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Effects of Water Quality, Stocking Density, Water Exchange Frequency, and Food, on Growth and Survival of the Green Mussel, *Perna viridis* Larvae

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Abstract

Experiments were conducted to determine the optimum conditions for growth and survival of the green mussel, *Perna viridis* larvae. Effects of various temperatures and salinities, stocking densities, water exchange frequency, and natural food preference of the larvae from D-hinged to pediveliger stage were investigated. The green mussel broodstocks were collected from the natural source, and spawning occurred in captivity. All experiments were conducted in triplicate. The results demonstrated that larvae from D-hinged to pediveliger stage had better growth and survival when the temperature was between 29ºC and 30ºC, and salinity ranging from 30-33ppt. Stocking density from 10-20 larvae/ml did not affect growth and survival of the larvae. Likewise, frequency of water replacement from daily to every 5 day interval did not influence the growth and survival until the pediveliger stage. During this stage, the larvae preferred a combination of *Isochrysis galbana* and *Chaetoceros calcitrans* as food in terms of better growth, and either *Isochrysis galbana*, *Chaetoceros calcitrans*, or their combination in terms of higher survival rate.

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Introduction

Green mussel, *Perna viridis* is a tropical mussel species native to the Indo-Pacific region and is abundantly found in the coastal waters of Indonesia, Malaysia, Thailand, China, India, and the Philippines where it is regarded as an important food source (Appukuttan 1977; Sivalingam 1977; Sidall 1980; Vakily 1989; Gosling 2004; Sallih 2005). *P. viridis* is widely recognized as an ideal aquaculture commodity due to its high and inexpensive protein source, rapid growth rate, early onset of maturity, high tolerance to a broad range of environmental conditions, and requiring minimal investment for farming (Vakily 1989; Rajagopal 2006).

In the Philippines, *P. viridis* is the only edible mussel species cultivated for commercial purposes (Aypa 1995). Following the establishment in 1955 of the first commercial mussel farm, mussel farming has since spread throughout the country, and various culture methods have been established including stakes and poles, rope web method, bottom and raft culture (Sitoy et al. 1988; Vakily 1989; Aypa 1995). However, despite its long culture history, the country's mussel industry is far from sustainable. Production of farmed green mussel has been inconsistent. Fluctuations in annual mussel production caused by limited seed or spat availability is recognized as one of the major constraints in the development of mussel industry.

The sustainability of mussel production is highly dependent on a reliable and continuous source of seed supply. In the Philippines, wild seed stock still constitutes the primary source of seed for mussel culture. Mussel seeds for grow-out culture are either gathered directly from natural breeding areas or collected through bamboo poles and wooden stakes employed in these areas for spat collection (Sitoy 1988; Vakily 1989;). However, wild seed stock is becoming scarce and insufficient, due to the rapid depletion of natural breeding stocks, destructive anthropogenic activities, predation, climate change, disease outbreaks, and massive mortality (Dailianis 2010; Segvic-Bubic et al. 2011; Callaway et al. 2012; Castinel et al. 2019). Insufficient seed supply can adversely affect development of the mussel industry. Hence, seed production in hatcheries is now seen as an alternative to collection of wild seeds (Helm et al. 2004), and augmentation of mussel seed supply in the country. Growing seeds under controlled conditions minimizes environmentally induced mortalities thus improving survival as well as production. It also helps reduce the pressure on the wild stock and provide livelihood opportunities for mussel farmers (Laxmilatha et al. 2011).

Green Mussel larval rearing involves growing larvae from fertilized eggs under laboratory conditions. Production of green mussel spats under controlled conditions has been successfully studied (AQUACOP 1979; Yap et al. 1979; Laxamilatha et al. 2011). However, setting up and running hatcheries is very costly (Helm et al. 2004; Gosling 2004), thus economic viability of hatchery seed production of *P. viridis* is yet to be determined for commercial production (Yap et al. 1979; Laxamilatha et al. 2011). Improvement of hatchery seed production technology is a big step towards an economically viable mussel hatchery. This involves better understanding of the biology of the species being cultured such as feeding mechanism, nutrition, larval development, growth, survival, and culture methods (Mann 1979; Helm et al. 2004).

Diet, temperature, and salinity play critical roles in the development, growth, and survival of bivalve larvae especially during its early stage (Gosling 2004; Helm et al. 2004; Rico-Villa et al. 2009). The influence of these factors, separately or jointly should be defined when considering large scale production of mussel seed. This study aimed to determine the water quality (temperature and salinity), favorable culture conditions (stocking density and water exchange frequency), and appropriate food for larval growth, development, and survival of the green mussel, *Perna viridis* from D-hinged to pediveliger stage.

Methodology

Broodstock collection and spawning.

Mussel broodstocks were obtained from traditional mussel growing sites in Roxas City, Capiz. Sexually mature green mussels measuring about 6 cm in length were used in spawning trials. Maturity of mussels was determined by taking 10-15 individuals from the batch, dissecting, and examining gonads. The presence of full and enlarged gonads characterized by orange to brick-red appearance for females and milky white for males indicates maturity.

The broodstocks were transported to the mussel hatchery by wrapping them in moist cloth and placing them inside a styrofoam box with ice. Upon arrival, they were placed in 300-liter capacity wooden tanks containing natural sand-filtered seawater (with the same salinity as that of the source) and provided with mild aeration. Prior to spawning, mussels were thoroughly cleaned by brushing off dirt and removing unwanted organisms from shells. Mussel broodstocks were desiccated for 1 hour by totally draining the water in the spawning tank. Thereafter, fresh seawater gradually filled the spawning tank. Upon indication of initial release of gametes, mussels were individually transferred to plastic containers with 250 ml of seawater. Sperm and eggs were collected separately and were subjected to quantitative microscopic analysis. Eggs were fertilized within one hour after being discharged using 1:50 egg-to-sperm ratio. Fertilized eggs were incubated in 1-ton capacity fiberglass tanks at 20 eggs/ml using UV-treated seawater.

Twenty-four hours after fertilization, water from the tanks was drained and fully developed D-hinged larvae were retained in 45 um nylon mesh sieves, washed with UV-treated seawater and transferred into a container. Number of larvae per mL of water was determined by taking three (3) 1mL samples from well-mixed larval concentrates, counted under the microscope at 4X magnification using a Sedgewick-Rafter counting cell.

Effects of food

Microalgae used in this experiment were cultivated in 1-L flasks under laboratoy conditions using F-medium for diatom (*Chaetoceros calcitrans*) and Conwy for flagellates (*Isochrysis galbana* and *Tetraselmis* sp.) as fertilizers. Larvae were fed twice daily with four diet treatments: *Isochrysis galbana* alone, *Chaetoceros calcitrans* alone, *Tetraselmis sp*. alone; and a combination of *Isochrysis galbana* and *Chaetoceros calcitrans* at 1:1 ratio. The amount of feed given was computed using the formula:

$$\text{Amount of feed (mL)} = \frac{\textit{feeding rate x total number of larvae}}{\textit{algal cell density}}$$

Below are the feeding rates followed:

1-4 d	(2,000 cells/larva/day)
5-9 d	(5,000 cells/larva/day)
10-14 d	(12,000 cells/larva/day)
15-19 d	(20,000 cells/larva/day)

Effects of water temperature

The effects of different water temperatures (from 28ºC to 31ºC) on larval development and survival were tested. The levels tested were based on the known temperature requirement for other bivalves. The required experimental temperature was maintained at its respective level using the PERIHA 300-watt heater installed in each experimental tank and in the stock water. Each temperature level was tested in triplicate.

Effects of salinity

The different water salinity tested ranged from 30-33 ppt based on the reported optimum salinity for other bivalves that influenced better growth and survival of the larvae. Desired salinity was obtained by mixing the freshwater and seawater. Each treatment was replicated 3 times.

Stocking density experiment

Three stocking densities were tested: 10, 15, and 20 larvae ml-1. The larvae were stocked at 0.8M, 1.2M, and 1.6M per tank at 3 replicates each. The number of individual stocked per ml was based from previous studies in other bivalve species.

Water exchange frequency experiment

In a separate experiment using another set of larvae, 3 water exchange frequencies were evaluated in triplicate: daily, every 3 days, and every 5 days, all at 100% of the water was changed.

Larval rearing conditions across experiments

D-hinged *P. viridis* larvae with initial height of 63.7 ± 0.8 µm and length of 82.4 ± 0.6 µm were reared for 18 days under static conditions in 100-L capacity fiberglass tanks, filled with UV treated seawater. Cultures were established at 10 larvae/ml stocking density (except for the experiment on stocking density) and provided with mild aeration. Each treatment had 3 replicates.

Water exchange (100%) as well as growth increments and survival estimates were carried out every day (except for the experiment on water exchange frequency). Mean shell length and height of larvae was determined by taking 25 ml sample from each replicate, fixed in Lugol's solution, and examined under the microscope (at 10X magnification) using a Sedgewick-Rafter counting cell where images from 10-20 individuals from each replicate were captured and analyzed using Motic 2.0 software. The larvae were measured along the two axes, the longer anteroposterior axis (APM), which is the shell length and the shorter dorsoventral axis (DVM), the shell height (Laxmilatha et al. 2011).

Statistical analysis

The data were presented as means±standard deviation (n=3 replicates) and analyzed with ANOVA using the SPSS 16. Significant differences between treatments were further determined by Tukey's test.

Results

Effects of food

The growth of the larvae as shown by the increase in their length and width, exhibited a similar pattern in all the dietary treatments (**Figure 1**). Highest increase both in length and height of the larvae was observed in the group fed with Ig + Cc which was significantly different from those fed with Ig alone and Cc alone. The larvae fed with *Tetraselmis* sp. did not survive after Day 8 (**Figure 1**). The results indicated that the combination of *Isochrysis galbana* and *Chaetoceros calcitrans* as food was best for the larvae from D-hinged to pediveliger or eyed stage). However, diet did not significantly affect the survival of the larvae.

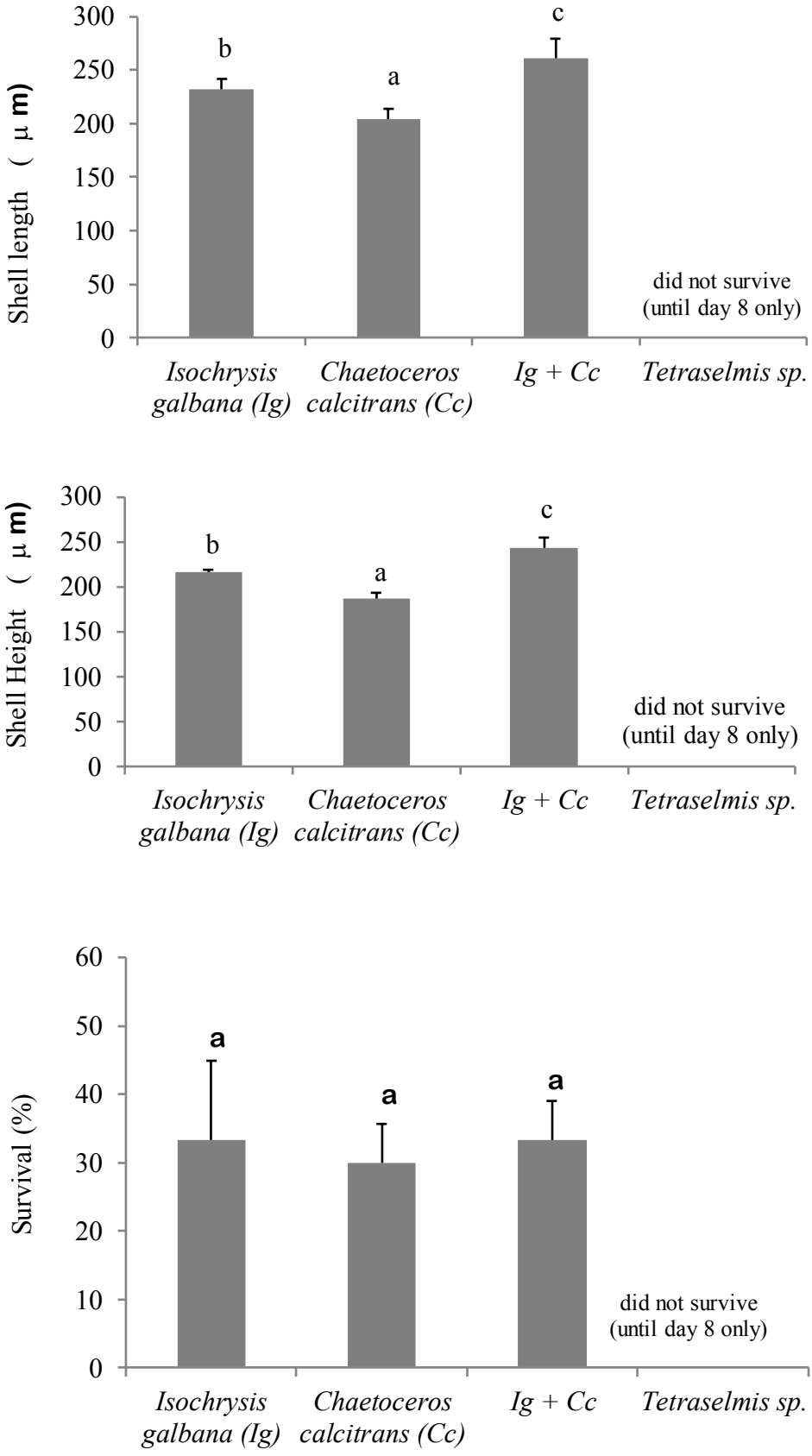

Figure 1 Growth and survival of green mussel from D-hinged to pediveliger stage fed different microalgae for 18 days. Bars are means ± SD (n=3). Means not sharing the same superscript letters are statistically different.

Effects of temperature

Growth of larvae was significantly improved when reared at 30ºC water temperature (**Figure 2**). Exposure of larvae to temperature lower and higher than 30ºC resulted in poor growth. Survival was better between 29 and 30ºC but no significant differences were observed due to the high variability of the data.

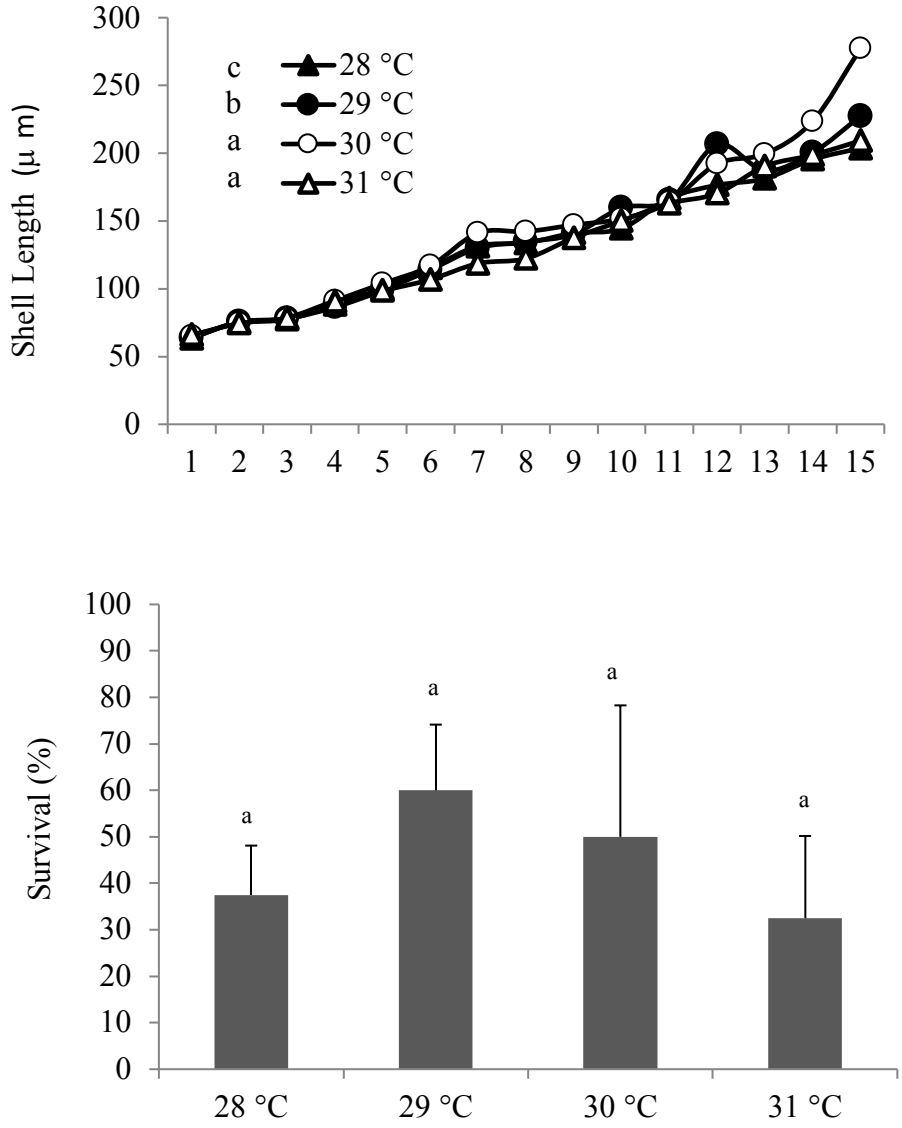

Figure 2. Effects of temperature on the growth and survival of the green mussel from D-hinged to pediveliger stage. Means ± SD (n=3) not sharing the same superscript letters are statistically different.

Effects of salinity

The growth of the larvae was significantly higher at 30–31ppt (**Figure 3**). The larvae exhibited lower growth at salinity higher than 31ppt. Survival showed a decreasing pattern with increasing salinity however, no significant differences were observed between the treatments.

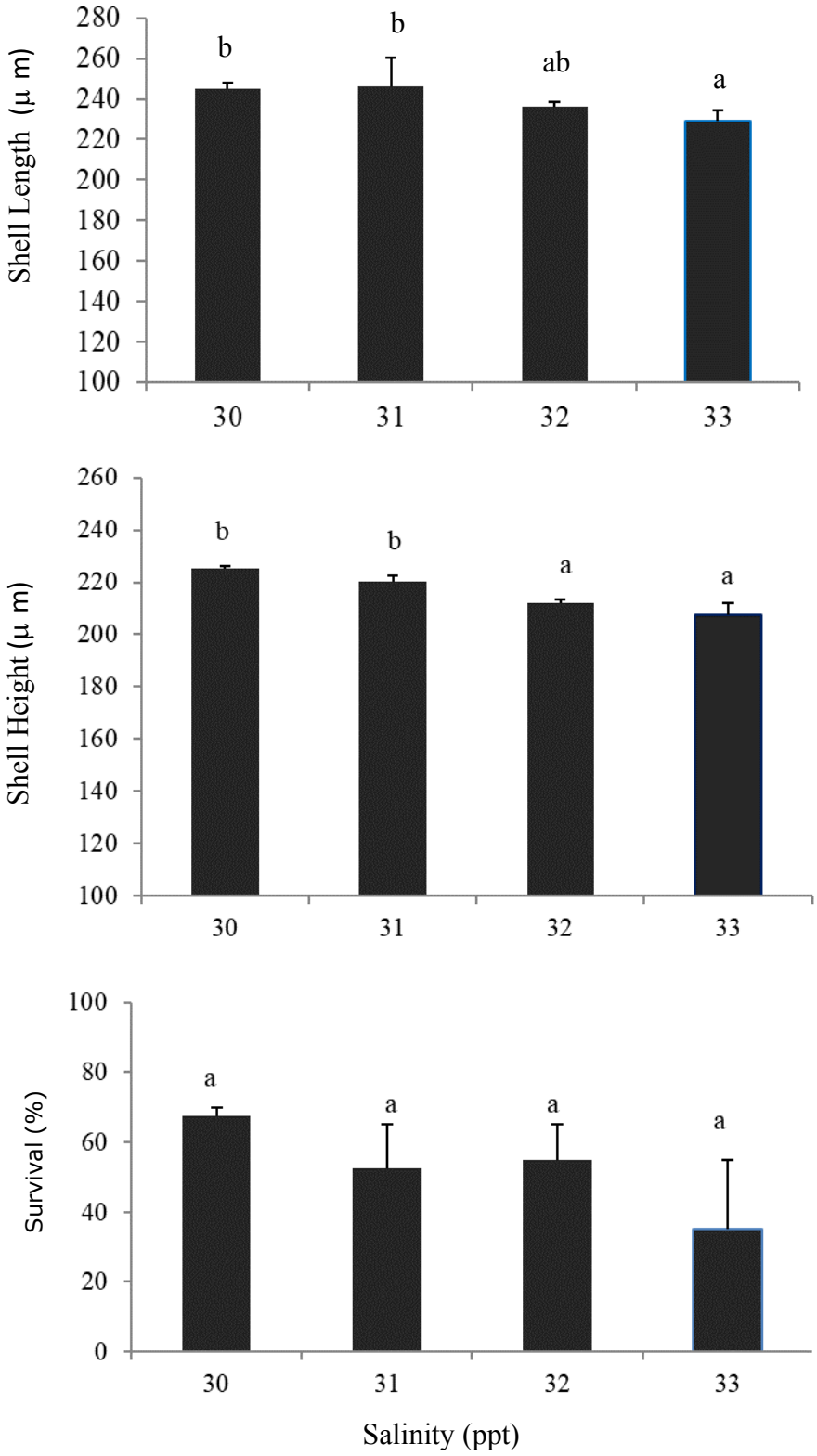

Figure 3 Influence of salinity on the growth and survival of the green mussel from D-hinged to pediveliger stage. Bars are means ± SD (n=3). Means not sharing the same superscript letters are statistically different.

Effects of stocking density and water exchange frequency

Stocking density of 10-20 larvae/mL (**Table 1**) and water exchange frequency of daily, every 3 days, or every 5 days (**Table 2**) did not affect the growth and survival of the larvae from D-hinged to pediveliger stage.

Table 1 Effects of stocking density on the growth and survival of the green mussel larvae*

Stocking Density (larvae/ml)	*Length (SGR, %/day)*	*Height (SGR, %/day)*	*Survival (%)*
10	8.4 ± 0.8	8.1 ± 1.0	69.3 ± 15.3
15	8.3 ± 1.0	7.3 ± 0.8	59.1 ± 10.6
20	8.2 ± 0.5	7.2 ± 0.5	45.9 ± 9.3

*Means ± SD (n=3) are not significantly different.

Table 2 Effects of water exchange frequency on the growth and survival of the green mussel larvae*

Water Exchange Frequency	*Length (SGR, %/day)*	*Height (SGR, %/day)*	*Survival (%)*
Daily	8.7 ± 0.4	7.4 ± 0.4	73.7 ± 16.3
Every 3 Days	8.4 ± 0.6	7.1 ± 0.6	62.3 ± 10.4
Every 5 Days	8.7 ± 0.3	7.3 ± 0.3	57.4 ± 6.1

*Means ± SD (n=3) are not significantly different.

Discussion

Rearing of filter-feeding bivalves requires more microalgal diets than any other aquatic organisms such as gastropods, crustaceans, and fish (Aji 2011). The requirement for microalgal diets varies depending on their developmental stage (e.g. broodstock, larval and post larval stage). In the hatchery, microalgae are considered the "heart of the system" since larval rearing activities are highly dependent on the production and availability of these organisms. The culture of natural food is deemed the most expensive and technically challenging aspect of all hatchery operations (Creswell 2010). However, not all algal species are successful in supporting growth and survival of a particular filter-feeding animal (Lavens and Sorgeloos 1996). Hence, finding the most appropriate algal species to culture is very critical. Suitable algal species should be selected based on the following factors: cell size, digestibility, production of toxic compounds, and biochemical composition (Lavens and Sorgeloos 1996; Guedes and Malcata 2012). The biochemical components of microalgae include lipids, proteins (amino acids), carbohydrates and vitamins, and highly unsaturated fatty acids (HUFA's). Particularly, algal species with HUFA's containing eicosapentaenoic acid (20:5n-3, EPA), arachidonic acid (20:4n-6, ARA), and docosahexaenoic acid (22:6n-3, DHA) are considered superior algal food (Lavens and Sorgeloos 1996; Gosling 2004; Creswell 2010; Guedes and Malcata 2012; de la Peña and Franco 2013). The cell size and biochemical composition of the microalgae are reported in de la Peña and Franco (2013).

Chaetoceros calcitrans (Cc), *Isochrysis galbana* (Iso), and *Tetraselmis* sp.(Tetra) are three of the most high-valued algal species commonly cultured and used as feeds in bivalve hatcheries due to their high lipid content and HUFA profiles (Lavens and Sorgeloos 1996; Helm et al. 2004; Creswell 2010).

In this study, *Perna viridis* larvae fed with the bi-species combination of diatom, *Chaetoceros calcitrans* and flagellate, *Isochrysis galbana* resulted to an improved larval growth performance. Similarly, the larvae of *Anomaocardia brasiliana* grew best when fed bi-algal diets, particulary a mixture of flagellates *Pavlova lutheri* and diatom *Chaetoceros calcitrans* (de Oliveira et al. 2016). Likewise, in *Crassostrea gigas*, the combination of *Isochrysis affinis galbana* and *Chaetoceros calcitrans forma pumilum* induced the best growth to the larvae (Rico-Villa et al. 2006).

The nutrient contents of *Chaetoceros calcitrans*, *Isochrysis galbana* and *Tetraselmis* sp. were reviewed by de la Peña and Franco (2013). As reported, protein content of these three algae ranges from 2.6-40% of dry weight with diatom having lower values. Highest lipid concentration, on the other hand was found on *Isochrysis galbana* with 21.2-36.2%. Furthermore, both DHA and EPA are present in all three algal species while ARA was lacking in *Isochrysis galbana*. Among these biochemical

components, the presence of essential fatty acids (EFAs) particularly the EPA and DHA in the diet were predominantly associated with high growth yields of bivalves (Lavens and Sorgeloos 1996; Gosling 2004; Creswell 2010; Guedes and Malcata, 2012; de la Peña and Franco, 2013). EPA and DHA are major membrane components and play an essential role for membrane function.

Chaetoceros calcitrans is an EPA-rich diatom with 2.9-14% whereas *Isochrysis galbana* is DHA-rich prymesiophytes with 2.1-16.7% (de la Peña and Franco, 2013). The presence of both algae in diet sufficiently supplies the DHA and EPA requirement for optimum growth of P. *viridis* larvae. Hence, high nutritional value of mixed diet (*Isochrysis galbana* and *Chaetoceos calcitrans*) is mostly related to high concentrations of both EPA and DHA.

Aside from the biochemical components, another important factor to be considered in bivalve nutrion is the size of algal cells. The processing of algal particles by bivalves highly varies in terms of bivalve size, algal species and algal concentrations (Berg et al. 1996). To ensure proper ingestion, larvae must be provided with algae of appropriate cell size in relation to its body size. Highest clearance rates of bivalve veliger larvae are normally between particle sizes 4.7 and 6.3um (Sommer et al. 2000).

The feeding of larvae with *Tetraselmis* sp. resulted in complete mortality on the 8th day of culture. Similar result was observed in oyster larvae fed with *Tetraselmis chui* where high mortality (>50%) occurred after nine days of culture (Blanchard et al. 2008). Both results suggest that high to 100% mortality observed in larvae fed with *Tetraselmis* genus was directly related to algal cell size rather than nutrition. *Tetraselmis* sp. used in this study have cell size of 8-11 um in width, 10-16 um in length and 4.2-5.0 µm in thickness while *Chaetoceros calcitrans* and *Iosochrysis galbana* only measures 5-6 µm in width (de la Peña and Franco, 2013). The relatively large cells of *Tetraselmis* sp. was poorly ingested by the larvae which resulted in complete mortality. Although this species has high nutritional value in terms of biochemical composition, it can only be fed on larvae with shell length >120 um (Lavens and Sorgeloos 1996; Helm et al. 2004).

Overall, results suggest that the mixture of *Isochrysis galbana* and *Chaetoceros calcitrans* is a superior diet for the larvae of *P. viridis* due to its appropriate cell size and balanced concentrations of important cellular components (protein, carbohydrate, lipid, energy, EFA's).

In this study, the growth of *P. viridis* from D-hinged larvae to pediveliger stage appears to be highly sensitive to even a small increase (1°C) in water temperature. A positive relationship between larval growth and temperature was found within the range of 28-30°C; yet, at temperature beyond 30°C, larval growth was reduced significantly. This finding is in agreement with that of most bivalve larval studies which highlighted that a rise in water temperature increases larval development and growth rates up to a certain threshold, but beyond which may have detrimental effects and subsequently jeopardized growth and survival rates (Sanchez-Lazo and Martinez-Pita 2012; McCormick et al. 2016; Huo et al. 2017). Temperatures may also have different effects on several bivalve species such as reduced growth and survival, enhanced growth but reduced survival or complete mortality. Davis and Calabrese (1969) observed a reduced growth and survival of European oyster, *Ostrea edulis* when temperatures were 30°C and 32°C. On the other hand, total mortality of the *P. viridis* larvae occurred after 24h at temperatures of 33ºC and 35 ºC (Nair and Appukuttan 2003). Peng et al. (2016) reported that larval growth rate increased while survival rate decreased when C. *iredalei* larvae were cultured in high temperature (34°C).

Extreme water temperatures (beyond tolerance limit of larvae) often lead to physiological disorder and may result in complete mortality before completion of larval development (Huo et al. 2017).

Particularly in this study, growth rate and survival at the highest temperature tested (31°C) were considerably reduced indicating that temperature has directly affected the larvae. This is in contrast to the results of Nair and Appukuttan (2003) in *P. viridis* with optimum larval survival at 31ºC. Discrepancy in the results could be influenced by many factors such as differences in experimental design and culture methods. Widdows (1991) has observed that any factor that reduces growth rate will

have a major effect on mortality and the chances of survival to settlement stage and beyond.

In general, temperature highly influences the rates of metabolic activity of bivalves. Improved growth is the result of increased feeding or ingestion rates and enhanced assimilation of enzyme for feed digestion brought about by the increase in water temperature (Rico-Villa *et al*. 2009; Sanchez-Lazo and Martinez-Pita 2011; Huo et al. 2017). In the present study, a temperature range of 29-30ºC was found suitable for the rearing of *P. viridis* from D-hinged to pediveliger stage which resulted in improved growth and survival of the larvae.

Salinity is an important abiotic factor that affects bivalve feeding, reproduction, growth, respiration and osmoregulation (Gosling 2004). *Perna viridis* are euryhaline, they are mostly found in environments with extremely fluctuating salinities such as in the mouths of the rivers and estuaries (Vakily 1989; Rajagopal et al. 2006). Their high degree of adaptability or tolerance towards salinity changes made them successful invaders. The normal salinity range of *P. viridis* is between 27ppt and 33 ppt (Gosling 2004). However, compared to adult mussels, the early larval stages have a much narrower salinity tolerance.

In the present study, optimum salinity for the green mussel larval rearing was observed at 30-31ppt at 30°C water temperature. This was based on larval growth and survival exhibited by the larvae on a 15-day rearing period. Similarly, growth and survival of the larvae of *Crassostrea virginica* and *Mulinia lateralis* were best at 19-30ppt (Lough 1975). Also, *P. japonica* larvae reared at 32 ppt exhibited rapid development, larger larvae, with high survival however, 100% mortality after day 12 was experienced when salinity was less than 25ppt (Huo et al. 2017). Fang *et al*. (2016) reported that lower salinities delay growth and development of *Crassostrea iredalei* larvae.

Generally, most findings suggest that a reduced salinity negatively affects larval performance. According to Jorgensen (1996), filtration rate, assimilation efficiency, and respiration of bivalve are compromised at low salinities which results in reduced growth, development, and survival.

Growth and survival of the larvae were not significantly affected by the stocking density from 10-20 larvae/ml. In *Ruditapes philippinarium*, stocking density of 10-20 larvae/ml did not also influence the growth of the larvae (Yan et al. 2006). Liu et al. (2006) reported that 10-40 larvae/ml similarly did not significantly affect growth of *Meretrix meretrix larvae*. In this study, survival of *P. viridis* larvae though not statistically different from other groups was slightly improved when reared at 10 larvae/ml.

The optimum stocking densities determined for various bivalve species were 2-5 larvae/ml (flat oyster), 5-10 larvae/ml (scallops), and 15-20 larvae/ml for *Crassostrea* and *Tapes* (Helm and Bourne 2004). Moreover, larval growth and survival may possibly be improved if stocking densities are adjusted as bivalves grow (by thinning out). Helm and Bourne (2004) provided a rough guide to applicable densities related to shell length: 15-10 larvae/ml (at larvae of less than 120 um), less than 5 larvae/ml (for 150-200um larvae) and 2 larvae/ml (for 250-300um).

In the present study, similar growth and survival data were obtained when D-hinged larvae were reared under different water management frequency. In general, commercial hatcheries routinely change the water in the culture tank at 48H interval or on alternate days. Earlier work of Helm and Millican (1977) on oyster *Crassostrea gigas* showed slow growth when water exchange was done daily rather than at 48H or at 72H interval. In addition, growth is enhanced when frequency of water exchange is set at every three days or at 72H interval (Yan et al. 2006). Highest growth of mangrove oyster *Crassostrea rhizophorae* was attained at 48H or 72H water exchange frequency (Antonio et al., 2009). Based on the results and literature search, current practice of water exchange being conducted by the project is set at 48H interval.

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