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Effects of Microalgal Diets on Growth, Development and Health Condition of *Holothuria scabra* Larvae

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Abstract - The study assessed the effect of using different microalgal diets on the growth, development, and health condition of *Holothuria scabra* larvae. Larvae were fed single diets (*Chaetoceros muelleri, C. calcitrans*, and *Isochrysis galbana*) and mixed diets Cm+Ig (50% *C. muelleri* and 50% *I. galbana*) and Cc+Ig (50% *C. calcitrans* + 50% *I. galbana*). On days 3 to 8, larvae fed *C. muelleri* and Cm+Ig consistently showed better performance than the larvae fed *I. galbana* alone and the unfed larvae. However, larvae fed *C. muelleri* alone or in combination with I. galbana had closely similar results to those fed with *C. calcitrans* and CC+Ig. Larvae fed *C. muelleri*, Cm+Ig, *C. calcitrans*, and CC+Ig developed late-auricularia with hyaline spheres from days 9 to 13. Neither larvae fed *I. galbana* alone nor unfed larvae developed any late auricularia with hyaline spheres. However, only larvae fed with *C. muelleri* followed by Cm+Ig showed greater percentage of late-auricularia with hyaline spheres compared to the other diets. In terms of size of hyaline spheres, those fed with Cm+Ig had the largest size compared to the other fed algae. Competent doliolaria was first observed on Day 11 in Cm+Ig, although *C. muelleri* fed larvae had greater percentage of doliolaria on day 16-17, closely followed by Cm+Ig fed larvae. In conclusion, the study showed *C. muelleri* alone or in combination with *I. galbana* were the most effective diets for rearing of *H. scabra* larvae. However, it was suggested to use *C. muelleri* in combination with *I. galbana*, for it produced the largest size of hyaline spheres in late-auricularia, which was an indicator of good health of larvae.

Key words: *Holothuria scabra*; growth; development; health condition; microalgae; hyaline spheres

INTRODUCTION

Tropical sea cucumbers processed into bêche-de-mer are valuable source of income for many Pacific islanders [1]. However, the increased demand in importing countries particularly China and the poor or lack of management of sea cucumber stocks in many countries, have decimated this resource. It is now apparent that depleted stocks will recover very slowly and that the usual boom and bust cycles will continue unless the proper management and restocking of stocks is introduced. The release of juvenile sea cucumbers produced in hatcheries is one way of enhancement wild stocks, more aptly called stock enhancement [2], which may involve reseeding or transplantation.

The culture of *Holothuria scabra* was already pioneered in India [3], then followed by the Solomon Islands [4], and currently being done at the Bolinao Marine Laboratory of the Marine Science Institute of the University of the Philippines. The major bottleneck in culture of sandfish during early stage is the nutritional requirement of larvae.

There were different methods recommended by various authors on feeding of *H. scabra* using different types of live microalgae. Pitt (2001) fed the sandfish larvae at 2 days post hatch using a mixed diatom culture [5]. Three species, *Chaetoceros muelleri, C. calcitrans*, and *Rhodomonas salina* were fed on an equal biomass basis. Giraspy and Ivy (2005) showed that *C. muelleri, C. calcitrans, R. salina,* and *Pavlova lutheri* are suitable microalgae for

the sea cucumber larvae [6]. Purcell et al. (2002) fed the auricularia larvae with a variety of microalgae including *R. salina, Chaetoceros muelleri, C. gracilis, C. calcitrans,* and *Platymonas sp.* [7]. In India, *Isochrysis galbana* and a mixed culture dominated by species of *Chaetoceros spp.* and *Skeletonema spp.* are used to feed auricularia larvae [8]. In Vietnam, *C. muelleri, C. calcitrans, Nanochloropsis occulata, Platymonas sp., I. galbana,* and *R. salina* are used as available [9]. Asha and Muthiah (2005) recommends *C. calcitrans* or in combination with *I. galbana* as the effective feed for the larvae of *H. spinifera*, a closely related species [10].

There is need to further study the effect of single algal species and/or its combination on the growth and development of *H. scabra* larvae. Hence, in this study, the effect of using single species (*Chaetoceros muelleri, Chaetoceros calcitrans* and *Isochrysis galbana*) and mixed species (*C. muelleri* + *Isochrysis galbana* and *C. calcitrans* + *Isochrysis galbana*) were tested simultaneously on the growth and development as well as health condition (i.e. presence of hyaline spheres as food reserves) of *H. scabra* larvae.

MATERIALS AND METHODS

Experimental Design and Lay-out

A feeding experiment was done to test the effects of using different species of microalgae, either singly or in combination, on the growth, development, and health condition of sea cucumber larvae. The treatments were as follows with 4 replicates using glass jars under a completely randomized design (Figure 1):

T1 – *Chaetoceros muelleri* (diatom)
T2 – *Chaetoceros calcitrans* (diatom)
T3 – *Isochrysis galbana* (flagellated algae)
T4 – *C. muelleri* + *I. galbana* (Cm+Ig)
T5 – *C. calcitrans* + *I. galbana* (Cc+Ig)
T6 – Control (Unfed)

Figure 1. Experimental set-up used in the study.

Locale of the Study

The study was conducted under laboratory conditions at the Bolinao Marine Laboratory of the Marine Science Institute of the University of the Philippines (UP-MSI) located in Barangay Luciente I, Bolinao, Pangasinan.

Collection and Maintenance of Broodstock

Broodstock were collected from Anda, Pangasinan. Collected individuals were brought to the hatchery and stocked in shallow rectangular 0.5 fiberglass tank. Thirty specimens were stocked in an area of $1m^2$. The bottom of the tank was not covered with sand or mud from the natural habitat since they were used immediately for spawning.

Spawning Induction

Spawning induction was done following the protocol of the UP-MSI. First method was desiccation wherein the broodstock were placed in spawning tank without seawater. This was done for 45 minutes to 1 hour. After desiccation, heat shock was done, the ambient water temperature was increased to 5ºC above ambient from 28-33°C. This was also done for 1 hour. After heat shock, food shock was done. Fifteen grams of spirulina powder was blended into 1 L tap water. Then, the spirulina was poured in the spawning tank. After 1 hour of food shock, the water in the tank was siphoned out and replaced with clean UV-filtered seawater and the behavior

of the broodstock was observed until they started to spawn.

Egg Collection and Fertilization

The eggs were collected directly from spawning females using beakers and placed in a plastic bin with clean UV-irradiated seawater. Then, the eggs were fertilized using collected sperm. Fertilized eggs were counted, estimated and was used in the study. The stocking density used was 0.5 ml^{-1}.

Experimental Units

The study was conducted using 20 units of 3-L glass jars filled with 2.5-L UV-filtered sea water with mild aeration. Each experimental container was covered with black plastic to eliminate the algal growth (Figure 1).

Larval Feeding

The cell density of 2-3 days outdoor culture of live *Chaetoceros muelleri, Isochrysis galbana* and *Chaetoceros calcitrans* was estimated using microscope and haemocytometer.

Estimation of algal cell density =

$$\frac{\text{Upper + Lower Chamber}}{\text{8 corners}} \times 10^4$$

Estimation of feed =

$$\frac{\text{Feed Concentration x Vol. of Container}}{\text{Cell Density}}$$

The feeding concentration started at 5,000 cells ml^{-1} and eventually increased up to 20,000 cells ml^{-1}. For the mixed diet, 50% - 50% of the feeding concentration for diatom and flagellated algae *(e.g. 50% C. muelleri* + 50% *I. galbana)* was given daily.

Feeding was done initially on January 15, 2019 at 5,000 cells.ml^{-1}. On the third feeding day (January 17, 2019), feeding concentration was adjusted to 10,000 cells.ml^{-1} and increased to 15,000 cells.ml^{-1} on the fifth day and 20,000 cells.ml^{-1} on the sixth day. Increased in feeding concentration was based on fullness of the gut as observed under the microscope. Routine culture of the three species of microalgae was regularly being done at UP-BML.

Water Management

During development, larvae produce excreted wastes, together with excess food. Poor water quality directly affects larval development and survival. Hence, proper water management was essential, which include regular siphoning of the experimental jars and partial water changes. During water change, a banjo sieve (80 µm) was used to prevent loss of larvae. About 30-50% water change was done daily.

Monitoring of Growth and Development

Larval development and growth were monitored daily starting on the second day of the experiment. Monitoring was done by sub-sampling of 10 larvae per glass jar using 80 microns sieve and glass pipette to determine the larval stage and length. Photos of the larvae were taken using Dinocapture to determine the development stage and growth in length. Larval stage was determined by observing the hydrocoel, left and right somatocoel and lateral folds or "arms" [11, 12]. Aside from larval growth, the presence of hyaline spheres and their size was recorded as good indicator of larval condition [13]. The formation and development of hyaline spheres and their size were believed to be strongly linked to the available nutrients to *H. scabra* larvae during its auricularia stage of development [13].

Monitoring of Physico-Chemical Parameters

Water parameters (salinity, temperature, and pH) were monitored daily after 30% to 50% of water was changed.

Statistical Analysis

One-way Analysis of Variance (ANOVA) was used to determine the effect of feed on mean length of the larvae. The Duncan's Multiple Range Test was used as the post-hoc test for pair-wise comparison of treatment means, if result of the ANOVA revealed significant

differences. Significant difference was set at 5% level of significance.

RESULTS AND DISCUSSION

Spawning of Sea Cucumber

The sexes in *H. scabra* are separate but it was not possible to distinguish the males and females by external examination. It was only possible to distinguish the sexes during spawning since the males and females have different spawning behaviors. The males were observed to lift the anterior end and exhibited swaying movements. After such behavior, the males started releasing sperm from its gonopore at the anterior end for several minutes. Then, the ripe females started to react by releasing their eggs. The eggs were ejected out through a single gonopore that can reach a distance of about 1 m that allows dispersal over wide area.

Larval Growth

Table 1 shows the growth data in terms of length of *Holothuria scabra* larvae at Day 3 (January 17, 2019) to Day 17 (February 1, 2019) after start of the feeding experiment.

On Day 3, larvae fed *I. galbana* (615.9 ± 14.5µm) had a significantly greater length than the other treatments except larvae fed *C. muelleri* (596.3 ± 9.3µm) and *Cc+Ig* (582.7 ± 5.4µm). The larvae fed *Cm+Ig* (554.3 ± 14.4µm) *C. calcitrans* (530.6 ± 31.2µm) and unfed larvae (523.4 ± 4.0µm) showed no significant difference from each other. Also larvae fed *Cm+Ig* had no significant difference to larvae fed *C. muelleri* and *Cc+Ig*.

On Day 4, larvae in all treatments showed no significant differences from each other. However, all fed larvae were significantly greater in length than the unfed larvae.

On Day 5, larvae fed *C. muelleri* (799.5 ± 17.1µm) had a significantly greater length from all other treatments except for larvae fed *Cm+Ig* (732.8 ± 7.0µm). On the other hand, the larvae fed *Cm+Ig*, *I. galbana* (713.0 ± 33.2µm), *Cc+Ig* (689.7 ± 15.3µm) and *C. calcitrans* (686.7 ± 16.6µm) showed no significant difference from each other; and the unfed larvae was significantly smaller than the fed larvae except to larvae fed *Cc+Ig* and *C. calcitrans* which did not differ significantly.

On Day 6, all fed larvae showed no significant difference from each other. However, the larvae fed *C. calcitrans* (712.1 ± 53.2µm) and *I. galbana* (695.5 ± 50.7µm) did not differ significantly to the unfed larvae.

On Day 7, larvae fed *C. muelleri* (843.0 ± 29.4µm), *Cm+Ig* (824.3 ± 34.3µm), *C. calcitrans* (796.5 ± 25.7µm) and *Cc+Ig* (760.9 ± 26.9µm) did not differ significantly from each other. However, larvae fed *C. calcitrans* and *Cc+Ig* showed no significant difference to larvae fed *I. galbana* (726.4 ± 30.0µm). The unfed larvae (568.7 ± 29.1µm) was significantly smaller than the fed larvae.

On Day 8, larvae fed C. *muelleri* (840.2 ± 12.3µm), *C. calcitrans* (802.2 ± 47.2µm), *Cm+Ig* (789.7 ± 70.5µm) and *Cc+Ig* (776.1 ± 31.0µm) showed no significant differences from each other. However, the larvae fed *I. galbana* (708.2 ± 30.3µm) was not significantly different from all other fed larvae except *C. muelleri*. The unfed larvae (568.7 ± 9.0µm) was significantly smaller than the fed larvae.

On Day 9, all fed larvae did not differ from each other. However, the larvae fed *C. calcitrans* (722.9 ± 65.5µm) and *I. galbana* (683.9 ± 41.2µm) showed no significant difference to the unfed larvae (580.7 ± 14.4µm).

On Day 10, larvae fed *Cm+Ig* (861.4 ± 60.9µm), *C. calcitrans* (844.1 ± 14.1µm), *Cc+Ig*, (809.0 ± 40.8µm) and *C. muelleri* (731.0 ± 20.7µm) did not differ significantly from each other. However, larvae fed *C. muelleri* showed no significant difference to larvae fed *I. galbana* (646.7 ± 55.6µm). Also, the larvae fed *I. galbana* was the only diet that showed no significant difference to the unfed larvae (537.2 ± 12.7µm).

Table 1. Length of sandfish *Holuthuria scabra* larvae fed single diet (*Chaetoceros muelleri*, *Chaetoceros calcitrans & Isochrysis galbana*) and mixed diet (50% *C. muelleri* + 50% *I. galbana* and 50% *C. calcitrans* + 50% *I. galbana*)

Treatment	**Day3** (µm)	**Day4** (µm)	**Day5** (µm)	**Day6** (µm)	**Day7** (µm)	**Day8** (µm)	**Day9** (µm)	**Day10** (µm)	**Day11** (µm)	**Day12** (µm)
C. muelleri	596.3 ± 9.3^{ab}	651.9 ± 9.9^{a}	799.5 ± 17.1^{a}	813.9 ± 46.3^{a}	843.0 ± 29.4^{a}	840.2 ± 12.3^{a}	837.4 ± 44.6^{a}	731.0 ± 20.7^{ab}	833.8 ± 96.3^{a}	872.5 ± 75.8^{a}
C. calcitrans	530.6 ± 31.2^{c}	659.5 ± 17.9^{a}	686.4 ± 27.0^{bc}	712.1 ± 53.2^{ab}	796.5 ± 25.7^{ab}	802.2 ± 47.2^{ab}	722.9 ± 65.5^{ab}	844.1 ± 14.4^{a}	811.0 ± 53.4^{ab}	662.6 ± 50.5^{abc}
I. galbana	615.9 ± 14.5^{a}	690.5 ± 15.3^{a}	713.0 ± 33.2^{b}	695.5 ± 50.7^{ab}	726.4 ± 30.0^{b}	708.2 ± 30.3^{b}	683.9 ± 41.2^{ab}	646.7 ± 55.6^{bc}	664.6 ± 23.1^{bc}	647.7 ± 21.1^{bc}
Cm + Ig	554.3 ± 14.4^{bc}	696.5 ± 5.0^{a}	732.8 ± 7.0^{ab}	784.0 ± 41.2^{a}	824.3 ± 34.3^{a}	789.7 ± 70.5^{ab}	841.4 ± 72.8^{a}	861.4 ± 60.9^{a}	899.4 ± 9.4^{a}	811.2 ± 99.8^{ab}
Cc + Ig	582.7 ± 5.4^{ab}	644.6 ± 27.0^{a}	689.7 ± 36.7^{bc}	754.0 ± 25.7^{a}	760.9 ± 26.9^{ab}	776.1 ± 31.0^{ab}	780.7 ± 34.9^{a}	809.0 ± 40.8^{a}	769.3 ± 19.9^{ab}	757.7 ± 46.9^{ab}
Control	523.4 ± 4.0^{c}	584.8 ± 16.1^{b}	625.8 ± 22.4^{c}	607.0 ± 33.1^{b}	597.9 ± 29.1^{c}	568.7 ± 9.0^{c}	580.7 ± 14.4^{b}	537.2 ± 12.7^{c}	558.0 ± 36.1^{c}	534.5 ± 36.4^{c}

Values are the mean ± SE. Significant differences in a column are indicated by different superscripts ($P \leq 0.05$).

On Day 11, larvae fed *Cm+Ig* (899.4 ± 9.4µm), *C. muelleri* (833.8 ± 96.3µm), *C. calcitrans* (811.0 ± 53.4µm) and *Cc+Ig* (769.3 ± 19.9µm) did not differ significantly from each other. Also, larvae fed *C. calcitrans* and *Cc+Ig* were not significantly different to larvae fed *I. galbana* (664.6 ± 23.1µm). The unfed larvae (556.0 ± 36.1µm) was significantly smaller to fed larvae except the larvae fed *I. galbana*.

On Day 12, larvae fed *C. muelleri* (872.5 ± 75.8µm), *Cm+Ig* (811.2 ± 99.8µm), *Cc+Ig* (757.7 ± 46.9µm), C. *calcitrans* (662.6 ± 50.5µm) were not significantly different from each other. Also, larvae fed *I. galbana* (647.7 ± 21.1µm) were not significantly different to all fed larvae except *C. muelleri.* The unfed larvae (534.5 ± 36.4µm) showed no significant differences to larvae fed *C. calcitrans and Cc+Ig.*

Larval Development

Observations on larval development are mentioned below. The eggs after spawning underwent development and reached the early auricularia stage after 24 hours (Day 2 – January 16, 2019), which constitute the first feeding stage and start of the experiment (Figure 2B). The early auricularia larvae were observed to have a buccal cavity, ciliary bands, cloaca and anus. As the days passed, the auricularia became more and more transparent and grew in length; also lateral projections became developed (Figure 2C, D).

In the late auricularia larvae, hyaline spheres appear at end of the four lateral projections on each side of the larvae (Figure 3). In the present study, late auricularia larvae with hyaline spheres were first observed in Day 9. Some of the auricularia larvae remained small. Few of the late auricularia larvae transformed in dolioaria on Day 11 (Figure 4).

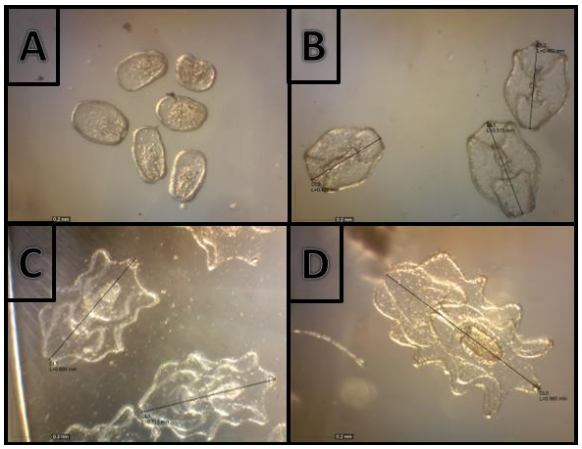

Figure 2. Larval development of sand fish (*H. scabra*). **A** Gastrula, **B.** Early Auricularia, **C.** Mid Auricularia, **D.** Late Auricularia.

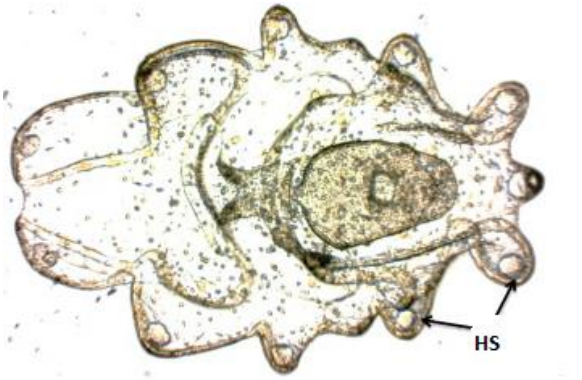

Figure 3. Late auricularia larvae hyaline spheres (HS).

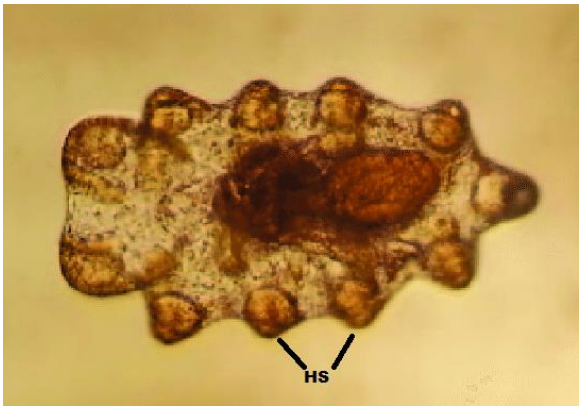

Figure 4. Doliolaria stage of *H. scabra*.

Percentage of H. *scabra* Larvae at Various Developmental Stages

Figure 5 shows the percentage number of early auricularia on Day 2. The larvae from all the treatments on Day 2 developed into early auricularia (100%). Samples from the different treatments were first taken before feeding starts.

Figure 6 shows the percentage number of auricularia (early, mid, & late) on Day 4. On Day 4, larvae fed *C. muelleri* developed into mid auricularia (85%) and late auricularia (15%). Those fed *C. calcitrans* still had early auricularia (20%), mid auricularia (80%) and no late auricularia. The larvae fed *I. galbana* developed into mid auricularia (83%) and late auricularia (17%). The larvae fed *Cm+Ig* also developed mid auricularia (81%) and late auricularia (13%) but few early auricularia (6%) was still observed. The larvae fed *Cc+Ig* had early auricularia (10%), mid-auricularia (90%) with no late-auricularia. The unfed larvae also developed mid-auricularia (72%) and early auricularia (28%) was still observed.

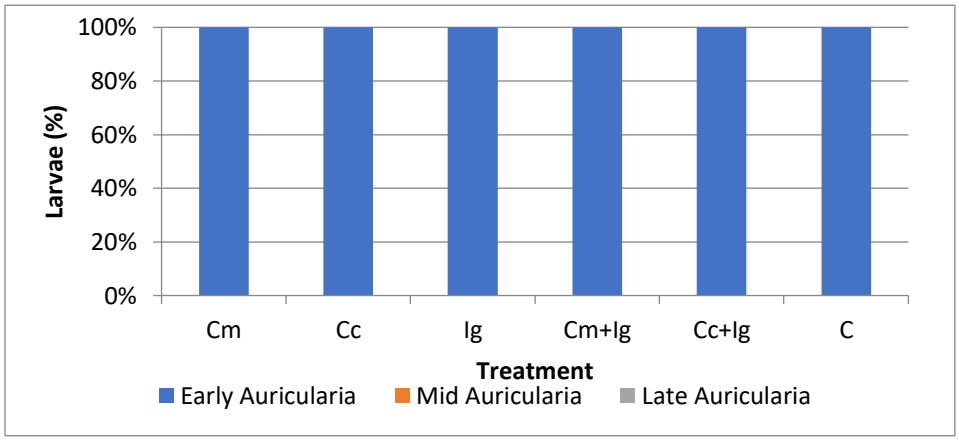

Figure 5. Percentage of early, mid, & late auricularia on Day 2.

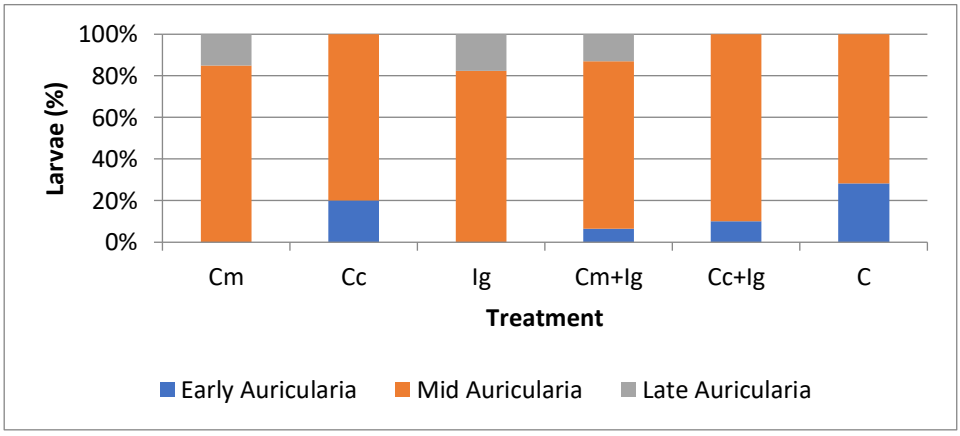

Figure 6. Percentage of early, mid, & late auricularia on Day 4.

Figure 7 shows the percentage number of auricularia (early, mid, & late) on Day 6. On Day 6, almost all developed into late auricularia (93%) in larvae fed *C. muelleri*. In larvae fed *C. calcitrans*, there were still a few early auricularia (3%) observed, but the percentage of late-auricularia (63%) increased and mid-auricularia (33%). Similar results were observed in *I. galbana* as compared with *C. calcitrans*. The larvae fed *Cm+Ig* had an increased development of late-auricularia (90%) and few mid-auricularia (10%). Same as *Cm+Ig*, larvae fed *Cc+Ig* also had an increased development of late-auricularia (80%) and few mid-auricularia (20%). The unfed larvae developed few late auricularia (8%) and dominated by mid-auricularia (86%) and a few early-auricularia (5%).

Figure 8 shows the percentage number of auricularia (early, mid, & late) on Day 8. On Day 8, no more early-auricularia were observed from all the treatments. Larvae fed *C. muelleri* had a slight decrease on the percentage of late auricularia (86%). Larvae fed *C. calcitrans* developed late auricularia (83%) and few mid auricularia (17%). The percentage of late auricularia (65%) remained the same as Day 6 in larvae fed *I. galbana*. The larvae fed *Cm+Ig* also decreased in percentage number of late auricularia (73%) compare to Day 6. The percentage of late auricularia (90%) for larvae fed *Cc+Ig* increased. The unfed larvae had developed mid-auricularia (90%) and increased development to late-auricularia (10%).

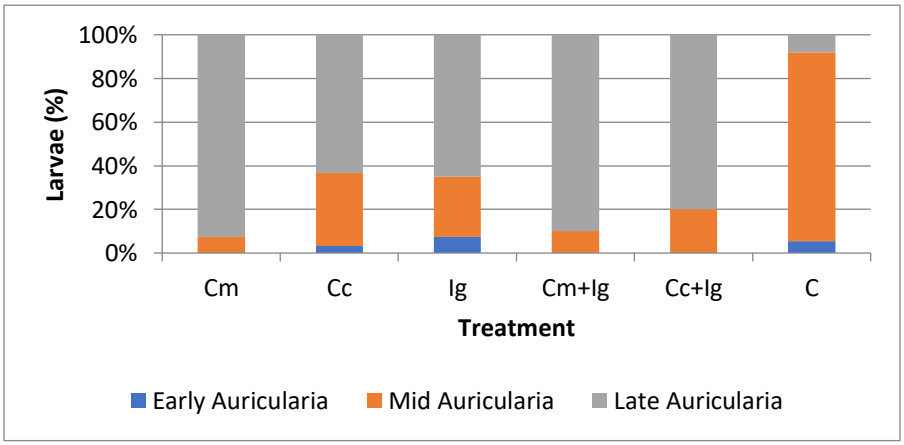

Figure 7. Percentage of early, mid, & late auricularia on Day 6.

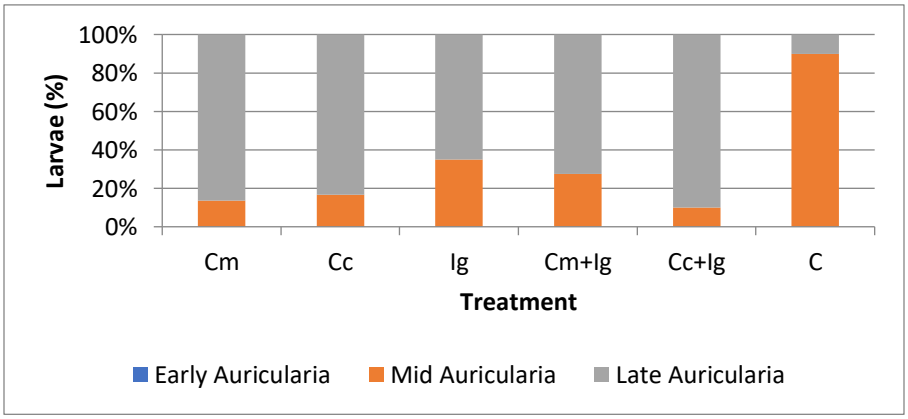

Figure 8. Percentage of early, mid, & late auricularia on Day 8.

Percentage of Late Auricularia with Hyaline Spheres

The late auricularia with hyaline spheres were first observed on Day 9 in larvae fed *C. muelleri* and *Cm+Ig;* followed by *C. calcitrans* on Day 11 and *Cc+Ig* on Day 12. The larvae fed *I. galbana* and the unfed larvae did not produce any late auricularia with hyaline spheres.

Figure 9 shows the percentage of mid-auricularia to late-auricularia with hyaline spheres present from Day 9 to 13. The percentage of late auricularia with hyaline spheres in larvae fed *C. muelleri* and *Cm+Ig* were 8% on Day 9. On Day 10, larvae fed *Cm+Ig* (16%) had a greater percentage compared to larvae fed *C. muelleri* (3%). On Day 11, the percentage of late auricularia with hyaline spheres increased for both *C. muelleri* (33%) and *Cm+Ig* (29%); also larvae fed *C. calcitrans* 7% of late auricularia was observed. On Day 12, same as Day 11 larvae fed *C. muelleri* (37%) had greater percentage of late-auricularia than larvae fed *Cm+Ig* (36%), *C. calcitrans* (7%) and *Cc+Ig* (3%). On Day 13, the highest percentage of late auricularia with hyaline spheres was observed in larvae fed *C. muelleri* (40%); in larvae fed *Cm+Ig* (28%), *C. calcitrans* (37%) and *Cc+Ig* (11%).

Figure 10 shows the average diameter of hyaline spheres of late-auricularia from Day 9 to Day 13. On Day 9 and Day 10, only larvae fed *C. muelleri* and *Cm+Ig* had late-auricularia that were observed. Late auricularia larvae fed *Cm+Ig* (Day 9 – 48.82µm, Day 10 – 54.04µm) had a greater diameter of hyaline sphere compared to *C. muelleri* (Day 9 – 47.17µm, Day 10 – 52.20µm).

On Day 11, as shown in the figure 9, late auricularia with hyaline spheres was also observed. The hyaline sphere of larvae fed *Cm+Ig* (57.40µm) had a greater diameter than larvae fed *C. muelleri* (54.04µm) and *C. calcitrans* (50.33µm).

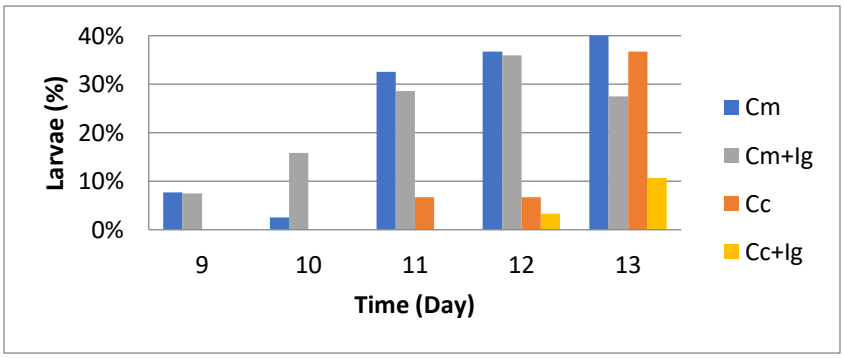

Figure 9. Percentage of late auriculae with hyaline spheres.

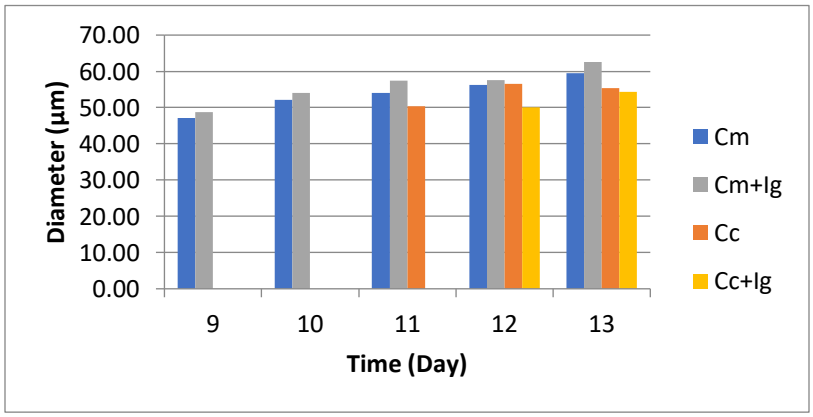

Figure 10. Average diameter (µm) of hyaline sphere of late auricularia larvae.

Percentage of Doliolaria

Figure 11 shows the percentage of doliolaria that appeared from Days 11 to 17. On Day 11 and Day 13, doliolaria was observed on larvae fed *Cm+Ig*. On Day 14, doliolaria were observed on larvae fed *C. muelleri* (3%) and *Cm+Ig* (3%). On Day 15, doliolaria was only observed in *C. muelleri* (7%). On Day 16, larvae fed *C. muelleri* showed a greater percentage of doliolaria (17%). Larvae fed *Cm+Ig* (10%) and *Cc+Ig* (9%), doliolaria were also observed. On day 16, doliolaria were observed in all the treatments. Larvae fed *C. muelleri* (15%), had greater percentage of doliolaria followed by *Cc+Ig* (11%), *Cm+Ig* (10%) and *C. calcitrans* (3%).

Growth of *H. scabra* larvae

Various authors had used different microalgal diets, either singly (diatoms & flagellates) or mixed diets. Battaglene (1999) fed *H. scabra* larvae with *Rhodomonas salina*, *Chaetoceros muelleri* or *Chaetoceros calcitrans*, and *Isochrysis galbana;* larvae failed to metamorphose if fed solely on *I. galbana* [4]. On the other hand, larvae grow best on diets of *R. salina and C. muelleri* or *C. muelleri* alone. It was also suggested that *C. muelleri*, *C. calcitrans*, *R. salina*, and *Pavlova lutheri* were suitable microalgae for the sea cucumber larvae [6]; also using mixed microalgal diet was suggested for better larval development. James (2004) observed best growth rates when early auricularia larvae were first fed *I. galbana* and mixed diet dominated by species of *Chaetoceros spp* [8]. Knauer (2011) tested four microalgae species, *H. scabra* larvae were fed with single diatom (*C. muelleri*, *C. calcitrans*), flagellate (*I. aff. galbana* (T-ISO), *P. salina*), or mixed diets comprised of 40% *C. muelleri*, 40% T-ISO, and 20% *P. salina*. Among the different microlage tested, *C. muelleri* was the most effective as a single microalagal diet for larval *H. scabra* [14].

The result of the present study showed the effectiveness of tested single microalgal diet (*Chaetoceros muelleri* & *Chaetoceros calcitrans* and *Isochrysis* galbana) and mixed diet (*C. muelleri + I. galbana* and *C. calcitrans + I. galbana*) as dietary microalgae for larval rearing of sandfish *H. scabra*. Larvae fed *C. muelleri* and *Cm+Ig* showed better performance than the larvae fed *I. galbana* alone and the unfed larvae. The larvae fed *C. calcitrans* or *Cc+Ig* was not significantly different to all fed larvae, but showed significantly greater length than the unfed larvae. On the other hand, larvae fed *I. galbana* showed better performance over the unfed larvae on Day 3 up to Day 8 and showed no significant differences to the unfed larvae on the succeeding days of the experiment.

Development of *H. scabra* larvae

Development of feeding auricularia to non-feeding doliolariae occurs generally by approximately 2 weeks [14, 15] and 2-20 days after hatching was reported by Al Rashdi et al. (2012) [16]. Likewise, in the present study, feeding started on Day 2 with feeding concentration of 5,000 cells ml^{-1}; only early auricularia larvae were observed from all treatments.

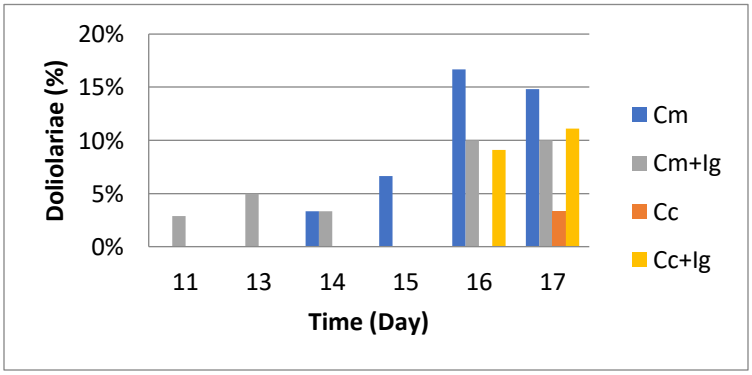

Figure 11. Percentage of doliolaria from Day 11 to 17.

Mid-auricularia were seen at varying levels in all treatments from Day 3 to Day 8 and late-auricularia was observed at Day 4 to Day 8.

Early-auricularia were observed less frequently for larvae fed *C. muelleri* and *Cm+Ig*. From Day 9-17, late-auricularia with hyaline spheres were observed (Figure 3). Development of competent doliolaria was first observed on Day 11 for larvae fed *Cm+Ig*, the percentage of competent doliolariae on larvae fed *C. muelleri* at Day 16 and Day 17 was higher compared to the other three microalgae followed by larvae fed *Cm+Ig*, where competent doliolaria was first observed on Day 11 and up to Day 17 (Figure 11.)

Abnormality among larvae from Day 2 to Day 8 was also observed. A minimal amount of folding and pointed apex, together with anterior-posterior contraction of the body length indicated an extreme of larval abnormality [17]. The unfed larvae had the highest percentage of abnormality from all the treatments which corresponds to 55±12%. The larvae fed *C. muelleri* (8±1%), *C. calcitrans* (11±2%), *I. galbana* (17±2%), *Cm+Ig* (15±3%) and *Cc+Ig* (16±3%) also had some abnormality.

Health Condition of Larvae

At the end of the auricularia stage, larvae develop hyaline spheres at the tip of the lateral processes (Figure 3) [11, 12]. Development of hyaline sphere during late auriclaria is a reliable indicator of successive larval competence for *H. scabra* [13, 18]. Although larvae fed *C. muelleri, Cm+Ig, C. calcitrans, Cc+Ig* or *I. galbana* did not differ significantly in terms of growth from Day 3 to Day 8 (Table 1), only larvae fed *C. muelleri, Cm+Ig, C. calcitrans* and *Cc+Ig* were able to produce late-auricularia with hyaline spheres (Figure 9). The percentage of late-auricularia with hyaline spheres on larvae fed *C. muelleri* was greater compared to the other three diets of microalgae; followed by larvae fed *Cm+Ig* (Figure 9). In terms of size of the hyaline spheres, late auricularia with hyaline spheres of larvae fed *Cm+Ig* had the largest size of hyaline sphere; followed by *C. muelleri* (Figure 10). The percentage of competent doliolariae on larvae fed *C. muelleri* at Day 16 and Day 17 was higher compared to the other three microalgae, followed by larvae fed *Cm+Ig,* where competent doliolaria was first observed on Day 11 and up to Day 17 (Figure 11).

Formation of hyaline sphere and their size during the auricularia stage of *H. scabra* larvae were greatly influenced by the nutrient available in the diet composition, and that there were significant positive correlations between hyaline sphere formation and dietary level of carbohydrate, EPA:DHA ratio [13] as well as neutral lipids which fuel the process of settlement [18]. The microalga *C. muelleri* contains 5-20% EPA and 0.2-1% DHA [19], *C. calcitrans* contains 2.9-14% EPA and 0.2-0.8% DHA, and I. *galbana* contains 0.1-0.9% EPA and 2.1-16.7% DHA [20]. The differences in biochemical composition, such as total amounts and proportions of essential nutrients may be accounted for the effectiveness of microalgae. It was established that dietary polyunsaturated fatty acids such as eicosahexaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) were essential for many invertebrate larvae [21]. In the present study, *C. muelleri* was an effective single-specie microalgal diet. However, the addition of *I. galbana* with *C. muelleri* may have contributed to the larger size of hyaline spheres in late auricularia larvae.

The use of a combination of microalgae is better able to meet the nutritional requirements of cultured sea cucumber auricularia larvae. It may be due to the fact that nutrients lacking in single-species diets may be supported by mixed microalgal diets [3, 4, 22]. Further studies on the nutritional value of these microalgae must be done to establish their effectiveness as larval diet of *H. scabra.*

Water Quality Parameters

Various authors recommended that the water temperature should be within the range of 26 to 30ºC and salinity from 32 and 36 ppt to ensure normal development [14, 16, 23]. In the present study, normal development was observed with water temperature ranging from 22.9-26.6ºC, salinity 30.4-30.7 ppt and pH 7.3-7.4.

CONCLUSIONS

Based on the result of the study, *C. muelleri* alone or in combination with *I. galbana* was the most effective microalgal diet for the larvae *H. scabra*. In terms of the health condition, microalgal diets *C. muelleri*, *Cm+Ig*, *C. calcitrans* and *Cc+Ig* were able to produce to late auricularia with hyaline spheres, which is an indication of larval competence. However, microalgal diets *C. muelleri* and *Cm+Ig* showed higher percentage of auricularia with hyaline spheres and largest size of hyaline spheres among the different microalgal diets. Mixed microalagal diets supports the nutritional requirement of *H. scabra* larvae. However, another study on this aspect should be done in order to establish the link between nutritional values of microalgae and the subsequent settlement and metamorphosis of *H. scabra*.

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