gonochoristic characteristics. This means that during spawning, the clam releases male gametes first, followed by female gametes (eggs). This characteristic leads to three gonad development phases in clams: immature gonads, male

gamete maturation, and female gamete maturation. The natural release of male gametes is influenced by spawning stimulation of mature clam eggs [14]. The first gamete release, sperm, yielded release times ranging from approximately 3 min, with the fastest sperm release at 3 min 21 s (T2), 3 min 41 s (T1), and 3 min 58 s (T3). Sperm release scoring was based on the number and opacity of filled containers, with T1 and T2 obtaining the highest scores, averaging three filled containers with high opacity, whereas T3 had the lowest score, with one container filled with moderate opacity. The second gamete release, eggs, occurred between 30 and 80 min, with the fastest egg release at 24 min 38 s (T2) and 84 min 31 s (T1), and T3 did not produce eggs. These results suggest a negative feedback effect from serotonin hormone induction at doses higher than 1.4 mM, affecting the inhibition of sperm gamete release and the absence of egg gamete release.

Based on the results of this study, fecundity values were obtained at different serotonin doses. Treatment with a 1.1 mM serotonin dose (T1) resulted in 10,455 eggs, while the 1.4 mM serotonin dose (T2) produced a higher quantity of 17,565 eggs. Conversely, the 1.7 mM serotonin dose (T3) did not yield any eggs, and the control group did not spawn. Egg quantity increased with increasing dose, as indicated by the linear regression results. However, the egg quantity exhibited an optimum range based on polynomial regression. The linear regression produced the formula "y =1821.1x + 1291.6," and the polynomial regression yielded "y = -37615x3 + 97846x2 - 57630x - 7E-09". The R-squared or coefficient of determination indicates the extent to which the independent variables influence the dependent variable. Coefficient of determination tests were conducted to measure the model's ability to capture the impact of the independent variable serotonin dose. The R^2 value for T1 (0.0468) was categorized as weak, as it was lower than 0.33, indicating that the linear regression analysis did not effectively illustrate the impact of different dose treatments. In contrast, the R^2 value for T2 (0.3885) was categorized as moderate, indicating that the coefficient of determination is adequate for describing a certain range where the given doses are effective [6, 10]. The most effective induction dose was in the range of 1.4 mM. Using the formula "y = -37615x3 + 97846x2 - 57630x - 7E-09", the most effective dose is found at 1.36 mM, resulting in a fecundity value of 7,980 eggs. In addition to serotonin dose effectiveness, induction results also revealed abnormalities in spawned eggs that may impede fertilization. However, the level of abnormality due to serotonin dose variability did not show a significant effect ($P>0.1$), with an average abnormality rate of 8.7%, characterized by external deformities and egg yellowing.

Excessive hormone induction can lead to imbalances and negative effects on organisms. Hormones serve as modulators of endogenous gametogenesis and play a role as neurohormones to enhance sexual maturation. Serotonin plays a role in physiological aspects of muscle function, tonic relaxation of smooth muscles, siphonal activity, and ciliated tissue in vivo. In addition to muscle physiology, serotonin is involved in immunocompetence as a regulator of immune system components [9]. This negative effect is a result of an imbalance in serotonin regulation within biological systems, as its increase can lead to abnormalities in physiological functions and even be fatal to cells [24].

The degree of egg fertilization or fertilization rate and the degree of egg hatching or hatching rate were interconnected aspects of the results. In the treatment with serotonin doses of 1.1 mM (T1) and 1.4 mM (T2), there was no significant difference ($P>0.1$). The results of treatment T1 showed a fertilization rate of 70.71±2.51% and a hatching rate of 70.07±2.72%, while treatment T2 exhibited a fertilization rate of 79.86±2.08% and a hatching rate of 79.81±8.55%. Generally, fertilization rates for giant clams fall within the range of approximately 80% [15, 18, 28, 7]. The findings of this study indicate that there was no impact of induction on egg fertilization results, as they still fell within this 80% range. Fertilization and hatching rates were not influenced by hormonal induction but were affected by water quality. The physicochemical values of water had a drastic impact on the degree of

fertilization and hatching rates, with these parameters decreasing as the water quality declined. Water quality parameters that affect fertilization and hatching rates include temperature and salinity. The optimal temperature for giant clam egg fertilization, observed with the first gastrula division at 9 h post-fertilization, falls within the range of 30-33°C, whereas at lower temperatures, such as 28°C, the first division is observed at 12 h post-fertilization. Similarly, for hatching, the optimal temperature range for giant clam eggs, with the first trochophore observed 18 h post-fertilization, is 30-33°C, and at lower temperatures such as 28°C, the first trochophore is observed 24 h post-fertilization [7, 33]. The maintenance temperature range of 24-27.5°C is less optimal for supporting egg fertilization and hatching. Salinity is another factor influencing egg fertilization and hatching, with a fertilization rate of approximately 46% obtained within the range of 30 ppt to 34 ppt, decreasing to 5%. The hatching rate was around 63% within the range of 30-34 ppt but decreased to 28% [22]. The salinity values of the maintenance water support high degrees of fertilization and hatching (approximately 30 ppt).

Based on the research findings, the survival rate values were obtained for treatment with serotonin doses of 1.1 mM (T1) at 70.07±2.47% and for treatment with serotonin doses of 1.4 mM (T2) at 80.93±3.37%. The results of the present study indicated that hormonal induction, as applied, did not have a significant effect ($P>0.1$) on larval survival rate. The overall survival rate of giant calm larvae is remarkably low, as is evident from previous research. The survival rate or survival rate, ranging from 1.4% to 4.2% [3, 13], significantly differed from the control treatment in this study's journal. The water quality was not a specific treatment in this study. Research comparing larval survival and development under the influence of different salinities and copper additions showed that the highest survival rate was achieved in the control treatment at a salinity of 32 ppt with a value of 4.2%. The salinity values in this study ranged from to 30-33 ppt, where giant clam larvae did not experience a decrease in survival rate or hindrance in larval development [3]. Larval density during the larval maintenance phase significantly affected the survival rate of the larvae. The highest value obtained in their study was 16.6% with a density of 0.5 individuals/mL in the maintenance container [13], whereas the lowest value in the control treatment was 1.4% with a density of 5-7 individuals/mL in the maintenance container. In this study, treatment T1 had a density of 0.22 individuals/mL in the maintenance container, and treatment T2 had a density of 0.41 individuals/mL in the maintenance container, supporting a high larval survival rate. Although water quality and larval density supported a high survival rate in this study, the elevated survival rate is attributed to the Standard Operating Procedure (SOP) for larval maintenance by PT. Dinar Darum Lestari, which aligns with Ambariyanto's study. The addition of antibiotics and nutritional supplements resulted in a survival rate of 57.4% compared to the control value of 32.4% [2].

5 Conclusion

Differential dosages of serotonin hormone induction on the spawning latency period and spawning performance of *T. squamosa* were effective at the optimal dosage of 1.36 mM for mating response and fecundity, with a polynomial regression coefficient determination of 0.3885. However, this induction had no effect on egg abnormalities, fertilization rate, hatching rate, or larval survival.

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