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Larval growth of green mussel, *Perna viridis* (Linnaeus, 1758) in hatchery

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Abstract
The study on the larval growth of *Perna viridis* (Linnaeus, 1758) from Sitaw was conducted at Shwekyungyi (Russell Island: Lat. 10º 15' N, Long. 98º 15' E) in June 2018. Induced spawning was done by a sudden rise of temperature from 18°C to 30°C and the larvae were successfully reared to setting in the laboratory. Average shell length measurements and the survivability of larvae among antibiotic treatments and control was significantly different (ANOVA; $F_{4,57}$=5.72, p=0.001<0.05 & $F_{4,57}$=4.96, p=0.02<0.05). Average shell length and survival of larvae treated with a mixture of penicillin and streptomycin were higher and significantly different than that of the other larvae ($F_{4,57}$=5.72, p = 0.013 < 0.05). The higher growth and survival rate were observed from the experiment of using a mixture of penicillin and streptomycin. This may be due to the synergic effect of the combination of these two antibiotics. The result of this study is encouraging as this can be successfully induced to spawn in a low cost yet effective way.

Keywords: Antibiotics, induce spawning, *Perna viridis*, Sitaw

1. Introduction
Perna viridis (Linnaeus, 1758) belongs to the Phylum Mollusca, Class Bivalvia and the Family Mytilidae. It is extensively cultured due to its high productivity, high tolerance to a wide range of environmental conditions and requiring less farm management [1]. It is currently being recognized as a cheap protein sources, containing high nutritional values and it is popular for its delicious taste [2]. Commercial cultivation of marine mussel *Perna* is extensively carried out in several countries especially in the Southeast Asian region [3]. Thailand and Philippines are the major green mussel producers followed by India, Malaysia and Singapore [4-6].
The fishery and culture of *P. viridis* around the world are generally dependent on natural spat settlement. This is leading to the conflicts between the mussel farmers and the wild mussel harvesters [7]. In addition, rainfall and other various environmental factors cause the rate of mortality and this make only a fraction of the larval population to get a chance to settle on the natural rocky beds [8]. Therefore, spat production through laboratory culture is necessary to develop the commercial production of *P. viridis* and the larval development of this species was carried out by several workers.
P. viridis is one of the important species in the estuary ecosystem of Sitaw. The ethnic population from Ye estuary regularly harvests bivalves for their own consumption and daily income. Currently green mussels are becoming more important than other molluscs in this area because they rank second prize to the oysters. Other threats such as habitat destruction due to overexploitation and environmental degradation can also directly affect the mussel population in this area. Therefore a reduction in the abundance of mussels can indicate a negative change in the estuary ecosystem. The conservation and restoration of mussel population need to be sustainable development of mussel population. Through the cultivation of mussel, the stock can be restored and therefore the sufficient mussel seed supplying is needed to develop the cultivation of this species.
The consuming of green mussel in Mon coastal area is only depended on the natural mussel beds. On the other hand, the mussel beds on the Sitaw coast are limited and scattered. The techniques for mass culture of *Perna viridis* are required to fulfill the food requirement of local people and their employment at Ye estuary. According to the previous research on the spatfall of this species at Ye estuary, the natural seed resource on the rocky intertidal beds cannot

support mussel culture. The hatchery production of green mussel seeds in the laboratory, therefore, is attempted to carry out to initiate mussel production in Myanmar. The present study is the very first observation of larval rearing of *P. viridis* for the seed production and the first step to develop mussel seed production through the hatchery technique in Myanmar.

Materials and Methods

Broodstock Collection: Adult *P. viridis* were collected from subtidal mussel bed at Sitaw (Lat. 15° 11' N, Long. 97° 48' E), Ye River Mouth, Mon State, Myanmar in May, 2018. Approximately 80 adult mussels were scrubbed off debris under running tap water and placed them in 0.03% sodium hypochlorite solution for 5 minutes to control protozoan and rotifer populations. Mussels were then rinsed with tap water for 2-3 minutes and placed them into an insulated box with ice. The adults were then transported to the pearl oyster hatchery of Belpearl Co.Ltd., Russell Island, Taninthayi Region, Myanmar, within 36 hours for artificial spawning in the laboratory (Fig. 1). Mussels were held in running seawater and mussel food was provided in the laboratory.

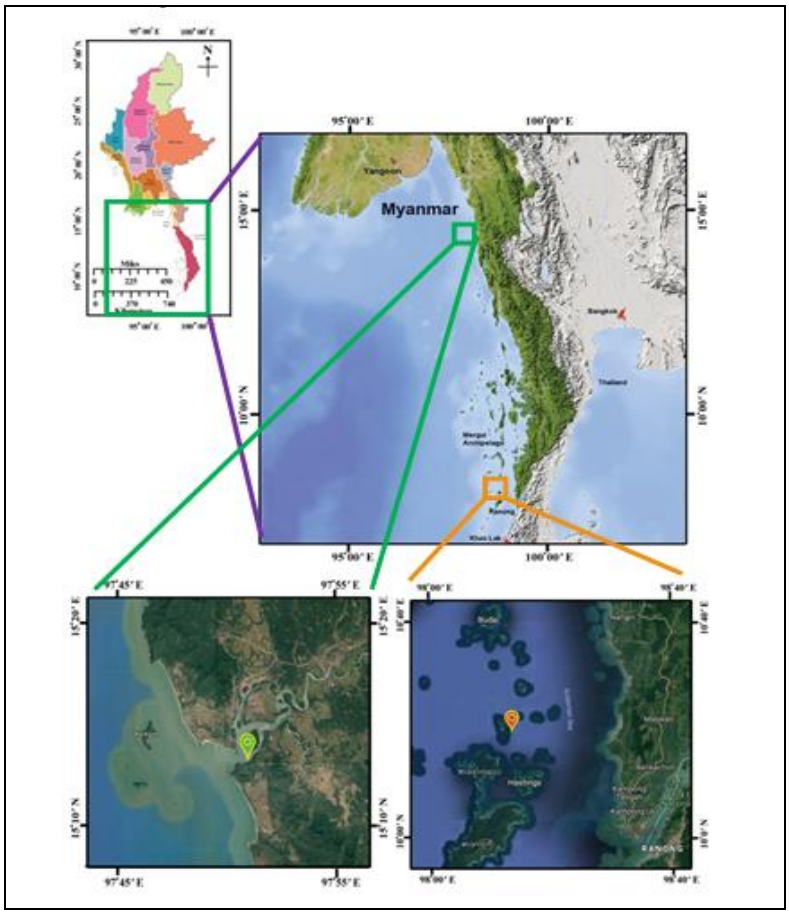

Fig 1: Map showing the broodstock collection site in Sitaw (green circle, lower right map) and larval rearing site in Russell Island (orange circle, lower left map) (Source: Google Maps).

Seawater preparation for culture: Seawater filtration system provided to the hatchery rooms is described as follows. Seawater was pumped from beyond low tide line and settled down in the first 500 L reservoir. Then it was filtered through 5 µ filter and 1 µ filter and then settled down in the second 500 L resevoir. Filtered seawater (FSW) was used for cleaning, larval rearing and mass production. For the algae culture, FSW was filtered again by using sterilized cotton filter. FSW was dispensed with P.V.C. pipes and air compressor provided aeration in the mussel larvae rearing.

Induced spawning and larval rearing: Induced spawning was done by a sudden rise of temperature from 18°C to 30°C. After fertilization, D-hinge larvae (2 days old) were placed into five 30 L tanks: four tanks were filled with antibiotic treated FSW (Fig. 2A) and one with natural seawater as a control experiment (Fig. 2B). The antibiotics used in the experiment were penicillin (P), streptomycin (S), a mixture of penicillin and streptomycin (PS), and chloramphenicol (Chlo.) with 0.02 mgL^{-1} concentration each. Before the D-hinge larvae were transferred to the respective larval rearing tanks, the antibiotic solution was added and strongly aerated. Antibiotics were added every three days during when the total volume of water in the culture tanks were changed. The seawater in the control tank was changed daily. Tanks were aerated for water circulation. Density in the tanks was 6 larvae mL^{-1}, in a total of c.a. 180,000 larvae in each tank.

The algae used to feed the larvae were *Isochrysis galbana*, *Chaetoceros calcitrans*, *Chaetoceros simplex*, *Chaetoceros gracilis* and *Chaetoceros ceratosporum*. Larvae were fed live cultures of the mixture of algae *I. galbana* and *C. calcitrans* starting from 2th day when the "D" shape was attained until the 7th day after which the larvae were fed five species of the mixture of the above-mentioned algae. Algal densities were gradually increased from 2.28-3.50 x 10^4 cells mL^{-1} to 5.2-6.3 x 10^4 celld per mL^{-1} as the larvae grew until 18 days rearing period. In this mussel hatchery, the monthly mean variation of the water temperature is ranged from 27.4 °C to 30.6 °C, salinity from 30.1‰ to 31.48‰, dissolved oxygen from 5.04 ml/L to 6.5 ml/L and pH from 7.7 to 8.4.

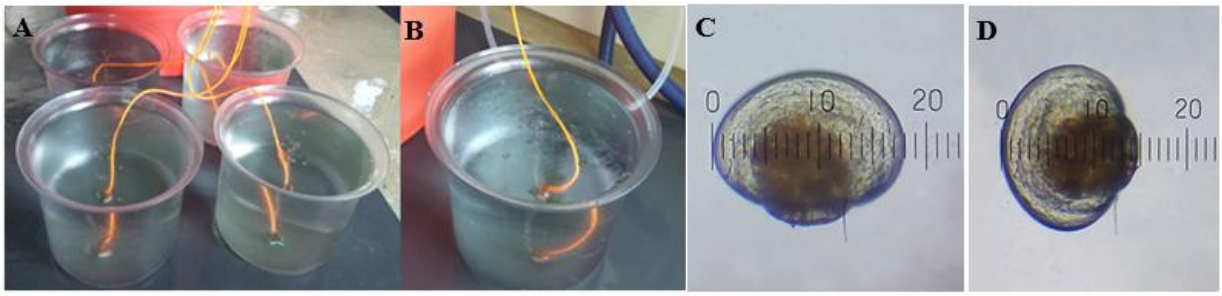

Fig 2: A-D. Larval rearing and biometric measurements of *Perna viridis*: A) Larval rearing in FSW treated with antibiotics; B) Larval rearing in natural FSW; C) larval shell length; D) larval shell height.

Sample measuring: To study morphological features of larvae during 18 days culture in the laboratory, 30 ml of seawater containing larvae was sampled and larvae were observed under optical microscope by using ocular meter. According to Bayne [9], shell length was measured as the greatest dimension in a line parallel to the hinge (anteroposterior axis) (Fig. 2C), and shell height was measured as the perpendicular dorsoventral axis (Fig. 2D). Mean values of larval growth were acquired from 33 measurements per tank under optical microscope. The morphological characteristics of each larval stage observed were compared to those of *P. viridis* described by Laxmilatha *et al*., [6] and Anil *et al*., [10]. The total number of eggs or larvae was calculated as follow:

$$\text{Number of egg or larvae} = \frac{\text{volume of rearing tank}}{\text{volume of sample}} \times \text{average eggs or larvae in sample}$$

Data analysis: Larval rearing during 18 days larval rearing period was analyzed at 5% level of significance. ANOVA was used to compare the amount of mussel larvae survival and growth rate in the different treatments of antibiotics. If significant and the variances showed to be homogeneous, average analysis according to Tukey test was applied using SPSS software program. For the other results, only descriptive statistical analysis was done. Survival rates were measured daily during the culture period.

Results and Discussion

Spawning took place after 2 hours of thermal stimulation from 18 °C to 30 °C. The male mussels released sperms first as a smoky stream (Fig. 3A) and females released millions of orange colored eggs (Fig. 3B). Laxmilatha *et al*., [6] and Sreedevi *et al*., [7] described the appropriate temperature regime for induced breeding and spawning of tropical green mussel *Perna viridis*. Anil *et al*., [10] spawned *P. viridis* and reared the larvae in the hatchery at Vizhinjam bay by rising the water temperature up to 30 °C-34 °C from the base temperature of 21 °C.

Induced spawning of bivalves is dependent on the availability of broodstock with mature gonads in ripe condition [11]. In the present study, the broodstock were collected before peak spawning seasons (June-August) and about 394,600,000 fertilized eggs were obtained from spawning. Of which 361,153,410 resulted in D-hinge larvae (Fig. 3M) about 16-20 hours after fertilization, representing a 90% yield in this phase of the hatchery. Of these only 900,000 were used for triplication in five experiments (one control and four antibiotic treatments) to observe the effect of antibiotics on the development and survival of the larvae. Laxmilatha *et al*., [6] reported that 2 million marketable spat-size mussels were produced from four experiments.

The embryonic development of *P. viridis* has a similar development compared to the previously studied bivalves by several workers [10, 12-19] although it was slightly faster than their results. The timing of developmental stages varies with different temperature, different species and different habitats.

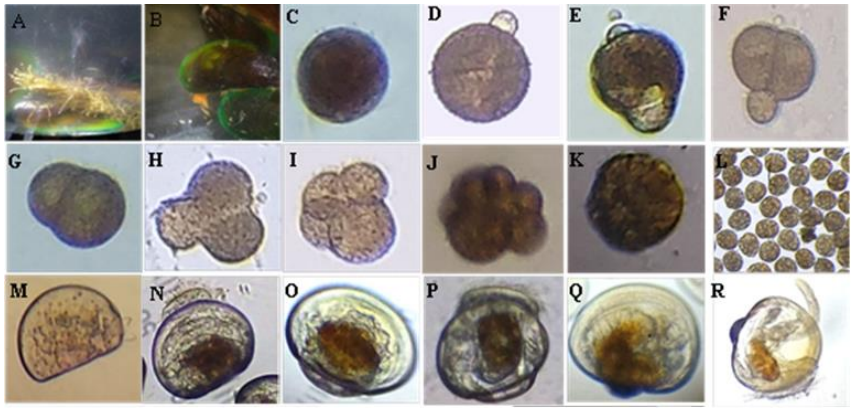

Fig 3: A-R. Spawning, Fertilization and development of mussel embryos A) Male green mussel releasing white clouds of sperms; B) Female green mussel releasing orange eggs; C) fertilized egg (50µm); D) fertilized egg with first polar bodies developing; E) Extrusion of the first and second polar bodies; F) First cleavaged egg showing the "trilobed" appearance; G) two celled stage; H) Formation of the second polar ; I) second cleavaged and formation of 4-celled stage; J) 8-celled stage; K and L) ciliated gastula stage (60µm); M) D-hinge stage; N) Early umbo showing overall organogenesis and microalgae (brown pigmentation) content in stomach (5 d); O) late umbo (7d); P) umbonate (9d); Q) Eye-spot stage larvae (13 d); R) Pediveliger larvae with developed foot (18 d).

Different larval stages of *Perna viridis* larvae in five experiments during 18 days rearing period is shown in table 1. Of these five culture experiments, only two experiments treated with a mixture of penicillin/streptomycin and chloramphenicol succeeded to develop to the pediveligers stage. The larval stages of *P. viridis* (Fig. 2M-2R) goes through similar larval stages as described for *P. viridis* by Aquacop and Cnexo-cop [12], CMFRI [20], Kamermans *et al*., [17], Hanyu *et al*., [18], Anil *et al*., [10]. Large variations in the growth rate of larvae of the same batch were observed in *P. viridis* as has been reported in several other bivalves.

The larval stage typically last 3 weeks and the larvae grew to a shell length between 300-400 µm. At this point, they are referred to as eye-spot larvae (pediveligers) because they have developed an 'eye-spot', and a 'foot' in addition to the velum (Fig. 3R). Larvae at this stage were capable of swimming with the velar cilia as well as crawling with the foot. The velum was reduced in size and was replaced by buds that will later form the gills. The average shell length was 332.83± 10.34 µm (300-400 µm) on day 18. The larvae have now transformed from the free swimming pelagic larvae to the creeping, crawling benthic stage ready to attach to the substratum.

Table 1: Different larval stages of *Perna viridis* in days (d)

Stage	P	S	PS	Chlo.	Cont.
D	3 d	3 d	3 d	3 d	3 d
EU	6 d	6 d	5 d	6 d	7 d
LU	-	9 d	7 d	8 d	11 d
U	-	17 d	9 d	10 d	-
E	-	-	13 d	14 d	-
PV	-	-	18 d	-	-

P=penicillin, S=streptomycin, PS=a mixture of penicillin and streptomycin, Chlo.=chloremphenicol, Cont.=control, D=D-hinge stage, EU= early umbo, LU= late umbo, U= umbonate, E= eye-spot, PV= pediveliger, d=day.

The growth of shell length and the survivality of mussel larvae during 18 days larval rearing period are shown in figure 4 and 5. Average shell length measurements among control and treatment larvae over 18-day rearing period were significantly different (ANOVA; $F_{4,\ 57} = 5.72$, p=0.001 < 0.05). Average shell lengths in the larvae treated with a mixture of penicillin and streptomycin were significantly different to the other treatments (p<0.001). However, this result were not significantly different from average shell length in the larvae treated with chlorempheciol during 18 days period (p = 0.684>0.05). The survivability (%) of larvae among antibiotic treatments and control was significantly different (ANOVA; $F_{4,57} = 4.96$, p=0.02<0.05). Average percent survival of the larvae treated with a mixture penicillin and streptomycin was significantly different to the other larvae (Turkey test; p=0.013< 0.05) however, there was no significant (Turkey test; p = 0.415>0.05) difference in percent survival between these larve and the larvae treated with chloremphenicol.

In the present study, the larvae treated with a mixture of penicillin/streptomycin showed highest larval development, survival rate and growth rate among five experiments. Average shell lengths and percent survival of these larvae were significantly different to the other treatments. These finding agreed with the result of Kesarcodi-Watason [21] although the concentration was less than those of their studies. Benzylpenicillin and streptomycin are allowed chemicals to use in aquaculture [22]. Combinations of antibiotics such as penicillin and streptomycin have been widely used in cultivation with positive survival and growth results in the species of interest. The synergist effect of combining drugs of different groups depends on the degree of sensitivity of the microorganism to these antibiotics [23].

Antibiotics are commonly used in the world used to avoid the adverse effect of pathogens in aquaculture [3, 24-27]. Chloramphenicol is totally banned for use in aquaculture and can be used only for research purpose [22, 27]. Benzylpenicillin and streptomycin are allowed chemicals to use in aquaculture [22]. Therefore the larvae treated with a mixture of penicillin and streptomycin were then transferred to the settlement tanks hung with collectors two days after 90 percent of larvae were with eye spots as well as the larvae were still actively free-swimming.

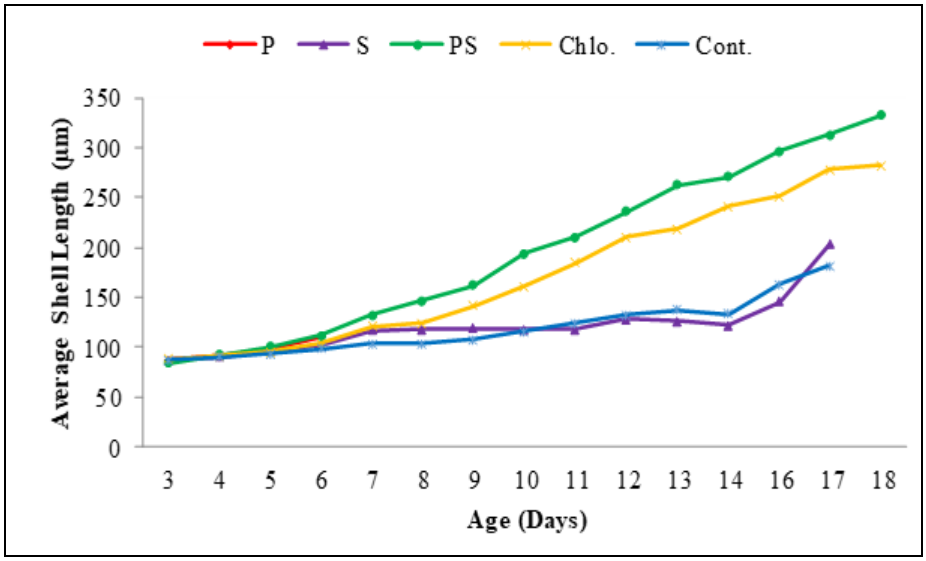

Fig 4: Average shell length of mussel larvae over 18-day rearing period when inoculated with different antibiotics. The larvae treated with penicillin had high mortality at day 7; therefore shell length was not attainable

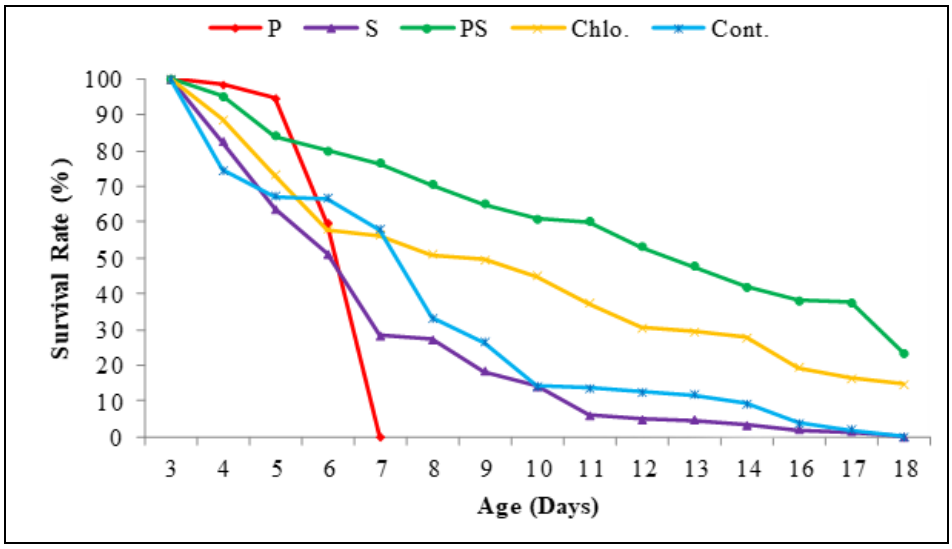

Fig 5: Average percent survival of mussel larvae over 18-day rearing period

Conclusion

Only one temperature jump was necessary to indue green mussels in this study. This may be because the broodstocks were collected in the maturity stage and just before the spawning season of mussels in the wild. The higher growth and survival rate of the larvae were obtained by using a mixture of penicillin and streptomycin. This may be due to the synergic effect of the combination of these two antibiotcs. The result of this study is encouraging as this can be successfully induced to spawn in a low cost yet effective way. Significant investments, however, are needed to develop hatchery production at a scale that would be economically viable. Though more works need to be done to establish a complete larval rearing protocol, this study shed positive lights on introducing new species to the local mariculture industry to Myanmar, empowering the local with simple yet effective bivalve culture technique and enable species restocking in the coastal ecosystem when necessary in future.

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