Appropriate Diet and Stocking Density for Sea Cucumber (Holothuria scabra) Larvae Rearing

N A Abidin¹, S R Muhamad Shaleh¹*, F F Ching¹, R Othman¹, M Manjaji-Matsumoto¹, S Mustafa¹ and S Senoo²

¹ Borneo Marine Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.
² Shirahama Aquaculture Research Institute, Kinki University, 3153 Shirahama, Wakayama 649-2211, Japan.

*Email: sittirae@ums.edu.my

Abstract. Overexploitation of sea cucumbers especially Holothurians is not a new issue, as this species has been highly demanded and traded at high price in Asian dried seafood market. As the wild seed supply has shown a substantial decline, hatchery production is urgently needed to supply the aquaculture industry. However, many are still struggling with the larviculture of Holothuria scabra. In this study, suitable diet (Nannochloropsis sp., Chaetoceros calcitrans and combined diet) and stocking density (1, 1.5 and 2 larvae ml⁻¹) for H. scabra larvae are tested in a factorial experiment. The experiment was carried out in plastic containers randomly arranged in a water bath at temperature 29-30°C. Result shows that, there was a strong interaction between diets and stocking density on the growth of the larvae (p=0.000). Single species of Nannochloropsis sp. at 1.5 larvae ml⁻¹ demonstrated the highest growth (109.69± 8.21). On the other hand, single species of C. calcitrans also at 1.5 larvae ml⁻¹ enhanced the metamorphosis of auricularia into doliolaria stage. However, the survival rate was not significantly affected by the diets and stocking density (p= 0.974) although single species of Nannochloropsis sp. at 2 larvae ml⁻¹ has the highest survival (1.2±1.05). For a stocking density of 1.5 larvae ml⁻¹, it is recommended to feed the larvae with Nannochloropsis sp. and C. calcitrans for higher growth and faster metamorphosis, respectively.

1. Introduction

Holothuria scabra, commonly known as sandfish was listed among the most highly valued tropical species in Asian markets. The price of the dried sandfish in the local market can reach up to RM1500-2000 per kilogram. Sandfish have high protein, low fat and contains many bioactive substances which exhibit the antibacterial, antifungal and anticancer properties. It also acts as a tonic and traditional remedy for asthma, hypertension, constipation and cut and burns [27]. In Malaysia, production of dried sandfish (beche-de-mer) is actively carried out in Sabah. This species can be found mainly in Kunak, Kudat and Semporna [5]. However, this sector depends solely on the wild-caught sandfish seeds. Based on the Annual Fisheries Statistics, 139 tonnes of sea cucumbers were landed in Sabah in 2005 [11].

Sandfish have been listed as an endangered species by the International Union for Conservation of Nature and Natural Resources (IUCN). The population of sandfish has declined at least 50% over the past 30-50 years [14]. Increasing demand and continuous exploitation of sandfish for export has leads to the extinction and depletion of broodstocks. Enforcement and regulatory measure by the authorities alone may not be sufficient to overcome the issue on the declining population. Therefore, expanded aquaculture activities of sandfish are necessary for the sustainability of this species.

Sandfish are the most commonly cultured species and bred extensively because it has high commercial value, widely distributed and currently the only tropical species that can be mass produced in hatchery system [23]. Sandfish has the benefits on aquaculture for the ability to be induced throughout the year compared to the other species which can only be induced once or twice a year. The spawning season was not influenced by the lunar periodicity [25].
According to [8], hatchery production of sandfish is seen as a way to restore the depleted wild stock through the stock enhancement program called sea ranching. Artificial spawning will enable continuous seed production of sandfish throughout the year for aquaculture and stock restoration. However, there are some challenges in the hatchery production of sandfish especially at the larval stage up to the juvenile rearing. Larval culture is the crucial aspect and the most critical stages in aquaculture. The main challenge in the larval culture is the low survival (1%) of the planktonic larvae up to the epibenthic stage [27]. There are many factors that influenced the success of the sandfish seed production such as quality of the broodstocks and fertilized eggs, water quality, feeding management, space availability and hatchery practice such as larval rearing techniques. This study was carried out to determine the effects of diet types and stocking density on the growth performance of sandfish larvae.

2. Materials and Methods

2.1 Broodstocks procurement and acclimatization
Healthy broodstocks (200-300 g; N=50) with no skin lesion were collected from Kampung Baru- Baru, Tuaran, Sabah (N6° 18’ 18.1656”, E 116° 17’43.1052”). The broodstocks were packaged in the oxygen-filled plastic bag and transported in the insulated polystyrene container. The broodstocks were acclimatized in Universiti Malaysia Sabah (UMS) hatchery for about 2 weeks prior to spawning induction. A two tonnes tank containing 5 cm depth of treated sand was used as the broodstock’s tank. Flow through water system equipped with biological coral filter was installed on the tank. Ground seaweed (Sargassum sp.) was given twice daily.

2.2 Spawning induction
Forty broodstocks were transferred into a 1 tonne high- density polyethylene (HDPE) tank with the absence of sand for overnight defecation to prevent any contamination of the foreign substances such as faecal matter during the spawning. Combination of 3 spawning induction methods: desiccation, thermal stimulation and feed stimulant were used to induce the sandfish. The broodstocks were left to desiccate for 30 minutes before being transferred into the spawning tank for the thermal stimulation. As for the thermal stimulation, the temperature of the water was raised by 3 to 5°C above the ambient temperature (28°C). The broodstocks that had been desiccated were transferred into the spawning tank containing heated sea water. The thermal stimulation was carried out for 2 hours until the broodstocks show the spawning behaviour. As no sign of spawning, the broodstocks were subjected to the third induction that is feed stimulant using Spirulina powder. HDPE tank with a capacity of 700 L containing 10 cm depth of sea water was used for Spirulina bath. Spirulina powder was added at a rate of 60 g L⁻¹ [1]. Moderate aeration was provided for homogenize mixing before the broodstocks were incubated in the bath for 30 minutes. Then, the broodstocks were rinsed to remove excess Spirulina before being transferred to the spawning tank. The spawning tank was filled at about 20 cm depth with filtered and UV treated seawater.

2.3 Egg collection
The fertilized eggs were siphoned from spawning tank and collected using 30 µm hand net. The eggs were rinsed with treated seawater to remove excess sperms that may cause polyspermy which leads to larval abnormalities. The fertilized eggs were collected in a 14 L bucket and stirred gently to ensure the eggs were distributed evenly. Then, 5 subsamples of 1 mL volume were collected and the density of the fertilized eggs in each subsample was determined under compound microscope using Sedgewick Rafter counting chamber. Total fertilized eggs collected were counted using the formula below:

\[
\text{Total fertilized eggs} = \text{average density of fertilized eggs in 1ml subsample} \times 14 \text{ l}
\]

2.4 Feeding trials
Two species of microalgae Chaetoceros calcitans and Nannochloropsis sp. used as diet were purchased from Japan and Taiwan, respectively. The cell density of the concentrated C. calcitans was 1×10⁸ cells ml⁻¹. Meanwhile, the cell density of the concentrated Nannochloropsis sp. was 1.1 × 10⁸ cells ml⁻¹. The concentrated microalgae were diluted according to the feeding rate required by the sandfish larvae. Four
diet types were tested; single species of *C. calcitrans* and *Nannochloropsis* sp., mixed species (50:50) and unfed (negative control). Thirty six plastic containers with the capacity of 4.5 L used as the experimental containers (triplicate experiment) were randomly arrange in a two tonnes HDPE tank (Figure 1). The tank which acts as a water bath was equipped with aquarium heaters to maintain constant temperature in the experimental containers at 29 to 30°C. The larval rearing tank was also covered with mosquito net to prevent any foreign substances and infestation of bloodworm in the tank. The two days after hatched (dAH) larvae were distributed into the experimental containers according to the required stocking density (1, 1.5 and 2 larvae ml⁻¹). All experimental containers were filled with 4 L filtered and UV sterilized seawater and supplied with aeration. The larvae were fed with respective tested diets twice a day at 9 a.m. and 4 p.m. The feeding rate was increased gradually from 20,000 cells ml⁻¹ to 40,000 cells ml⁻¹. Partial water changes (20%) were carried out every two days [1].

**Figure 1.** Experimental containers were kept in fibreglass tank which acted as water bath to maintain the temperature at 29-30°C.

2.5 **Data collection**

The measurement on the growth of the larvae was carried out every two days. Twelve larvae were randomly sampled from each experimental tank. The larval development was recorded and the total length of the larvae were measured using Zeiss Axioplan 2 IE Microscopic System (40x magnification). The survival of the larvae was determined at the end of the experiment. At the end of this experiment, the growth in terms of total length increment and survival rate of the larvae was calculated using the equations below:

\[
Growth \ (size \ increment \ in \ %) = \frac{final \ length \ (\mu m) - initial \ length(\mu m)}{initial \ length \ (\mu m)} \times 100\%
\]

\[
Survival \ (\%) = \frac{final \ number \ of \ larvae}{initial \ number \ of \ larvae} \times 100\%
\]

SPSS (Statistical Package for Social Sciences) software version 21 was used for the statistical analysis. Two way ANOVA were used to determine the significant difference between the treatments and any interaction between the factors tested.

3. **Results and discussion**

Size increment of sandfish larvae fed with *Nannochloropsis* sp. was maximum (109.7%) at 1.5 larvae ml⁻¹ followed by 1 and 2 larvae ml⁻¹ (Figure 2a). There was no significant difference on the size increment of larvae fed with single *Nannochloropsis* sp. at 1 and 1.5 larvae ml⁻¹, but at 2 larvae ml⁻¹ it was significantly low (p<0.05). Meanwhile, stocking density of 2 larvae ml⁻¹ demonstrated the highest
percentage of size increment (97.9%) when fed with C. calcitrans (Figure 2b). For the combined diet, no significant differences (p>0.05) was observed on the size increment in all stocking density tested (Figure 2c) and it was similar to the unfed larvae (Figure 2d). The lowest survival was recorded in the larvae fed with combined diet at stocking density 1.5 larvae ml\(^{-1}\) (Table 1). However, there were no significant difference on the survival among the treatments (p>0.05). Table 2 shows the diet types has significant effect on the growth of the larvae (p= 0.00), inversely the growth of the larvae was not significantly affected by the stocking density (p= 0.515). The two way ANOVA analysis also indicated the strong interaction between diet types and stocking density on the growth of the larvae (p=0.00), but not on the survival of the larvae (p= 0.974).

**Figure 2.** Mean of size increment of sandfish larvae fed with different diets at different stocking density. a) *Nannochloropsis* sp., b) C. calcitrans, c) Combined diet and d) Unfed (negative control). Vertical lines represent standard errors. The different letters indicate significant different (p< 0.05) among treatment, (n=24).
Table 1. Mean (± SE) survival (%) of sandfish larvae fed with different diets and stocking density.

| Diet                        | Stocking density (ml⁻¹) | Survival (%) |
|-----------------------------|-------------------------|--------------|
|                            | 1                       | 0.73 ± 0.47  |
| *Nannochloropsis* sp.       | 1.5                     | 0.36 ± 0.29  |
|                            | 2                       | 1.2 ± 1.05   |
| *Chaetoceros calcitrans*    | 1                       | 0.6 ± 0.5    |
|                            | 1.5                     | 0.53 ± 0.34  |
|                            | 2                       | 0.9 ± 0.25   |
| Combined diet               | 1                       | 1.07 ± 0.79  |
| (*Nannochloropsis* sp. + *C.* calcitrans) | 1.5                     | 0.22 ± 0.12  |
|                            | 2                       | 1.2 ± 0.57   |
| Unfed                      | 1                       | 0.4 ± 0.4    |
|                            | 1.5                     | 0.49 ± 0.12  |
|                            | 2                       | 0.9 ± 0.06   |

The different letters indicate significant differences (p<0.05).

Table 2. Two way ANOVA showing the interaction between diet types and stocking density on the growth and survival of larvae.

| Two way ANOVA                        | Growth (%) | Survival (%) |
|--------------------------------------|------------|--------------|
| Diet types                           | p= 0.00    | p= 0.945     |
| Stocking density                     | p= 0.515   | p= 0.202     |
| Interaction                          | p= 0.00    | p= 0.974     |

The level of significant difference was p<0.05.

The highest percentage of doliolaria was observed on the larvae fed with single species of *C. calcitrans* (Figure 3b). The sandfish larvae started to undergo metamorphosis into doliolaria on day 6 with 12.5% at 2 larvae ml⁻¹, followed by 8.3% both at 1 and 1.5 larvae ml⁻¹. However, no metamorphosed larva was observed at day 8 in all stocking densities tested. 45.8% of doliolaria was recorded at 2 larvae ml⁻¹ but no doliolaria was found at 1 and 1.5 larvae ml⁻¹ on day 10. On day 12, 16.6% and 25% doliolaria were observed at stocking density of 1.5 and 2 larvae ml⁻¹ respectively. For the larvae fed with combined diet (Figure 3c), doliolaria was observed only at 1 larva ml⁻¹ on day 6 (8.3%) and no doliolaria was found on day 8. The percentage of doliolaria appeared on day 10 at stocking density of 1 and 1.5 larva ml⁻¹ were 8.3% and 4.2% respectively. On day 12, doliolaria was only recorded at stocking density 2 larva ml⁻¹ (4.2%). Larvae fed with single species of *Nannochloropsis* sp. started to metamorphose into...
doliolaria at day 12 (4.2%) (Figure 3a). The metamorphosis of the larvae was delayed compared to the other treatment. No larvae metamorphosed into doliolaria in the negative control (unfed larvae) throughout the experimental period.

**Figure 3.** Percentage of doliolaria at different diet type and stocking density. a) *Nannochloropsis* sp, b) *C. calcitrans* and c) Combined diet

Nutrient requirements for all holothuroid species might be different. Combined diet was not only effective for the larval rearing of *H. scabra*, but also effective for other species such as *S. japonicus, H. atra* and *H. spinifera* [2]; [32]; [15]; [29]. [8] reported the growth performance of the sandfish larvae was better when fed with the mixture of *Rhodomonas salina* and *C. muellerii*. [32] recommended a mixture of *Dicrateria, Platymonas, Nitzschia, Dunaliella* and *Toru lopsis* as the feed for *S. japonicus* larvae. Meanwhile, combination of *C. calcitrans* and *I. galbana* resulted in better growth performance of *H. spinifera* larvae [3].

However, mixture of algal diets does not necessarily promote the best growth performance in marine invertebrate species [30]. In the present study, single species of *C. calcitrans* supported the highest percentage of larval transition into doliolaria even though the size increment was lower than *Nannochloropsis* sp. fed larvae (Figure 3). The combination of *C. calcitrans* and *Nannochloropsis* sp. was not significantly improved the growth (Figure 2) and survival (Table 1) of sandfish larvae in comparison with *C. calcitrans* alone. Single diet of *C. calcitrans* was also recommended in the larval rearing of *Bohadschia marmorata* and *H. spinifera* [3]; [18]. Higher growth and survival rate were also observed on the larvae of *Actinopyga echinites* when fed with single species of *I. galbana* [10].
contrast, [16] stated single species of C. muellerii was the most effective diet for the growth performance of sandfish larvae.

The difference in the nutritional composition of the microalgae resulted in the varying growth, survival and metamorphic rate of the sandfish larvae [30]. High lipid content of microalgae was required by the larvae to enhance the development of the lipid-riched structure called hyaline spheres. The hyaline spheres contain nutrients which will be used by the larvae to complete the metamorphosis into doliolaria [12]. According to [6], C. calcitrans has higher protein and lipid content (protein= 41.6% and lipid= 26.8%) compared to Nannochloropsis oculata (protein= 32.8% and lipid= 13%). In this study, highest metamorphic rate was recorded on the larvae fed with single species of C. calcitrans followed by combined diet and single species of Nannochloropsis sp (Figure 3).

Even though the growth performance of the unfed larvae (negative control) was poor and metamorphosis of the larvae into doliolaria was not observed but there was a slightly increased in the size increment percentage of the larvae (Figure 2). [9] and [24] reported that the energy required for the growth, development and metamorphosis of echinoderm species with planktonic larval development mainly obtained from exogenous food sources. They also stated endogenous reserves in the larva itself was not sufficient for the metabolic demand. There might be some nutrients uptake by the unfed larvae from dissolved organic compounds [17]; [22]; [35]. In sandfish aquaculture, stocking density is one of the exogenous factors that will influence the production of a hatchery. It directly gives impact on the growth performance, food availability, health and yield of cultured organisms [31]. [20] reported high density-rearing condition also leads to the size variation and abnormalities such as rotten stomach and low metamorphosis rate at the larval stage. Stocking density also gives significant effects on the juvenile of sandfish. It is highly vulnerable especially at the early stage of juvenile rearing and highest mortality was reported due to the high stocking density [33]. [7] also stated large area was necessary in the seed production of holothurians through the hatchery system.

The optimum stocking density for H. scabra versicolor and H. scabra was 0.75 and 1 larva ml⁻¹ respectively [13]. However, lower optimum stocking density was recorded for Apostichopus japonicus which were 0.1-0.2 larvae ml⁻¹, 0.3-0.4 larvae ml⁻¹ and 0.5 larvae ml⁻¹ [34]; [19]. [4] also stated that, the species wise variations in the size of auricularia larvae leads to the difference in the optimum stocking density for the larval rearing. Lower stocking density may cause an unnecessary waste of space and not efficient for the mass seed production of sandfish through hatchery system.

The current study shows that the possibility to carry out the larval rearing of sandfish at the stocking density higher than 1 larva ml⁻¹, as long as a sufficient supply of feed that meet the nutritional requirement of the larvae was provided. The larvae fed with a single species of C. calcitrans and combined diet were still capable to metamorphose at the stocking density of 1.5 and 2 larvae ml⁻¹ (Figure 3). Table 1 shows the stocking density of 2 larvae ml⁻¹ also shows the highest survival (1.2 ± 1.05%) even though there was no significant difference (p>0.05) with the other stocking densities. High stocking density may affect the growth rate of the larvae, but it rarely affected the survival rate [28]. A major factor that leads to the growth variation of the larvae was the genetic factor and not only caused solely by the factor of stocking density [21]; [26]. According to this feeding trial, a single species of C. calcitrans at the stocking density of 2 larvae ml⁻¹ was recommended because highest percentage of auricularia metamorphosis into doliolaria was recorded (day 8= 12.5%, day 10= 45.8% and day 12= 25%) (Figure 3).

4. Conclusion
This study provides the basis information on the best diet type and optimum stocking density to enhance the growth performance of sandfish larvae. Optimal condition for the larval rearing must be established to achieve maximum hatchery seed production of sandfish. Consequently, it allows higher yields of hatchery produced juveniles for the stock enhancement program.
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Acknowledgement
This research was funded by Ministry of Higher Education of Malaysia under the Niche Research Grant Scheme (NRGS0002).