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## **Verification of a hatchery protocol for green mussel *Perna viridis* spat production in the Philippines using industry-scale facilities**

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### **Abstract**

Hatchery seed production of mussels has been identified as a sustainable method to address the problem of low production due to insufficient seed supply. We conducted the first hatchery production trials of the Asian green mussel *Perna viridis* spats in the Philippines to demonstrate the feasibility of producing a sufficient and reliable seed supply for grow-out operations. However, results of small-scale experiments cannot be directly rolled-out commercially unless these are verified in bigger-scale facilities approximating those of commercial hatcheries. Thus, techniques on broodstock collection, spawning, and larval rearing, developed by the project in the laboratory during the experimental trials, were applied and verified in a production run using industry-scale tank facilities. Mature broodstocks collected from traditional mussel growing areas were successfully spawned in the hatchery. Eggs were fertilized, and these developed into D-hinged larvae, pediveliger, and metamorphosed into early spat before fully developing into the spat stage. Successful larval rearing up to the spat stage required the use of appropriate algal species and rates of feeding, close monitoring of larval stages, and water quality management. The survival from eggs to D-hinged larvae, D-hinged larvae to pediveliger, pediveliger to early spat, D-hinged larvae to early spat, and early spat (1 mm) to fully grown spat was 77, 64, 6.4, 3.1, and 72%, respectively. This study has established the feasibility of producing *P. viridis* in commercial-scale hatchery facilities.

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## Introduction

Among the commonly cultivated genera of bivalves, *Mytilus* and *Perna* are widely studied in efforts to increase production. Representative species include *Mytilus edulis*, *Mytilus galloprovincialis*, *Perna perna*, *Perna canaliculus*, and *Perna viridis*. In 2018, over 26 million tons of bivalve mollusks were produced worldwide, showing that these commodities are indispensable food items (Kamermans and Capelle, 2018; PSA, 2019) with increasing demand (FAO, 2018). Among Asian countries, China leads in the production of *M. galloprovincialis*, while Thailand registered the highest production of *Perna viridis* (Kamermans and Capelle, 2018). In the Philippines, the green mussel *P. viridis* is an economically-important bivalve and is the only farmed mussel species. However, although its production increased from 16.0 thousand metric tons in 2015 to 26.3 thousand metric tons in 2018, it contributed only a measly 1% to the total aquaculture production of the country (PSA, 2019). Owing to the absence of commercial hatchery facilities in the country (Napata and Andalecio, 2014), mussel growers obtain natural seeds from the wild, which is considered to be a highly unpredictable and unreliable source, and thus a major constraint in increasing production and/or expansion of areas for farming.

New Zealand has been producing seeds for the green-lipped mussel *P. canaliculus* in hatcheries since the 1990s (Alfaro et al., 2010). In 2005, the Blueseed Project, a collaborative effort among European countries such as Spain, France, and the Netherlands, was initiated, which focused on hatchery production of temperate species such as the blue mussel *M. edulis* and Mediterranean mussel *M. galloprovincialis* (Kamermans et al., 2013). Optimization of the larval-rearing techniques for *M. edulis* was also carried out, as reported by Galley et al. (2010).

India was among the first countries to develop hatchery techniques for the green mussel *P. viridis*. Rao et al. (1976) described the spawning, fertilization, and larval development of *P. viridis*. Successful spawning and larval rearing of this species were also reported in Malaysia (Sivalingam, 1977). In French Polynesia, AQUACOP refined the larval rearing technology, spat production, and mass production scheme for the green mussel (AQUACOP, 1980). Manoj et al. (2003) examined the effects of temperature on the growth and development of the species. The foregoing contributed in developing a more detailed and successful green mussel spat mass production scheme in India from spawning, larval rearing, and settlement (Laxmilatha et al., 2011; Anil et al., 2017).

In the Philippines, the Asian green mussel *P. viridis* was successfully spawned in the laboratory (Young, 1979). However, mass mortality due to bacterial contamination from the algal food was encountered during the larval rearing trial resulting in poor growth and low survival of the larvae. After the above report, no further attempts were reported to develop the hatchery techniques to a level that can be taken up by the industry.

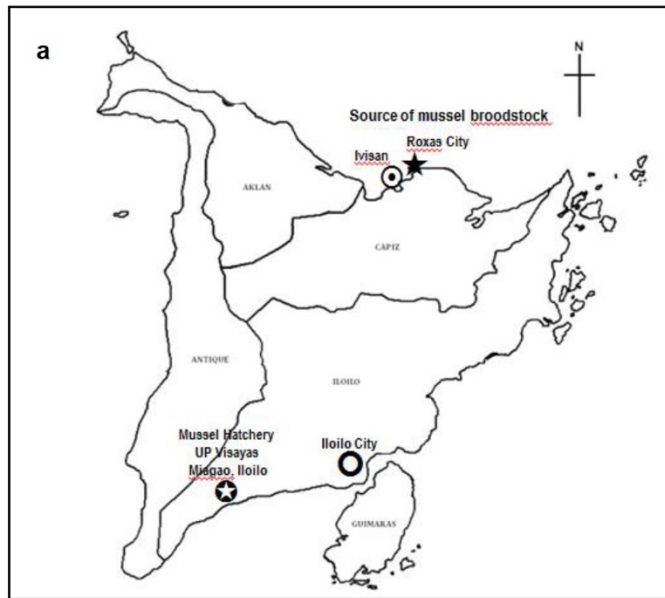
Recently, acknowledging the potential contribution of the mussel industry to Philippine aquaculture production, the Philippine government, through the Department of Science and Technology-Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development (DOST-PCAARRD), funded the green mussel hatchery research project as a fresh initiative towards the establishment and possible commercialization of the green mussel hatchery technology in the country.

This paper highlights the efforts in developing the green mussel *P. viridis* hatchery techniques within the Philippine setting. Detailed procedures employed from broodstock collection, transport, and spawning, larval rearing from D-hinged larvae until the spat stage developed in laboratory scale experiments were applied in large-scale green mussel spats mass production trials).

## Materials and Methods

### Location of the green mussel hatchery

The green mussel hatchery was located at the University of the Philippines Visayas, College of Fisheries and Ocean Sciences, Institute of Aquaculture, Multispecies complex in the southern part of Iloilo Province, approximately 40 kilometers from Iloilo City, Philippines, 10°38'21" N; 122°13'33" E (earth.google.com/web; **Figure 1**).



**Figure 1** Panay Island map (a) showing the location of the green mussel broodstock source and the hatchery, (b) Site of the green mussel hatchery within the multispecies hatchery complex of the Institute of Aquaculture, University of the Philippines Visayas (Source: earth.google.com/web).

### *Broodstock Collection and Spawning*

Before broodstock collection, mussel samples were taken to check the reproductive maturity or readiness of the bivalves to spawn. Mature green mussel broodstocks with shell length (SL) of >5 cm were collected from their natural growing areas in Roxas City, Capiz, Philippines. They were transported to the mussel hatchery following the method described in Apines-Amar et al. (2020). The broodstocks were wrapped with a moist cloth and placed in a closed-cell extruded polystyrene foam box. Ice was provided in the box to maintain the low temperature. Upon arrival, all the mussels were stocked and left undisturbed overnight in 250L-capacity conditioning tanks filled with approximately 200 L of seawater provided with aeration. The following day, approximately 100 mussels were cleaned of epibionts, shell encrustations, and other clinging debris, transferred into dry, clean tanks (4 ft x 8 ft x 1ft deep), and left desiccated (exposed to air) for 1 h. Thereafter, fresh seawater with slightly elevated temperatures ranging from 28-30°C was introduced to the mussel tanks (Apines-Amar et al., 2020; Piñosa et al., 2020). Close observations showed that mussels started to release gametes within an hour after water was introduced. At the onset of gamete release, mussels were individually transferred into 250 mL capacity plastic containers filled with UV-treated seawater. The individual containers facilitated the collection and counting of the gametes, whereas UV-treated seawater ensured the exclusion of possible mussel pathogens. Egg and sperm cells were then collected separately and counted under the compound microscope using a Sedgewick Rafter Counting Cell at 40–100x magnification and a hemocytometer at 400x magnification, respectively. Sperm cells were added into the eggs at a ratio of 50:1 within an hour after release, then the gametes were mixed in a 50-100L-capacity container and were allowed to stay for 10-15 minutes to fertilize before being stocked in 500L to 1-ton capacity circular tanks at 20-40 eggs mL<sup>-1</sup> with very minimal aeration (Apines-Amar et al., 2020; Piñosa et al., 2020). Salinity and temperature during incubation were 30±1 ppt and 28-30°C, respectively (Apines-Amar et al., 2020; Piñosa et al., 2020). Fertilized eggs hatched and developed into D-hinged larvae were harvested 24-48 h post-fertilization.

### *Larval Rearing*

#### *D-hinged to pediveliger stage*

D-hinged larvae were grown until the pediveliger stage following the method used in Apines-Amar et al. (2020). Harvested D-hinged larvae were stocked in 250 L to 1 ton-capacity fiberglass tanks using UV-filtered seawater at a stocking density of 10-20 larvae mL<sup>-1</sup> provided with gentle aeration. Partial water change (50%) was done every three days. The ambient salinity and temperature during the culture period were 30 ± 1 ppt and 26 ± 1°C, respectively. Feeding strategies were based on the estimated number of algal cells an individual larva could ingest in a 24-h period, as determined previously. Algal diets used were *Isochrysis galbana* and *Chaetoceros calcitrans* given at a 1:1 ratio twice daily. The algal foods were given at a rate of 2,000 cells larva<sup>-1</sup> day<sup>-1</sup> from day 1-4; 5,000 cells larva<sup>-1</sup> day<sup>-1</sup> on day 5-9; 12,000 cells larva<sup>-1</sup> day<sup>-1</sup> on day 10-14; and 20,000 cells larva<sup>-1</sup> day<sup>-1</sup> on day 15-19 as described in Apines-Amar et al. (2020) which was modified from Manoj et al. (2003) and Laxmilatha et al. (2011). The amount of algal food given corresponded with the larval development from a D-hinged larva to a pediveliger larva. The detailed descriptive morphology of these larval stages was described in Laxmilatha et al. (2011). Growth in terms of larval shell length (SL) and shell height (SH) in the present study was measured from 30 larval samples.

#### *Pediveliger to early spat stage (1 mm)*

During the metamorphosis stage, larvae were harvested and stocked at 2 larvae mL<sup>-1</sup> in 250-500 L-capacity fiberglass tanks filled with sand-filtered and UV-treated seawater and supplied with mild and later strong aeration. Partial (50%) water change every three days was observed. The culture tanks were provided with either black or green nylon net as

substrate before settlement. Larvae were fed initially at 12,000 algal cells larva<sup>-1</sup> day<sup>-1</sup>, gradually increasing to 100,000 algal cells larva<sup>-1</sup> day<sup>-1</sup> at the end of the 30-35 days rearing period (Wang et al., 2018; Mero et al., 2019; Maquirang et al., 2020).

#### *Early spat to spat stage (1 cm)*

Spats already visible to the naked eye (size range, 1-2 mm) were transferred to the nursery tanks for further rearing. The estimation of settled spats was difficult. Spat collectors covered with well-distributed growth of spats were manually counted. However, spats in clumps were gently scraped from the surface of the substrate and the walls and bottom of the tanks. After weighing a certain number of spats, the total count was estimated by the weight-to-number ratio (e.g., 1g spat = 360 pcs). Spats were then stocked in 1-ton-capacity concrete nursery tanks filled with sand-filtered seawater at a stocking density of 0.2-0.3 g L<sup>-1</sup> (72-108 spats L<sup>-1</sup>) provided with aeration. Partial (50%) water change was done on alternate days, and totally drained once every week to monitor the overall condition of the culture (e.g., presence of other organisms, mortality, and settled dead algal food). Feeding was based on the weight of spats held in tanks at 40% biomass (Helm and Bourne, 2004). A large flagellated green prasinophyte *Tetraselmis tetrahele* was added to the diet of the spats to accommodate its bigger size (Helm and Bourne, 2004). Thus, mixed algal food consisting of *Isochrysis galbana*, *Chaetoceros calcitrans*, and *Tetraselmis tetrahele* was provided to the spats to ensure a more balanced nutritional profile for the larvae.

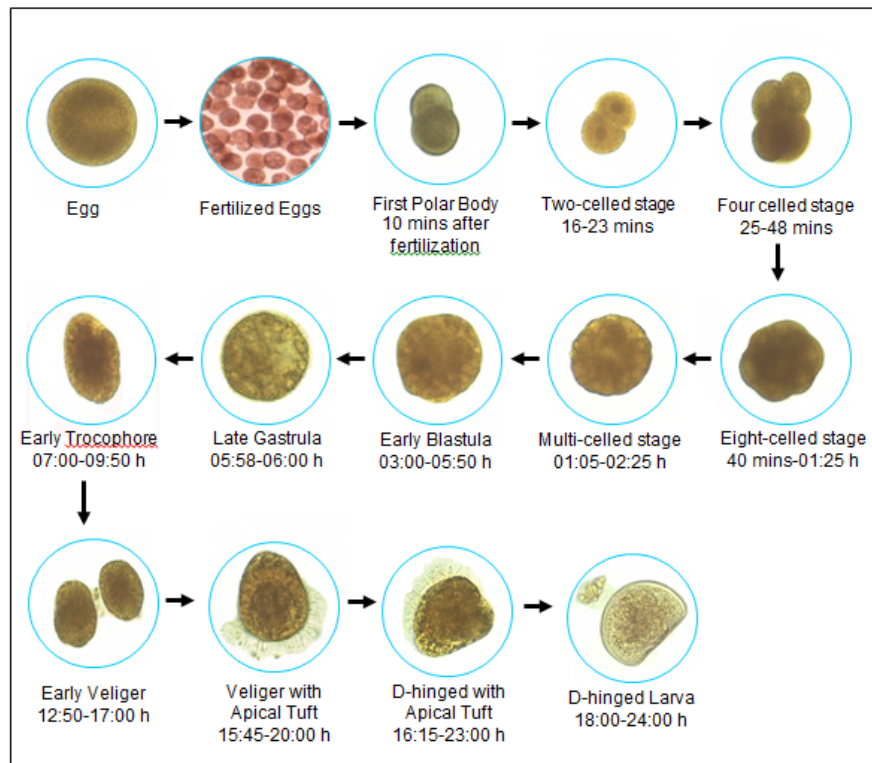
## Results

#### *Broodstock collection and spawning*

High survival of 99.7-100% was recorded (**Table 1**) during the transport of green mussel broodstocks from the natural grounds to the hatchery (**Figure 1**). In this study, thermal stimulation coupled with desiccation proved to be an effective method of stimulating the release of gametes in the Asian green mussels. The eggs produced were brick red in color, spherical, and with a diameter of 40-50 µm. The number of eggs released ranged from 1.32 x 10<sup>5</sup> to 14.77 x 10<sup>6</sup> cells depending on the gonadal maturity of the female broodstock. The sperm suspension was cloudy and milky-white with actively moving sperm when viewed under the microscope. The number of sperm released per individual mussel broodstock was between 1.75 x 10<sup>8</sup> and 6.81 x 10<sup>9</sup> cells. After fertilization, embryonic development was documented, as presented in **Figure 2**. The average survival at D-hinged from fertilized eggs was 77% (range: 63-88%; **Table 2**).

**Table 1** Survival of broodstock during transport from the source to the hatchery

Broodstock Source	Travel time (No. of hours)	Survival (%)
Source A	6 - 7	100
Source A	6 - 7	99.7
Source B	5 - 6	100
Source B	5 - 6	100



**Figure 2** Embryonic development in green mussel *Perna viridis*.

**Table 2** Survival of the hatchery-produced green mussel spats

Larval Stages	Survival (%)	
	Range	Average
Fertilized eggs to D-hinged larvae <sup>a</sup>	63-88	77
D-hinged larvae to Pediveliger <sup>b</sup>	50-89	64
Pediveliger to Early Spat <sup>c</sup>	3.1-9.6	6.4
D-hinged larvae to Early Spat (~1mm) <sup>d</sup>	2.7-3.4	3.1
Early Spat to Spat (~10mm) <sup>e</sup>	60-84	72

<sup>a</sup> n=9 trials; <sup>b</sup> n=8 trials; <sup>c,d</sup> n=4 trials ; <sup>e</sup> n=6 trials

### Larval rearing

**Growth and development from D-hinged larvae to early spat stage (1 mm)** D-hinged larvae, named after their resemblance to the letter D, measured 75-95  $\mu\text{m}$  in shell length (SL) and 60-75  $\mu\text{m}$  in shell height (SH) (**Table 3**). When observed under the microscope, larvae moved actively either through swimming or spinning with the aid of cilia which enabled them to capture available food in the water. The larvae developed into the umbo stage on days 5-6. The Umbo stage, as the name implies, is the larval stage associated with the onset of an umbo, the earliest and primary source of the shell for the larvae. At this stage, the larvae measured 135-200  $\mu\text{m}$  in SL and 120-165  $\mu\text{m}$  in SH.

**Table 3** Green mussel larval growth\*

Larval Stages	No. of Days	Shell Length (µm)	Shell Height (µm)
D-hinged	1	75-95	60-75
Umbo	5-6	135-200	120-165
Eyed-spot	9-11	153-260	134-220
Pediveliger	13-16	260-330	225-300

\*Values were measured from 30 larvae

Then, the umbo larvae became an eye spot on days 9-11. This stage is characterized primarily by the presence of a black rounded spot around the food mass of the larvae. The shell appearance becomes more rounded and globular, measured from 153-260 µm in SL and 134-220 µm in SH. The clear appearance of a muscular foot and ctenidial gill indicate larval development at the pediveliger stage, which is usually observed from day 13<sup>th</sup>-16<sup>th</sup> and measured 260-330 µm in SL and 225-300 µm in SH. Survival from D-hinged to pediveliger larvae ranged from 50-89%, with an average of 64% from the 8 trials conducted (**Table 2**). This is the last stage before the larvae become benthic. In this stage, the larvae started to find a suitable substratum to settle. During the 15 days of rearing, constant observation revealed that the larvae tended to swim in groups and clumped on a particular part of the substrate or areas in the tanks such that clumps of settled spats could be seen on substrates and within the tank sides and bottom. However, in some trials, even after the masses of the larvae had settled, some larvae were still swimming and had yet to find a suitable substrate. In such a case, the culture period was extended for another 5 days or until no planktonic larvae were left. The survival rate from pediveliger to early spat in this study was from 3.1-9.6%, with an average of 6.4%, while from D-hinged larvae to early spat stage was 3.1% on average, with values ranging from 2.7-3.4% in 4 trials (**Table 2**).

### *Spat Rearing*

Settled spats with SL of about 1-3 mm (**Figure 3**) were harvested from pediveliger culture tanks and transferred into the nursery tanks. The spats are oblong-shaped with a brownish color resembling that of an adult mussel. Transfer of spats started from the 30<sup>th</sup> to the 40<sup>th</sup> day, depending on the onset of metamorphosis. Spats were grown until they reached 10 mm in SL for them to be able to adapt totally to the harsh natural conditions in the grow-out site. It took approximately 4 weeks to grow the spats from 1 to 10 mm. Survival was between 60 and 84%, with an average of 72% in 6 trials (**Table 2**).

## Discussion

Larval and spat production technologies for bivalve species have been reported worldwide (Loosanoff and Davis, 1963; Appukuttan et al., 1987; Muthiah et al., 2002; Galley et al., 2010; Kamermans et al., 2013; Wong and Arshad, 2013). However, a particular technology should be verified, tested, and optimized under a prevailing environment which may include but not be limited to salinity, seasons, or different species (Helm and Bourne, 2004). Thus, it was necessary to test the feasibility of green mussel hatchery operations in the country.

Thermal stimulation, a combination of air exposure and water temperature fluctuations (Loosanof and Davis, 1963), was an effective method to induce spawning in green mussels. This method is natural and chemical-free and minimizes stress on the organisms (Helm and

Bourne, 2004). A similar method was employed in the spawning of *Mytilus (Perna) viridis* in French Polynesia (AQUACOP, 1979). Likewise, this method proved to be efficacious on tropical rock oyster *Striostrea prismatica* (Argüello-Guevara et al., 2013), on scallop *Pecten sulcicostatus* (Arendse et al., 2018), and on several clam species including the Manila clam *Ruditapes philippinarum* (Hur et al., 2005). However, Manoj et al. (2003) and Laxmilatha et al. (2011) did not perform any induction procedures but achieved natural spawning of *P. viridis*. Uninduced natural spawning was not experienced in this study, probably because of the difference in the gonadal maturity of the organisms.

Fertilization and egg incubation was performed following the method of Piñosa et al. (2020). The egg-to-sperm ratio of 1:50 lessened the probability of polyspermy, a lower stocking density of 20-40 eggs mL<sup>-1</sup> reduced intra-specific competition for space, and optimized egg-to-sperm contact to avoid gamete degradation. Likewise, salinity and temperature were set at 28-31 ppt and 28-30°C, respectively, based on previous results in small-scale experiments. In this study, the average survival of eggs at D-hinged larvae was 74% (range, 63-88%). This is a satisfactory figure, although lower than the 92-95% result recorded by Laxmilatha et al. (2011).

For larval rearing, optimized techniques based on our previous results performed in small-scale culture (Apines-Amar et al., 2020) were applied during the mass production trials. Both *I. galbana* and *C. calcitrans* were utilized as algal food throughout the culture period. However, due to intermittent culture crashes, either species was sometimes given a single diet. Similarly, Manoj et al. (2013) and Laxmilatha et al. (2011) used the same species of algae as food for *P. viridis*, either singly or combined, from the larval stage until settlement and metamorphosis. Stocking density was reduced (10-20 larvae mL<sup>-1</sup>) to avoid crowding and intra-specific competition as the larvae grew. Partial (50%) water change was done every 2-3 days to avoid mechanical/physical stress. Mild aeration was provided in the tanks as a source of oxygen and for adequate mixing of suspended algal cells.

The transition/metamorphosis stage, together with the availability of algal food, is considered one of the main bottlenecks in bivalve culture since it is associated with a high mortality rate (Helm and Bourne, 2004). Thus, effective strategies are necessary at this stage of the culture. In these production trials, methods for the culture of pediveliger larvae were adapted from our previous work (Mero et al., 2019; Maquirang et al., 2020). Reduced stocking density (2 larvae mL<sup>-1</sup>) was used during the study for optimum survival based on our previous experimental trials (Apines-Amar et al., 2020). Culture tanks were provided with mild to strong aeration since previous observations in small-scale studies showed that areas provided with strong aeration had a higher settlement rate. Pediveliger or the settlement stage is critical in the larval stages of green mussel resulting in the low survival until the early spat stage as shown in this study. Black nylon mesh nets were used as substrate based on previous findings on the suitable substratum (unpublished data). Likewise, during the D-hinged larvae culture, partial water change was observed on alternate days but not to exceed three days to maintain good water quality and avoid stress to the larvae. Algal diets fed singly did not adversely affect survival during this transition stage (Maquirang et al., 2020). Thus, depending on the availability of stock culture, either of these two species was used as feed during pediveliger rearing.

Newly settled spats were continuously cultured in the nursery tanks until they were ready to be seeded in the natural environment. As in fish culture, the nursery acts as an interphase between hatchery and grow-out culture. Specifically, in bivalve culture, nurseries enable spats to adapt to the environmental conditions of the on-growing areas gradually. Nurseries would keep small and vulnerable spats from predators or fouling organisms until they are big enough and ready for transfer (Kamermans et al., 2013). In this study, the maximum spat size that the nursery could hold was set at the limit of 10 mm shell length, considering the cost required to rear them indoors. At this stage, spats were more stable and sturdier than their larval counterparts.



Nevertheless, food and environmental conditions should be optimized for fast growth and high survival. Among the algal food available and tested in this study, the combination of *I. galbana*, *C. calcitrans*, and *T. tetrahele* is the recommended diet during this stage. However, the diet can be enriched with equally nutritious algae (e.g., *Skeletonema* sp.) whenever available. A stocking density of 0.2 g L<sup>-1</sup> (~72 early spat 1 mm larvae L<sup>-1</sup>) is recommended to avoid overcrowding and competition for food among larvae.

Mass production of mussel seed has been described in the present study as a strategy to lessen the impact of overharvesting and total reliance on natural spat fall, as well as to reduce conflict among farmers (Alfaro et al., 2010; Laxmilatha et al., 2011; Anil et al., 2017). More importantly, hatchery-produced spats can be a source of clean/healthy seeds for possible introduction into new growing areas or for mussel culture area expansion instead of wild seeds of unknown health status that may introduce disease agents or contaminants. In the Philippines, the hatchery production of green mussel seeds in commercial quantities can help the country address the challenge of low and inconsistent grow-out production due to insufficient seed supply. The verification trials in this study confirmed the results obtained earlier in our laboratory-scale experiments. The pilot testing of this technology, the green mussel spat hatchery production, is being conducted in Palawan, in the southwestern part of the Philippines.

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