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FULL PAPER

Growth, development and survival of *Holothuria scabra* larvae in different microalgal regimens and water rearing media

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ABSTRACT

Different aspects of *Holothuria scabra* larval production, including feeding regimen and water treatment, were investigated under experimental conditions. This study highlights the optimization of techniques and simplification of the requirements of sea cucumber larval rearing. The growth performance, development, and survivorship of *H. scabra* larvae were measured to assess which treatment provides optimum results. *Chaetoceros gracilis* (*Cgr*) and *Chaetoceros calcitrans* (*Cc*) were administered singly and in combination (*Cgr-Cc*) to sea cucumber larvae. Growth was highest in combined *Cgr-Cc* feed with mean final length of 2088µm, followed by *Cc* with 1855 µm and *Cgr* with 1800 µm, but with no significant difference ($p>0.05$). Similarly, survival rates among treatments were not statistically different (*Cgr-Cc* = 2.23%; *Cgr* = 1.6%; *Cc* = 1.3%) ($p>0.05$). However, larval development was better in combined *Cgr-Cc* and *Cc* single diet, with 90% and 100% composition of early juveniles on Day 30. Slower development was observed in *Cgr* single feed, with only 90% early juveniles observed later on Day 35. Different microalgal concentration of *Cgr-Cc* (10,000, 30,000 and 50,000 cells.mL^{-1}) were also tested. Juveniles (~3 mm) yielded from 50,000 cells.mL^{-1} microalgal concentrations were five times larger than when fed at 10,000 cells.mL^{-1} microalgae. Development of larvae was also faster in 50,000 cells.mL^{-1}, yielding harvestable juveniles in 25 days. However, water replenishment in tanks with high microalgal density should also be regularly done at 50-70% rate in two days interval to mitigate fouling. In addition, sand-filtered, chlorinated, and UV-treated seawater were also tested for their efficiency as culture media. Growth rates were significantly highest in sand-filtered seawater (68.3 µm.d^{-1}), followed by UV-treated seawater (52.4 µm.d^{-1}), and by chlorinated seawater (34.8 µm.d^{-1}) ($p<0.05$). Larval development did not differ in sand-filtered and UV-treated seawater, yielding ~1 mm juveniles as early as Day 25. Likewise, sand-filtered seawater rendered highest survival of larvae (10.24%) followed by UV-treated seawater (6.24%); chlorinated seawater yielded lowest (2.60%) ($p<0.05$). Although a sterilization process is advised, findings on sand-filtered seawater as a rearing medium were notable.

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1. INTRODUCTION

Sea cucumbers are among the most important and highly-priced marine invertebrate resources in the Philippines (Olavides et al. 2010). Out of 1,717 species known to exist, there are about 41 commercial species of sea cucumber in the country, and 25 of these are regularly collected for bech-de-mer, trepang, or dried sea cucumber product (Gamboa et al. 2004; Paulay 2014; Schoppe 2000). The increasing demand for trepang, active harvesting from local fishers, and inadequate management of sea cucumber natural stocks have resulted in severe overexploitation and imposed fishing pressure on the natural population of this commercially important species (Battaglene 1999; Hamel et al. 2001). The release of hatchery-reared juveniles has been suggested as a good solution to restore depleted populations in the wild (Battaglene and Seymour 1998; Trinidad-Roa 1987).

Larval rearing is probably the most critical phase of sea cucumber seed production. According to Duy (2010), stocking density and microalgal feed

regimen are vital to the growth and survival of the larvae. Generally, different hatcheries tend to use different microalgal species such as *Chaetoceros muelleri*, *Chaetoceros calcitrans*, *Isochrysis galbana*, *Rhodomonas salina*, and *Tetraselmis sp.* to suffice nutritional requirements for larvae (Agudo 2006). These microalgae can be used in different proportions, singly or in combination, and in different concentrations. A mixture of algae was found to be better than single species for optimal larval rearing of sandfish. *Chaetoceros* spp. have shown good results on the survival of *H. spinifera* larvae when administered singly and also in combination with *I. galbana* (Asha and Muthiah 2006). However, the result of survival rates depends on the type of sea cucumber species being cultured in the hatchery. The type of rearing water has also not been well-explored, although the manuals of Agudo (2006) and Al Rashdi et al. (2012) suggest the use of UV-treatment to sterilize the rearing medium for larval production. Despite this, sand-filtered seawater has been traditionally used at the Bureau of Fisheries and Aquatic Resources – Guiuan Marine Fisheries Development Center (BFAR-GMFDC) hatchery.

Today, optimization of hatchery techniques includes reduction of live feed requirements of larvae such as utilizing mono-species microalgal feed, culturing a low stocking density of 0.3 larvae/mL, and using *Spirulina*-coated settlement plates instead of natural conditioning of benthic diatoms on plates. These techniques reduced the complexity of culture systems and have led to substantial increase in production (Mills et al. 2012). The simplicity of methods, particularly in the feed regimen would be beneficial for small-scale hatcheries. Hatchery experiences from the Philippines and Vietnam suggest a current benchmark in seed production of 2.5% survival rate from egg to 5-mm juvenile (Mills et al. 2012). The University of the Philippines Marine Science Institute Bolinao Marine Laboratory (UPMSI BML) reported survival rates ranging from 0.7 – 2.10% (egg to <10-mm first stage juvenile) from 2007-2010. They employed *C. calcitrans* and *I. galbana* combined feed at concentration 20,000–30,000 cells/mL for larval rearing (Juinio-Menez et al. 2012). At the BFAR-GMFDC, *Chaetoceros* species are currently being employed as feed for the larvae of *H. scabra.* However, the growth, development, and survival rates in the hatchery have not been well-investigated under experimental conditions.

With the need to improve the larval production of BFAR-GMFDC hatchery, this study was aimed at determining the optimum microalgal diet and concentration, and optimum water rearing medium for sea cucumber larvae.

2. MATERIALS AND METHODS

2.1. Spawning

H. scabra broodstock with an individual wet weight of 255.9 ± 29.3 g (mean ± SD, n=30) were collected from the marine protected waters of Guiuan (11° 1' 7.7" N, 125° 42'39.4" E) and were maintained in sea pens at the BFAR-GMFDC hatchery one week prior to spawning. F1 broodstock from Salcedo, Eastern Samar (11° 5' 33.7" N, 125° 34' 46.9" E) were also used in some of the spawning trials. Spawning activities were conducted monthly during days of the full or new moon as considered putative schedule for sea cucumber spawning. Healthy broodstock, which have not eviscerated and have no visible skin lesions were selected for spawning. Induced method for spawning *H. scabra*, as described by Juinio-Meñez et al. (2012) was adopted in this study employing dry treatment and food shock techniques while omitting thermal shock. Individuals were blotted with towel and air-dried for 45 minutes, and afterward were immersed in a basin with 2 g *Spirulina* and seawater mixture for 45 minutes. After spawning induction, the broodstock were placed in rearing tanks and were allowed to spawn. Eggs were fertilized with the sperms and were then observed for fertilization and development via microscopy. Egg/larval density was also determined.

2.2 Larval Rearing

Developing eggs and larvae were reared in 60-L capacity transparent plastic mega box containers at stocking density of 500 ind.L-1. Aeration and sand-filtered seawater were provided, and tanks were covered with a black net as shade. Corrugated plastic settlement plates (1x1 ft) smeared with *Spirulina* paste were placed in the containers for the attachment of larvae when they developed into pentactula stage. Larvae were only fed with microalgae after two days of incubation in rearing tanks. Replenishment of seawater in the larval rearing tanks was done by siphoning 50% of the amount at two days interval. Larvae were maintained in tanks at water temperature of 27.5 – 32.0°C, salinity of 3.5 – 3.6%, pH of 8.5 – 9.0, and dissolved oxygen of 4.6 – 8.0g.L^{-1}. Larval rearing experiments were conducted for 35 days after which the larvae were expected to have grown approximately >1 mm in length. Different broodstock were used every month, hence, for rearing trials conducted at different schedules. The same protocols were used for all of the experimental setups conducted in this study.

2.3 Rearing Experiments

Three aspects of larval rearing were investigated in this paper. These include experiments on single and combined microalgal feeds, different microalgal feed concentrations, and different types of rearing water. The experiments were conducted independently from each other.

Single and combined microalgal feeds. Chaetoceros gracilis (Cgr) and *Chaetoceros calcitrans* (Cc) larval feeds were tested individually and in combination. Mass cultures of the microalgae were acquired from the Phycology Laboratory of BFAR-GMFDC. Combined *Cgr-Cc* feed was prepared at 50–50 density of each species. The larvae were reared in sand-filtered seawater and were fed twice daily with the assigned microalgae, each at concentration 30,000 cells.mL^{-1}. The experimental treatments were conducted in triplicates, and three trials following the same methodology were conducted during May 2016, December 2016, and August 2017.

Different microalgal diet concentrations. A separate rearing experiment was conducted to determine which among 10,000, 30,000, and 50,000 cells. mL^{-1} microalgal concentrations would yield optimum growth, development, and survival of *H. scabra* larvae. In this experiment, mixed *Cgr-Cc* microalgal feed was utilized since it is the accustomed diet for sea cucumbers at the BFAR-GMFDC hatchery. Larvae from the same broodstock as the previous experiment were utilized for rearing. As a hatchery protocol, the larvae in all the setups were primarily fed at lower microalgal density (10,000 cells.mL^{-1}) from Day 2 to Day 5. On Day 6, the larvae were administered with their assigned microalgal concentration. Microalgal density was monitored daily to ensure that the respective treatment concentrations were maintained. Three replicates were produced for each treatment, and three similar trials were also conducted in May 2016, December 2016, and August 2017.

Different types of rearing water. Sand-filtered, chlorinated, and UV-treated seawater were also studied for their efficiency in larval rearing. Sand-filtered and UV-treated seawater were readily available at the hatchery through sand-filtration and UV-treatment systems. Chlorinated seawater (5 ppt) was prepared by dissolving 0.25 g of chlorine powder in 50 L seawater and then was neutralized with sodium thiosulfate 24 hours before usage. Water treatments were replenished on alternate days at a 50% rate. The larvae were fed with *Cgr-Cc* mixed feed at 30,000 cells.mL^{-1} concentration. Three replicates were prepared for each water treatment, and trials following the same procedures were done in September 2016, January 2017, and April 2017.

2.4 Growth, Development and Survival Assessments

Larvae were sampled from each treatment to determine their growth performance and development. Larval body length was measured via digital microscopy during 3rd, 5th, 10th, 15th, 20th, 25th, 30th, and 35th sampling days. Length measurements were measured for doliolaria larvae only on Day 15, during which most doliolaria occur based on hatchery experience. The growth performance of the larvae was determined by calculating total length gain (TLG) and average daily growth rate (ADGR) during the pre-metamorphosis and post-metamorphosis development. Pre-metamorphosis includes the phase from the occurrence of early auricularia up to late auricularia larval stage, while post-metamorphosis includes the onset of doliolaria larvae until the early juvenile stage. The growth parameters were calculated based on Tolon et al. (2016) as follows:

$$\text{Pre-metamorphosis TLG } (\mu m) = A_f - A_i$$

$$\text{Pre-metamorphosis ADGR } (\mu m.d^{-1}) = \frac{(A_f - A_i)}{t}$$

$$\text{Post-metamorphosis TLG } (\mu m) = D_f - D_i$$

$$\text{Pre-metamorphosis ADGR } (\mu m.d^{-1}) = \frac{(D_f - D_i)}{t}$$

where,

A_i – mean length of early auricularia sampled at Day 3, as initial length;
A_f – mean length of late auricularia sampled at Day 10, as final length;
D_i – mean length of doliolaria larvae sampled at Day 15, as initial length;
D_f – mean length of early juveniles sampled at Day 30 or 35, as final length; and,
t – number of rearing days.

Three 1000 mL aliquots were collected from the larval tanks of each treatment to assess development and metamorphosis. Larval samples were counted via microscopy and were classified into auricularia, doliolaria, pentactula, early juveniles, and deformed eggs/larvae. The percent composition of each larval stage was determined every sampling day. The metamorphic rate of the larvae at a particular sampling day was represented by the mean percent composition of observed doliolaria larvae (Ren et al. 2015). The survival rate of the larvae was determined by calculating the percentage of the harvested viable early juveniles

(~1 mm) at the end of larval production.

2.5 Data and Statistical Analyses

Descriptive statistics included mean, standard deviation, and standard error of the mean, which were presented in tables and graphs. Results of the growth performance, development, and survivorship were averaged from the three trials conducted for each experiment. Log transformation was applied to growth data, while arcsine or square-root data transformation was applied to survivorship data. Statistical differences among the different treatments were determined using single-factor ANOVA, and a Duncan test was run as a post hoc test. Descriptive and inferential analyses of data were done using IBM® SPSS® Statistics version 21.

3. RESULTS AND DISCUSSION

3.1 Single and combined microalgal diets

Larvae fed with combined *C. gracilis*-*C. calcitrans* (*Cgr-Cc*) had consistently and significantly highest mean length during the pre-metamorphosis phase (Day 3-10) and post-metamorphosis phase (Day 15-35) (Figure 1). However, the differences in average length during these phases were significant only on Day 5 ($p<0.05$), Day 10 ($p<0.05$), Day 15 ($p<0.001$), and Day 25 ($p<0.01$).

Mean final length was also highest in *Cgr-Cc* with 2088 ± 134 µm followed by *Cc* with 1855 ± 142 µm and lastly by Cgr with 1800 ± 115 µm, but the means show no statistical difference ($p>0.05$) (Day 35 in Figure 1).

Furthermore, the calculated growth parameters in Table 1 show that larvae fed with combined *Cgr-Cc* microalgae have the best growth performance in terms of length increment (TLG) and growth rate (ADGR) during the pre- and post-metamorphosis assessments. However, the differences among the mean results of the growth parameters among different treatments were also not significant ($p > 0.05$).

Photomicrographs of the different larval stages of *H. scabra* are shown in Figure 2. Figure 3 shows the percent composition of each larval stage in different treatments throughout the rearing period. Auricularia larvae occurred up to Day 20 in setups fed singly with *C. gracilis* and *C. calcitrans*, while in the setup fed with combined *Cgr-Cc* microalgae, they persisted up to Day 25. Doliolarian larvae were first observed in all of the treatments on Day 15. Metamorphic rates (% doliolaria) of the larvae fed with single-species feeds, *Cgr* and *Cc*, were at peak on Day 20 with 63% and 33%, respectively. However, doliolaria larvae have not completely metamorphosed into pentactula stage in the *Cgr* feed until Day 30. On the other hand, metamorphic rate peaked on Day 25 in the combined *Cgr-Cc* feed treatment. Pentactula larvae have developed as early as Day 20 in the setup fed with *C. calcitrans*, but

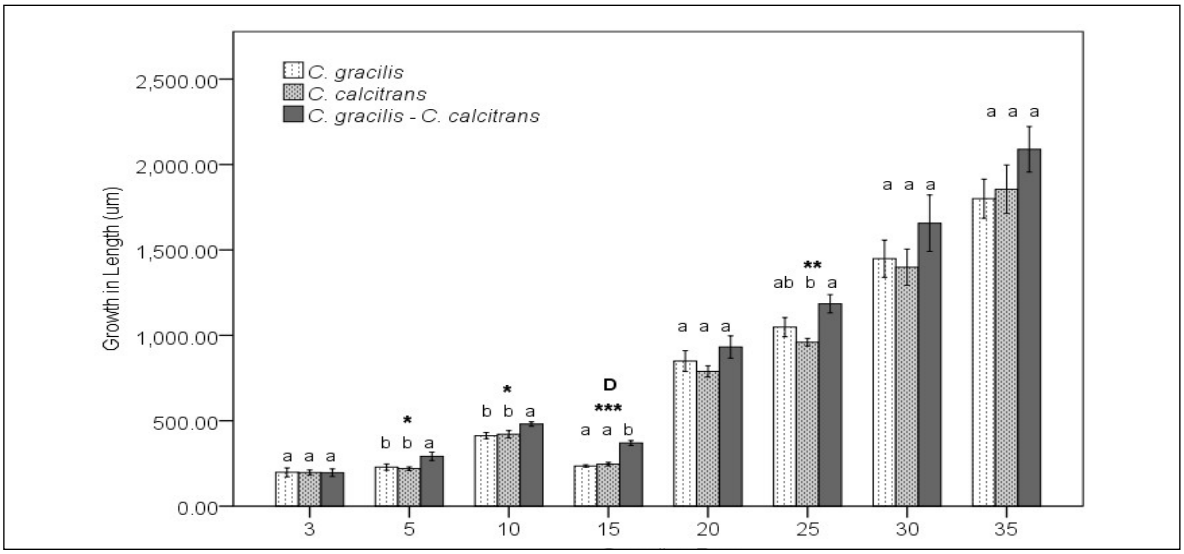

Figure 1. Mean growth in length of *H. scabra* larvae fed with *C. gracilis*, *C. calcitrans*, and combined *C. gracilis* – *C. calcitrans* during 35-day rearing. Length measurements were averaged from results of trials in 2016-2017. Note: Values (mean ± SE) with different letters show significant difference at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. D denotes the onset of metamorphosis into doliolaria stage; hence, measurements refer to doliolarian larvae only.

Table 1. Mean results of total length gain (TLG) and average daily growth rate (ADGR) of pre- and post-metamorphosis *Holothuria scabra* larvae fed with different microalgal diets from experiments conducted in 2016-2017.

Microalgal Diet	Pre-metamorphosis [A]		Post-metamorphosis [B]	
	TLG (μm)	ADGR (μm.d^{-1})	TLG (μm)	ADGR (μm.d^{-1})
C. gracilis	214.6 ± 39.5 [a]	26.8 ± 4.9 [a]	1564.6 ± 116.4 [a]	97.8 ± 7.3 [a]
C. calcitrans	224.7 ± 31.2 [a]	28.1 ± 3.9 [a]	1609.1 ± 137.4 [a]	100.6 ± 8.6 [a]
C. gracilis-C. calcitrans	285.2 ± 32.8 [a]	35.6 ± 4.0 [a]	1719..0 ± 134.9 [a]	107.4 ± 8.4 [a]

Note: Values (mean ± SE) which have similar superscripts show no significant difference ($p > 0.05$).
[A] Pre-metamorphosis growth parameters were calculated from length increments from early auricularia to late auricularia larvae.
[B] Post-metamorphosis growth parameters were derived from length increments from doliolaria to the early juvenile stage.

were first observed later on Day 25 in set-ups fed with *C. gracilis* and *Cgr-Cc* combined feed. Larvae have developed into early juveniles on Day 30 in both setups fed with *C. calcitrans* and *Cgr-Cc* combined feed with 90% and 100% composition, respectively. On the other hand, early juveniles were observed later on Day 35 in setup fed with *C. gracilis*.

Although larval development was erratic and not synchronized for each treatment, the rate of development can be gauged from the metamorphic rates (metamorphosis of doliolaria larvae) and the time of occurrence of early juveniles. Doliolaria larvae in both *Cc* single feed and combined *Cgr-Cc* feed have metamorphosed earlier, and early juveniles were already present on Day 30. Therefore, larval development was more favorable in these diets than in *Cgr* single feed.

In terms of survivorship, combined *Cgr-Cc* feed yielded the highest mean survival rate with 2.23 ± 0.79%, followed by *C. gracilis* with 1.6 ± 0.78% and *C. calcitrans* with 1.3 ± 0.69%. However, the differences in the means were not statistically significant ($p > 0.05$) (Figure 4).

Preliminary results in survivorship of *H. scabra* larvae (May 2016) had relatively lower survival rates (<1%). Although similar methods were applied in the following verificatory trials, survival yields have increased during experimental runs conducted in December 2016 and August 2017. The combined *Cgr-Cc* microalgal feed had the highest mean survival rates of larvae during the last two runs, with 2.93% and 3.22% for December 2016 and August 2017, respectively. However, these results were not significantly different from the survival yields of the mono-species feeds.

Chaetoceros sp. was reported to yield better results on the growth and survival of the larvae compared to *Pavlova luther, Tetraselmis chuli, Nanochloropsis salina* (Asha and Muthiah 2006). The result of combined species *Cgr-Cc* microalgal feed in this study is concordant with the result of Asha and Muthiah (2006). Furthermore, Battaglene (1999) reported that mixture of microalgae mainly composed of *Chaetoceros* sp. helped improve the larval growth of *H. scabra*. Combining different microalgal species for sea cucumber larvae has become a practice in the hatchery of UPMSI (Juinio-Menez et al. 2012) and was also recommended by Agudo (2006) and Al Rashdi et al. (2012). The UPMSI used *C. calcitrans* and the diatom *Navicula ramossissima* and additional nutrition from *Sargassum* extract during larval rearing.

In this study, results in growth performance and survivorship of larvae in combined feed (*Cgr-Cc*) treatment were similar when fed with *C. calcitrans* or *C. gracilis* singly. However, in terms of larval development, only *C. calcitrans* single feed yielded similar results with the combined microalgal feed treatment. Mono-species feeding for holothurian larvae was supported by Gamboa et al. (2004) using only *C. calcitrans*. Gamboa et al. (2004) concluded that single-species feeding was satisfactory and cost-effective, and was able to yield utmost 2.2% survival of larvae. In this study, maximum average survival rates of larvae were 2.84% for *C. gracilis* (August 2017) and 2.12% for *C. calcitrans* (December 2016).

3.2 Microalgal Diet Concentrations

The highest mean final length during pre-metamorphosis phase was observed in larvae maintained at 50,000 cells.mL^{-1} concentration with 488 ± 10 μm (Figure 5). This was followed by larvae maintained at 30,000 cells.mL^{-1} with 312 ± 13 μm, and at 10,000 cells.mL^{-1} with 237 ± 9 μm. ANOVA results show that the differences in means were significant ($p < 0.05$). Moreover, the growth of post-metamorphosed *H. scabra* larvae was also notable in the setup maintained at 50,000 cells mL^{-1} microalgal concentration. The mean final length of larvae in 50,000 cells mL^{-1} microalgal concentration was significant-

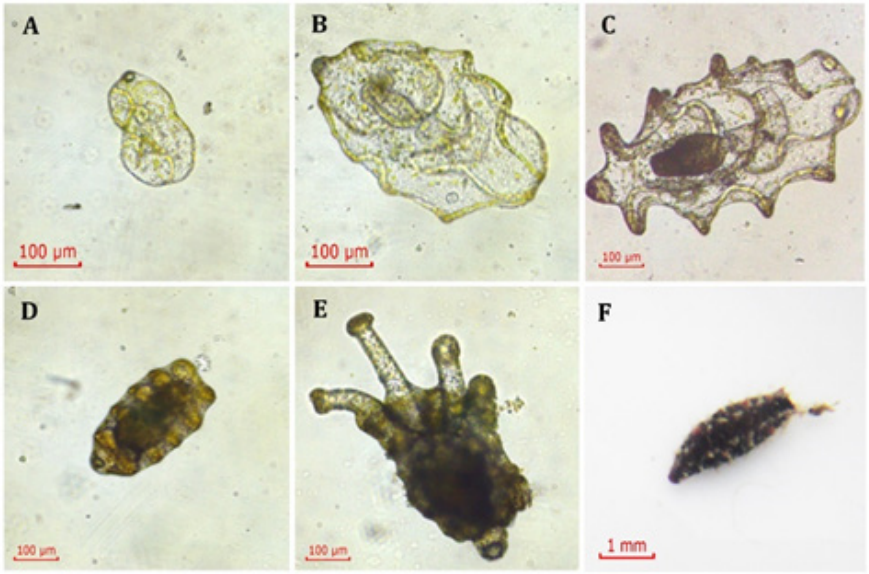

Figure 2. Photomicrographs of the different larval stages of *H. scabra*: (A) early auricularia, 2 days old; (B) mid-auricularia, 5 days old; (C) late auricularia, 10 days old; (D) doliolaria, non-feeding stage, 15 days old; (E) pentactula, 20-25 days old; and (F) early juvenile (photomacrograph), 30-35 days old.

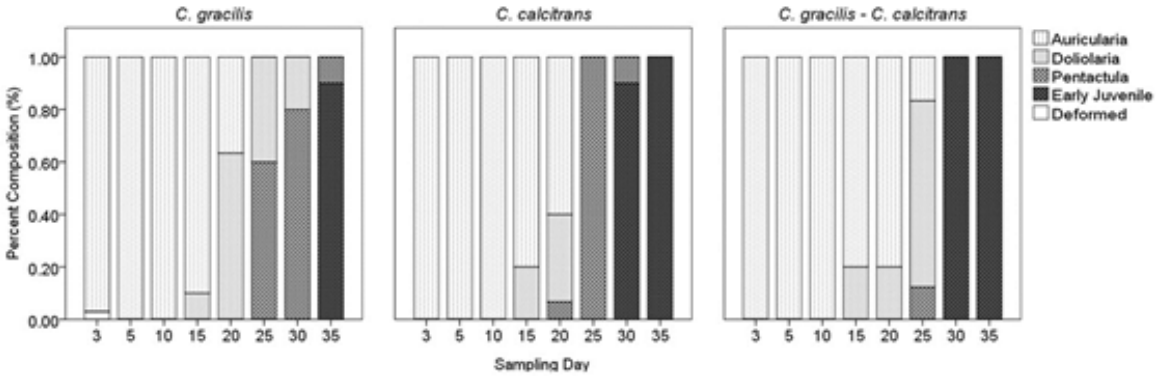

Figure 3. Development of *Holothuria scabra* over 35 days of rearing in different microalgal diets. Graphs showing the mean percentage composition of the larval stages from auricularia, doliolaria, pentactula, and early juvenile stage.

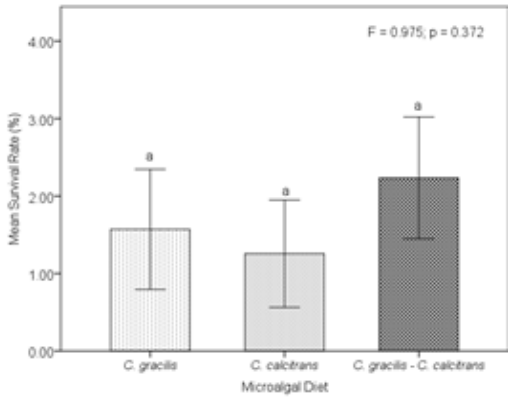

Figure 4. Mean survival rates of *H. scabra* larvae fed with different microalgal diets; results averaged from experiments conducted in 2016-2017.

ly highest, with 3767 ± 523µm ($p < 0.001$). This was followed by the larvae maintained at 30,000 cells mL^{-1} concentration with a mean final length of 1559 ± 233µm, and lastly by 10,000 cells mL^{-1} with 878 ± 22µm. However, there is no significant difference between the mean length results for 10,000 and 30,000 cells.mL^{-1} concentrations ($p>0.05$).

Growth parameters calculated for pre- and post-metamorphosis phase of the larvae are shown in Table 2. Correspondingly, total length gain and average daily growth rates were highest in setups maintained at 50,000 cells.mL^{-1} and lowest in setups maintained at 10,000 cells.mL^{-1} microalgal concentration.

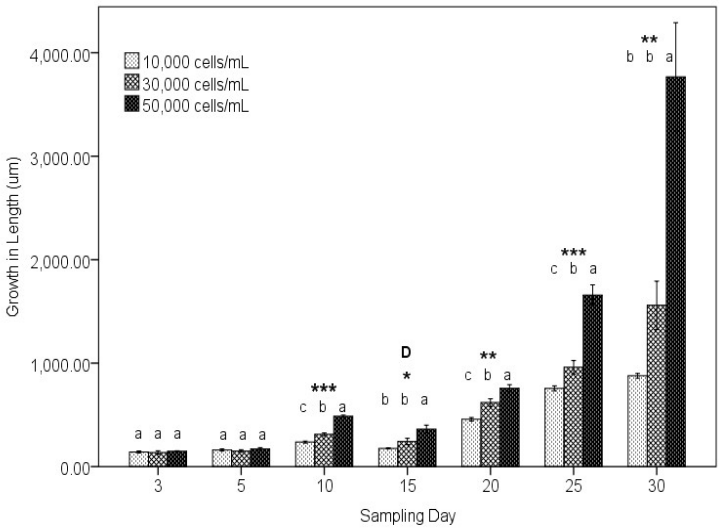

Figure 5. Mean growth in length of *H. scabra* larvae fed at different microalgal concentrations during the 35-day rearing. Length measurements were averaged from the results of trials in 2016-2017. Note: Values (mean ± SE) with different letters show significant difference at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. D denotes the onset of metamorphosis into doliolaria stage; hence, measurements refer to doliolarian larvae only.

In terms of larval development, the appearance of doliolaria larvae was first observed immediately on Day 10 in setups maintained at 30,000 and 50,000 cells.mL^{-1} microalgal concentrations (Figure 6). Metamorphic rates peaked on Day 15 in 50,000 cells. mL^{-1} concentration with 90% doliolaria composition; on Day 20 in 30,000 cells.mL^{-1} with 60%; and, on Day 25 in 10,000 cells.mL^{-1} with 80%. Furthermore, larvae reared in 50,000 cells.mL^{-1} had metamorphosed into >1 mm juveniles as early as Day 25, whereas, in the lower concentration treatments, early juveniles were observed later on Day 30.

Although, growth and development were favorable in the 50,000 cells.mL^{-1} setup, the mean survival rate was lowest (6.05 ± 2.90%) in this treatment (Figure 7). Highest survival rate (8.4 ± 1.35%) was observed in the setup fed at 10,000 cells.mL^{-1}, followed by 30,000 cells.mL^{-1} with 7.80 ± 0.47% survival rate. Nevertheless, the difference in mean survival rates among treatments were not statistically significant ($p<0.05$).

The hatchery manuals of Agudo (2006) and Al Rashdi et al. (2012) suggest increasing the algal concentration up to 40,000 cells.mL^{-1}. Although microalgal concentration in the larval tanks is not daily quantified at the BFAR-GMFDC hatchery, microalgal requirements were ensured by feeding the larvae with 3-day old cultures that provide adequate density to feed the larvae. Nevertheless, it is necessary to measure algal concentration since algal bloom is dependent on the season and environmental factors, and feed consumption rate of the larvae is often neglected in the actual hatchery.

The harvested juveniles observed in 50,000 cells.mL^{-1} treatment were relatively larger in sizes compared to the other treatments, but the survival rate was lower. This is consistent with the findings in other studies that showed inverse relationship between growth performance and stocking density (e.g. Seeruttun et al. 2007; Asha and Diwakar 2013). Lower stocking density increases available space and food; hence, promoting good nutrition. Nevertheless, it is also important to note that growth increments were still highest in the 50,000 cells.mL^{-1} treatment during the earlier days of larval rearing (Day 10-15) even though in this period, larval density was still higher. Thus, better growth performance in higher microalgal feed concentration could not be explained within the context of density-dependence alone.

The high mortality of larvae in the 50,000 cells.mL^{-1} treatment could also be a consequence of overfeeding or accumulation of toxic ammonia from excess food (James 1999). However, this factor has not been measured and investigated in this study. From experience during the experimentation, possible fouling in larval tanks was mitigated by increasing the rate of water replenishment in the tanks.

Table 2. Total length gain (TLG) and Average Daily Growth Rate (ADGR) of pre- and post-metamorphosis *Holothuria scabra* larvae fed with different microalgal concentrations.

Microalgal Concentration (cells.mL-1)	Pre-metamorphosis [A]		Post-metamorphosis [B]	
	TLG (μm)	ADGR (μm.d^{-1})	TLG (μm)	ADGR (μm.d^{-1})
10, 000	95.4 ± 18.9 [c]	11.9 ± 2.4 [c]	701.5 ± 23.2 [b]	43.8 ± 1.5 [b]
30, 000	175.9 ± 26.7 [b]	22.0 ± 3.4 [b]	1316.5 ± 244.0 [b]	82.3 ± 12.3 [b]
50, 000	338.8 ± 9.4 [a]	42.4 ± 1.2 [a]	3403.9 ± 487.3 [a]	212.7 ± 30.5 [a]

Note: Values (mean ± SE) which have similar superscripts show no significant difference ($p > 0.05$).
[A] Pre-metamorphosis growth parameters were calculated from length increments from early auricularia to late auricularia larvae.
[B] Post-metamorphosis growth parameters were derived from length increments from doliolaria to the early juvenile stage.

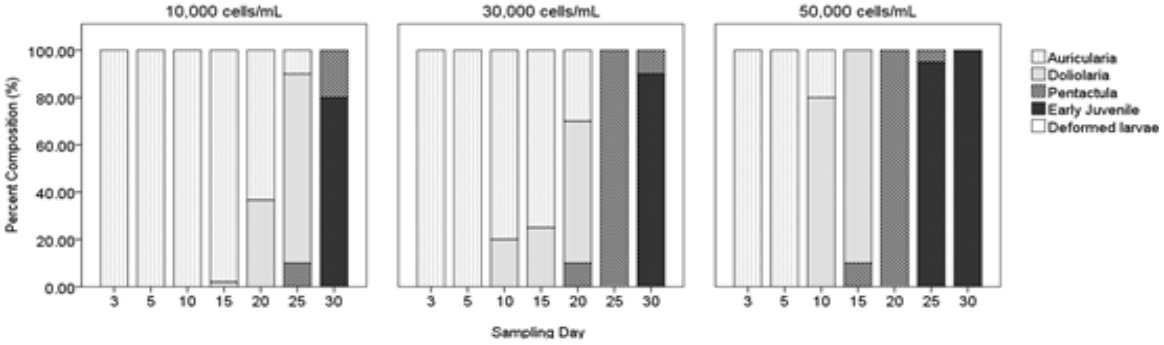

Figure 6. Development of *Holothuria scabra* over 35 days of rearing in different microalgal diet concentrations. Graphs show mean percentage composition of larval stages from auricularia, doliolaria, pentactula, and early juvenile stage.

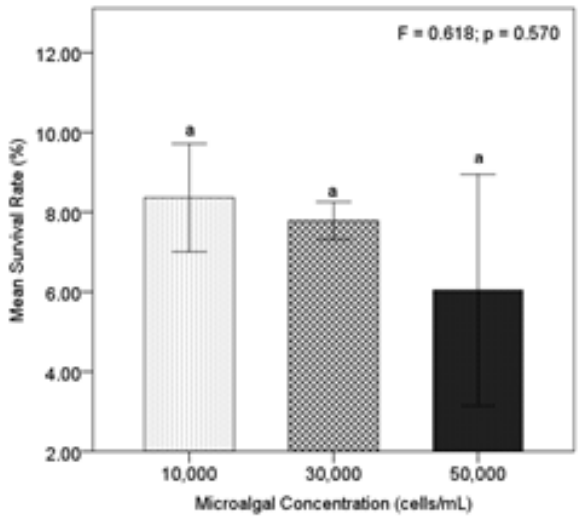

Figure 7. Mean survival rates of *H. scabra* fed with different microalgal diet concentrations: 10,000, 30,000, and 50,000 cells mL-1. Bars labeled with similar letters have no significant difference ($p>0.05$).

3.3 Water Treatments

H. scabra larvae reared in sand-filtered seawater consistently had the highest mean final lengths on the pre- and post-metamorphosis phases with 472 ± 16 µm (Day 10) and 1690 ± 171 µm (Day 35), respectively (Figure 8). This was followed by larvae reared in UV-treated seawater with mean final lengths of 383 ± 27 µm and 1374 ± 155 µm correspondingly for pre- and post-metamorphosis phase. On the other hand, larvae grown in chlorinated seawater had stunted growth in both the pre- and post-metamorphosis phases (mean final length of 333 ± 18 µm and 939 ± 14 µm, respectively). The differences in mean final lengths of larvae in the different setups were statistically significant (Day 10, $p<0.001$; Day 35 $p< 0.01$).

Larval growth performance in sand-filtered seawater was notable (Table 3). The larvae in this setup have gained an average length of 1366.66 µm and had an average daily growth rate of 68.3 $\mu m.d^{-1}$. In contrast, poor growth performance was observed in larvae reared in chlorinated seawater with only 695.0 µm total length gain and 34.8$\mu m.d^{-1}$ average daily growth rate. The results in the growth parameters evaluated for the different water treatments were significantly higher in sand-filtered seawater compared to UV-treated and chlorinated seawater as shown in Table 3.

The development of larvae in different setups is shown in Figure 9. Some larvae in sand-filtered seawater and UV-treated seawater already developed into doliolaria as early as Day 10, while in chlorinated seawater, doliolaria larvae were first observed later on Day 15. Larval metamorphic rates on Day 10 was 50% in sand-filtered seawater and 40% in UV-treated seawater. Early juveniles were already observed on Day 25 in setups of sand-filtered and UV-treated seawater (28% and 13%, respectively), suggesting earlier time for harvest. On the other hand, early juveniles were observed in chlorinated seawater later on Day 30 (47%).

Survivorship in sand-filtered seawater was consistently highest during the three runs conducted from 2016 to 2017, with a mean survival rate of 10.24 ± 1.84% (Figure 10). On the other hand, UV-treated and chlorinated seawater had 6.24 ± 0.93% and 2.60 ± 0.67% mean survival rates, respectively. ANOVA results show that the differences in the means among the three treatments were statistically significant ($p <0.001$). However, mean survival rates in the UV-treated and chlorinated seawater did not significantly differ ($p > 0.05$).

Decontamination and disinfection of seawater media for larval culture is critical for the success of rearing. Conventionally, seawater for aquaculture is clarified by using high-efficient sand-filters and is followed by sterilization with ultraviolet light treatment, ozonization or electrolization (Hisae et al. 2002). UV-treatment for seawater is advised by Agudo (2006) to achieve success in larval rearing of sea cucumbers. This sterilization method ensures that the rearing medium is void of pathogens and contaminants that could cause diseases in the larval stock. According to Hisae et al. (2002), UV-treatment has disinfectant effect on aquatic pathogens including gram-negative bacteria and fish rhabdoviruses, herpesviruses, and iridoviruses.

Chlorine concentration in seawater can be produced through electrolization of saltwater (Hisae et al. 2002). In this present study, chlorine concentration was introduced by dissolution. Although preliminary results were not favorable in chlorinated seawater, survivorship of larvae was improved when chlorine was neutralized with sodium thiosulphate, and was used in lower concentration (5 ppt).

Despite the sterilization methods done for the culture water, it remains unclear why survival rates of sea cucumber larvae in UV-treated and chlorinated seawater were not even at par with that of sand-filtered seawater. The sterilization process had no apparent effect on the growth and concentration of the live microalgal feeds or the physico-chemical properties of the water since these factors were daily observed and monitored during the experimentation. Other aspects of UV treatment and chlorination are still unknown to substantiate the findings of this present study.

Sand-filtration is typically employed in many hatcheries to minimize contamination from siltation, organic particles, and other diminutive marine organisms such as copepods, which cause an increase in mortality of the larvae. Sand-filtration system at the BFAR-GMFDC hatchery employs rapid filtration, which encompasses primarily by mechanical filtration (sand and rubbles) and secondarily by biological and chemical purification (charcoal). This enhances and ensures the efficiency of clarification of the seawater sourced from the waters adjacent to the hatchery. Although several hatchery manuals recommend rearing water sterilization such as UV-treatment and chlorination, it is interesting to note that sand-filtration alone rendered satisfactory results.

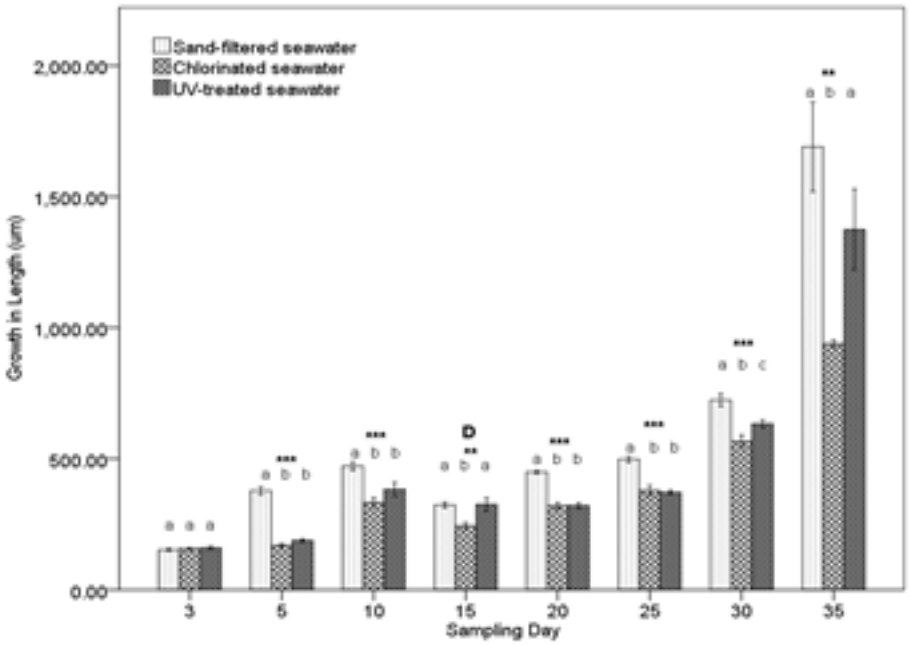

Figure 8. Mean growth in length of *H. scabra* larvae reared in different water treatments during 35-day rearing. Length measurements were averaged from the results of trials in 2016-2017. Note: Values (mean ± SE) with different letters show significant difference at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. D denotes the onset of metamorphosis into doliolaria stage; hence, measurements refer to doliolarian larvae only.

Table 3. Total length gain (TLG) and Average Daily Growth Rate (ADGR) of pre- and post-metamorphosis *Holothuria scabra* larvae reared in different treatments on water rearing medium.

Water Treatment	Pre-metamorphosis [A]		Post-metamorphosis [B]	
	TLG (μm)	ADGR (μm.d^{-1})	TLG (μm)	ADGR (μm.d^{-1})
Sand-filtered seawater	319.6 ± 18.8 a	40.0 ± 2.4 a	1366.6 ± 175.0 a	68.3 ± 8.8 a
Chlorinated seawater	176.2 ± 21.0 b	22.0 ± 2.6 b	695.0 ± 12.4 b	34.8 ± 0.6 b
UV-treated seawater	222.9 ± 29.4 b	27.9 ± 3.7 b	1048.6 ± 144.2 ab	52.4 ± 7.2 ab

Note: Values (mean ± SE) that have different superscripts show significant difference ($p > 0.05$).
A Pre-metamorphosis growth parameters were calculated from length increments from early auricularia to late auricularia larvae.
B Post-metamorphosis growth parameters were derived from length increments from doliolaria to the early juvenile stage.

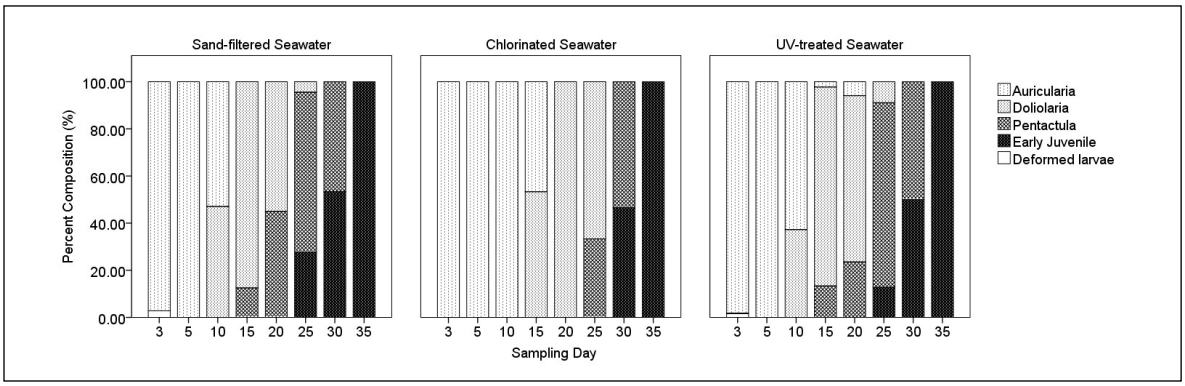

Figure 9. Development of *Holothuria scabra* over 35 days of rearing in different water treatments. Graphs show the mean percentage composition of larval stages from auricularia, doliolaria, pentactula, and early juvenile stage.

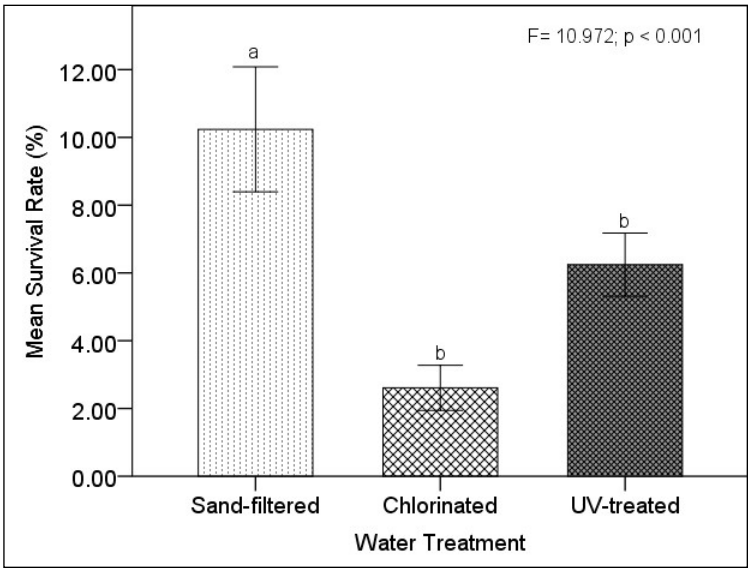

Figure 10. Mean survival rates of *Holothuria scabra* reared in different water treatments. Results averaged from experiments conducted in 2016-2017.

4. CONCLUSION

Feeding regimen and water treatments are critical to the success of hatchery production of sea cucumbers. Feeding regimen for sea cucumber larvae typically involves a variety of benthic microalgal species. This study reduced the feeding regimen into utilizing *C. gracilis* and *C. calcitrans* only, which were administered singly and in combination. Although mixed *C. gracilis- C. calcitrans* diet had better results in the growth and survival of larvae, the results did not significantly differ with that of the single-species diet, especially with that of *C. calcitrans* single feed. From this, either *C. gracilis* or *C. calcitrans* mono-species diet could suffice nutrition for the larvae. Nevertheless, the rate of larval development was slower in *C. gracilis* single-species feed.

A higher concentration of microalgal diets resulted in notable growth performance, development, and survivorship of sea cucumber larvae. Yield sizes (~3 mm) of juveniles fed at 50,000 cells.mL^{-1} were five times larger than when fed at 10,000 cells.mL^{-1}. The development of larvae was also faster at 50,000 cells. mL-1, yielding harvestable juveniles as early as Day 25. However, water replenishment in tanks should also be regularly done every two days at 50-70% rate to mitigate fouling from high microalgal concentration.

Significantly best growth performance and survivorship of larvae were observed in sand-filtered seawater. Sterilization with UV-treatment and chlorination had lower yields of viable juveniles. Typically, rearing water for larval culture is sterilized to lessen mortality from diseases and pathogens. However, it is interesting to note that the clarification of rearing

water using sand-filtration system alone rendered better growth performance of larvae and higher quantity of harvestable juvenile sea cucumbers. At present, sand-filtration system aids in water treatment for culturing sea cucumber larvae at the multi-species hatchery of BFAR-GMFDC, Eastern Samar. Nevertheless, hatchery manuals for sea cucumber aquaculture (Agudo 2006; Al Rashdi et al. 2012) highlight the use of these sterilization processes.

The investigation on some aspects of sea cucumber larval production was conducted independently although a multi-variable design is much preferred. Despite this limitation, the study underscores the refining of techniques and simplification of requirements in the production of juvenile *H. scabra*, which is a crucial phase in the provision of viable seeds for grow-out culture and restocking programs.

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