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Research Paper

Yield and product quality of processed sandfish *(Holothuria scabra)* using different processing techniques

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Abstract. Four different techniques in processing sandfish: a) MB-method described by Brown *et al.* (2010), b) MN- from NFRDI, per Pardua *et al.* (2018), and two variations of method from Purcell (2014), c) MP1, and d) MP2 which were slightly modified and tried to compare resulting product quality and yield from freshly gutted sandfish. This demonstration was to encourage sandfish ranch managers to add value to their sandfish and ensure better earnings. Twenty-four sandfish individuals (350 to 870 g) harvested from the sea ranch site at Maliwaliw Island were used for processing at six individuals per technique. Results show that live sandfish weight was reduced to 45% after gutting. MB had the highest weight percent recovery from gutted weight at 10.75%, not significantly different ($p > 0.05$) from MN at 9.65% but significantly higher ($p < 0.05$) from MP1 (9.38%) and MP2 (9.50%). In terms of product quality, bigger sandfish >600 g produced good quality products when processed correctly. *Bêche-de-mer* is priced based on three criteria: quality of the product, size length, and weight. Although there is no single best-practice method for processing sandfish, sea ranch managers may opt to adopt the MB and MN for a simpler procedure to save time and resources, and higher product yield for better income.

Keywords: Sandfish, processing techniques, yield, balat, product quality.

INTRODUCTION

Sea cucumbers are worm-like echinoderms that live on the seafloor. Species like sandfish (*Holothuria scabra*) are regarded as one of the highest-valued commodities globally where they are utilized mostly for food and perceived medicinal benefits (Esmat *et al*., 2013). People across Asia have been using sea cucumbers to treat joint problems such as arthritis (Bordbar *et al*., 2011; Kiew and Don, 2012). More recently in Europe, sea cucumbers are also used to treat cancers and to reduce blood clots as sea cucumbers are found to exhibit anticoagulant, antioxidant, anticancer, antimicrobial, and antiviral properties (Rasyid *et al.*, 2021; Kareh *et al.*, 2018; Khotimchenko, 2018). According to Purcell *et al.* (2018), the price of sea cucumbers in the international market increased sharply as demand for this Asian delicacy, as well as the new interest from western pharmaceutical businesses, increased.

Sandfish and other sea cucumber species have been harvested and sold as dried, non-perishable items for a long time (Ram *et al*., 2016; Purcell *et al.*, 2014). Dried sea cucumber products are called *bêche-de-mer* (BDM) in India and the Pacific, *trepang* in Indonesia and northern Australia, and *balat* in parts of Malaysia and the Philippines (Akamine 2013). Premium-size and premium-quality dried sandfish have an average price of 369 US dollars per kilogram and extra-large premium-quality specimens can fetch up to USD 1898/kilo in the international market (Bassig *et al*., 2021). The thick body wall of sandfish, which primarily makes it more valuable (Akamine, 2002), is composed of bio-medically important compounds such as peptides, collagen, gelatin, polysaccharide, and saponin (Oh *et al*., 2017). China, Hong Kong, Taiwan, Singapore, and Malaysia are among the major Asian markets for Trepang (Purcell *et al.*, 2018; Perez and Brown, 2012).

The Philippines whose coastal waters are home to about 200 sea cucumber species, plays a major role in the dried sea cucumber trade in the world market (Juinio-Meñez and Samonte, 2016; Brown *et al.*, 2010). Among the 35 commercially important species listed in the Philippines National Standards for sea cucumber (BAFPS, 2013), sandfish is the most expensive at Php 5326.00 or USD108/kg in the local market (Pardua *et al*., 2018). In the central Philippines, particularly in the Eastern Visayas, sandfish are commonly found in the coastal waters of Samar and Leyte (de la Cruz *et al.*, 2015). However, spurred by high international market prices, fishing pressure has increased in the poorly managed sea cucumber fishery nationwide (Purcell *et al.*, 2018; Perez and Brown, 2012). The unregulated gathering of sea cucumbers depleted the resource and consequently a decline in export volume (Choo, 2008).

To enhance the recovery of depleted stocks of sandfish in the wild, extensive research on the production technologies for sandfish have been carried out in the Philippines (Juinio-Meñez *et al.*, 2017). In the North-western part of the country, the University of the Philippines-Marine Science Institute (UP-MSI) piloted sea ranching to provide a supplemental source of income to municipal fishers and at the same time allow the wild stocks to recover (Juinio-Meñez *et al.*, 2012).

Replicating good practices employed by UP-MSI, Maliwaliw Island in Eastern Samar became the pilot site of the sandfish sea ranching in the Eastern Visayas to address the collapsed sea cucumber fisheries in the area (Villamor *et al*., 2021). The Maliwaliw community that once experienced local extinction has successfully brought back the sandfish in the area seven years after the implementation of the ACIAR-funded community-based sandfish sea ranching. Recently, sea ranch co-managers are able to produce marketable size (320 g) to premium size sandfish (>600 g). However, the lack of knowledge in the proper processing of sandfish to satisfy the export market has forced them to sell their sandfish fresh at low prices. Hence, middlemen and local processors take advantage of the fishers selling their sandfish fresh. To help the sandfish ranch managers, four different processing techniques were tried to evaluate the resulting product quality and yield of processed sandfish.

METHODOLOGY

Study site

This study was conducted on Maliwaliw Island, Salcedo, Eastern Samar (11.098714°N, 125.585800°E), the pilot site of sandfish sea ranching in Eastern Visayas (Figure 1).

Sample collection and preparation

A total of 24 live and uninjured sandfish individuals weighing 300 to 870 g collected from the sea ranch were bought from a sandfish co-manager. Six pieces of sandfish were used per processing technique. Sandfish were washed clean using seawater and arranged in a single layer on a flat surface for about 5 minutes to allow the animals to relax and expel water before their wet weights were recorded. Sandfish were then patted dry using a paper towel and weighed individually before degutting and then re-weighed individually using a kitchen scale.

The materials and utensils needed for processing such as a weighing scale, sharp knife, large wok, wooden ladle with a long handle, strainer with a long handle, commercially available brush with plastic bristles, containers with cover, a container with slits, basins, polypropylene resealable bags, papaya leaves, coarse-grade salt, and clean seawater were made ready.

Processing

Four different established techniques in processing sandfish: a) MB (Brown *et al*., 2010), b) MN (NFRDI, per Pardua et. al., 2018), and two variations of the method from Purcell (2014), c) MP1, and d) MP2, were employed to compare resulting product quality and product yield from fresh/gutted sandfish. MB processing steps were adopted from the commonly used techniques of sea cucumber processors in Palawan, Philippines. Steps involved in the MN technique were based on a procedure developed by the National Fisheries Research & Development Institute (NFRDI) of the Department of Agriculture also in the Philippines. Whereas MP1 and MP2 were based on the Manual for processing sea cucumbers into *beche-de-mer* designed for fishers in the Pacific Islands (Purcell, 2014). Detailed steps of each processing technique are shown in Figure 2.

Post-harvest processing requires a relatively simple and traditional technique that involves post-capture handling, cleaning, cooking, and drying. Suggested steps for processing sandfish from the four techniques were followed with slight modifications.

Degutting

At the ventral side of the anus, a 1-inch incision was made using a sharp stainless knife. The internal organs and water were then squeezed out of the opening. Subsequently, gutted samples were placed on a flat surface to allow them to retain their original shape. Each sandfish was again weighed to obtain the gutted weight.

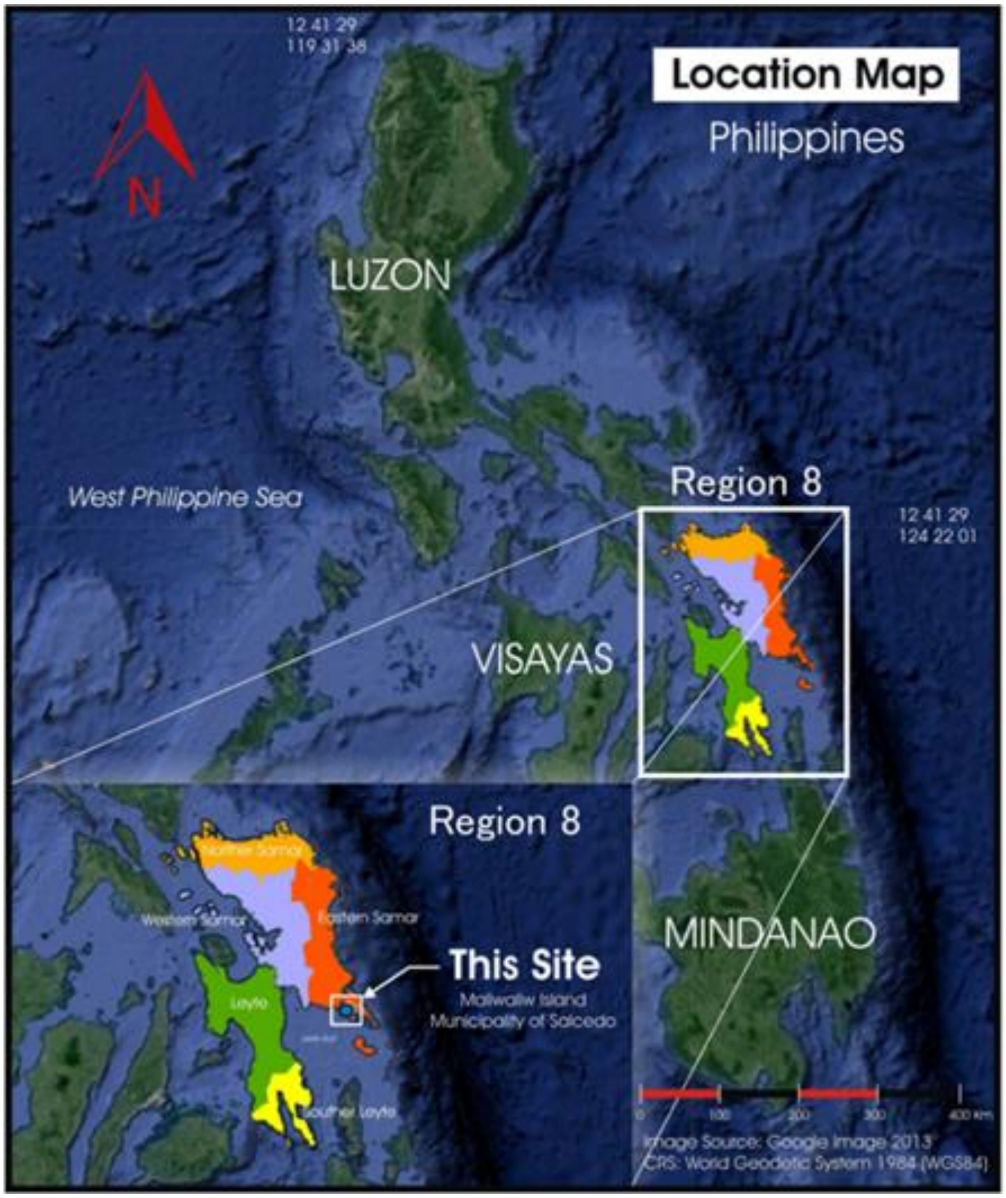

Figure 1. Location of sandfish sea ranch site in Maliwaliw Island, Salcedo, Eastern Samar, Philippines. Map image source: Google image 2013. CRS: World Geodetic System 1984 (WGS84).

Cooking

All samples in the four techniques have undergone the process of first and second cooking at varying times. MN and MB samples were first cooked in a wok with pre-heated seawater at 60°C until the temperature gradually increased to 100°C for one and two hours, respectively. MP1 and MP2 samples were first cooked in pre-heated seawater at 60°C for 30 min. For the second cooking, MN and MB samples were cooked again in pre-heated seawater for an hour while MP1 and MP2 were cooked for another 30 min only. However, MP1 and MP2 samples were subjected to third cooking for another 30 minutes after smoke-drying for three hours. The total cooking time for MB was three hours, MN was two hours, and MP1 and MP2 were an hour and a half each. Clean seawater was used in cooking the sandfish for all four processing techniques. According to Purcell (2014), saltwater retains the color of sea cucumber and keeps the skin from being damaged. All cooking processes were also done with constant stirring using a wooden ladle.

Ossicle removal

All techniques vary when it comes to softening and removing sandfish ossicles or calcareous deposits. After the first cooking, samples were taken out of the wok and

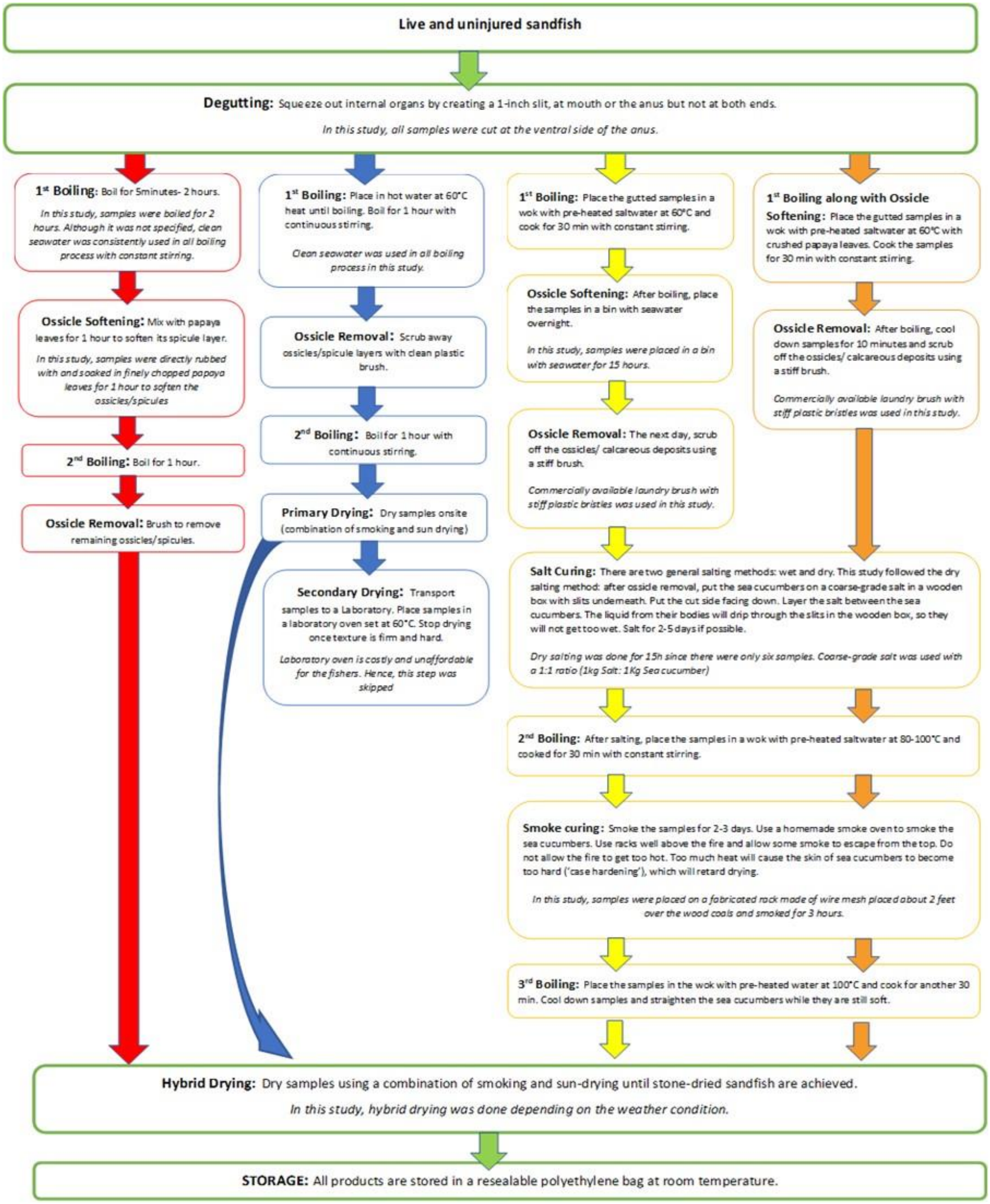

Figure 2. The steps followed were adopted from the four established techniques in processing sandfish: MB (Brown *et al.*, 2010) in red, MN (NFRDI) in blue, MP1 (Purcell, 2014) in light yellow, and MP2 (Purcell, 2014) in orange. Boxes and arrows in green means that all samples were subjected to the same action. Fonts in italics are some of the modifications applied step.

cooled for 5 min. For MB, samples were directly rubbed with and then soaked in finely chopped papaya leaves for an hour to soften the ossicles after the first cooking. Scrubbing off the ossicles was carried out after the second cooking. For the MN technique, ossicles were directly scrubbed off right after the first cooking using a commercially available brush with stiff plastic bristles. In MP1, samples were soaked in a container with seawater overnight after the first cooking. The ossicles were then scrubbed off the next day using a stiff brush. In MP2, crushed papaya leaves were added during the first cooking to soften the ossicles. The cooked sandfish were then allowed to cool before scrubbing the ossicles off. There are four ways of removing calcareous deposits under Purcell's technique, but only two variations were adopted in this study.

Salt-curing

Among the four techniques, only the two variations reported by Purcell use salt-curing. Two general salting methods are highlighted in Purcell’s (2014) manual, but in this study, dry salting was employed for MP1 and MP2. After the first cooking, sandfish were placed in a container with slits underneath. A plastic container was fitted with bamboo slats and coarse-grade salt was layered on the slats. After the first cooking, the sandfish were arranged on the layered salt with the cut side facing down. Salt was layered again between each sample and on top at a ratio of 1:1 (1 part salt to 1 part sandfish) by weight. Moisture from the cooked sandfish will drip through the bamboo slats to the container. In Purcell’s (2014) manual, salt-curing is done for 2 to 5 days, but in this study, salt-curing was done overnight (15 hours) since there were only six samples per procedure.

Drying

All techniques employed hybrid drying which is a combination of smoke curing and sun-drying depending on the suitability of prevailing weather conditions. However, MP1 and MP2 samples were also smoked for three hours after the second cooking and alternately smoke-cured and sun-dried after the last cooking process. Hybrid drying was done until the stone-dried product was produced (Figure 2).

Smoke curing.

After the last cooking process of each technique, samples were placed in a fireplace over a fabricated rack positioned about 2 feet over the burning wood coals. The 2-foot distance between the wood coals and the rack was maintained to avoid case hardening, which retards drying (Purcell 2014) and hasten the decay of the product. Smoke curing helps sea cucumber products to dry properly during the rainy season.

Sun drying

During fine weather, samples were laid outside on a clean rack and placed on an elevated platform. Samples were kept in storage after sundown.

Storage

Stone-dry sandfish were placed in labeled dry resealable bags separately by technique and stored at room temperature.

Product evaluation and pricing

Three local buyers/processors from three municipalities in Eastern Samar were requested to evaluate the product quality from the trials in terms of its sensory attributes such as size (length and weight), appearance, odor, color, texture, etc. using a descriptive sensory evaluation form adapted from *Beche-de-mer* grading features used in Hongkong (Bassig *et al*., 2021). Information on selling prices for live sandfish and buying prices for different grades of dried sandfish was also obtained from informal interviews with local buyers/processors. Limitations on the product quality evaluation include measurement of moisture content using a moisture analyzing device and structural microscopy of tissue samples.

Data and statistical analysis

Descriptive statistics including mean, standard deviation, and standard error of the mean, were presented in a table and graph. The results of the percentage recovery for the final weight were averaged ($n = 6$) per processing technique. The statistical difference among the four processing techniques was determined using One-Way ANOVA, and a Tukey HSD test was run as a post hoc test. Analyses of data were done using IBM® SPSS® Statistics version 21.

RESULTS AND DISCUSSION

Product yield

Despite allowing sandfish to relax to be able to expel water before weighing, a variable amount of water was retained in the live animal. After degutting, all live weights of the sandfish were reduced by 53-58%, or roughly 57% on average (Table 1). It was observed that the gutted weight of bigger sandfish was generally higher. Water makes up around 60% of the sea cucumber's body wall and a big percentage of this is lost during processing (Ram *et al*., 2016). However, the thickness of the body wall, which is mostly protein (Dong *et al*., 2011), also affects gutted weight. As shown in Table 1, samples from MB and MN showed high gutted weight recovery. However, MP1

Table 1. Sandfish weight recovery after degutting (n = 6).

Techniques	Live wt. ± SD (g)	Gutted wt. ± SD (g)	% wt. Recovery
MB	3585 ± 137.47	1655 ± 66.36	46.16
MN	3420 ± 190.39	1600 ± 54.01	46.78
MP1	2080 ± 36.29	976 ± 19.62	46.91
MP2	2435 ± 95.21	1014 ± 32.21	41.63

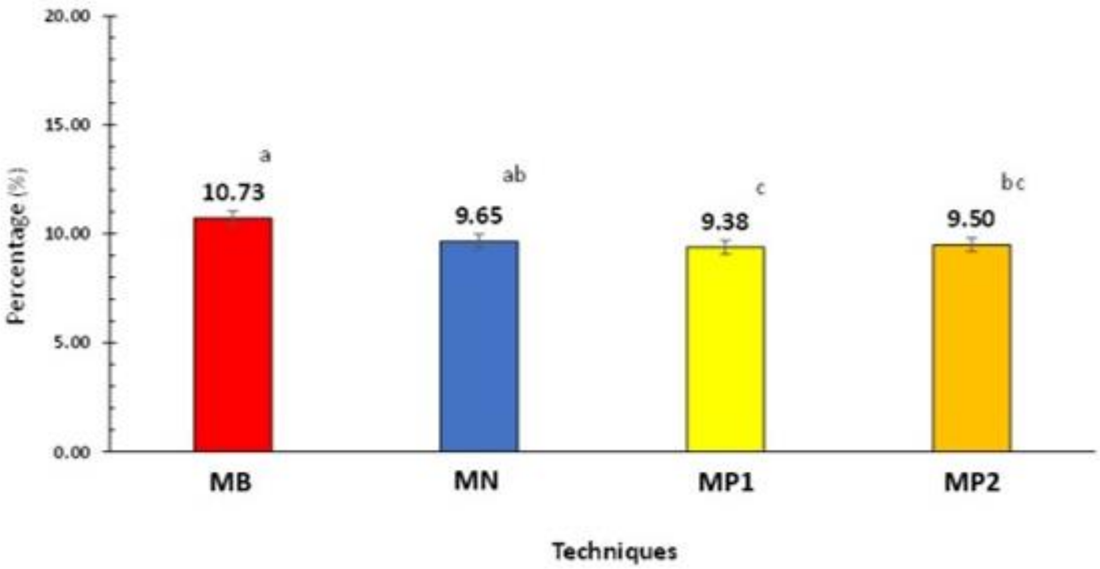

Figure 3. Percent recovery of sandfish (*n*=6) from its gutted-fresh weight after the application of four processing techniques: MB=Brown *et al.* 2010, MN= NFRDI, MP1= Purcell 1, and MP2= Purcell 2. Different letters labelled on the values show significant differences using the Tukey's honestly significant difference (HSD) post hoc test (subset for alpha = 0.05).

samples which are smaller than MP2 and much smaller than MB and MN, produced the highest % gutted weight recovery. It was also observed that sandfish with more pronounced grooves (usually attributed to age or nutrition), had thicker body wall and higher gutted weight recovery. This suggests that only sandfish >600 g with more pronounced grooves should be harvested and processed to maximize profits from the BDM trade.

The stone-dry product yield significantly differs (ANOVA, $p < 0.05$) for some techniques (Figure 3). Product yield from the MB technique had the highest weight percent recovery from gutted weight at 10.75%. Product recovery in the MB technique is significantly higher (Tukey's Test, $p < 0.05$) from MP1 and MP2 but did not significantly differ (Tukey's Test, $p > 0.05$) from the MN technique at 9.65%. The two methods by Purcell (MP1 and MP2) which yielded 9.38 and 9.50% recovery, respectively did not significantly differ from each other. Both MP1 and MP2 required salt-curing, a process that makes the sea cucumber product heavier (Purcell 2014). Ram *et al.* (2016) explained that salting causes salt-soluble proteins to leach and allow the salt to enter the tissues. The salt entering the tissues binds to the triple-helix collagen structure resulting in the increased weight of the final product (Gómez-Guillén *et al.*, 2011; Bao *et al*., 2010; Dong *et al.*, 2011, 2008). Even though it was noted that MP1 had the highest gutted percentage weight recovery among all sample sets despite having the smallest animals and having undergone salting which helps increased product weight, the MP1 product still had the lowest percentage yield among the four techniques at the end of the drying process. Therefore, harvesting larger sandfish >600 g is preferable to maximize income in the sea cucumber trade.

Product quality

The results of the sensory evaluation of BDM products from the local traders and processors in Eastern Samar are shown in Table 2 and backed with photographs in Figures 4 and 5. Among all the sensory attributes evaluated, size, appearance, and texture were found to have varying results.

BDM products were larger in MB and MN (70 to 80 mm and 26 to 30 g) compared to MP1 and MP2 (55 to 60 mm and 15 to 16 g) which could imply that larger sandfish have

Table 2. Descriptive evaluation of dried sandfish (*H. scabra*) from the buyers/processors in Eastern Samar.

Attribute	Product quality of sandfish subjected in four processing methods			
	MB	**MN**	**MP1**	**MP2**
Size				
Ave length (mm)	80	70-80	55	55-60
Ave weight (g)	29.6	25.7	15.26	16.04
Appearance	Straight, distinct groves, 2 out of 6 samples have slightly dented upper side, clean	Straight, distinct groves, slightly wrinkled, dented upper side, clean	Straight, 1 out of 6 samples is wrinkled, slight presence of chalky deposits	Slightly bent, mostly wrinkled, slight presence of chalky deposits
Odor	Slight smokey odor	Slight smokey odor	Slight smokey odor	Slight smokey odor
Color				
Upper side	Black	Black	Black	Black
Underside	Dark Brown	Dark Brown	Dark Brown	Dark Brown
Texture	Hard and almost dry	Hard and almost dry	Hard and Dry	Hard and Dry
Cut	1 small slit through anus	1 small slit through anus	1 small slit through anus	1 small slit through anus

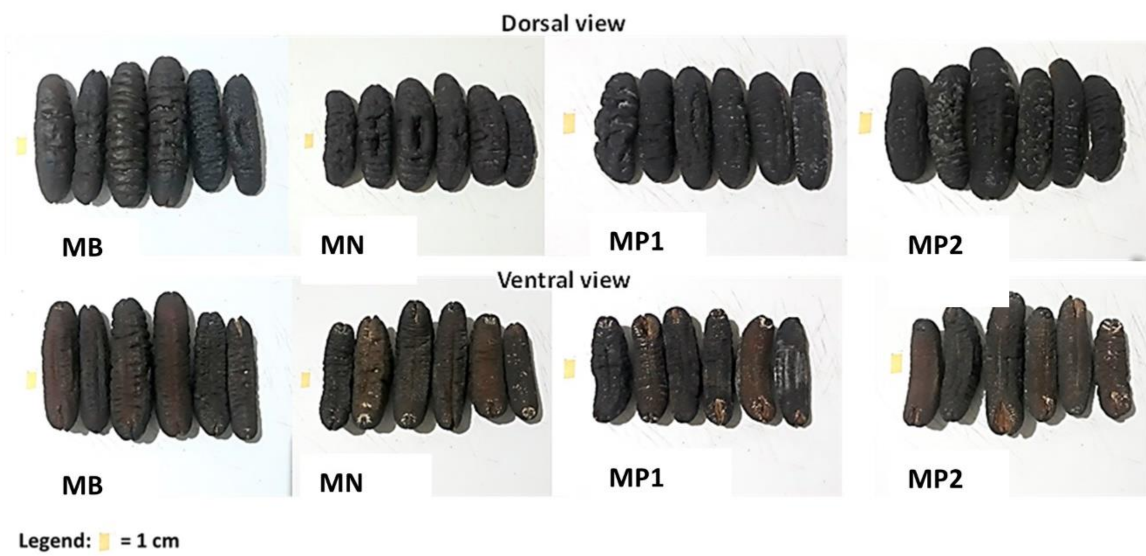

Figure 4. Stone-dried sandfish after the application of four processing techniques: MB = Brown *et al.* (2010), MN = NFRDI, MP1 = Purcell 1, and MP2 = Purcell 2.

thick body wall with lesser water content compared to smaller ones. In terms of appearance, most of the products were straight except for P2 which was slightly bent. Grooves were also found to be distinct in larger products (70-80 mm). Some of the product samples were also noted to have hollow dents (MN) as well as a wrinkled dorsal side most especially in MP2. However, during the cooking process, it was observed that the hollows or dents formed at the dorsal part of the sandfish may have been the result of not being submerged in the water during the first cooking and not due to the specific processing method used.

Smaller products most especially in MP1 and MP2 were also harder to clean as a slight presence of chalky deposits was noted. Among the four processing techniques, the MB technique required the least amount of time for the removal of ossicles or calcareous deposits. After the second cooking, it was observed that sandfish which were directly

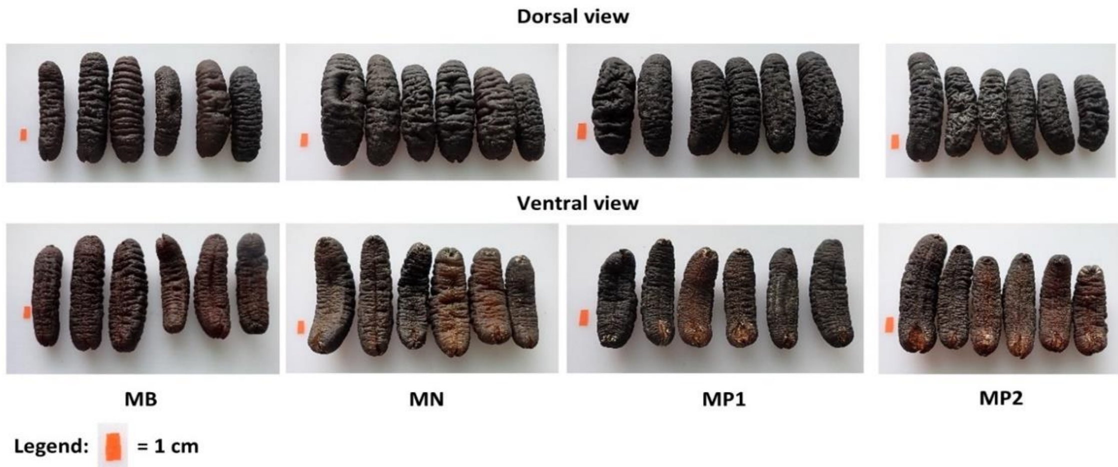

Figure 5. Stone-dried sandfish at 1.3 years after the application of four processing techniques: MB = Brown *et al*. (2010), MN = NFRDI, MP1 = Purcell 1, and MP2 = Purcell 2.

Table 3. Price of sandfish in Eastern Samar as of April 2021.

Gutted weight (per piece)	Price (fresh)	Price (dried)/kg.	
		(Php)	(USD)
<200-300 g	Php70.00/kg	1,200	24
500 g	Php100.00/pc.	2,000-2,500	40-50
600 g	Php200.00/pc	3,500-4,000	70-80

rubbed with and soaked in finely chopped papaya leaves had almost completely lost their ossicles. Only minimal brushing was needed to completely brush off the remaining ossicles. Papaya leaves have been used to remove ossicles not only in the Philippines (Gajelan-Samson *et al*., 2011; Brown *et al*., 2010), but also in Indonesia, Malaysia (Choo, 2012), and Madagascar (Lavitra *et al*., 2008; Rasolofonirina *et al*., 2004), among other places. Papaya leaves contain papain, a proteolytic enzyme that is commonly used to tenderize meat (Moy 2003; Quaglia and Gennaro 2003). However, Lavitra *et al*. (2008) also noted that papaya leaves must be used with care since the integumentary layer of the sandfish can be damaged by the papain in it.

Traders also noted that there was a slight smoke odor from all the products. It was expected since all products were subjected to hybrid drying which included smoke-drying. For color attribute, all products had uniformly black and dark brown colors on their dorsal and ventral side, respectively. Evaluators also noted that P1 and P2 products were completely dried compared to M1 and M2. It is most probable that larger products (70 to 80 mm) take more time to dry compared to smaller ones (55 to 60 mm). Both MP1 and MP2 were also subjected to salt-curing, other than making the product heavier, it was also found that salting increases the drying rate of BDM products (Purcell, 2014).

Studies also showed that salt-curing helps minimize both length and weight losses during processing (Lavitra *et al.*, 2008), protects the product from spoilage, and prolongs shelf life (Ram *et al*., 2016). Yaptenco *et al*. (2017) found that properly processed sandfish packed in polypropylene plastic and stored at 30 to 35°C may have a shelf life of 5 to 12 months. Interestingly, like those of MP1 and MP2 samples, all samples from the MB and MN techniques that did not undergo a salt-curing process have not shown visible signs of molds and decay even over a year of storage (Figure 5). This may imply that the natural salt in seawater used in cooking the sandfish is also sufficient to preserve the product for more than a year. In addition, hybrid drying may also be a factor in effectively reducing the moisture content of the product thereby prolonging its shelf life. According to Purcell (2014), smoke curing is a good preservation method when sea cucumbers are not salted.

Pricing

Live sandfish can be sold on a per individual or kilogram basis (Table 3). Based on the information obtained from the sandfish traders in Eastern, Samar, fishers/collectors receive the lowest net income by selling their sea cucumber fresh or processing it poorly (Tables 3 and 4). According to the local sea cucumber buyers and processors, dried sea cucumbers or BDM products are

Table 4. Dried sandfish prices, size, and grade at local traders in Eastern Samar, Philippines as of April 2021.

Code	Measurement in length (mm)	No. of pieces to reach 1 kilo	Price good quality		Price Class B/Reject	
			(Php)	(USD)	(Php)	(USD)
XL	≥85	40	4000-5000	80-100	1800	36
L	80	40-60	3500	70	1700	34
M	70	60-80	3000	60	1500	30
S	55	80-120	2500	50	1200	24
PSS	30	120-160	1800	36	600	12
PSSS	25	160-240	1300	26	300	6
PSSSS	20	240-280	800	16	300	6

priced based on the quality of the product, size length and weight. Deformed and damaged individuals are considered “rejects” and priced at 30 to 50% lower than specimens that are in better condition. The way by which the primary processing of sandfish is carried out is very important as it determines the resulting product quality and consequently the price that the product can command in the market (Perez and Brown, 2012). The market offers a high price for well-dried premium-size/large sandfish (Pardua *et al.*, 2018; Ram *et al*., 2016). Having tested and experienced the different processing methods, ranch co-managers can now carry out primary processing properly. Dried sandfish can be stored for a period until sufficient volume is accumulated before selling for a better price thus, adding value to their sandfish and earning a better income.

CONCLUSION

Properly processed, stone-dry premium-size sandfish fetch a very good price in the export market. However, sandfish gatherers and small sandfish sea ranch owners opt to sell their sandfish fresh at low prices because 1) they lack the knowledge on how to properly process sandfish to meet export market requirements, and 2) they feel that processing is too tedious and requires a lot of time and effort. The different methods tested during this study showed that sandfish sea ranch owners can earn more by processing premium-size sandfish. Although there is no single best-practice method for processing sandfish, MB and MN may be adopted, as the process is simpler and produces a higher yield. Since the product can be kept for over a year, sandfish can be processed by batch, stored, and sold when a good volume is reached to get the best price for the product.

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DISCLOSURE STATEMENT

All authors have read and approved the final manuscript and declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that all relevant data and supporting information are within the manuscript.

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HARVESTING AND HANDLING ON BOARD

Sea cucumbers are easy to harvest from reefs, lagoons and deeper coastal waters. They are harmless, move slowly and do not resist being collected. It is important, however, to catch and handle the animals carefully because the quality of the final dried product and therefore the market value depend on how well this is done.

There are a number of procedures for harvesting sea cucumbers. The one used will depend on their habitat and the equipment available to the collector. In shallow water, particularly at low tide, sea cucumbers can be collected easily on foot. In deeper waters down to 10 m, snorkeling or free-diving from a boat or canoe is necessary. Alternatively, if the water is clear, a shooting-lead or rope-spear (also called a ***dri-bomb*** in Fiji) can be used to spike and collect sea cucumbers from a boat. The shooting-lead is a 3–4 kg weight tied to a rope, with a 3 cm straight barbed spike firmly attached underneath. This is lowered into the water over a selected sea cucumber on the seabed and the weight allowed to drop the last few centimetres so that the spike penetrates the skin. The hooked animal should be lifted carefully into the boat and removed gently from the spike; try not to damage it further.

During harvesting it is important to take only the large sea cucumbers. The small ones should be left to grow and collected later when they have reached a good size. There are two reasons for this:

(1) Harvesting small sea cucumbers will eventually result in the loss of the resource; there will be none left;

(2) Small animals produce dried products that will not meet the required size grading and therefore will not fetch good prices.

There may also be regulations in force setting a limit for each of the commercially important species on the size of sea cucumber that can be harvested. It is important to know these regulations. The local Fisheries Department will have this information. These regulations are designed to protect the long-term health of the fishery.

It is important to keep the harvested sea cucumber alive and clean until ready for processing. Any pieces of coral and sand stuck to the skin must be removed by washing in seawater. These particles can become embedded in the soft body wall, damaging the skin. Away from their natural aquatic environment sea cucumbers become flaccid and soft, taking on the shape and fitting into whatever they are laid on or in, possibly marking their surface permanently. They should therefore be placed on a flat, smooth surface in a single layer. Shallow plastic fish boxes with smooth surfaces are ideal. If sea cucumbers are stacked on top of each other, the outer skin of the body wall is likely to break down. This will cause tears to appear after processing; the product will be down-graded because of these. Sandfish are an exception because they have a tougher body wall with many spicules. These animals can be placed on top of each other. They will flatten out, remaining alive. The prickly redfish requires special care as the large pointed teats are easily damaged if the animal is not handled properly.

Keep harvested sea cucumbers shaded and damp. This can be done by covering them with sacks or leaves kept wet with seawater (not fresh water, as this can damage the skin). If the harvest has not been good and the animals are to be kept overnight or for extended periods, they can be placed in a deep pool in the reef close to shore, or a small seawater pond can be constructed to hold them until enough animals have been collected to start processing. For enclosed ponds a regular change of seawater is necessary.

PROCESSING

EQUIPMENT

Only simple equipment is required:

1. A ***large container for boiling the beche-de-mer*** and a ***stirrer***. A shallow container is best since it allows more uniform heating and makes it easier to inspect and stir the sea cucumbers. A 200 litre (44 gallon) drum cut in half lengthwise and **thoroughly** cleaned is the most commonly used container. In Fiji it has become more popular to cut the drum in half crosswise. This apparently leads to more efficient heating and is less wasteful on fire-wood. Ideally a thicker-walled cast-iron container should be used. This would retain more heat and therefore help provide steadier, more continuous heat for more efficient boiling.

2. A ***wire mesh basket*** for easy inspection and removal of the beche-de-mer during boiling, or a ***mesh scoop***. There should be no sharp projecting ends of wire anywhere in the mesh since these would damage the delicate skin of the product.

3. A ***very sharp knife*** (and a ***sharpening stone*** to keep the knife sharp) for cutting, slitting and gutting.

4. A ***smoke-drying shed*** or ***copra dryer*** with ***drying trays*** of steel mesh on wooden frames. This consists of a lower chamber where the fire is maintained and an upper chamber where the cooked sea cucumbers are placed on mesh trays for smoke-drying. The dryer can be made from a wooden or galvanised steel frame and sheets of corrugated or flat iron sheets for the outer walls. A door in the front provides access for loading trays of product. The trays are either wood or steel-framed and the mesh is most commonly made from chicken wire. Other types of steel mesh are available, which are better than chicken wire since this can damage the skin of the product, especially when the wire is broken.

5. ***Drying racks*** or ***iron sheets*** for sun-curing the smoked beche-de-mer.

6. ***Miscellaneous equipment and materials:***

   - dry clean sacks made of hessian or woven polypropylene,
   - firewood and coconut husks for fuel,
   - buckets for carrying seawater,
   - string or vines and small sticks (2.5 to 4 cm long).

PROCEDURE

This is simple but must be carried out with care if good-quality products are to be obtained. The following technique is used for teatfish but can generally be applied to all species (Fig. 1), except sandfish which requires a special treatment (see page 31). Depending on the species of sea cucumber, minor changes in procedure may be needed to suit requirements of certain markets.

Boiling is the most important step in processing. Incorrect cooking can irreversibly damage the product by causing splitting of the skin or other faults that will only show up at the drying stage.

First boil

Fill the container with clean seawater and bring it to the boil. It is very important to bring the water to the boil **before** adding the live sea cucumbers to the container. Sort the sea cucumbers according to size. Only boil those of a similar size together, as the cooking time needed varies according to size. If the sea cucumbers have been kept in seawater for some time, allow them to naturally evacuate any internal water. Gently squeezing the body will assist this process. Immediately put the animals into the boiling water one by one while stirring, making sure that each one is completely covered with water. Do not allow any to remain partly out of the water as the skin could split. To ensure uniform heating do not put too many animals into the water at once.

Stir continuously and examine frequently. Cooking time for the first boil depends on the size of the animals and may be as short as a few minutes. The best way to judge the cooking time is by inspection. If the sea cucumbers start to swell too much you will need to pierce them. This can happen quickly, so you must keep a close watch. If they are left to boil too long at this stage they will burst.

Piercing the body wall during the first boil (optional) — This procedure releases the build-up of pressure caused by too much water and air inside the cooking animal. Make a short longitudinal slit about 4 cm long, along the centre line of the back, to let the water out. For ***Thelenota ananas*** (prickly redfish) the slit must be made on the underside, with the animal laid flat on its back. For species that should not have a slit along the length of the body a small cut in the posterior end through the anus will suffice.

After piercing continue to boil for about another 10 to 15 minutes. Continue to stir gently and frequently, to ensure uniform cooking. When the ends of the sea cucumber become rubbery like a rubber ball, the first boiling is complete. This will not be hard to recognise. Remove the cooked sea cucumbers from the container and immerse them in fresh seawater to cool down.

Slitting and removal of gut

Make a straight slit down the centre line of the animal (along the back for teatfish, and the underside for prickly redfish), to within 3 cm of each end, incorporating the cut made during the first boiling. Open up the beche-de-mer and empty out the loose gut contents. Cut out the organs that run through the centre, removing the anal teeth and any loose tissue, using the hands only. Do not remove the strips of tissue running down the length of the inner walls of the body cavity (the longitudinal muscle bands). Wash out with clean seawater.

Second boil

Follow the same procedure as for the first boil. Cook for 15–40 minutes, stirring continuously. Exact boiling time will again depend upon the size of the animal. The sea cucumbers will shrink slightly and gradually become hard. This hardness is the best way to gauge cooking time, so inspect them frequently. Check the cut edge of the animal too; if it no longer feels slimy, but rather dry and rubbery, the second boiling is complete. Remove the sea cucumbers quickly from the container and put them into cold seawater to cool. They are now ready for smoke-drying or sun-drying.

Smoke-drying

The fire in the smoker can be made with coconut husks or firewood. The fire should be small, producing constant heat. If the fire is too hot, it will overcook the sea cucumbers, reducing their value. The fire should therefore be tended regularly.

To prepare sea cucumbers with long body slits for smoking (teatfish, prickly redfish, etc.), open them up one at a time and place a short stick, not more than 2.5 to 4 cm long, across the cut to keep the sides apart. This helps speed up the drying process. Place the cooked sea cucumbers on the smoking tray with their slit side down so that the inner part of the body is exposed to the heat of the fire. Do not turn them over during smoking — always leave the split side facing down. Place the cooked sea cucumbers (including the uncut ones) on the trays in such a way that they do not touch each other and that similar sizes are put together on the same tray. Put the trays of smaller ones at the top and the larger ones at the bottom closer to the fire.

Periodically rotate the trays around in the dryer. Move the tray on the bottom rung to the top rung and move all other trays down a rung. This ensures uniform drying.

The sticks holding open the long slits should be removed about half-way through the drying process, when the insides of the animal feel dry to the touch. The sea cucumbers can now be tied up with string or vines to help close up the slit edges, especially if they are misshapen.

Smoke-drying is usually completed within 24–48 hours. The exact drying time will depend upon many factors, such as the drying temperature, the size of the animals and the weather. Judge the dryness of the product by feeling the inside surface. Take particular care to check inside the ends, as they will be the last areas to dry completely.

Sun-curing

Brush off any soot, ash or dirt that has accumulated during the smoking. Place the product in the sun on a clean, dry surface. It is preferable to use drying racks for sun-curing because the product can then dry on both sides simultaneously. These are simply wood-framed tables with a fixed mesh top (chicken wire, steel mesh, plastic mesh or fishing nets). Alternatively, mesh trays, similar to the ones used in the smoker, can be placed directly onto the table frame.

Curing can take as few as four or five days, or as long as one to two weeks, depending on the size and species of sea cucumber, and, of course, the weather. It is important to avoid exposing the product to rain at any time — contact with fresh water is not good for it. Use plastic sheets to cover the curing product if rain threatens, or, if using trays, move them indoors. When sun-curing is complete, a powdery substance will appear on the surface of the beche-de-mer.

Remove the string from the tied beche-de-mer and brush off any dirt or sand.The product should now be ready for packing and storing. If, after examining the product, you find it somewhat soft and damp, you will have to repeat the smoking and sun-curing process. A properly dried and cured beche-de-mer is easy to recognise with a little experience.

Note: *Smoke-drying may be replaced by sun-drying. This depends on the target market for which the beche-de-mer is being prepared. Some buyers prefer beche-de-mer without the smoky odour and flavour. The buyer of the product is the person to provide guidance on this.*

Special method for processing sandfish

A different procedure must be used for sandfish (***Holothuria scabra*** and ***H. scabra*** var. ***versicolor***) in order to remove the chalky spicules in the body wall (Fig. 2).

Gutting This species usually loses its guts after harvesting. If this has not happened (especially for the ***versicolor*** variety), make a short slit, 2–3 cm long, in the anus (posterior end). Press the water and guts out by firmly squeezing the body wall.

First boil This step is the same as for the other species. Boiling time can range from a few minutes to one hour. When the sandfish are cooked, take them out to cool.

Burying The traditonal method of removing the chalky spicules in the body wall is to bury the boiled and cooled sandfish in a 20–30 cm deep sand-pit. A clean, uncontaminated part of the beach above the tidal line is ideal. Pack the sandfish into the pit, cover them with a hessian/jute sack soaked in seawater and finally cover them with sand.The outer layer of the body wall will decompose by bacterial action. This will take about 12 to 18 hours. Remove the sandfish, then wash them in sea-water and rub them vigorously to remove the outer decomposed layer containing the hard chalky spicules. If the decomposition process has not removed all the hard spicules, repeat the boiling and burying processes.

Second boil The sandfish are boiled again in seawater for about 40–45 minutes, with continuous stirring.

Smoke-drying Most markets prefer dried sandfish to be **unsmoked**. If the buyer requests a smoked product, follow the procedure described on pages 29–30.

Sun-curing The sandfish should be arranged in a single layer, preferably on drying racks that allow the air to circulate freely above and below them. Follow the procedure described on page 30.

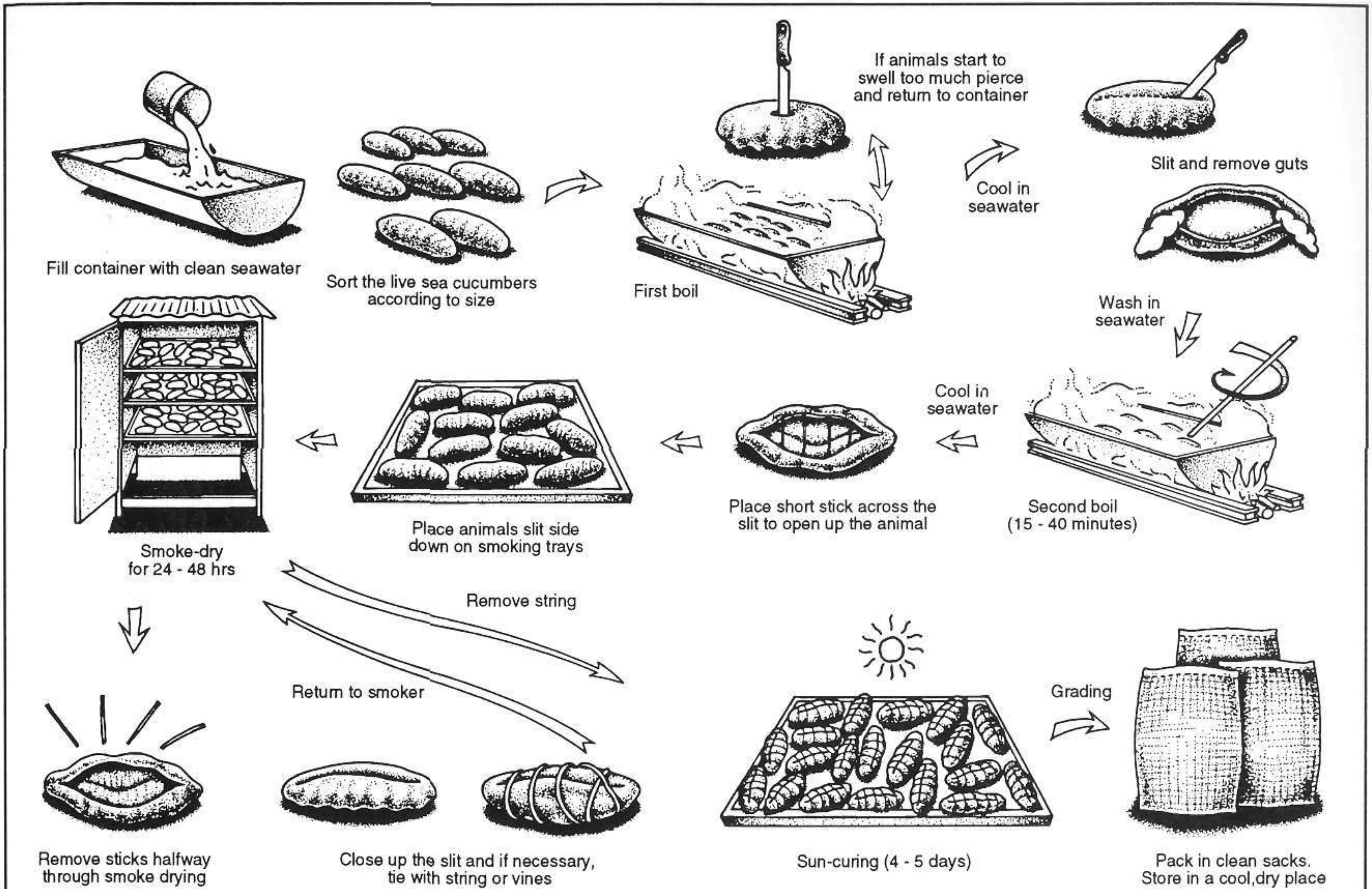


Figure 1. Processing sea cucumbers

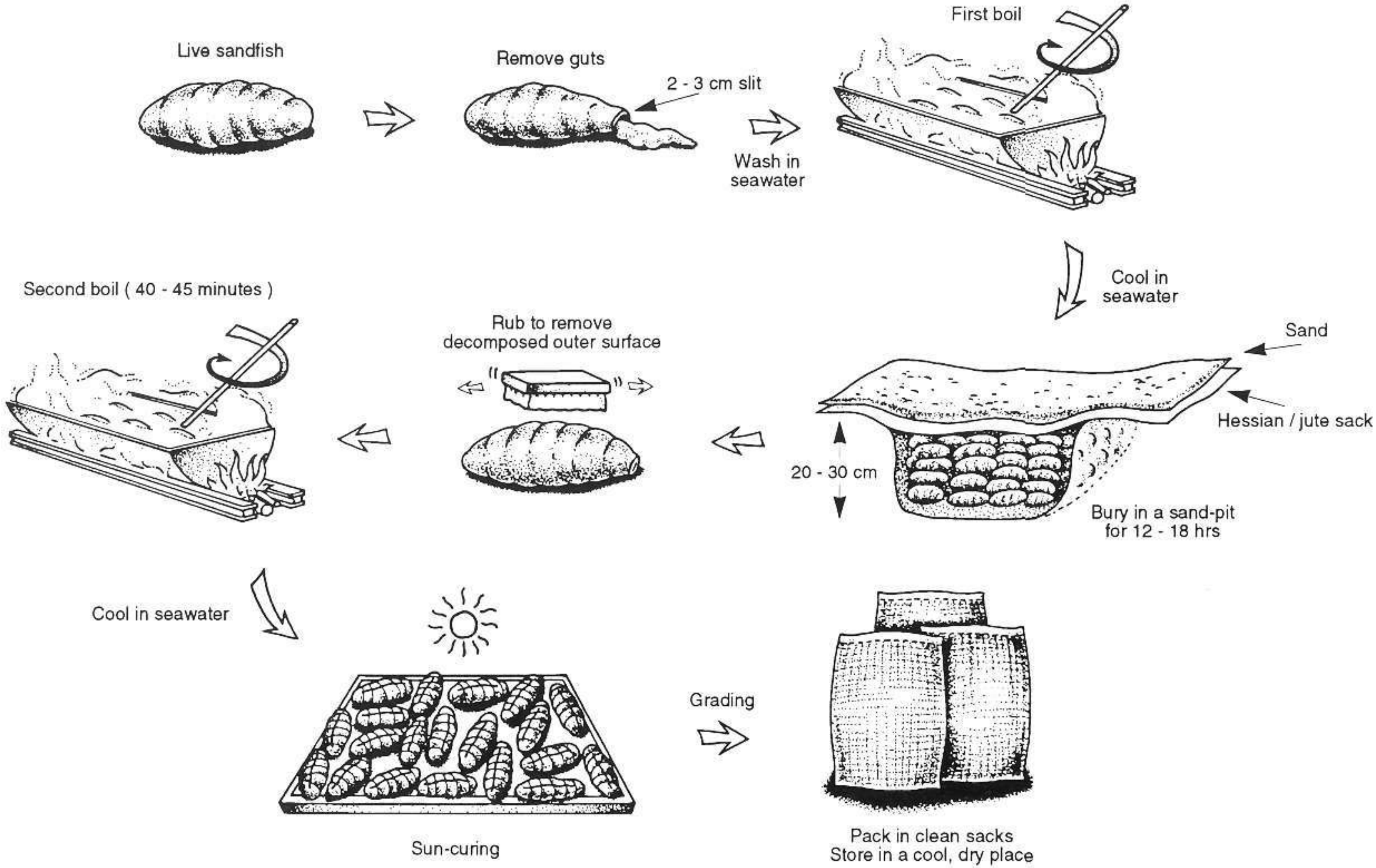


Figure 2. Processing sandfish

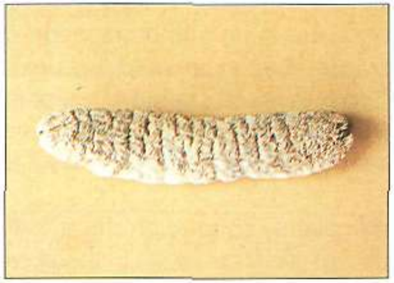
CHANGES IN LENGTH AND WEIGHT DURING PROCESSING

Processing causes a considerable decrease in both length and weight. Shrinking starts during the boiling processes and continues during smoke-drying and sun-curing. Weight loss is high because of gutting and dehydration, both essential for a properly preserved beche-de-mer product. Decreases in length and weight vary with the species, the size of the individual sea cucumbers, the shape of the species and the thickness of the body wall. Average values, based on various trials, are given below.

SPECIES		LENGTH (CM)			WEIGHT (GM)		
Common name	Latin name	Initial state	Dry product	%	Initial state	Dry product	%
White teatfish	*Holothuria fuscogilva*	52	23	44	4200	320	8
Black teatfish	*Holothuria nobilis*	37	19	51	1800	150	8
Sandfish	*Holothuria scabra*				370	20	5
Sandfish	*H. scabra* var *versicolor*	37	14	38	1600	100	6
Deep-water redfish	*Actinopyga echinites*	19	9	47	330	37	11
Deep-water redfish	*Actinopyga echinites*				470	15	3
Blackfish	*Actinopyga miliaris*				2500	76	3
Prickly redfish	*Thelenota ananas*	58	22	38	4000	190	5

GRADING

To produce the best possible product, carry out every processing step very carefully. You will then have beche-de-mer that are all top quality and will fetch the highest price. Common defects that will cause the product to be downgraded are insufficient drying, small sizes, mixed sizes, twisted shape, smoke-dried when sun-cured is preferred (e.g. sandfish), bad flavour, incorrect incisions, damaged body wall, and inadequate cleaning (see photos). If the drying time was too short or moisture was absorbed later there is a danger that the beche-de-mer will become mouldy. When this happens, clean the surface of mould and repeat the smoking and drying processes.



Outer spiculy layer not removed (sandfish)

Misshapen (surf redfish)

Shrunk too much due to overcooking (black teatfish)

Badly cut, cut has not closed properly (white teatfish)

Examples of poor-quality beche-de-mer

The following are the main factors that determine the commercial grade of beche-de-mer products:

Species

Separation into species is the first step in grading. The three grades given here are based on prices paid for quality product and consumer preference:

High grade	Sandfish, white teatfish, black teatfish
Medium grade	Prickly redfish
Low grade	Deep-water redfish, stonefish, surf redfish, blackfish, brown sandfish, lolly fish, pinkfish, elephant's trunk fish, greenfish, curryfish, amberfish

Size

As a rule, the larger the size the better the grade. Unfortunately there is no standardisation of size grades. They vary with different markets and even with different retailers. They are often expressed as very large (XL), large (L), medium (M), small (S), very small (XS). Each size class is defined by a length range, a weight range or (now more common), the number of indvidual beche-de-mer per kilogram. Small sizes attract very low prices. It is best not to harvest sea cucumbers that yield small product. Leave these to grow larger for collecting later.

Appearance

Regular shapes are preferred to distorted, twisted, shrunken or unevenly shaped products. All cuts must be clean, straight and in the right place and on the correct side of the body. For example, a slit in the upper part of the prickly red fish or a long slit appearing in a species which requires no cutting will result in a lower grade.

Moisture content

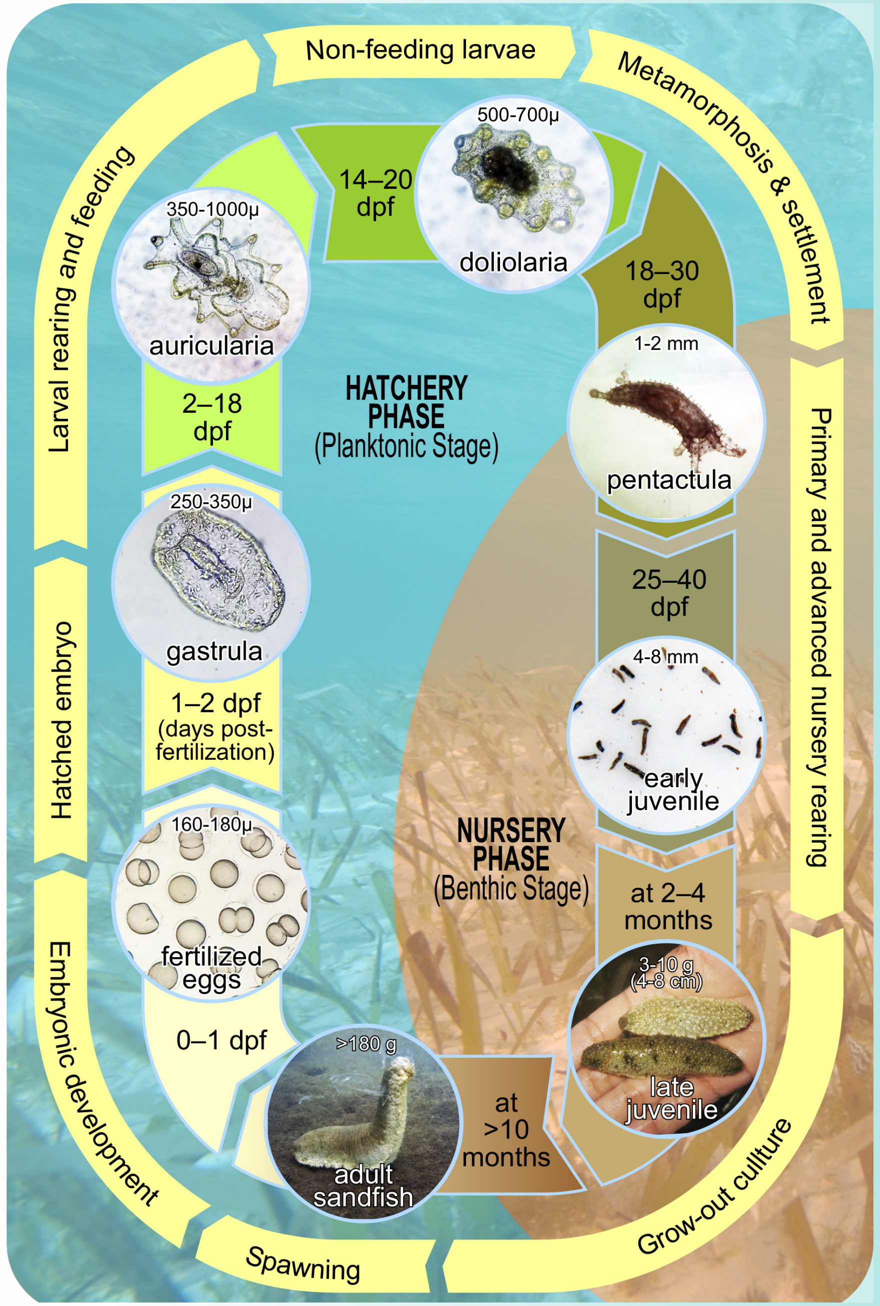
The product must be hard and **completely dry**. The most common quality problem encountered in the South Pacific is inadequate drying, which can make the product become misshapen and mouldy. If the damage is not too severe it can be re-dried, otherwise it will have to be thrown away. Beche-de-mer stored in a humid atmosphere can also absorb moisture and become damp and mouldy. If this happens they should be re-dried and the storage method improved.

Grading characteristics of some of the most common beche-de-mer species

SPECIES	APPEARANCE/SMELL	CUTS	COLOUR	SIZE
Sandfish	Straight or slightly bent. Large numbers of grooves around the body. No smoky smell.	Small slit only in the posterior end through anus.	Upperside – brown-black to black; underside – greyish brown.	Large = 8–12 pieces/kg, 10–15 cm in length
White teatfish	Flat and straight with teats clearly visible. Powdery surface a desirable attribute. Distinct smoky smell with no off-odours.	One single, long straight cut in the upper part of the body. The cut must be fully and evenly closed.	Different shades of grey-brown.	Large = 3–6 pieces/kg, 18–24 cm in length
Black teatfish	Flat and straight with teats clearly in view. Powdery surface a desirable attribute. Distinct smoky smell with no off-odours.	One single long straight cut in the upper part of the body. The cut must be fully and evenly closed.	Powdery cover is greyish brown, but the skin surface underneath is black.	Large = 4–6 pieces/kg, 18–24 cm in length
Stonefish	Flat, roughly oval shape. Shallow grooves around the body. Pleasant smoky smell.	None	Brown-black	Size = 6–12cm
Surf redfish	Flat, long oval shape. Upper surface has a rough appearance, while the lower surface looks smoother with a distinct downward pointing mouth at one end. Pleasant smoky smell.	None	Brown-black	Common size = 7.5–15 cm
Blackfish	Chunky, roughly oval-shaped body with pointed ends with white anal teeth visible at open end. Upper surface has a slightly rougher appearance than the lower surface. Distinct mouth on lower surface. Pleasant smoky smell.	None	Black	Common size = 8–12 cm
Amberfish	Long and straight, covered in small warty growths on top and sides. Mouth is on the underside. Pleasant smoky smell.	Small slit in anus	Brown-black	Size around 12 cm

Adapted from *Beche-de-mer grading features as used in Hong Kong* by David C. Cook and Julie Palaso Cook, Hong Kong Pacific.

Production Phases and Life Cycle of **SANDFISH *Holothuria scabra***



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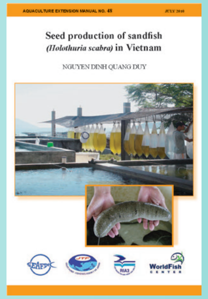

AEM 48 Seed production of sandfish (*Holothuria scabra*) in Vietnam *Nguyen Dinh Quang Duy* (2010) An extension manual describing broodstock management, larval rearing, and management of nursery systems.
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Why culture **SEA CUCUMBER**?

Sea cucumbers are highly valued marine commodities, with prices reaching up to US$ 2,000 per kilo, when processed and dried into *trepang* or *beche-de-mer*. A great majority of traded sea cucumbers comes from wild harvest causing severe decline in stocks. Sea cucumber mariculture using hatchery-bred juveniles can offer an alternative income source especially for coastal communities, while protecting the remaining wild populations.

Expensive dried sea cucumbers for sale in Chinese markets

What is **SANDFISH**?

Sandfish is the common English name for one particular tropical species of sea cucumber called *Holothuria scabra*. It is one of the most threatened tropical species because of its high price and ease in collection. It is typically found in shallow intertidal sandy-muddy shores, commonly associated with seagrass beds and sand flats. Sandfish has one of the highest potential for aquaculture because hatchery production technology of this species is established. SEAFDEC/AQD is one of the leading institutions developing the production technologies for sandfish.

Sandfish *Holothuria scabra* in their natural habitat

The SEAFDEC/AQD Sea Cucumber Hatchery

The SEAFDEC/AQD maintains a small-scale sea cucumber hatchery facility for sandfish spawning, larval rearing and juvenile culture. Continuous life support systems like flow-through seawater and aeration are maintained. Natural microalgal food such as *Chaetoceros* sp. and *Navicula* sp. are produced within the facility as well. Various experimental research are being conducted here in order to enhance sandfish production.

SEAFDEC/AQD sea cucumber hatchery, established in 2010

How to breed **SANDFISH**?

Broodstock conditioning

Broodstock collected from the wild are conditioned in tanks with sandy-mud substrate and flow-through seawater. They are fed with a mixture of powdered *Sargassum*, formulated feed and *Navicula* sp. slurry. After spawning, they are returned to the field where they were collected for natural recovery.

Cleaning and preparation of sandfish broodstock for spawning induction

Spawning induction

A pre-defecated spawning group of 20-60 sexually mature sandfish are induced to spawn using non-lethal thermal and food stimulations. Males are expected to spawn first by releasing a steady stream of white milt with sperm through the gonopore – a small genital opening above the anterior or front end of the body. This may last up to 3 hours. Females spawn by releasing quick bursts of eggs after a characteristic bulging around the gonopore. Females typically perform 2-3 bursts at about 5 minutes interval. Although at SEAFDEC/AQD, we have recorded as much as 22 bursts from a single female within 1 hour.

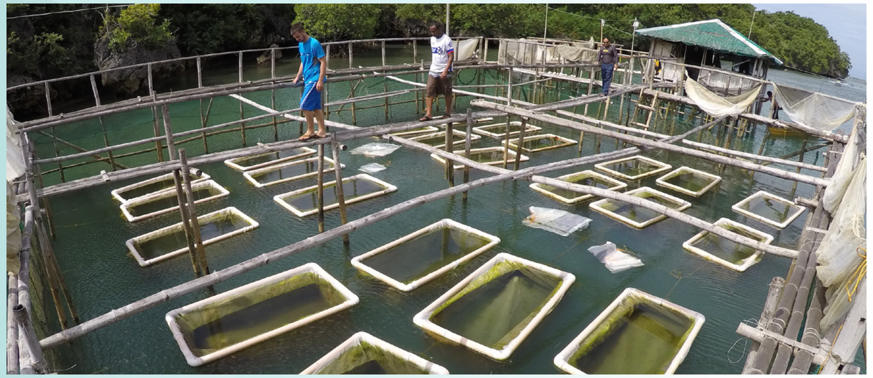
Larval Rearing

Fertilized eggs are stocked in tanks filled with filtered and UV-treated seawater at 100-500 eggs/L at optimum temperature (26-30°C) and salinity (28-33 ppt). Auricularia stage larvae are fed daily with *Chaetoceros calcitrans*. Water exchange (20-50%) is done every two days while siphoning out wastes from the tank bottom. At Doliolaria stage, corrugated plastic sheets painted with *Spirulina* paste are added into the rearing tank to induce settlement. Metamorphosed Pentactula are fed with *Navicula* sp. slurry.

Larval development monitoring (left) and preparation of settlement plates (right)"

Nursery Rearing

Post-metamorphic or early juvenile sandfish (4-10 mm), at 30-45 days old, are transferred from larval tanks in the hatchery into floating hapa nets (1 m x 2 m x 1.2 m) in sea-based or tank-based nurseries. Nursery hapas are made of fine-meshed (>1 mm) net suspended with a floating PVC frame. In good sites, sandfish juveniles grow to 2-4 g within 1-2 months, depending on season and sea conditions. At this size, they are ready for advanced nursery rearing in pens or ponds.



Sandfish nursery pen in a protected cove at SEAFDEC/AQD's Igang Marine Station in Nueva Valencia, Guimaras, Philippines

SANDFISH

ATTRIBUTES

- Sandfish is an Echinoderm.
- There are more than 1000 species of sea cucumber known to exist.
- The sea cucumber *Holothuria scabra* has an elongated body with leathery skin whose color ranges from gray to black.
- They live in sandy-muddy substrate and feeds on organic matter which is mainly composed of mud or sand, bacteria and benthic algae.
- Males and females are not distinguishable unless they have spawned.
- At least 100 species are found in the Philippines, 25-30 species are commercially traded.
- They can be found throughout the shallow tropical waters of the Indo-Pacific region.

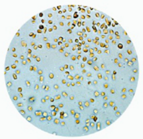
WHY VENTURE INTO SEA CUCUMBER FARMING?

- Stock enhancement is one major reason of farming.
- Bech-de-mer , trepang or dried sea cucumbers are being exported to Asian markets due to its pharmaceutical, nutraceutical and culinary uses.
- It is being sold at price ranging from 1000 to 6000 pesos per kilogram.

THE HATCHERY

1 NATURAL FOOD | Phytoplankton Culture

Phytoplankton species commonly cultured:
Chaetoceros gracilis
Chaetoceros calcitrans
Isochrysis galbana
Navicula ramosissima



Mass culture of Microalgae

- Prepare 180 Liters UV filtered sea water in clean containers.
- Fertilize with: 200ml manusol, 200 ml Algafer and 200 ml sodium silicate.
- Pour 20L of inoculants into the fertilized sea water.
- Harvest cultured microalgae during its peak exponential growth when cells are of good quality which is 3 days after culturing.

2 BROODSTOCKS | Collection and Selection

Sea cucumber broodstock may be bought from local dealers or collected from the wild. It should weigh **200 to 500 grams.** Broodstock should be **healthy, undamaged and with no visible skin lesion.**

Management and Maintenance

- Sea cucumbers can be held in tanks, sea pens or ponds.
- The stocking density should be <500g /m².
- Blended shrimp starter feed is used at 1-1.5g/m3.
- Broodstock held in pens, ponds or tanks for several months are easier to spawn than the ones taken directly from the wild.

3 SPAWNING | Induction Method

Spawning is usually done 3 to 2 days before full moon or new moon of the month. The hatchery uses the combined effect of dry treatment (dessication) and food shock technique using *Spirulina*.

Dry Treatment

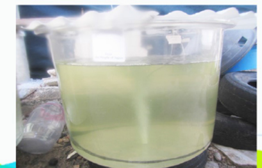
- Broodstock are placed in a tank without water and left to dry for 45 minutes.

Spirulina Bath

- After dry treatment, the tank (or basin) is filled with sea water and broodstocks are immersed.
- Blended *Spirulina* powder (15 grams) is then added.
- Allow 45 minutes of bathing.

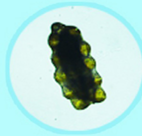
Spawning & Egg Collection

- Broodstocks are allowed to spawn in tanks.
- Spawning usually takes an hour or more.
- Sperms appear whitish while eggs appear in orange.
- Collect eggs separately in a large container and sperms are added to fertilize them.
- Study eggs under a microscope to determine if fertilization has already occurred.



4 LARVAL REARING

Sandfish transforms into different stages as it develops. Below is a table showing the different stages and the appropriate management techniques to be applied during a specific larval stage.
Larval tanks with sand-filtered seawater and aeration system, and natural food are basic requirements in larval rearing.

No. of Days	*Stage*	*Feeding*	*Water Management*
0	 *Fertilized egg*	Non-feeding stage	No water exchange.
2-5	 *Early Auricularia*	After 2 days, larvae are fed with microalgae (Cgr-Cc) with concentration of 20,000 cells/ml.	50% water exchange is done every 2 days.
7-13	 *Late Auricularia*	Microalgal feed (Cgr-Cc) concentration is increased to 40,000 cells/ml	50% water exchange is done every 2 days.
13-18	 *Doliolaria*	Doliolaria larvae are non-feeding stage. However,feeding is maintained since larvae do not metamorphose evenly.	50% water exchange is done every 2 days. Tanks are provided with corrugated plastic plates smeared with *Spirulina*.
18-25	 *Pentactula*	Pentactula larvae are fed with Cgr-Cc combined feed.	50% water exchange is done every 2 days.
25-30	 *Juvenile*	Juvenile sandfish are fed with extracts of seaweeds such as *Sargassum* sp., *Gracilaria* sp. or *Laurencia* sp.	Juveniles are tansfered intanks with flow-through system where water is actively replenished.

The techniques and methods used at GMFDC Sea Cucumber hatchery are based on the protocols of University of the Philippines Marine Science Institute.

5 JUVENILE REARING

When juveniles reach 1-2mm (23 - 35 days after fertilization) they are transferred from larval tanks to floating hapa nets. Growth and survival of juveniles are very density-dependent and thus grazing area is an important consideration in nursery rearing.

Primary Nursery

- Juveniles are carefully harvested and detached from the larval tanks by siphoning them or individual picking.
- Stocking density: 1000 first stage juveniles per hapa net.
- Juveniles are regularly monitored.

Secondary Nursery

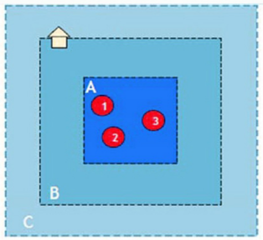
- After a month in primary nursery, the juveniles are thinned-out and transferred in the secondary nursery hapa/ floating hapa.
- Stocking density: 500 second stage juveniles per hapa net.
- Floating hapas are regularly monitored and cleaned.

- From ocean nurseries/ floating hapa, juveniles (20 mm or >1g) are harvested after two months and transferred to sand-conditioned tanks in the hatchery.
- Muddy-sandy substrate collected from sites that supported wild sandfish population is used in sand conditioning.

6 GROW-OUT CULTURE

After sand-conditioning, juveniles are transferred for grow-out culture in a sea ranch. Sea cucumbers are allowed to grow for several months until they reach at least 300 g for harvest.

The ranch has the following zones and dimensions as suggested by the UP MSI:



- Zone A is 50 x 50 m with 3 100 sq. m. circular monitoring pens.
- Zone B is 1-hectare nursery area. This is for sea cucumber monitoring only.
- Zone C is a 5-hectare buffer area. No-take area for sea cucumbers.

This information is made for you by the

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AND DEVELOPMENT CENTER

SEA CUCUMBER CULTURE

Hatchery Techniques and Protocols of the Seed Production of the Sea Cucumber *Holothuria scabra* at GMFDC, Guiuan Eastern Samar.

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Growth, Survival, and Behavior of Early Juvenile Sandfish *Holothuria scabra* (Jaeger, 1883) in Response to Feed Types and Salinity Levels under Laboratory Conditions

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Aquaculture of the tropical sea cucumber *Holothuria scabra* or sandfish is still at a developing stage, especially in the Philippines. In Mindanao, early juveniles of sandfish were successfully produced at the Mindanao State University at Naawan (MSUN) sandfish hatchery for 5 yr now. However, on-site growing of these early juveniles in ocean nurseries often suffered very low survival rates. Two separate laboratory experiments were conducted for 60 d to test for the effects of feed types (*Navicula* sp., powdered *Sargassum*, and *Sargassum* extract) and salinity levels [ambient seawater at 32–35 (as control), 20, 25, and 40 ppt] on the growth, survival, and behavior of 7-wk-old early juvenile sandfish (2–15 mm in length). Juveniles fed with *Sargassum* extract significantly produced the highest increase in width and length, followed by powdered *Sargassum* and *Navicula* sp. with a survival rate of at least 71%. The highest growth rate (GR) and survival were observed with ambient salinity, followed by 40, 25, and 20 ppt. Unusual pale coloration, sluggish movement, and destroyed integument in some parts of the body were observed in some juveniles exposed to lower salinity indication of an unhealthy individual. Overall, *Sargassum* extract is an ideal feed for sea cucumber juveniles. Low salinity is stressful to early juvenile sandfish and that growth and behavior were adversely affected.

Keywords: biological factor, environmental factor, hatchery-reared juveniles, laboratory experiments, sandfish

INTRODUCTION

Sea cucumbers are essential keystone species, bioturbators, and recyclers in many coastal ecosystems and provide vital and often overlooked ecological services in the habitats they occupy (Yuval *et al.* 2014). They are a significant component of the marine ecosystem as they help in the local buffering of ocean acidification and can be hosts for different parasites and commensals (Purcell *et al.* 2016).

In an economic sense, sea cucumbers provide substantial resources of livelihood in coastal communities because they are harvested locally as a source of food and traditional medicine (Subaldo 2011) or as export products

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(Choo 2008; González-Wangüemert 2014). Accordingly, the sea cucumber fisheries have been generating income for over 1,000 years along the Indo-Pacific region (Omar *et al.* 2013), and have now been expanding worldwide driven by the Chinese demand for dried sea cucumbers or *beche-de-mer* (Kinch *et al.* 2008). There were approximately 24–35 species that are commercially exploited (Jun 2002; Ram *et al.* 2010). Sea cucumbers with rigid body walls such as *Holothuria scabra* or sandfish were selected as high valued species and preferred by exporters (Purcell *et al.* 2012). The increasing demand for this organism leads to a depleted state of its population (Eriksson and Bryne 2013). As of now, *H. scabra* is listed as endangered species based on IUCN red list together with other high-valued sea cucumber species such as *Apostichopus japonicus*, *Holothuria lessoni*, and *Holothuria nobilis* (Conand *et al.* 2014).

To provide the demand of the Asian market, sandfish production is highly recommended, and restocking of juveniles is one solution for the depleted wild fishery (Giraspy and Ivy 2009; Giraspy and Walsalam 2010; Ghobadyan *et al.* 2012). Pond grow-out of juvenile sandfish to market size is being developed in Vietnam (Pitt and Duy 2004; Bell *et al.* 2007), and several programs and projects were made to enhance sandfish production, sea ranching, and stock enhancement – especially in the Philippines (Hair *et al.* 2011).

Several problems encountered in sea cucumber farming are often the result of drastic changes in environmental conditions and increases in parasites and predators (Lavitra *et al.* 2009). Stocking densities and periphyton abundance are also other factors for the fast growth and high survival of sandfish juveniles in floating hapas (Gorospe *et al.* 2019; Sinsona and Juinio-Meñez 2019; Altamirano and Noran-Baylon 2020). However, in Naawan, appropriate stocking densities are strictly observed yet high mortality is still experienced. The decline of the periphyton abundance might be one reason for the high mortality of sandfish juveniles during the nursery stage; thus, we are looking into the possibility of subjecting juveniles to different feed types to maximize their growth and survival. And if feeding is to be introduced, we must find alternatives to the usual feed used in the hatchery, which is the live microalgae, to minimize the cost as well as its negative impact on the environment. We looked between powdered *Sargassum* and *Sargassum* extract, as *Sargassum* species are still readily available in the coastal area of Naawan, Misamis Oriental. Depending on the formulated diet and feeding rate, these could affect the growth and survival of sea cucumbers such as in *Holothuria atra* and *H. scabra* (Seeruttun *et al.* 2008; Pattinasarany *et al.* 2014). Studies were conducted to formulate the effective diets of *H. scabra* larvae and early juveniles (Agudo 2006; Duy 2010). One of the most effective diets for *H. scabra* larvae and early juveniles is the *Chaetoceros calcitrans* (Abidin *et al.* 2019). However, during the preliminary experiment conducted last June–July 2016, it was found out that the *C. calcitrans* produced the lowest growth and survival among powdered *Sargassum* and *Sargassum* extract and the cost of production and maintenance for this microalgae is expensive, which is a bottleneck for small scale hatcheries. That is why introducing new diets that are cost-efficient yet could enhance growth performance and produce high survival is necessary for culturing this organism. One alternative for live microalgae that is easily produced in the hatchery is the *Navicula* sp. Another accessible feed is the *Sargassum* found to be a good supplemented feed for live microalgae during the early juvenile stage (Edullantes 2015). *Sargassum* species and floating mats of *Sargassum* contain a wide range of biologically active compounds and have a high ash content, which provides minerals and trace elements that are beneficial in both fertilizer and animal feed (Milledge and Harvey 2016). These also contain minerals and vitamins (Cajipe 1990) and macro and micronutrients that promote growth (Divya *et al.* 2015; Milledge and Harvey 2016).

Similarly, another physicochemical factor is salinity, which is one of the principal components that affect the physiological performance (Yu *et al.* 2013) and regulate the growth and reproduction of marine animals (Purwati and Luong Van 2003). Below or above the preferred normal salinity level of the organism could result in abnormal development and deformation, which may cause larvae mortality (James *et al.* 1994; Meng *et al.* 2011) and low specific GR like in the *H. atra* (Seeruttun *et al.* 2008).

In Northern Mindanao, Philippines, an ACIAR (Australian Centre for International Agricultural Research) funded project on the "Expansion and Diversification of Production and Management System for Sea Cucumbers in the Philippines, Vietnam and northern Australia" (Project No. FIS/2010/042) – also called the Sandfish project –was implemented at MSUN. With the help of this project, sandfish juveniles have been successfully produced in the hatchery. However, the juveniles experienced high mortality rates during nursery culture in sea-based floating hapas. Before the juveniles are released to the natural environment, they have to grow up to 3 g, thus requiring at least another 2 mo of nursery rearing. During this period, their feeding efficiency and responses to eco-physiological factors are most crucial for the success and high survival of the sandfish juvenile. We have an insufficient understanding of the role of feed types and salinity during this crucial stage. Investigating these aspects can shed more light on more effective resource management. To address this knowledge gap and to generate useful information on the growth, survival, and behavioral response of *H. scabra*, a 60-d study was conducted using the hatchery-reared *H.scabra* early juveniles subjected to different feed types (*Navicula* sp., powdered *Sargassum*, and *Sargassum* extract) and exposed to various salinity levels [ambient seawater at 32–35 (as control), 20, 25, and 40 ppt] under laboratory conditions.

MATERIALS AND METHODS

Test Organism

The hatchery-reared sandfish early juveniles measuring 2−15 mm in length and weighed less than 1 g were used in the study and were provided by the MSUN sandfish project. The source broodstock of the juveniles was from Kauswagan, Lanao del Norte, which was spawned on 15 Sep 2016 at the MSUN sandfish hatchery. The 60-d experiments started on 16 Nov 2016 until 16 Jan 2017.

Experimental Design

Two experiments (feed types and salinity) were conducted indoors at the extension facility of the MSUN sandfish hatchery. Experiments were carried out simultaneously in a 50-L glass aquarium with a dimension of 59 cm x 29 cm x 30 cm (L x W x H). Nine glass aquaria were used for feed types and 12 for salinity experiments with three replicates per treatment. Glass aquaria were placed on three white tables to easily observe the juveniles. Sediments were not added to avoid other contributing factors such as organic matter, which may affect the growth of the juveniles. In each experiment, replicate aquaria were haphazardly arranged across the experimental table to discount potential spatial effects such that of light, temperature, and other factors. A total of 1,050 juveniles (length: 2–15 mm; weight: < 1 g) were selected and transferred to the aquarium. For each treatment, 50 individuals were randomly stocked per aquarium to avoid overcrowding, since the growth of *H. scabra* juvenile is being affected by high density (Battaglene *et al.* 1999). The seawater that was used was passed through an ultraviolet (UV) water filtration system. Each aquarium was filled with 40 L of UV-filtered seawater, where each set up was provided with a complete aeration system for water circulation ensuring a continuous supply of dissolved oxygen. Water management was done every 3 d by partially siphoning ~ 80% of water in the aquarium.

Feeding Experiment

Feed types experiment was conducted using three different feed types (Table 1). The amount of feed subjected to the juveniles in this experiment was based on the preliminary experiment in feed types we conducted last June–July 2016. In the first 30 d of the experiment, *H. scabra* juveniles were fed with each of the experimental feed types twice a day, *i.e.* 6:00 AM and 4:00–6:00 PM. Adjustments were done on Days 31–60 of the experiment by increasing the feeding amount and feeding only once a day at 6:00 AM. For example, F1 was fed with 500 mL of *Navicula* sp. twice a day during the first 30 d, and 2,000 mL of the former succeeding Days 31–60 of the experiment. One (1) mL of *Navicula* sp. contains ~ 20,000–40,000 cells. For F2, approximately 2 g of powdered *Sargassum* on the first 30 d and ~ 8 g on Days 31–60. Powder *Sargassum* was diluted in 50 mL of UV-filtered seawater before being administered to each treatment. For F3, 100 g of fresh chopped *Sargassum* and 100 mL of UV-filtered seawater from the tanks (1:1 ratio) were blended for 2–3 min, then strained through a 45-µm mesh size sieve. Each replicate was fed with 30 mL of the extract on the first 30 d and 120 mL on Days 31–60.

Salinity Experiment

Four salinity treatments with three replicate each treatment was conducted. Treatments that were used were 32–35 (ambient; as the control), 20, 25, and 40 ppt. Desired lower salinities were achieved by diluting UV-sterilized filtered seawater mixed with filtered freshwater. The highest salinity level (40 ppt) was obtained by adding commercial sea salt to the seawater. Juveniles were fed using powdered *Sargassum* suspended in seawater before adding, with ~ 2 g on the first 30 d in the morning and afternoon and adjusted into ~ 8 g on Days 31–60 and fed

Table 1. Different feed types/diets used in the study.

Treatment	Feed type	Description	Feeding rate per day
F1	*Navicula* sp.	Pure cultured benthic diatom	1,000 mL (Days 1–30) 2,000 mL (Days 31–60)
F2	Powdered *Sargassum*	Powdered dried brown *Sargassum* seaweed	4 g (Days 1–30) 8 g (Days 31–60)
F3	*Sargassum* extract	Blended fresh brown *Sargassum* seaweed	60 mL (Days 1–30) 120 mL (Days 31–60)

in the morning only. Powdered *Sargassum* was chosen as feed to the juveniles in the salinity experiment since it is used as an alternative feed for early juveniles cultured in tanks at MSUN hatchery.

Data Collection

During monitoring (Days 0, 30, 45, and 60), juveniles were carefully siphoned from the tank using an aeration tube to a white plastic tray, where a ruler was provided as a scale for length and width measurement using Coral Point Count with Excel Extensions (CPCe 4.1) software (Kohler and Gill 2006). The length was measured from the mouth to the anus. The widest portion of the body was measured to get the width of the juveniles. Juveniles that were curved or curled in the photo were noted in the comments and estimations of the length and width were done during photo analysis. The actual length and weight of juveniles at 60 d of culture or upon the termination of the study were determined using a celluloid ruler and a pocket weighing scale with 0.1-g accuracy, respectively.

During water management (every 3 d), survival was determined by manual counting of the number of surviving live juveniles. Dead juvenile in each tank was noted as indicated by the presence of a lesion of the integument and paleness of body color. A dead juvenile was removed but not replaced in treatments where mortality was observed. Different physical and behavioral responses of juveniles were also monitored every before and after feeding. These include skin condition, location in the tank, and activity (Table 2).

Table 2. Physical and behavior response criteria for *H. scabra* juveniles with activity response modified from Wolkenhauer and Skewes (2008).

Responses	Definition
Skin condition	
Thin	Pale/ lighter color; veins and internal organs may be seen from the skin
Medium	Medium; not too dark nor pale; gray skin coloration
Thick	Dark color; a large amount of black is mixed into the skin
Location	
On the wall	Juveniles found in the wall surface of the tanks
On the bottom	Juveniles found on the floor of the tanks
Activity	
Resting	The animal is inactive with no movement observed
Feeding	The animal is actively feeding on the floor or walls; tentacles are exposed and the head performs sweeping movements
Moving	The animal is slowly moving and not resting

Statistical Analysis

One-way analysis of variance (ANOVA) was used to test the differences in length, width, GR, survival, and FPR (feces production rate) among treatments of the two experiments at a 5% level of significance. Data were log-transformed when the assumption of homogeneity of variance was not met. When a significant difference was found, Tukey's test was performed to determine the source of variation. Average values were determined and range descriptive statistics were also employed. All analyses were performed using SPSS software (18.0).

RESULTS

Length and Width

The length and width of juveniles fed with powdered *Sargassum* and *Sargassum* extract showed a rapid increase within 30 d but not those fed with *Navicula* sp. (Figures 1A and B). Juveniles fed with *Sargassum* extract consistently showed the highest increase in both length and width across all sampling times, followed by powdered *Sargassum* and *Navicula* sp. as the lowest. All feed type treatments were significantly different in length and width (one-way ANOVA, both, $p < 0.05$). Juveniles in different salinity levels showed a linear increase in length and width and were significantly different in each day of culture (one-way ANOVA, $p < 0.05$) (Figures 1C and D). The highest length of juveniles was observed in 40 ppt while the lowest was in 20 ppt during 60 d. In contrast, juveniles under 25 ppt had the highest width while 20 ppt are still the lowest.

GR

The weights of juveniles administered with different feed types and salinity levels increased gradually until the end of the experiment. After the 60-d feed-type experiment, the GR of *H. scabra* juveniles differed significantly among treatments (one-way ANOVA, $p < 0.001$; Figure 2A). Juveniles fed with *Sargassum* extract showed the highest GR of 0.026 g d^{-1}, followed by powdered *Sargassum* and *Navicula* sp. having GR values of 0.010 and 0.003 g d^{-1}, respectively (Figure 2A). In contrast, the GR of juveniles did not differ significantly among salinity treatments (one-way ANOVA, $p > 0.05$). The highest GR of the juveniles was observed in the ambient salinity (32–35 ppt), followed by 40, 25, and 20 ppt with a range of 0.016– 0.026 g d^{-1} (Figure 2B).

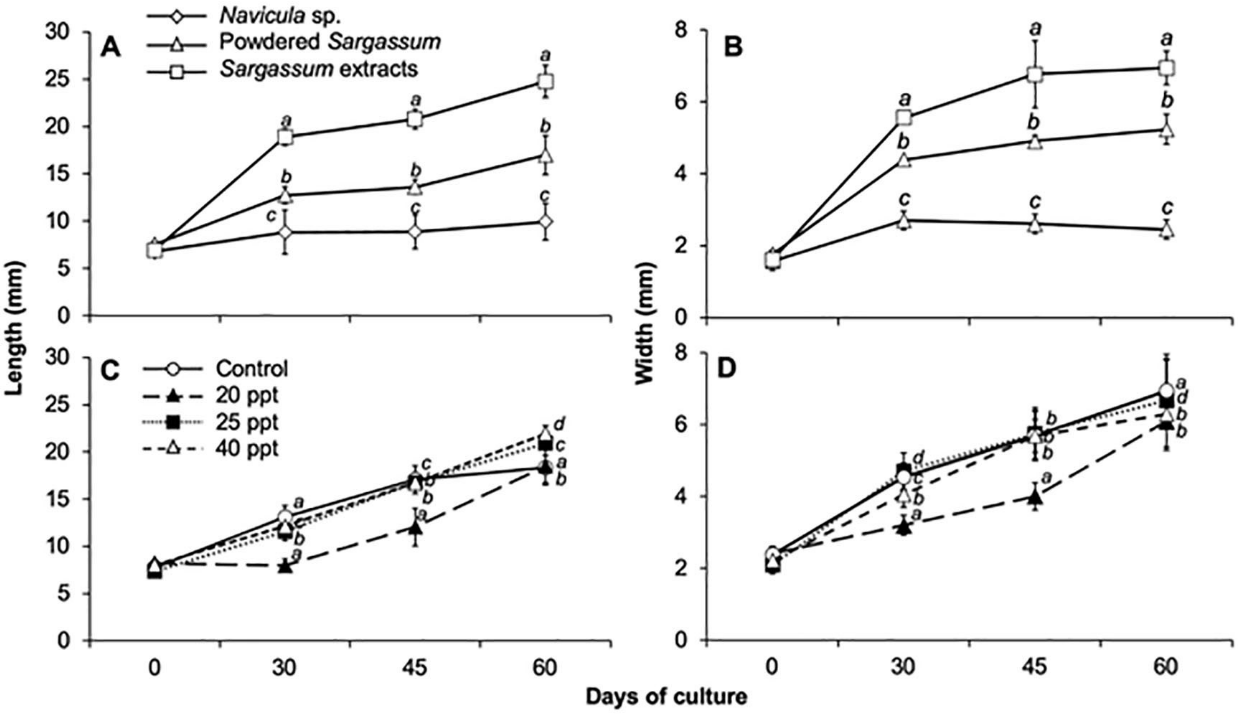

Figure 1. Growth of *Holothuria scabra* juveniles in terms of length and width in different feed types (A, B) and salinity (C, D) experiments.

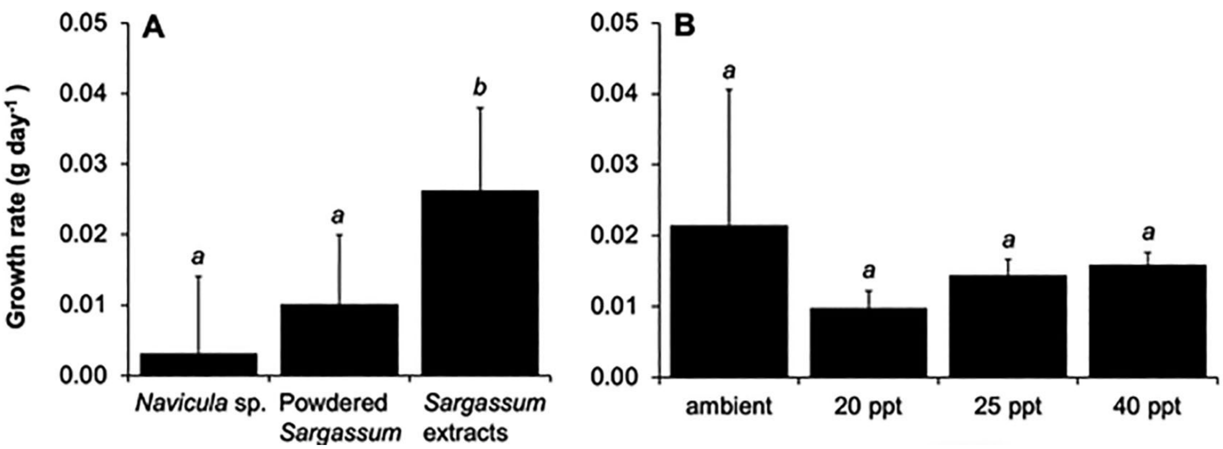

Figure 2. The GR of *Holothuria scabra* juveniles in different feed types (A) and salinity (B) experiments. Error bar represents the standard deviation of the experiments in three replications.

Size Distribution

Size ranges of juveniles administered in different feed types were approximately the same at the start of the experiment. All treatment size ranges were within the range of 2–12 mm. At the end of the experiment, the length of juveniles in all treatments greatly varied and diversified (Figure 3A). Juveniles fed with *Navicula* sp., powdered *Sargassum*, and *Sargassum* extract obtained wide size ranges of 0–28, 2–38, and 2–54 mm, respectively. Moreover, all treatments showed a different higher frequency of size ranges. Juveniles fed with *Navicula* sp. had a higher frequency in the range of 2–10 mm while in powdered *Sargassum*, the length-frequency was higher at 10–22 mm and in *Sargassum* extract at 20–34 mm. The size ranges of juveniles grown in different salinity levels were also approximately the same at 6–15 mm at the start of the experiment. The length of juveniles cultured in the ambient reached 3–39 mm and juveniles in 20, 25, and 40 ppt reached 6–45 mm at the end of the experiment (Figure 3B). The highest frequency of juveniles was found at 24

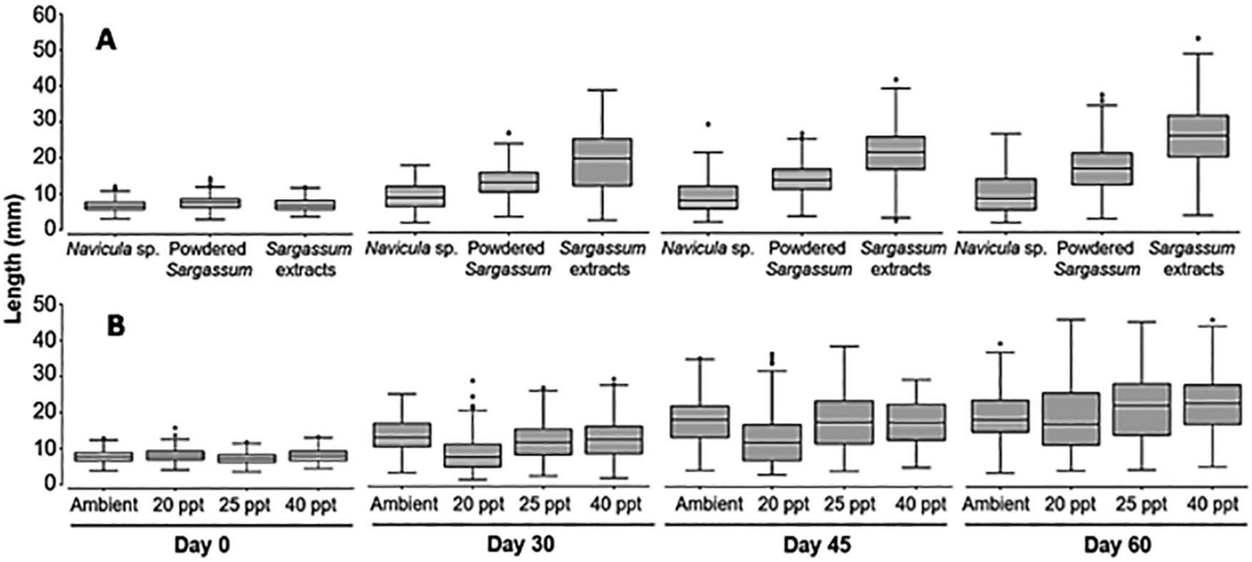

Figure 3. Size distribution of *Holothuria scabra* juveniles in different feed types (A) and salinity (B) experiments. The number of individuals indicated on each bar.

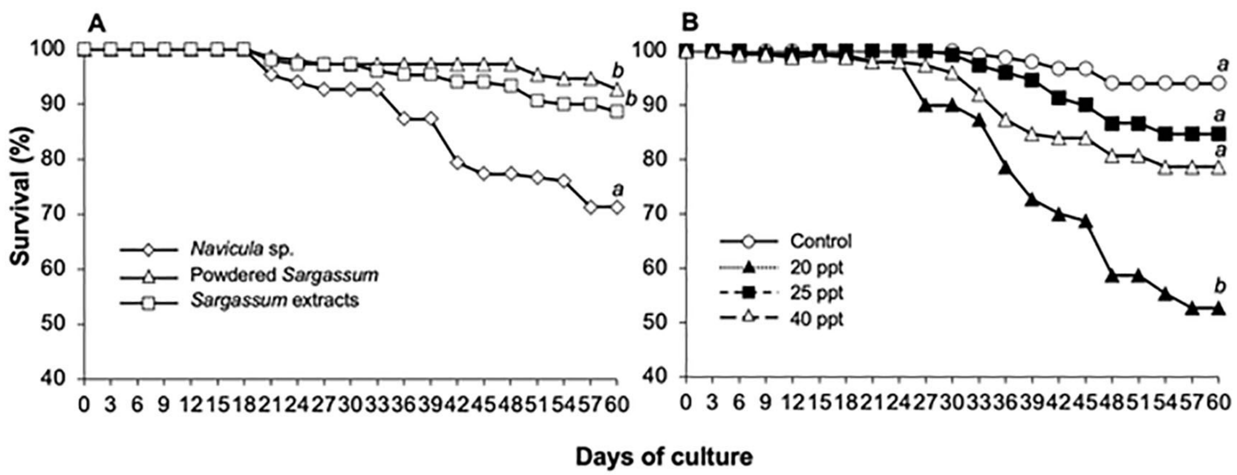

Figure 4. Survival of *Holothuria scabra* juveniles in different feed types (A) and salinity (B) experiments.

mm exposed at 25 and 40 ppt, followed by ambient and 20 ppt at 18 and 12 mm, respectively.

Survival

Percent survival of juveniles in feed types and salinity experiments differed significantly among treatments (one-way ANOVA, $p < 0.001$, both experiments; Figures 4A and B). The survival of juvenile was highest on powdered *Sargassum* at 92.67%, followed by *Sargassum* extract at 88.67%, and lastly, those fed with *Navicula* sp. at 71.33% (Figure 4A). The start of mortality occurred at 21 d of rearing and continuously decreasing until 60 d of the experiment. In contrast, the mortality of juveniles in different salinity levels began on Day 6 in 40 ppt, Day 15 in 20 ppt, Day 30 in 25 ppt, and Day 33 in ambient salinity. The trend showed the highest mean number of live juveniles was in the ambient salinity and lowest in 20 ppt at the range of 52.87–94.00% survival (Figure 4B

Physical and Morphological Behavior

The change of skin condition of the juveniles was observed inside the aquaria in both experiments, in which the number of the thin and medium juveniles decreases while the thick skin condition increases until Day 60 (Figures 5A and B). Aside from the change in skin condition, some of the *H. scabra* juveniles exposed to the lower salinity treatments (*i.e.* 20 ppt) had a sluggish movement and a disintegration of some body parts during the middle

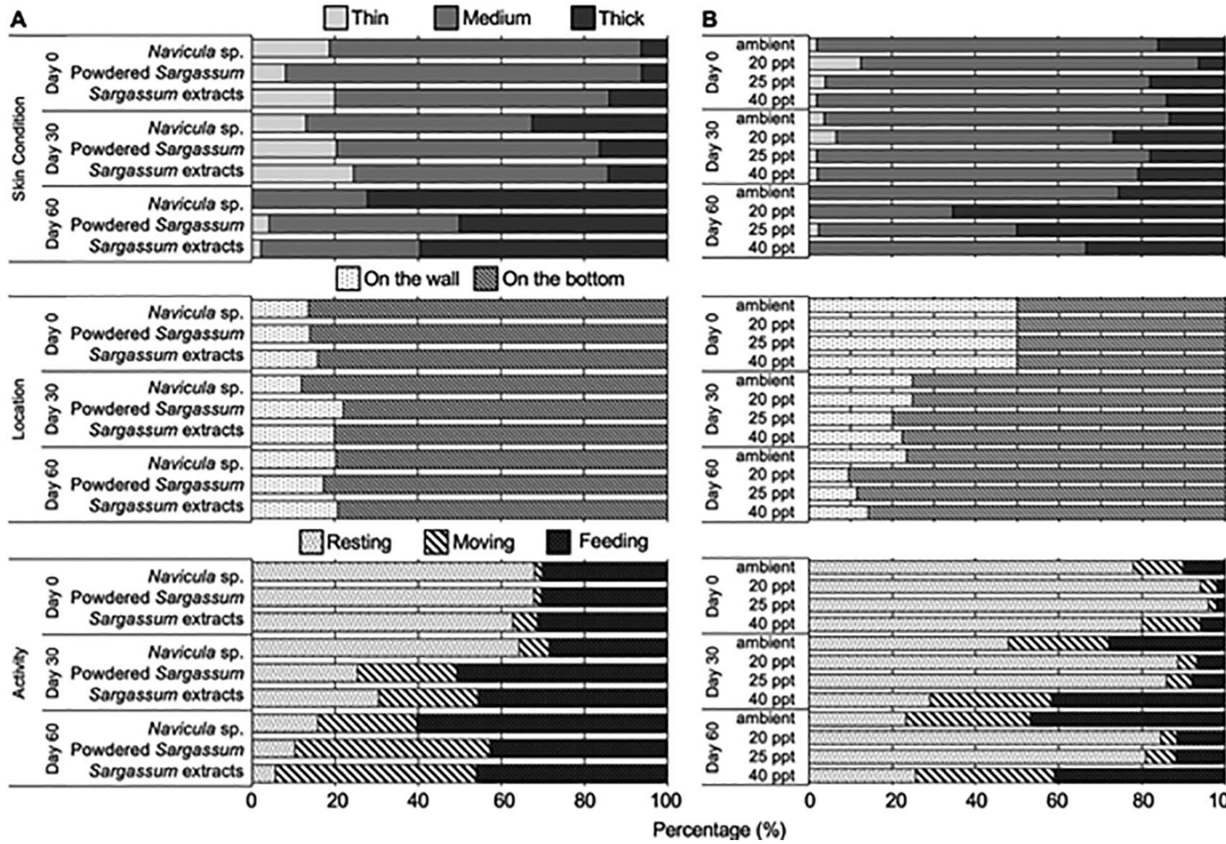

Figure 5. Percentage of skin coloration, location, and activity responses of *Holothuria scabra* juveniles in different (A) feed types and (B) salinity levels during Days 0, 30, and 60.

until the end of the salinity experiment. In the feed types experiment, most of the juveniles in *Sargassum* extract were observed to be abundant on the walls of the aquarium tank during feeding. In contrast, juveniles in the *Navicula* sp. and powdered *Sargassum* treatments were abundant at the bottom of the tank. *H. scabra* juveniles exposed to different salinity levels were mostly observed at the bottom rather than on the walls of the tank during feeding. Juveniles activity in both experiments were found to spend more time resting on the first 30 d and actively feeding thereafter; however, less movement on lower salinity of 20 ppt was observed (Figures 5A and B).

FPR

The FPR in the feeding experiment did not differ significantly among treatments (one-way ANOVA, $p > 0.05$). The highest FPR was found in powdered *Sargassum* (1.38 g g^{-1} d^{-1}), followed by *Navicula* sp. (1.01 g g^{-1} d^{-1}), and lastly, *Sargassum* extract (0.72–1.38 g g^{-1} d^{-1}). *Sargassum* extract has the highest total weight of animal casting at the end of the experiment, yet they exhibited the lowest FPR of 0.72%.

DISCUSSION

Length and Width

Here, we systematically demonstrated the effects of feed types and salinity on the growth and survival of *H. scabra* juveniles. The feed types significantly affect the increase in length and width of the juveniles. When juveniles fed with *Sargassum* extract, it showed the highest increase in length and width, not only because *Sargassum* contains bioactive compounds plus micro and macronutrients that are beneficial as animal feed (Milledge and Harvey 2016) but also because brown algae such as *S. thunbergii* and *S. polycystum* are widely used as a source of alginate, a compound with high viscosity (Wu *et al.* 2002). This greater viscosity enables sea cucumbers to handle more easily and feed more efficiently on it (Xia *et al.* 2012). The low viscosity makes it hard for sea cucumbers to handle such as the powdered *Sargassum*, which may cause the sea cucumber to reject the feed or reduce its consumption. In the case of the live feed like *Navicula* sp., Watanabe *et al.* (2011) found that *Navicula ramosissima* resulted in the lowest growth than shrimp tank detritus and shrimp feces. They are only favorable as supplemental feed if mixed with *Sargassum* extract for better results in early juveniles. Tuwo *et al.* (2012) mentioned that salinity is one of the important factors affecting the growth of *H. scabra* juveniles. In this study, the length and width of *H. scabra* juveniles were significantly affected when exposed to different salinity levels. The highest lengths were observed at the highest salinity (40 ppt) compared to the lower salinity treatment (20 ppt). Similar results were also reported by Yuan *et al.* (2010), which concluded that sea cucumber grew better in higher salinity rather than low salinity at its optimum temperature, although this result is species-specific. Possibly, the effect of lower salinity causes stress to juveniles, resulting in slower growth. The findings of the study may also suggest that juveniles exposed to ambient salinity may limit their energy in increasing its length and width and spend their energy more in growing other aspects such as their weight since salinity is an indirect factor that modifies numerous physiological responses such as the growth of marine organisms (Hu *et al.* 2010).

GR

Juveniles fed with *Sargassum* extract showed the highest GR followed by powdered *Sargassum* and *Navicula* sp., which concurred in the study of Battaglene (1999), in which juveniles fed with *Sargassum* resulted in a GR of 0.2 ± 0.02 g d^{-1}. In this study, juveniles fed with *Sargassum* extract reached larger sizes. Nutrients extracted from *Sargassum* such as *S. latifolium* gave the best growth and high survival rates on early juveniles of *H. scabra* (Lavitra *et al.* 2009; Dabbagh and Sedaghat 2012). Many biologically active compounds like terpenoids, flavonoids, sterols, sulfated polysaccharides, polyphenols, sargaquinoic acids, sargachromenol, and pheophytine can be isolated from different *Sargassum* species (Yende *et al.* 2014). This could be the reason for the higher growth of the juveniles produced when fed with *Sargassum*. Also, some macro- and microorganisms are still present in the *Sargassum* extract than the powdered *Sargassum*, which has undergone drying and pulverizing. The results of the study also showed that ambient seawater around 32–35 ppt was best for the rearing of the *H. scabra* juveniles, which is consistent with the findings of Mills *et al.* (2012) and Seeruttun *et al.* (2008). Asha *et al.* (2011) observed the highest GR in early-stage juvenile *H. scabra* at 30 ppt with 0.008 g d^{-1} and lowest at 0.0032 g d^{-1} at 20 ppt while juveniles did not grow in 40 ppt. Lower GRs were observed in the lower salinity treatment. This finding corroborates with other studies which reported that lower salinities reduced the GRs and increased mortality in sea cucumbers (Meng *et al.* 2011; Mills *et al.* 2012). Furthermore, reduced salinity would be a difficult environment for the juveniles to adapt and grow, which causes stress that eventually affects their burrowing behavior and feeding cycles (Mercier *et al.* 1999, 2000).

Size Distribution

Food preference experiments are widespread in the ecological literature (Roa 1992). However, algal diets in several studies were not selected based on nutritional value (Xia *et al.* 2012), as well as in this study. The peak of the length sizes of the juveniles in response to different feed types was observed at 8, 16, and 32 cm in *Navicula* sp, powdered *Sargassum,* and *Sargassum* extract, respectively. As observed, the peak in sizes in different feed types doubled and quadrupled from the lowest peak because most animals have evolved senses that enable them to discriminate against foodstuffs, resulting in dietary preferences (Xia *et al.* 2012). The length frequencies of juveniles exposed to different salinity levels do not greatly vary between treatments in this study, which may suggest that juveniles spent more energy on survival rather than growth. Similarly, Yuan *et al.* (2010) found *Apostichopus japonicus* subjected to lower salinity have more energy allocation spent on consumed food on feces, respiration, and excretion but less energy on growth. Findings of Liu *et al.* (2004) indicated that different sizes of *A. japonicus* juveniles thrive to different salinity levels, indicating that salinity tolerance of juvenile holothurians is size-specific. In this study, juveniles exposed to 40 ppt and ambient salinity exhibited unimodal patterns while 20 ppt and 25 ppt showed polymodal patterns, although these were not clearly shown on Day 60. These patterns showed that juveniles under 40 ppt and ambient were not as a variable in size compared to lower salinities treatments. The juveniles under lower salinity treatments have different adaptive capability probably results in their varied sizes, leading to a polymodal pattern. Aside from the ambient salinity, the other treatments had a wide representation of small and large individuals on Day 60. But still, all treatments are normally distributed.

Survival

In this study, a clear difference was observed in the survival of all feed-type experiments. Mortality substantially started to occur during the 3rd week of the experiment. Although Lavitra *et al.* (2009) reported that the highest mortality could occur during the first 2 wk for < 5 mm juveniles (Lavitra *et al.* 2009), both food availability and feed types affect the survival of early juveniles of *H. scabra*. The results of the salinity experiments showed that juveniles could tolerate higher salinity around 40 ppt and reduced salinity up to 20 ppt for about 24 d. However, after 24 d, rapid mortality in the juveniles occurred. The mean percentage survival of *H. scabra* juveniles varied in all treatments, indicating the influence of salinity on the survival of juveniles. The study of Tuwo *et al.* (2020) revealed that the mortality of H. *scabra* exposed to lower salinity is evident regardless of whether the drop is due to a sudden or gradual change. It was highly unfavorable to culture the sea cucumber in low salinity (Seeruttun *et al.* 2008), especially below the body salinity of saltwater bony fish, which is 18 ppt (Tuwo *et al.* 2020). *Holothuria scabra* could tolerate reduced salinity (20 ppt) for a short period (Pitt *et al.* 2001; Agudo 2006), but it does not show preferences for brackish waters (Mercier *et al.* 2000). These observations may substantiate the findings of the present study, wherein ambient water has shown to be the preferred salinity level for the rearing of juveniles. Several sandfish nursery systems were successfully developed in marine ponds (Duy 2012) and successful grow-out trials for monoculture in marine earthen ponds (Bell *et al.* 2007). In this study, sandfish juveniles were found to have no potential for brackishwater pond nursery due to their limited tolerance to lower salinity of lesser than 20 ppt. Moreover, there are other physicochemical factors aside from salinity that need to be considered in putting a brackish-water pond nursery production of sandfish in the Philippines such as silty muddy sediment that seems to be unsuitable for sandfish cultures (Altamirano *et al.* 2017).

Physical and Behavioral Responses

Juveniles showed and exhibited no marked abnormal physical and behavioral responses on both experiments except for some instances in the low salinity treatments. The change in the skin condition of juveniles observed in both experiments was due to the increase in the body length of the juvenile in which such changes were observed when organisms were growing and performing adaptation in the environment. However, there are some juveniles in low salinity treatments that have a sluggish movement with the integument destroyed in some parts of the body and unusually thin (pale coloration), which could be an indication for their inability to adapt to the lower salinity levels. These observations concurred with the study of Purcell and Eeckhaut (2005) that juveniles become unhealthy when they become sluggish and have an unusual pale body coloration. The motility of juveniles was only a normal behavior/adaptation in searching for food where they stayed on the bottom or on the walls depending on the availability of food. This is also the same normal behavior for the activity of the juveniles, in which they spend more time resting at the start of the experiment as they are still adjusting to the environment. Aside from the 20 ppt treatment, all juveniles in the remaining treatments in the two experiments were adapted to the environment, resulting in their active feeding and movement. Sea cucumbers exhibit general plasticity of behavioral and feeding strategies in response to variations in resource availability (Roberts *et al.* 2000) and to animals that spent time feeding and spent periods on burying in sediments (Robinson *et al.* 2015).

FPR

The findings of the feed experiment showed that *Sargassum* extract had the highest total feces weight followed by powdered *Sargassum* and *Navicula* sp. However, the result showed FPR was greatly affected by the initial and final wet weight of the juveniles. *Sargassum* extract had the highest average wet weight of juveniles; thus, the FPR was being affected by the wet weight of the juveniles, resulting in *Sargassum* extract having the smallest FPR value. Results also showed that diets affected feces production, as well as the growth and energy budget. Compared with other echinoderms, in holothurians, the energy deposited in growth is lower and the energy loss in feces accounts for the majority of the ingested energy (Yuan *et al.* 2006). The inorganic component of the feces is primarily undigested dietary elements that also depend on its dietary supply (Rose *et al.* 2015). This claim explains the reason why lower FPR resulted in high growth because more dietary elements were digested rather than excreted.

CONCLUSION

This study tested two factors, *i.e* the effect of different feed types and salinity levels on the growth and survival of the hatchery-reared sandfish juveniles towards the improvement of its nursery stage production. Results of the feed types experiment showed that *H. scabra* juveniles fed with *Sargassum* extract yielded the highest length, width, GR, and survival, as well as no abnormal behavioral responses. Thus, *Sargassum* extract is the most suitable feed for achieving higher growth and survival of *H. scabra* juveniles. This brown alga is readily available from the wild as drifters in the local coastal area of Naawan, Misamis Oriental, and has never been used or exploited by local communities. It should be noted, however, that in small-scale aquaculture operations, this dependence on *Sargassum* is potentially viable but not at an industrial level because of the potential risk of overexploitation.

Ambient seawater salinity (32–35 ppt) resulted in a higher GR and the highest survival, although it could only reach a length of 39 mm after 60 d of the experiment. Nevertheless, *H. scabra* juveniles can still grow and survive at higher salinity of 40 ppt and lower salinity of 20 ppt but only for a short duration, *i.e.* 24 d. However, some of the *H. scabra* juveniles exposed to lower salinity were observed to have a sluggish movement, destroyed integument in some parts of the body, and unusual thin pale body coloration, which can be concluded to be unhealthy. Hence, it will be challenging to culture sandfish juveniles in brackish-water systems.

RECOMMENDATION

Further research needs to be conducted to identify an appropriate formulated feed diet for *H. scabra* juveniles. In addition, salinity experiments still need to be done to determine the level of tolerance and exposure limit of juveniles to lower salinity that may address the viability of sandfish production in brackishwater ponds. These studies will enhance the state of knowledge in making this activity economically and environmentally sustainable for the improvement of the rearing conditions suitable for mass production of the sandfish juveniles for stock restoration.

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NOTES ON APPENDICES

The complete appendices section of the study is accessible at https://philjournalsci.dost.gov.ph/

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APPENDIX

Data Collection

The feces production rate was analyzed in the feed types experiment only to determine the digestibility of the feed to know the best feed type that could result in higher growth of the sandfish juveniles. Water management was also done every three days to ensure the physico-chemical conditions of the water such as salinity maintained throughout the experiment. Animal fecal matter was collected every 3 d during water management by siphoning using an aeration tube and placed in a filtration paper. The collected feces was air-dried for 2–7 d and then oven-dried at 60° C to constant weight for each replicate. After drying, casts were weighed using an analytical weighing scale. Upon termination, all replicated feces were pooled for each treatment.

The water parameters monitored during the experiments were salinity, water temperature, light intensity, dissolved oxygen, and ammonia. Salinity was monitored every 3 d using a portable hand refractometer before and after water management. Water temperature and light intensity were monitored every day using a data logger (HOBO® Pendant Temperature/Light 64K Data Logger). Dissolved oxygen and ammonia in water were analyzed using the Winkler's method and the Phenate method, respectively (APHA 1995).

Data Analysis

The average lengths of *H. scabra* juveniles were converted to average weight using the formula of Purcell and Agudo (2013):

$$W = 0.000614 \times L^{2.407} \quad (1)$$

$$L = 22.826 \times w^{0.370} \quad (2)$$

where L is the length in mm and W is weight in g. The values obtained were also used to determine the length-frequency of juveniles in each day of the culture of the two experiments. The length and width of the juveniles in the two treatments were presented in linear graphs while the length-frequency in box plots.

The growth rate indicates the gain in biomass in different experiments. Absolute growth rate (GR) was calculated using the formula:

$$GR(gd^{-1}) = \frac{(W2 - W1)}{T} \quad (3)$$

where *W1* and *W2* are the mean initial and final wet weight of sea cucumbers in each aquarium, and T is the duration of the experiment in days. The growth rate of the juveniles was presented using bar graphs.

Survival was calculated using the formula:

$$S = \frac{final}{initial} \times 100\% \quad (4)$$

wherein S is the survival (%), "final" is the number of live juvenile sandfish, and "initial" is the number of all juvenile stock in the aquarium. The survival of the juveniles in the two experiments was represented using linear graphs.

The FPR was calculated as follows (Yuan *et al.* 2006):

$$FPR\ (g\ g^{-1}\ d^{-1}) = \frac{F}{\left[\frac{T(W2 + W1)}{2}\right]} \quad (5)$$

where W1 and W2 are the initial and final body weight of sea cucumbers in each aquarium, T is the duration of the experiment (60 d), and F is the dry weight of feces.

Physicochemical Parameters

The physico-chemical water parameters during the experiments were in a normal range in both experiments throughout the 60-d experimental period (Table I).

Table I. The physico-chemical parameters of the feed types and salinity levels experiments in 60 d (NA = not applicable).

Physico-chemical parameters	Feed types	Salinity levels
Salinity	32–35 ppt	NA
Water temperature	25.58–29.75 °C	25.5–29.7 °C
Light intensity	0.14–186.68 lum/ft²	0.07–310.80 lum/ft²
Dissolved oxygen	5.18–9.36 ppm (30 d) 6.13–9.14 ppm (60 d)	6.44–7.36 ppm (30 d) 6.25–7.24 ppm (60 d)
Ammonia	0.07–0.08 ppm (30 d) 0.09–0.23 ppm (60 d)	NA

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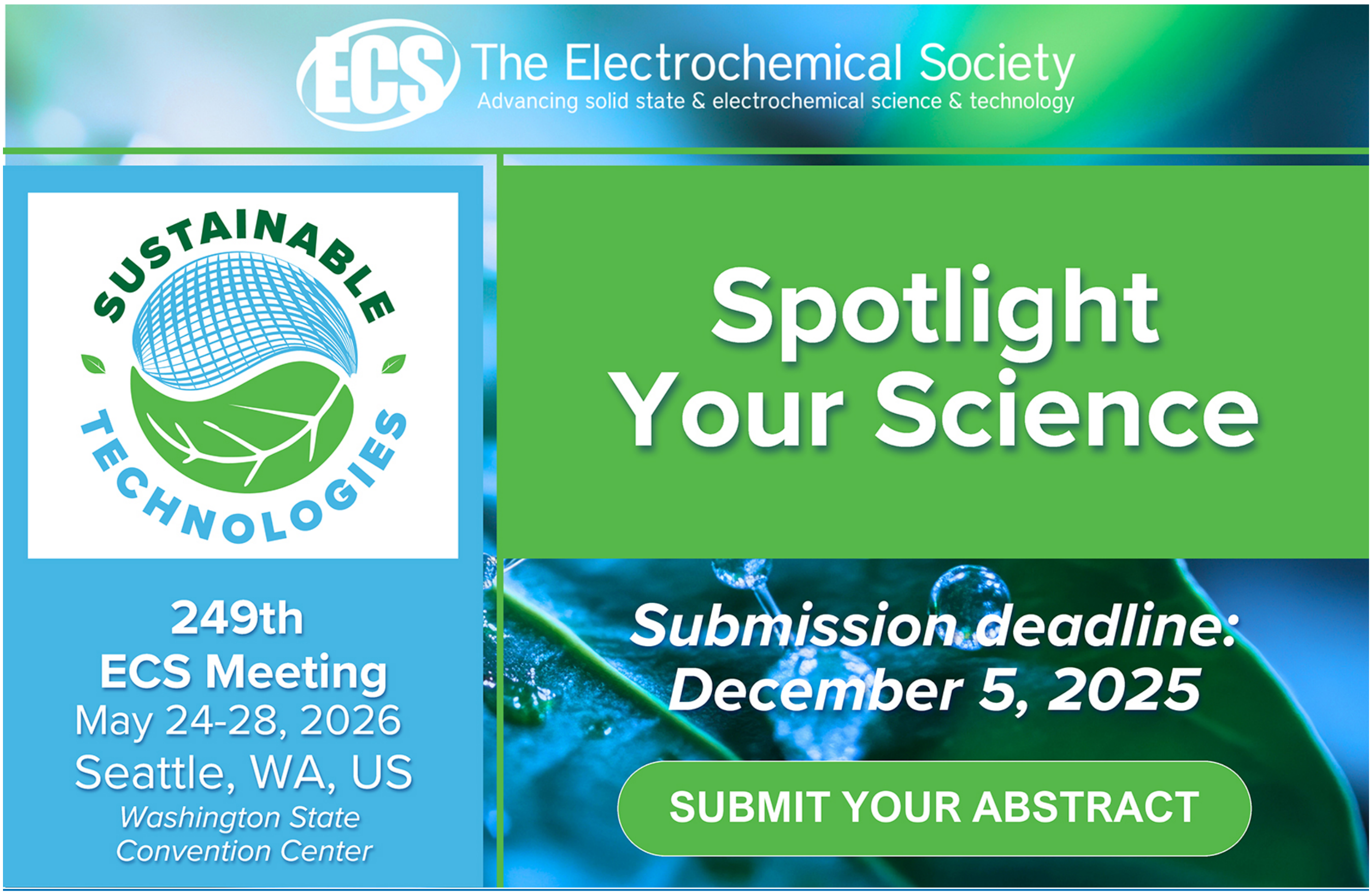

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Low salinity reduces survival rate of a commercially important sea cucumber (Sandfish: *Holothuria scabra*)

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Abstract. Sandfish culture had been implemented for more than 20 years but has frequently failed to yield the expected results due to low survival rates. A decrease in salinity during the rainy season was suspected as a cause of sandfish mortality. This study aimed to assess the influence of salinity on sandfish survival rates. Treatments included sudden shock and gradual acclimation trial methods. Even though the results of this study indicate a mean survival rate of 50% at 14.6 ppt, the survival rate fell drastically as water salinity was reduced below the salinity of saltwater bony fish body fluids (around 18 ppt). Sandfish cannot live in water with salinity lower than the osmotic body fluid of freshwater bony fish (around 14 ppt). Therefore, for the cultivation of sandfish in coastal ponds, salinity should not be allowed to fall below a minimum of 18 ppt.

1. Introduction

Southeast Asia is the principal source of sea cucumbers worldwide, with around 80,000 tons harvested each year from tropical regions [1], while Indonesia has been (and remains) the top producer of dried sea cucumber (*teripang*) [2-8]. Many species of sea cucumbers from this region have a high economic value [9-11]. Sea cucumber fisheries provide an income to many poor coastal fishers [12]. In many islands and coastal villages, income from sea cucumber fishery is used to constitute a significant portion of the total income of many families [13].

Sandfish is one of the largest catches of sea cucumbers in the world [14]. This commodity, as well as other commercial sea cucumber species [15] is an important source of income for marginal fishermen in poor coastal villages of Southeast Asian countries, especially in Indonesia [9]. Sea cucumbers, as bycatch, can contribute as much as 41% to the daily take-home income of fishers in The Philippines [16]. In these countries, sandfishis highly exploited to the point where overfishing has been causing declines in the wild populations and in sandfish production [17, 18]. Current populations of commercially sea cucumber in many shallow coastal areas have been overfished [14], and the income derived from sea cucumber gleaning has, therefore, become less important [19]. This situation requires an alternative strategy to preserve the sea cucumber populations, increase sea cucumber production, and improve coastal community welfare. Aquaculture is one alternative for maintaining the sustainability of sandfish production [20]. In Southeast Asian countries, sandfish is the only sea cucumber species that currently cultivate [9, 21].

Sandfish have been cultivated in coastal ponds for more than 20 years [22], but this effort has often not yielded the expected results [20, 23]. The lack of success in culture is due to low survival rates, especially for the culture of sandfish in brackish-water ponds [24]. It has been suggested that low sandfish survival rates in these coastal ponds might be due to excessive fluctuations of salinity. This hypothesis was based on previous research on the effects of sub-lethal stress on the salinity responses of marine organisms [25, 26]. Salinity stress could affect marine invertebratesina variety of mechanisms [27, 28]. Although there have been many results of previous studies that support directly or indirectly for the success of cucumber cultivation [29-33], advance research is still needed to be able to make sea cucumber ecologically and economically feasible.

The effects of salinity on the physiology of marine invertebrates have been documented for several species of echinoderms [34-36]. Echinodermsare generally considered as one of the stenohaline phyla in the animal kingdom, but several species show remarkable abilities to acclimate and survive in euryhaline habitats [37]. Yuan (2006) had shown that sea cucumbers could tolerate a range of salinity from 22 to 36 ppt. However, salinities of 28–34 ppt are preferred for the growth of sandfish [26]. In addition to their economic value, sandfish are better suited for cultivation compared to other sea cucumber species [38], owing to their capacity to tolerate salinity fluctuation [9].

Sandfish cultivation has been attempted for many years; however, until the present time, it has not been economically viable due to high mortality rates, especially during the rainy season. It is suspected that sandfish deaths in coastal ponds culture may be due to decreases in salinity. Even though previous research has reported that sea cucumbers, including sandfish, can be found in estuarine areas [26], from a biological perspective, sea cucumbers are primitive animals that do not have good regulatory systems [28, 39, 40]. There is a lack of studies in assessing the capacity of sandfish to adapt to salinity changes or other environmental stress factors. The present study aims to assess the effect of salinity changes to sandfish survival rates, both in a sudden event or in a situation where the salinity decrease gradually.

2. Materials and methods

Sandfish were collected during low tide at night-time in the waters of Liukang Tuppabiring Village, Pangkep Regency, South Sulawesi, Indonesia (4°42’13” S, 119°37’04” E). There were a total of 120 sandfish used in two separate experiments. The sandfish were acclimatized for 30 days in a holding tank with a volume of two tons of seawater with salinity 36ppt. During the acclimatization and experimentation process, dried spirulina and artificial feed are given every 6 AM and 6 PM,respectively, in an amount equivalent to 2ppm in the experimental tank. The experimental tanks are cylindrical in shape, with a 55cm diameter and a height of 45cm, filled with seawater to a depth of 40cm which made up to 95 l volume. During the experiments, all of the tanks were kept outdoors.

To experiment on the sudden decrease in salinity, 21 tanks were filled with seawater with seven different treatments, each with three replicates. The seven salinity treatments are 35, 30, 25, 20, 15, 10, and 5 ppt, chosen based on the fact that the salinity in coastal ponds during the rainy season ranges from 35 to 5ppt (personal observations). Apart from the salinity difference, all other physical aspects such as temperature and pH were kept the same. The experimental animal used in a range of 5.0−13.5 cm (8.65±2.34 cm in average), while the gutted body weight rangesfrom 13.92−106.33 g (49.40±29.20 g in average). The experiment began when four sandfish were put into each tank and ended when 100% mortality was reached. Observations were carried out everyday at 6 AM and 6 PM just before feeding.

The second experiment was aimed to observe the survival rate during a gradual decrease in salinity. Using nine experimental tanks, filled with seawater in 36ppt salinity. A total of 36 sandfish, with an average length of 3.5−10.0 cm (6.11±1.50 cm in average) and a gutted bodyweight of 5.62−56.54 g (19.47±12.66 g in average),were placed evenly into the tanks, resulting in four sandfish for each tank. The treatment was obtained by replacing the experimental tank water by 7.5%, 5%, and 2.5% with fresh water for treatment a, b and c, respectively. This water replacement is carried out every 12 hours during the study. The study was ceased when 100% mortality was reached. The survival rate (Sr) was calculated using Sparre, Ursin, and Venema [41] equation: $Sr = \left(\frac{Nt}{No}\right) x100$, where N_o is the number of sandfish at the beginning of the treatment, and N_t is the number of sandfish at a given time. The significance level of the difference between means was tested using a One-sample t-test [42]. The relationship between length and gutted body weight as well as the theoretical curve of decreasing salinity were both determined using Scherrer [42] quadratic equation: $Y = ae^{bX}$. The salinity value at 50% survival rate was estimated using Scherrer [42] linear regression equation: $Y = a + bX$ (3).

3. Results

3.1. Sandfish size

The length-gutted bodyweight relationsfor the samples used in the sudden decrease of salinity experiment was $Y = 4.322e^{0.258X}$, with $R^2 = 0.847$ (Figure 1A), while the length-gutted body weight relationof sandfish used in gradual decrease salinity experiment was $Y = 1.767e^{0.362X}$with $R^2 = 0.804$ (Figure 1B).

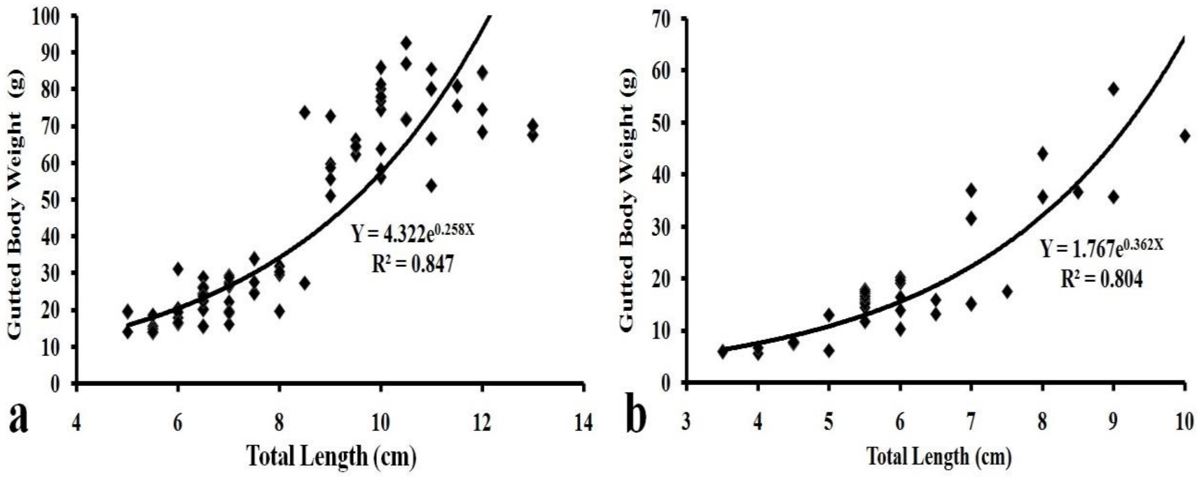

Figure 1. The curves of length-gutted body weight relation of sandfish *Holothuria scabra* used in sudden salinity decrease experiment (A) and gradual salinity decrease experiment (B).

3.2. Survival rate

3.2.1. Sudden salinity decrease experiment. In the sudden salinity decrease treatment, at salinities of 5, 10, and 15 ppt the sandfish had not moved from their original place by the third day of the treatment and did not produce feces. On the first day, some sandfish had their ventral face-up, indicating an attempt to turn around, while the mouth and anus were tightly closed. On the second day, the condition of the sandfish remained the same as on the first day, showing no signs of life. On the third day, the skin appeared crushed, indicating that the sandfish had been dead for two days; based on our observations over many years, sea cucumber skin begins to disintegrate two days after death. At salinities of 20, 25, 30, and 35, sea cucumbers appeared to be actively moving and passing feces. From the start of the treatment until the end of observation, the survival rate was still 100% (Table 1). Sandfish were still alive in these tanks after 30 days of treatment.

Table 1. Survival rate of sandfish *Holothuria scabra* exposed to sudden salinity decrease treatments.

Days	35 ppt	30 ppt	25 ppt	20 ppt	15 ppt	10 ppt	5 ppt
1	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
2	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

3	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
4	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
5	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
6	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
7	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
8	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

3.2.2. Gradual salinity decrease experiment. Under gradual salinity decrease treatment, the first mortality occurred on the sixth day of treatment for 7.5% freshwater substitution per 12 hours, which coincides with a decrease in water salinity below 18 ppt (Table 2).

Table 2. Survival rate of sandfish *Holothuria scabra* exposed to gradual salinity decrease treatments.

Day 1	Hour	Treatments (amount of water substitute)					
		2.5%		5.0%		7.5%	
		Salinity (ppt)	Survival rate (%)	Salinity (ppt)	Survival rate (%)	Salinity (ppt)	Survival rate (%)
1	0	36.0±0.0	100.0±0.0	36.0±0.00	100.0±0.0	36.0±0.00	100.0±0.0
	12	36.0±0.0	100.0±0.0	36.0±0.00	100.0±0.0	36.0±0.00	100.0±0.0
2	24	34.7±0.3	100.0±0.0	34.3±0.6	100.0±0.0	33.5±0.5	100.0±0.0
	36	32.7±0.6	100.0±0.0	32.7±0.6	100.0±0.0	29.3±0.3	100.0±0.0
3	48	31.7±0.6	100.0±0.0	30.7±0.6	100.0±0.0	29.0±0.0	100.0±0.0
	60	31.3±0.3	100.0±0.0	29.5±1.7	100.0±0.0	27.7±0.3	100.0±0.0
4	72	30.7±0.6	100.0±0.0	27.7±0.6	100.0±0.0	25.0±0.0	100.0±0.0
	84	30.3±0.6	100.0±0.0	26.7±0.6	100.0±0.0	24.3±0.6	100.0±0.0
5	96	30.0±0.0	100.0±0.0	26.0±0.0	100.0±0.0	23.2±0.3	100.0±0.0
	108	30.0±0.0	100.0±0.0	25.0±0.0	100.0±0.0	21.0±0.0	100.0±0.0
6	120	28.3±0.6	100.0±0.0	23.3±0.6	100.0±0.0	17.7±0.6	100.0±0.0
	132	26.7±0.6	100.0±0.0	21.7±0.6	91.7±14.4	16.2±0.3	91.7±14.4
7	144	26.7±0.6	100.0±0.0	20.0±0.0	91.7±14.4	15.0±0.0	91.7±14.4
	156	25.0±1.0	100.0±0.0	19.7±0.6	83.3±28.9	15.0±0.0	91.7±14.4
8	168	25.0±1.0	91.7±14.4	18.0±0.0	83.3±28.9	14.7±0.6	75.0±25.0
	180	24.5±0.5	91.7±14.4	17.3±0.3	83.3±28.9	14.7±0.6	0.0±0.0

Mortality increased and reached 100% on eight days after treatment started (Figure 2a), with salinity below 15 ppt. Under the 2.5% and 5.0% freshwater substitution treatments, mortality was not significantly different ($P < 0.05$) between treatments, with survival rates still above 80% at the time treatment was stopped. This indicates that up to 80% of sandfish can remain alive at salinities of 15 ppt and above for at least a week. The salinity level at which survival rate would be 50% was estimated at 14.561 ppt (Figure 2b).

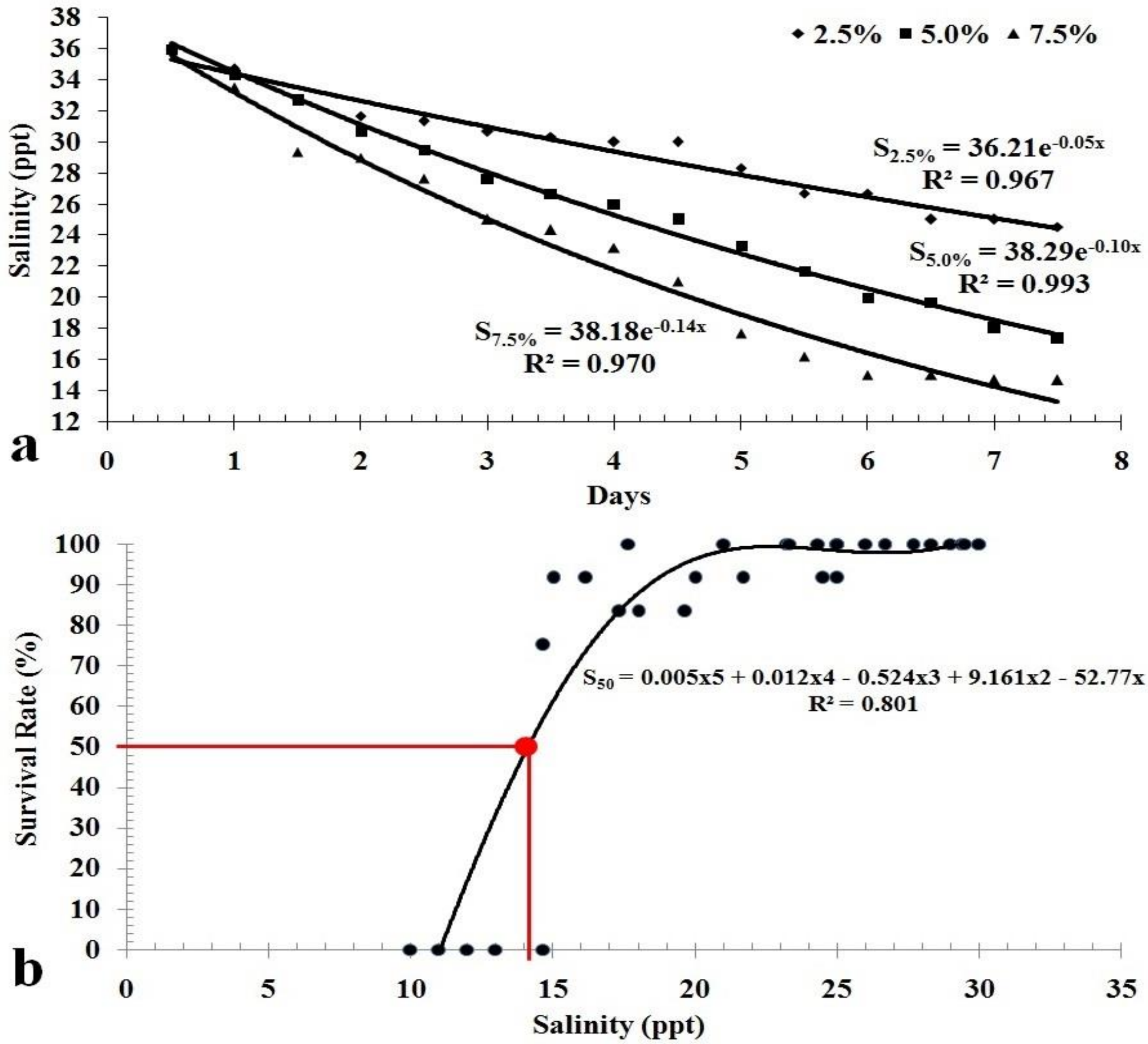

Figure 2. Salinity decrease curves for 2.5%, 5.0% and 7.5% freshwater substitution at 12 hourly intervals (A), and survival rate of sandfish *Holothuria scabra* (B) under the gradual salinity decrease treatment.

Water quality parameters other than salinity, such as temperature, pH, and dissolved oxygen, were maintained within or very close to the optimum ranges for sandfish (Table 3). Temperature, dissolved oxygen and pH of the treatment medium were measured in the morning and evening during the study and varied significantly ($p < 0.05$) between times of day but not between treatments ($p > 0.05$).

4. Discussion

Poor water quality can affect normal sandfish development, and therefore good water quality is a key factor for success in sandfish culture [43]. During the study, water quality parameters (temperature, dissolved oxygen, and pH) were in the recommended range for sandfish culture and rearing (Table 3); it is therefore unlikely that these parameters influenced the salinity treatments. The optimum water temperature range for sandfish culture 26-30°C; while the optimal temperature range for sea cucumber hatcheries is 27-29°C [44, 45]. In sandfish hatcheries, dissolved oxygen should be maintained above 5-6 ppt [45]. Although dissolved oxygen was below 5 ppt on one occasion, this is not considered likely to have been a problem because the tanks were aerated continuously throughout the study. The pH range was in the optimal pH range for sandfish throughout the study (Table 3).

Table 3. Water quality parameters during the experiments.

	Temperature (^oC)		Dissolved Oxygen (ppm)		pH	
	06:00am	06:00pm	06:00am	06:00pm	06:00am	06:00pm
Sudden salinity decrease treatment						
N	9	9	9	9	9	9
Range	28.3-28.5	31.1-32.5	5.00-5.60	4.80-5.00	7.7-7.9	7.7-7.9
Average	28.4±0.1	31.5±0.5	5.20±0.21	4.92±0.07	7.8±0.1	7.8±0.1
Gradual salinity decrease treatment						
N	9	9	9	9	9	9
Range	27.0-28.5	29.0-30.0	5.60-6.70	4.80-5.60	7.5-7.7	7.6-7.8
Average	27.7±0.6	29.3±0.4	6.1±0.4	5.2±0.3	7.6±0.1	7.7±0.1
Optimum						
Range	26-29 (Pitt et al. 2004; Giraspy and Ivy 2005)		5-6 (Pitt et al. 2004)		6-9 (Pitt et al. 2004)	

Salinity is one of the factors that can limit the distribution and survival of marine invertebrates through a variety of mechanisms [46]. Salinity can affect marine invertebrate immune systems, especially in sea cucumbers [47]. The immune system in echinoderm is in the form of coelomocytes, which are found in coelomic, water, and circulatory systems. These cells are known to be activated when sea cucumbers are exposed to environmental changes or other stressors that cause stress [48-54]. The concentration of coelomocytes increased when the animals were exposed to lower (25 ppt) or higher (45 ppt) salinity levels [48].

Salinity has a positive effect on immune responses ROS (reactive oxygen species) of Echinoderms. *Asterias rubens*, amoebocyte ROS production increased at low salinity [36]. Salinity can also affect sea cucumbers through its influence on the activity levels of superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), and lysozyme (LSZ). Limited tolerance of salinity stress has been reported in *A. japonicus*; where SOD, MPO, and LSZ activity were significantly affected by salinity change, while CAT activity decreased rapidly after exposure to the salinity of 20 ppt [55]. Decreasing salinity can also interfere with breathing and excretion systems in *Apostichopus japonicus*. *A. japonicus* respiration and excretion rates were lowered at salinities of 22 and 27 ppt, and within one hour, the immune capacity was significantly showed a difference between salinity levels of 25 ppt and 35 ppt [56]. This is consistent with the lack of movement and excretion observed prior to mortality in *H. scabra* during the present study.

The results of this study are in line with the hypothesis that salinity not only affects mortality in sandfish larvae and juveniles but also in adult sandfish. Previous research has reported 100% mortality of sandfish larvae within three days at a salinity of 15 ppt [45]. Mercier, Battaglene, and Hamel (2000a; 2000b) reported that decreasing salinity from 35 ppt to 30 ppt, 25 ppt, and 20 ppt would make all juvenile sandfish bury themselves into sediment within minutes. This study indicated that sandfish mortality is more due to an organism's minimum tolerance limit for salinity, rather than the process of decreasing salinity whether suddenly or gradually.

In nature, salinity fluctuations are relatively small and, in general, did not last very long, so that sandfish can overcome the situation by burying themselves into the sediment. These conditions are different from the situation in coastal ponds, where a decrease in salinity can be much greater and tends to last for a long time. Sandfish cannot overcome the decrease in salinity with their natural burying behavior.

Changes in salinity will affect all organisms in the sea, especially invertebrates, but some types of animals are equipped with mechanisms that help to minimize these effects. Observations by Chen and Chen [25] found that mollusks abundance and population size in low tide zone were not much different compared to the one in 10 m depth. This was due to their ability to prevent or limit osmotic stress by closing their shell or by burying themselves into the sediment. In contrast to what happens to mollusks, the diversity and abundance of echinoderms are very dependent on fluctuations in the salinity of the environment. This might be due to the nature of the outer layer of the sea cucumber, which has high permeability [34]. Like other echinoderms, although sensitive to salinity change, sandfish is more tolerant compare to some other sea cucumbers. The metabolism of *Lytechinus variegatus* is disrupted by salinity levels below 25 ppt [57]. Although the calculated salinity, where the survival rate reached 50%, is 14.6 ppt (Figure 2B), the studies indicate strong evidence that *H. scabra* appeared to be able to tolerate salinity fluctuation as lower as 18 ppt, for at least several days with relatively low levels of mortality.

The body fluid salinity of freshwater bony fish is around 14 ppt [27], while that of saltwater bony fish is around 18 ppt. The results of this study indicate that sandfish have limited resistance to fluctuations or decreases in salinity. The lower limit of salinity tolerance of sandfish seems to be located within the range of salinity of bony fish body fluids. At salinity levels below that level, sandfish seem to begin facing osmotic problems; while at salinity levels below the body fluid salinity of freshwater bony fish, sandfish mortality quickly reaches 100%.

5. Conclusion

The current study indicates that the mortality of *H. scabra* exposed to low salinities is due to environmental salinity has exceeded its lowest tolerance limit, regardless of whether the decrease is due to a sudden or gradual process. Although the calculated salinity, where the survival rate reached 50%, is 14.6 ppt, the studies indicate strong evidence that the survival rates will decrease when salinity reaches below the body salinity of saltwater bony fish, which are 18 ppt. The survival rate of sandfish reached 0% when approaching the body salinity of freshwater bony fish, which is around 14 ppt. Therefore, to ensure the sandfish cultivation in coastal ponds is succeeding, the salinity should not be allowed to fall below 18 ppt. The first paragraph after a heading is not indented.

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Hatchery Manual for Sea Cucumber Aquaculture in the U.S. Affiliated Pacific Islands


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Masahiro Ito

Hatchery-Based Sea Cucumber Farming

It is common knowledge that aquaculture farms will result in employment opportunities for island communities and provide potential source for exports. A hatchery-based sea cucumber production is to make available for stock enhancement program and for aquaculture-based farming enterprises. The author (Masahiro Ito) is an independent sea cucumber hatchery consultant and a former director of aquaculture research and extension of COM. He has propriety technology in possession which can significantly boost the sea cucumber juvenile production in the hatchery. He has agreed to write this manual to contribute to the benefit of the U.S.-Affiliated Pacific Islands and its future industry development. The main objective is to provide the advanced methodologies and to improve the hatchery technology for the holothurian sea cucumbers, particularly the sandfish (*Holothuria scabra*) in the U.S.-affiliated Pacific islands.

Status of World Sea Cucumber Trading

The sandfish sea cucumber business was once prosperous and has been a valuable source of income for decades in the tropical and subtropical coastal communities, but it was based on "boom and bust" business resulting over-fishing to the level of extinction of this high-valued species. Similar phenomenon on almost all sea cucumber fishery have occurred worldwide. Despite of these facts, a sustained demand for bêche-de-mer (processed sea cucumbers) from China and other Asian sea food markets has pushed up the price of this favored *holothurian* sea cucumber species. Most of the sandfish product which has been regarded as one of the most valuable tropical sea cucumber is traded and sold in the dried form in the Asian market mainly in Hong Kong where the products are distributed into mainland China. Dried sea cucumbers are brought from all over the world to be bought and sold in Hong Kong. Traders and wholesalers are located along Nam Pak Hong Street in the Sheung Wan area in the north-west of Hong Kong Island. Hong Kong and Guangzhou in Guangdong province, China, have been tightly connected since the birth of Hong Kong in the 19th century. Through this channel, most of the dried marine products imported into Hong Kong are re-exported to Guangdong, from where they are traded throughout China. Currently, retail prices of the sandfish in Hong Kong are from around US$50 for the low quality with small sized products to US$300 per kg for high quality with larger size and the highest quality sandfish fetches between US$500 and US$800 per kg. The "Australian" or "Australian-made" sandfish have always been regarded as the highest quality and price in Hong Kong wholesale and retail markets.

This hatchery manual includes the following topics; i.e. broodstock management and juvenile production work of the sea cucumber sandfish, notes on microalgae culture, complete larval development as well as descriptions of post-larvae and juveniles of the sandfish and the black teatfish (*Holothuria whitmaei*):

1) quarantine culture of the broodstock, recovering them from spawning stress and conditioning for spawning induction by using down-weller "Habitat Simulator" system;
2) microalgae culture of benthic diatoms and knowledge of heterotrophic algae (micro-organisms) for feeding the settled pentactula and early juvenile stages;
3) spawning induction methods with disinfection of spawners, fertilization, collection and incubation of eggs;
4) larvae rearing including specific knowledge of feeding capability of larval stages and combination of feed, calculating amount of larval feed mix, controlling algal cell suspension, adjusting feeding amount and rearing water volume, and knowledge of optimal larval development by expecting

proportions of larval and post-larval stages between day-1 and day-11;

5) settlement techniques including preparations of settlement plates and tanks, maintenance of benthic diatom culture and water quality, nutrient media preparation and culture techniques of benthic diatoms and/or naturally occurring epiphytes, knowledge on the types of benthic diatoms (*Navicula* sp. & *Cocconeis* sp.) and symbiotic heterotrophic micro-organisms in the mangrove ecosystem for feeding pentactula and early juveniles;
6) culture of pentactula and early juvenile stages in the settlement tank (the 1st phase nursery culture) from day-11 to day-56 or 8 weeks after spawning (approximately 2-month-old juveniles of 6 – 15 mm, 0.2 – 1g size), including calculation of feeding mix amount for the juvenile culture and preparation of feed mix;
7) grow-out culture using the down-weller "habitat simulator" tanks from day-56 or 8 weeks (onset of the 2nd phase nursery culture) until 5-6 months old (approximately 20 – 50mm, 5 – 20g size).

Broodstock Management

Elsewhere, the sandfish broodstock are usually held either in FRP (fiber-reinforced plastic) raceways, concrete tanks or earthen ponds for spawning work (Figs. 1a-c). The COM's hatchery in Pohnpei, Federated States of Micronesia, uses freshly caught sandfish broodstock from nearby its hatchery a day prior to the spawning induction work without doing any conditioning work.

Figure 1a. Habitat simulators.

Figure 1b. Concrete tanks.

Figure 1c. Earthen ponds.

The sandfish habitat is characterized by a seagrass bed of the tidal flat along the mangrove-covered shoreline from low-tide line to 10 – 20m deep in subtidal zone with soft muddy or sandy substrate. Seagrass bed is characterized by turtle-grass such as *Thalassia* spp. or by eel-grass such as *Zostera* spp. in the Indo-Pacific region (Fig. 2a-b). It is said that stocking density of the sandfish grow-out in a pond is one or two animals per square meter and the broodstock may be stocked at 3 – 5 per m-2 in a tank for conditioning if they are provided good aeration, water flow (water exchange rate at 400% per day), ample feeding with periodic tank cleaning at least once a fortnightly or renewal of tank with fresh muddy sand substrate (Duy, 2011; Purcell et al., 2012).

A key technological innovation developed by the author is a land-based broodstock culture system with a "down-weller" or "habitat simulator" tank system. The system uses a combination of closed recirculating seawater and partial flow-through method, which enables a long-term holding and domestication of healthy broodstock for selective breeding programs rather than relying on wild-caught parents on each hatchery operation. The tank system holds 5 - 10 broodstock per m-2 by providing with good air, water circulation (100% daily water exchange) and enabled to feed without periodic tank cleaning or renewal of

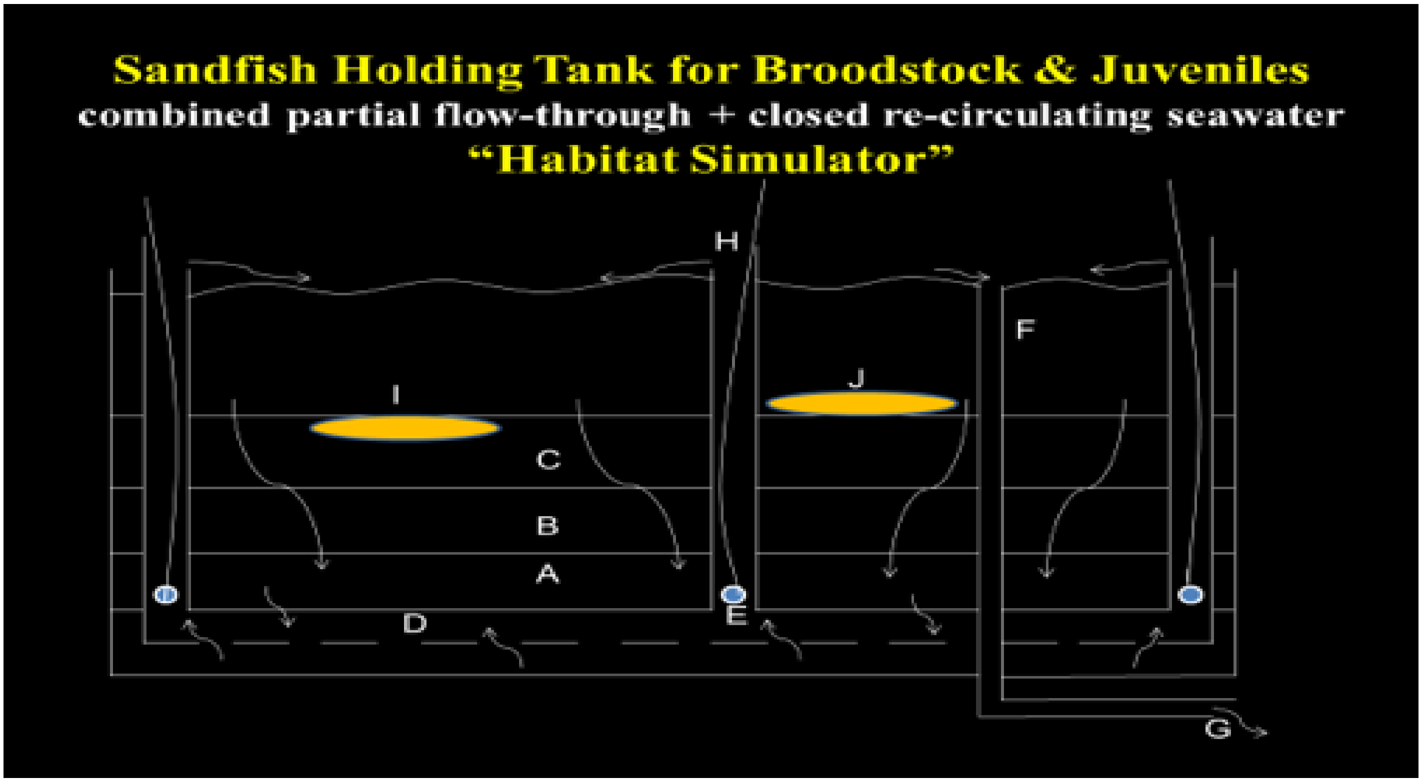


Figure 3. Diagram of down-weller "Habitat Simulator" tank. A: coral rock, B: coral gravel & sand, C: fine sand & silt, D: perforated pipe, E: air stone, F: overflow standpipe, G drain, H: airlift pipe, I & J: sandfish

tank. The down-weller was consisted of two or three layers of substrates to form a false-bottom structure; sand and mud, which are collected from tidal flat areas in the mangrove shore. Water re-circulates through the surface muddy and sand layer by air-lift pump which also maintains aerobic condition of the tank bottom and substrates (Fig. 3).

For quarantine purpose, an Australian private hatchery uses this tank system to prevent spreading potential disease among the wild-caught or domesticated broodstock (Fig. 4). Furthermore, this system has been used for recovering the spawners which had been injured or stressed during the transportation and/or spawning induction work. In Pohnpei of the Federated States of Micronesia, the COM built 2,500L rectangular tanks with down-weller system were made for broodstock recovering (Fig. 5) and juvenile grow-out (Fig. 6). Routine maintenance of the down-weller system for the broodstock is to: 1) avoid the macro-green algae (e.g. *Enteromorpha* spp.) over-grown on the tank surface; and 2) adjust aeration so as not to give strong air to drag too much seawater into the perforated piping system. To prevent green algae over-grown, use shade-screen to cut too much sunlight onto the tank. Continuously strong air-lift causes hardening the sandy substrates from strong downward water movement.

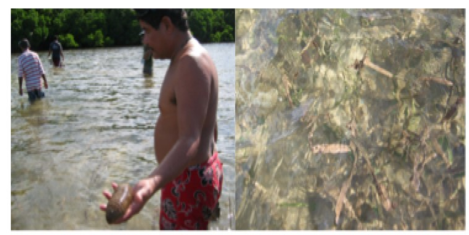


Figure 2a. Seagrass beds of turtlegrass (*Thalassira* species).

Figure 2b. Seagrass beds of eelgrass (*Zostera* species).

Figure 4. Down-wellers for commercial

Figure 5. Quarantine and recovery for broodstock in Pohnpei, Micronesia.

Figure 6. Down-wellers for juvenile grow-out in Pohnpei, Micronesia.

Figure 7a. Transporting in bags.

Figure 7b. Selecting broodstock for spawning.

1-1. Feeding broodstock

The broodstock in the “down-weller” tanks are fed daily with 1~2 % BW (body wet weight) per individual depending on the purposes; i.e. grow-out or quarantine at 1%, fattening or recovery at 1.5~2%. For practical reason, it is recommended to feed them weekly. The feed consists of dried alga Spirulina, fishmeal and seaweed of which ratio varies depending on the sizes and conditions of the animals; approximately 1 vs. 20 vs. 10. Mud/silt collected from tidal flat zone of the mangrove-covered shore is also included as an important food for both broodstock and juveniles. The amount of mud is equivalent to a total weight of other three foods. The amount of food should be adjusted by increasing or decreasing according to their average body weight. Therefore, the body weight needs to be measured at least monthly or bi-monthly. Feeding the broodstock in the Habitat Simulator can be done without a renewal of sandy and/or muddy substrates because this down-weller system itself contains organic matters such as seaweed and detritus. Currently with no mortality has been recorded by using with a combination of dried seaweed, fishmeal and mangrove-silt.

1-2. Transporting broodstock

When transporting broodstock for a long distance (4 hours or more) from wild habitat to the hatchery, the animals should be packed individually in a plastic zip-bag in a polystyrene box or ice-chest (Fig. 7a-b). It is better using ambient seawater the same water collected at the habitat and better inserting ice-gel pack (s) in the box to keep temperature at lower than 25 °C during the transportation. If the animals are found eviscerated (vomited the gut/internal organ), they should be removed from the spawners and held in the recovery-fattening tank for at least six months period for the next spawning work.

2. Microalgae Culture Management

For microalgae culture of benthic diatoms refer to "Trainer's manual for hatchery-based pearl farming" (Ito, 2005), "Development of pearl aquaculture and expertise in Micronesia" (Ito, 2006) or "A hatchery operations manual for rearing sandfish, *Holothuria scabra*, in Tarawa, Republic of Kiribati." (SPC, 2015). Detailed descriptions of the microalgae culture management and techniques had been written by the author during the COM Land Grant Program's pearl project in 2001-2013, which offered basic but practical knowledge on the microalgae culture in the tropical conditions.

2-1. Precaution for microalgae culture

- soak in freshwater and wash with detergent, brushing off dirt/wastes. Although it is not always necessary, hydrochloric acid (5 - 10% HCl solution) can be used for cleaning flasks by soaking when the dirt is difficult to clean off. Collect the used hydrochloric acid in a glass bottle for re-use.
- rinse with freshwater 5 - 10 repeats, completely wash off residue of detergent or chemicals.
- dry flasks upside down and avoid air-born dirt inside the flask.
- spray alcohol (isopropyl-alcohol or ethanol 75 % solution), rinse with distilled /filtered rainwater, and wait for dry upside down.
- put the lid on (aluminum foil) or place them in a dust-free cabinet for longer storage.
- rinse with filtered (0.2 µm or 1µm) seawater and, if available, UV-sterilized seawater before use.
- make sure washing your hand, particularly dirty finger nails and oily fingers, with soap and rinse off any residue of soap/chemicals, and then spray alcohol before commencing work.
- spray alcohol on the surface of culture flasks/containers/fittings/ working bench when entering the room.
- keep the floor and bench clean and dry and, if necessary, clean a floor with chlorinated freshwater.
- soak your feet in the chlorine bath before stepping into the room.
- periodically check and clean air filter/air outlet of air pump, air-conditioner and ventilator.

- always keep the room door/windows closed and avoid unnecessary entry into the room.

The hatchery staff tend forget general precautions for the microalgae culture work and how to properly operate the autoclave. Usually, hatchery operation elsewhere uses 121 °C for 45-60 minutes for larger flasks such as 3~5L high density culture, and small 100~250mL flasks for stock culture are sterilized for 10-15 minutes at 121°C. Periodical maintenance of autoclave is also necessary by changing or refreshing water in the chamber.

2-2. Culture methods for the sea cucumber hatchery

Sea cucumber hatchery work involves microalgae culture of planktonic and benthic diatoms. The author also uses mud/silt collected for the tidal flat zone of the mangrove shore for feeding the settled pentactula and early juvenile stages as well as broodstock. This kind of mud contains nutrient rich, particularly Omega-3 (ω3) fatty acids, derived from heterotrophic algae (micro-organisms).

Live microalgae are not required for feeding broodstock (adults) of the sea cucumbers. During the larval and post-larval rearing, however, live and/or dried microalgae are used toward settlement stage (pentactula stage) and after settlement to juveniles. The author simplified feeding methods for the larval rearing to reduce workload of culturing live microalgae. With higher survival rate at 30 - 40 % from day-1 to the settlement stage, the author has been using a single live planktonic diatom species of *Chaetoceros muelleri* together with dried form of microalga, Spirulina sp. For settlement phase and post-settlement rearing of juveniles, the author developed to use two kinds of live benthic diatoms (*Navicula* sp. and *Coconeis* sp.) by combining with dried Spirulina during the pentactula and early juvenile stages and during the juvenile stage by combining fishmeal, seaweed, Spirulina and tidal flat mud. Note that there are eight types of benthic diatoms and *N. ramosissima* (Type-A benthic diatom) and *C. scutellum* (Type-B benthic diatom) are commonly used at abalone hatchery for post-settlement juvenile culture in Japan (Kawamura, 1998). The author has been using two types as live epiphytes on the settlement substrates for the sea cucumbers, such as *N. jeffreyii* for type-A and *Coconeis* sp. for type-B. Master stock culture of these benthic diatoms can be purchased commercially such from Commonwealth Scientific Industrial Organization (CSIRO) in Australia or elsewhere. Culture media of these benthic diatoms or naturally occurring epiphytes are same as planktonic diatom such as *C. muelleri* with nutrient media strength varies from 1/100th to 1/10th. Starter high density (3L - 5L flasks) & mass culture (20Lcarboys - 100L polycarbonate tanks) are used for the above three diatom species. For these benthic diatom culture techniques and work plan, refer to Chapter 4 (Larval Rearing) and 5 (Settlement Techniques).

For specific knowledge of heterotrophic algae, refer to some of many publications such as "*Schizochytrium limacinum* sp. nov., a new thraustochytrid from a mangrove area in the west Pacific Ocean" (Honda et al., 1998), "Fatty acid composition and squalene content of the marine microalga *Schizochytrium mangrovei*" (Jiang et al., 2004), "Effects of dried algae *Schizochytrium* sp., a rich source of docosahexaenoic acid, on growth, fatty acid composition, and sensory quality of channel catfish *Ictalurus punctatus*" (Li et al.,2009), and "Heterotrophic cultivation of microalgae as a source of docosahexaenoic acid for aquaculture" (Taberna, 2008).

Figure 8a. Cold water treatment using ice cubes.

Figure 8b. Cold water treatment in algae room.

Figure 9a. 1ppm iodine bath.

Figure 9b. Disinfecting (1 min.)

Figure 9c. Rinse with freshwater.

Figure 10a. Thermal shock.

Figure 10b. Gently stirring.

Figure 10c. Siphoning droppings.

Figure 11a. Sp*irulina* bath (12g/60L seawater)

Figure 11b. Monitoring water temp.

Figure 12a. Spawning.


Figure 13a. Collecting eggs.


Figure 13b. Sampling for counting eggs.


Figure 13c. Cutting broodstock.


Figure 13d. Stripping gonads.


Figure 14. Incubating eggs in 1,000L

3. Spawning Induction

Spawning induction work involves; conditioning and disinfection of spawners; inducing by stimulations or stressing such as exposing to the air, changing water temperature, water pressure and/or salinity, and chemical or food; fertilization and washing of eggs; and collection, sampling, counting and incubation of eggs (see Figs 8-14). When using a 2,500L tank for a small-scale juvenile production work, about 50 - 60 broodstock (spawners) are used for single larval run. Prior to spawning induction work before transferring from cold water treatment to spawning tank, all the spawners are disinfected by iodine, in which the animals were immerged in 1 ppm iodine bath (freshwater) for 1 minute. Spawning induction are usually done by: 1) stress by handling with exposure to the air, 2) thermal shock from cold (20-22 °C) to warm water (32-34 °C), 3) chemical stimulation by dried microalga Spirulina (20g/100L) in seawater for 30 minutes, 4) changing water pressure (decreasing/increasing water level), and/or changing salinity (decreasing salinity to about 30 ppt.

Collection of the spawned eggs are usually two-step approaches; 1) the first batch by scooping the spawned eggs by beakers and 2) the second batch by draining spawning induction tank. For a small-scale work, the former method is better to obtain cleaner with enough number of eggs. This also requires careful and continuous observation of female spawning posture. Noticeable change is observed in gonophore shape by swelling outwardly. Therefore, swift and timely scooping actions to collect eggs are required. If the latter method is used, collection of eggs should be commenced soon after several females spawned before the spawning tank becoming cloudy from too many sperms.

For incubating the fertilized eggs, stocking density should be less than 10 eggs per mL. Seawater is filtered to 1 µm by using filter-bags or cartridge filters, which does not necessarily required sterilization by in-line UV sterilizer unless virus infection or other disease has been reported from the surrounding environment. A combination of plankton screen (50 µm and 80 µm or 90 µm) is essential for collecting eggs.

3-1. Spawning procedures

The following describes timeline of spawning induction work which is based on a combination of 1) physical stress; 2) exposure to air, 3) thermal shock from cold to warm water, 4) chemical (dried algae Spirulina-bath) and/or 5) changing water pressure.

- Start preparing boiled seawater in deep pan (20-40L) using firewood (or use immersion heaters in the spawning tank) to maintain the induction tank water temperature at 33-34 °C.
- Cleaning off dirt from the body surface, measuring body weight (BW) and selecting spawners to be at least 200g, so the smaller ones should be returned to the broodstock holding tank. Before transfer to a cold water, quickly rinse with filtered seawater.
- Prepare cold seawater (1 µm filtered) beforehand, in the preceding day by placing it in the algae room. Transfer spawners to 100L cold treatment tank at about 20-22 °C and keep them for at least 2~3 hours, preferably for overnight.
- Transfer spawners to iodine (freshwater) bath at 1ppm of iodine (or 100ppm of Betadine®*) for 1minute (= 60 seconds). *Betadine® contains 1%W/V iodine. Therefore, 1g Betadine contains 0.01g iodine. To make 1ppm iodine solution (or 0.1g iodine per 100L), add 10g Betadine® per 100L to make 1ppm iodine (freshwater) bath.
- Start spawning induction work immediately after the iodine bath, rinse off iodine with filtered seawater and transfer to the spawning tank (2,500L raceway with approximately 1,000L water volume) at 33-34°C.
- Wait spawning (male and female) for at least an hour and keep cleaning droppings on the tank floor by siphoning. Use a plunger to stir gently spawning tank water and keep mixing the warm water.
- If the spawners dose not respond to the above thermal shock, transfer them to "Spirulina bath" for 30 minutes at 12g of Spirulina in 60L of filtered seawater. Spirulina is dissolved faster and better in freshwater (or rainwater), so prepare 12g Spirulina in about 500mL rainwater before making 60L seawater solution.
- Rinse off the Spirulina with filtered seawater and introduce them again to the spawning tank.
- Wait for the spawning. Males usually spawn before females release eggs.
- Observe spawning posture of female(s) and scoop the eggs with beakers when the female releases the eggs. If excess eggs are needed after confirming the females finished spawning, drain the spawning tank to collect remaining eggs inside the tank. Use a combination of 50 and 80 µm-pore size mesh screen to collect and wash the eggs. If no female responded after two hours, return all the spawners to broodstock holding tank.
- After washing/rinsing off sperms for 10-20 minutes, transfer the eggs into a 20L bucket to make 15L volume of 1 µm filtered seawater.
- Take at least two samples of 2mL volume while stirring the bucket by a plunger.
- Count the eggs under microscope with an aide of Rafter Counting Chamber and estimate total number of eggs obtained. While counting the eggs, check the fertilization by confirming the 1st polar body or more advance embryonic development such as 2-cell stage, 4-cell stage, and so on.
- Stock the eggs in incubator tanks, maximum stocking density of the eggs being 10 eggs per mL.

3-2. In vitro Fertilization (Gonad Stripping Method)

At present, a method using thermal shock with or without Spirulina bath treatment has been effective, but it has not always resulted in 100% success rate of the sea cucumber spawning induction work. Sooner or later, it is inevitable to develop an effective method for spawning both males and females. A Japanese group of scientists (Kato et al., 2009) found that neuronal peptides induced oocyte (ovum) maturation and gamete spawning of the Japanese sea cucumber *Apostichopus japonicus*. They extracted the neuronal peptides and so synthesized it chemically, which was effective to mature 150 µm diameter or larger eggs. They also experimented to inject this synthesized hormone into the body cavity of the sea cucumbers, resulting the male and female spawned 60 minutes and 80 minutes later, respectively. Unfortunately, they did not describe how far the eggs developed as embryos and whether their hatchery work went through to settlement as pentactula stage. Therefore, no information was available from their study for fertilization and hatching rates as well as survival rates during the larval and post-larval rearing works. They also stated that this chemical did not work effectively on immature ova and, thus, they concluded that the maturation mechanisms and process of ova/spermatozoa still needed further studies. Synthesizing and producing such a neuro-hormone commercially could be very expensive and won't be available for the Indo-Pacific region in foreseeable future. The important fact is that developing techniques of artificial maturation of oocytes (ova/spermatozoa) and activation of gametes (sperms) are the keys to success in obtaining the fertilized eggs. In this end, a gonad stripping method* could be an alternative to spawning induction work near future, either using synthesize neuronal hormone or other chemicals such as ammonia-seawater which has been used for commercial pearl oyster hatcheries in Japan, or just use of natural seawater.

**Note that the gonads are removed from the parent animal by cutting a small portion of the body and the gametes are obtained by stripping/squeezing the gonads (Fig. 13c-d). This is called "gonad stripping method".*

Although no one has been successful for in vitro fertilization of the sea cucumbers, the author thought that it was worthwhile for the hatchery technicians to understand principle and procedures of this method. During the hatchery training workshop in May 2015 at the Fijian Government's hatchery in Galoa, the author used filtered seawater (1 µm nominal pore) without using any other chemicals for the gonad stripping method for the sandfish (Ito, 2015). As a result, fertilized eggs subsequently underwent embryonic and larval development to the settlement as pentactula stage. Although the number of eggs and resultant pentactula were very small, several hundred, and low survival rate at less than 10 % to day 11, this method may be economical and could be the first step towards future improvements for a large-scale juvenile production on a regular basis.

4. Larval Rearing

Larval rearing of the sandfish sea cucumber requires knowledge of feeding capability of larval stages, suitable combination of food, calculating amount of feed mix, controlling algal cell (feed mix) suspension, adjusting feeding amount and rearing water volume, water quality control, and knowledge of optimal larval development in changing proportions of larval and post-larval stages; i.e. from hatching as auricularia stage to settlement as pentactula stage. Approximately 18-24 hours after spawning depending on water temperature, larval rearing work commences by draining the incubator to collect gastrula and/or auricularia larvae. For collecting larvae and post-larvae, a combination of 80 µm and 100 µm mesh screen is to be used. Larval rearing tanks should be completely drained every other day, on days 1, 3, 5, 7, 9 and 11. The larval specimens need to be kept alive but immobilized/anesthetized by isopropyl alcohol for counting, measurements and microscopic photographs. For longer term preservation, use formalin (10% seawater formalin). Water temperature in the larval rearing could be better between 27-30 °C.

Hatchery facility should be maintained good conditions in terms of hygiene and efficiency for larval and post-larval rearing work:

- animals such as cats need to be kept away in- and out-side the hatchery, around the indoor and under-cover tanks and indoor storage areas;
- microalgae culture room should not be a sort of storage room with scattered lab supplies and equipment on the culture benches, dusty air-conditioners and air pumps without filter maintenances;
- air supply system needs to be functioning effectively, with sufficient air pressure in the under-cover areas as well as in the microalgae culture room;
- in-use or used tools should not be scattered on the floors, e.g. filter cartridges, filter-bags, hoses, pipes, buckets, air-stones, airlines, pipe-fittings, nets, plumbing machines; air leaks from many outlets with unnecessary accessories and fittings;
- sea water and freshwater supply piping are better to be simple and do not need unnecessary connections, diversions and outlets fittings;
- the hatchery staff understand general hygiene procedure before and during the hatchery operation.

4-1. Precautions before, during and after working at hatchery

For precautions of the onsite hatchery work, refer to "Trainer's manual for hatchery-based pearl farming" (Ito, 2005) or "A hatchery operations manual for rearing sandfish, *Holothuria scabra*, in Tarawa, Republic of Kiribati." (SPC, 2015). The following were also described in those manuals.

- make sure your hands are clean. Wash your hand with soap, particularly dirty finger nails, before starting work.
- Soak your feet in "chlorine-bath" before entering in the larval and/or microalgae room.
- don't work with you own shoes. Always ware designated boots or work with barefoot.
- always rinse again the cleaned and dried equipment with filtered rainwater (1 µm) before use.
- for the used equipment/tools, wash first with chlorinated (public) water to wash out the waste/dirt.
- second-wash by using detergent and wash-off the dirt thoroughly with a soft sponge or brush.
- rinse with 1 µm-filtered rainwater and completely wash out residual soap/detergent.
- soak in chlorine-batch (a diluted Sodium Hypo-chloride, NaHClO) for overnight. Don't mix with soap or this may release Cl2 (chloride gas).
- rinse completely with filtered rainwater (1 µm). Make sure "no residual chlorine".

Figure 15. Seawater filters and UV sterilizer.

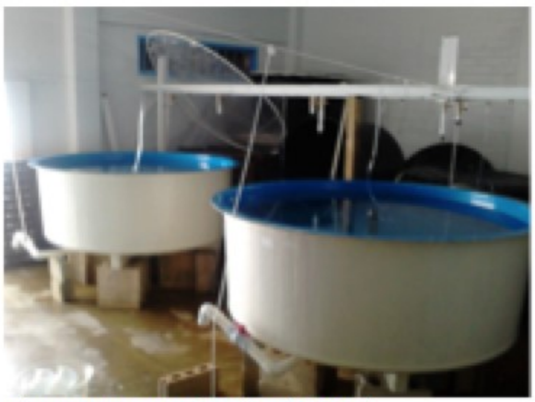


Figure 16a. Larval rearing (2,000L).

Figure 16b. Larval rearing (5,000L).

Figure 17. Sieves for collecting larvae.

- always hang and dry the equipment after being cleaned. Do not leave them on the floor or dirty bench.

- if necessary, use isopropyl-alcohol spray (75 % solution) and wait for it to dry. *Note that the use of methanol (methyl-alcohol) will become a health hazard in a small microalgae culture room.

- don't touch inside of the cleaned surface of equipment and tools such as bucket/ container/tank/tub/flask, etc.

- wash filter bags, cartridges and housings after every use. Wash out the dirt with pressurized freshwater, filtered rainwater (1µm), soak in chlorine-bath, rinse with filtered rainwater (1 µm) and dry them on a designated bench. Keep the filter bags, cartridges in sealable plastic bags each with alcohol-sprayed inside. For the filter housings, spray alcohol inside and store them upside-down on the bench.

- make sure always clean the floor; wash with freshwater (chlorinated town-water or rainwater). It is the best that the floor is a "dry" condition when you start working in the following morning.

- don't disturb animal (larvae/juveniles/broodstock) and minimize giving shock or stress to the animals. Avoid unnecessary entry to the microalgae culture room and larval rearing unit.

4-2. Preparation for the larval rearing

The seawater for the larval rearing should be filtered to 1µm with a bag filter or cartridge filter. In-line UV-sterilizer is not necessarily required (Fig 15). When the day-1 larvae exceeded 0.35 larvae per mL in a rearing tank, the stocking density should be adjusted to make acceptable number of larvae in each tank: e.g. between 0.5 - 0.7 million larvae in a 2,000L tank or 1.25 - 1.75 million larvae in a 5,000L tank (Figs. 16). Gentle aeration is given throughout the larval rearing and the tank must be protected by a lid (tank cover) from debris from the ceiling. If the stocking density is less than 0.25 larvae per mL, the rearing water volume must be adjusted (reduced) to maintain required range of algal cell suspension based on *C. muelleri* culture density and number of larvae in each day. A combination of sieves is usually 80/100 µm throughout larval rearing and each sieve must be deep and wide enough (30 cm deep x 50 cm wide) to do sieving efficiently from a larger diameter drain pipe e.g. 25.4 cm (2 inch) pipe (Fig. 17).

Apart from tools and equipment, preparation of the live microalgae species (*C. muelleri, Navicula sp.* and *Cocconeis* sp.) need to be cultured at least two weeks before commencing spawning and larval run. For continuous culture and feeding the larvae, several subcultures should be made after commencing larval rearing, instead of starting from new stock culture (Figure 18). Larval rearing period with microalga *C. muelleri* feeding would finish within two weeks on day-14 after spawning. When a hatchery operation is planned a single spawning0larval run, therefore, it is not useful to start any new culture of *C. muelleri*. Generally, 7 days needed for a 2 - 5L high density starter cultures to be ready for starting 20 - 100L mass cultures, and these mass culture needs further 4 - 5 days to use for feeding the larvae. The author uses dried microalga Spirulina to mix with live diatom *C. muelleri* for larval and post-larval rearing. *Navicula* sp. and *Cocconeis* sp. are used for feeding settled pentactula and early juvenile stages up to two months old in settlement tanks. If live microalgae are used for sea cucumber hatchery, therefore, it is necessary to culture diatoms.

Figure 18. Microalgae (diatoms) for feeding larvae and post-larvae.

4-3. Feeding methods for larval rearing

Mixture of *C. muelleri* and Spirulina are given for feeding the larvae twice a day. Feeding is maintained by estimating algal cell suspension in each larval tank, starting from approximately 1,000 up to 20,000 cells per mL during the two-weeks larval rearing. Total cell suspension in each day is attained by combining these two feeds and expressed as the number of *C. muelleri* cells, where amount of Spirulina is computed and expressed as the number of *C. muelleri* cells. Feeding ratio of *C. muelleri* and Spirulina is approximately 80 % and 20 %, respectively. Feeding is divided into two; one in the morning between 8 and 10 AM, and the other in the late afternoon between 4 and 6 PM. The author developed daily feeding tables with simplified data-inputting methods, therefore, the hatchery staff are usually trained to use such feeding tables.

A hatchery protocol with daily work schedule for larval rearing was summarized and shown in Table 1. The hatchery staff needs to count the number of living larvae and post-larvae after tank draining every two days and *C. muelleri* to input daily culture density (million cells per mL). The feeding amount of both *C. muelleri* and Spirulina are obtained instantly in those feeding tables. All feeding amount were converted and expressed as *C. muelleri* cells. For the Spirulina, the hatchery staff simply measures the dry-weight according to the feeding table, prepare for AM or PM feeding amount, dissolve in the freshwater (about 500 mL) and wait for an hour before adding to the rearing tanks. It is advised the stocking density of larvae from onset of larval rearing on “day 1” to be between 0.25 - 0.35 larvae per mL.

Table 1. Hatchery protocols for juvenile production of the sandfish sea cucumber.

HATCHERY PROTOCOL **(WORK SCHEDULE)** **days of run (size.; µm)** **Algal cell suspension** **(as *C. muelleri* cells mL^{-1})**	***larval & post-larval development based on the water temperature at 29 ± 1 °C** ***larval rearing tank (LR) = 1 x 5,000L** ***settlement tank (ST) = 4 x 5,000L (bottom area = 5,000 m^2); settlement plates=50cm x 50cm x 800~1,000 plates per 5,000L tank** ***plates = corrugated plastic plates** ***juvenile down-weller tank (JHS) = 4 x 10,000L (bottom area = 10,000 m^2); sandy bottom covered with mangrove mud (dried mud sieved through 100 µm screen)**
-3d before spawning	Start conditioning settlement tanks and plates; add benthic diatoms (i.e. *Navicula sp.* & *Cocconeis sp.* 200L each in 5,000L settlement tank) & nutrient (100% strength) to culture in the settlement tanks
0 (egg: 150-160)	Collecting (sieves 50 & 80 µm mesh screen), washing, counting & incubating eggs (up to 50 million eggs per 5,000L incubator)
1 (420 x 320) 900-1,500 cells/mL	Draining incubator & collecting gastrula & early auricularia (sieve 80 & 100 µm) approx. 20 hours after fertilization; sampling, counting & stocking to larval rearing tanks (1.5 million larvae in 5,000L tank); start feeding larvae with live microalga *Chaetoceros muelleri* and dried microalga *Spirulina* sp. as soon as stocking larvae.
2 - 4,000-7,500 cells/mL	
3 - 7,000-12,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% Water Exchange); early auricularia (20%) + mid-auricularia (80%)
4 - 8,000-14,000 cells/mL	
5 (800-1000) - 8,500-15,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); mid-auricularia (20%) + late-auricularia (8
6 - 9,000-17,000 cells/mL	
7 (800-1200 x50-80) 9,500-18,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); late-auricularia + fully developed late auricularia; remove settlement plates to dry; drain settlement tanks & refill the settlement tanks with new benthic diatoms (200L each in 5,000L tank) & nutrient (1/20th strength)
8 - 10,000-20,000 cells/mL	Spray *Spirulina* on the settlement plates (*Spirulina* 30g per liter freshwater solution) & dry the plates
9 - 8,000-18,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); late-auricularia + fully developed late auricularia + post-late auricularia (= metamorphosing to doliolaria) + doliolaria (~ 5%)
10 - 6,000-16,000 cells/mL	Return the plates to the settlement tanks
11 (400-1200 x 60-80) 4,000-13,000 cells/mL	Larval tank draining (80 & 100 µm), collect larvae & post-larvae & transfer them to the settlement tanks; late-auricularia + fully developed late auricularia + post-late auricularia + doliolaria + pentactula; *survival rate from day-1 = ave. 30% (approx. 0.5 million each in 5,000L settlement tanks)
12 - 2,000-6,000 cells/mL	*feeding only with *C. muelleri* for the swimming stages (no need of using *Spirulina*)
13 - 500-2,000 cells/mL	*feeding only with *C. muelleri* for the swimming stages (no need of using *Spirulina*)
14 - 200-1,000 cells/mL	Finish using *C. muelleri* & *Spirulina* for feeding larvae; fully developed late auricularia + post-late auricularia + doliolaria + pentactula
15 (~ 1mm)	Start flow-through over 24 hrs. 100% WE; (inlet water = 1 µm filter & outlet water = 100 µm screen); post-larvae; Start feeding daily with 3 foods (fishmeal 5g + seaweed 5g + mud 12.5g) per 100,000 post-larvae; *Sieve mud, fishmeal & seaweed with 100 µm screen for feeding; *no need of giving *Spirulina* as the settlement plates still covered with *Spirulina*
21 (1 ~ 2mm)	flow-through 24 hrs.100% WE; (inlet = 1 µm filter & outlet = no screen); continue daily feeding at 0.25% of BW with 3 foods (fishmeal 10g + seaweed 10g + mud 25g) per 100,000 early juveniles; *sieve feed-mix with 100 µm screen for feeding
28 (1-mo) (2~5 mm, 0.01~0.1g)	flow-through 24 hrs.100% WE; start daily feeding at 0.25% of BW with 4 foods (*Spirulina* : fishmeal : seaweed : mud = 1 : 2 : 2 : 5); *start giving *Spirulina*; *settlement success rate from day-11 to 1-mo = 20-50%
30	Start weekly feeding at 0.25% of BW with 4 foods (*Spirulina*, fishmeal, seaweed & mud)
Day **35**	Continue weekly feeding
Day **42**	Continue weekly feeding
Day **49**	Start transferring juveniles (2-mo = ave. 1g BW, 10-20mm BL) from settlement tank (ST) to down-weller “habitat simulator” for juveniles (JHS);
Day **56** (2-mo = J2) (0.2~2g)	*increase proportion of fishmeal and seaweed (Spirulina, Fishmeal & Seaweed = 1 : 2.5 : 2.5); *stocking density of JHS = approx. 10,000~1,500 x J2 per m^2 (= 10,000~15,000 xJ2 in a 10,000L JHS tank);

(Example 1)

If the rearing tank volume is 2,000 L (=2,000,000 mL),
C. muelleri culture density is 3.0 million cells/mL, and
today's total amount of *C. muelleri* required is 2,000 mL

"total *C. muelleri* cells in the rearing tank" =
"culture density of *C. muelleri* " x " today's required amount of *C. muelleri* "
(= 3 million x 2,000 = 6.0 billion cells)

Therefore, today's " *C. muelleri* cell suspension " in the 2,000L tank
= 6 billion cells / 2,000,000 mL = 3,000 cells/mL

(Example 2)

If today's required total feeding amount of larva is 12,000 cells/larva/day,
number of larvae in 2000L tank is 500,000,
today's required algal cell suspension is 3,000 cell/mL, and
today's algal culture density is 3 million cells/mL

"required total algal cells in the 2,000 L rearing tank"
= 12,000 (cells/larva/day) x 500,000 (larvae) = 6 billion cells
*algal cell suspension in this 2000L tank = 6 billion cells / 2 million mL = 3,000 cells/mL

Therefore, today's "total amount of algae" = 6 billion cells / 3 million cells/mL = 2,000 mL

For feeding the larvae, the hatchery staff must take algal samples (= *C. muelleri*) and counts the culture density; input each counting results into the designated cell in the MS Excel spreadsheet ("HIROITO CONSULTING's Sandfish Feeding Tables"), of which table automatically calculates the daily (in the morning-AM and afternoon-PM) feeding amount of both *C. muelleri* and Spirulina. "estimated available food per larva as CM" is based on the past results of larval feeding experiments of the sandfish and other sea cucumber species by the author and others in Japan and elsewhere, which ranges from 3,000 to 60,000 cells/larva/day.

Note that a commercially available dried alga Spirulina contains certain amount of dried form of cells (e.g. 2.6 billion cells per 1 g). The author found about three different sizes (e.g. 10 µm x 15 - 50 µm) and shapes (e.g. rectangular). Average size of *C. muelleri* is about 10 x 5 µm. 1 g of Spirulina cells are approximately to 42.8 billion *C. muelleri* cells. Therefore, it is necessary to estimate approximate volumes of those different sizes of Spirulina cells and to convert to the number of cells to *C. muelleri.* This is because total feeding amount is expressed as the number of *C. muelleri* cells and the feeding ratio (based on the number of cells) of *C. muelleri* vs. Spirulina is 80 % vs. 20 %.

4-4. Example of two spawning-larval runs in September 2017 at COM's Nett Point Hatchery in Pohnpei, FSM.

During the work, 1.4 million eggs (trial 1) and 5.3 million eggs (trial 2) were stocked in the 1,000L incubators. Approximately 20 hours after spawning, each yielded 0.42 million larvae (trial 1) and 2.5 million larvae (trial 2). As the two 2,000L round tanks were needed each with approximately 600,000 larvae for the trial 2. The followings were the results of larval runs between day 1 and day 11:

- trial 1 at survival rate of 10.9 % with 63,750 swimming stages on day 11 including late auricularia, doliolaria and pentactula from 416,250 larvae on day 1;
- trial 2-1 with 67,500 from 620,625 at survival rate of 15.3%;
- trial 2-2 with 221,250 from 620,625 at survival rate of 35.6%

Average survival rate of the three larval runs was 20.6% from day 1 to day 11. Total 353,500 larvae and post-larvae were transferred to three 2,500L settlement tanks.

4-5. Larval Development

Detailed morphological descriptions of the complete larval and post-larval development for both sandfish (*H. scabra*) and black teatfish (*H. whitmaei*) were given in Appendix 1 with notes on larval growth and durations in Appendix 2. Comparisons of morphological features of larval and post-larval stages of these two holothurian sea cucumbers were also given in Appendix 3.

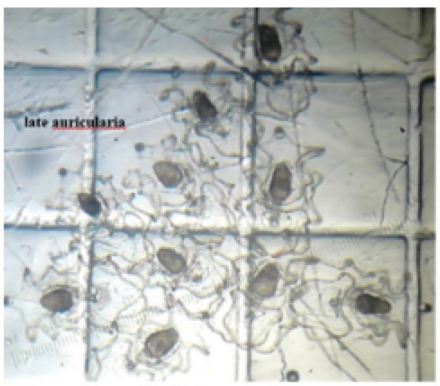


Figure 19. Late auricularia.

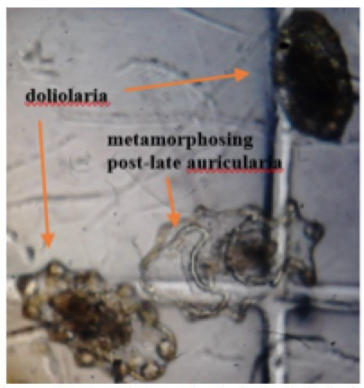


Figure 20. Post-late auricularia & doliolaria.

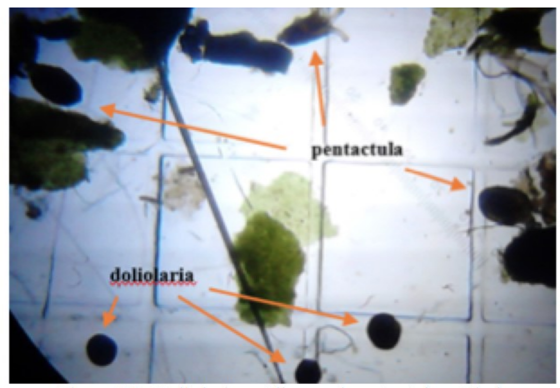


Figure 21. Doliolaria settling and pentactula settled.

5. Settlement Techniques

Settlement techniques involves conditioning of settlement plates and tanks, culturing benthic diatoms. The pentactula generally settles on the sandy sea grass bed such as *Talassira* spp. (for sandfish) in tidal flat zone, coralline gravels and rocks in the intertidal or littoral zone (e.g. black teatfish, greenfish). In the hatchery, the pentactula settle anywhere in the tank and so corrugated plastic plates are usually used as settlement substrates. Surface of settlement substrates such as plastic plates and inside the tank are conditioned with dried alga Spirulina and/or with epiphytes either naturally occurring or cultivated. The author uses two species of benthic live diatoms (*Navicula* sp. & *Cocconeis* sp.) as epiphytes on the settlement substrates. On day 11, larval rearing tank was drained and all swimming stages including fully developed late auricularia, metamorphosing post-late auricularia and doliolaria were collected onto a 100 µm screen (Figs. 19-20). A small percentage of pentactula up around 5% (Fig. 21) might have also settled on this day, so soft brushes were used to collect those settled individuals. Samples were taken for counting to estimate number of living individuals of all stages. Then, they were transferred to settlement tanks.

Table 2. Dimension of settlement plates used at COM's Hatchery in Pohnpei, FSM.

Settlement Plates (type)	Length (m)	Width (m)	Surface Area - both sides (square meters)
Large A (LA) =	**0.65**	**0.44**	**0.57**
Large B (LB) =	**0.44**	**0.44**	**0.39**
Medium (M) =	**0.39**	**0.33**	**0.26**

5-1. Conditioning settlement substrates.

Conditioning settlement plates and tanks should commence at least two weeks prior to settlement. To start culturing benthic diatoms *Cocconeis* sp. and *Navicula* sp. in the settlement tank, 2,500L tank for example, 100L each of mass-cultured those diatoms and a full strength of nutrient media (i.e. medium F2 in Australia and Fiji, OFCF/JICA's medium in Kiribati, MI medium and Kent's F2 media at COM in FSM) together with sodium meta-silicate 15 - 30 g were added to the 2,500L tank. About 10 days after the initial diatoms culture, the settlement plates were removed for drying and the tanks were drained to renew the culture water (1 µm filtered seawater) and to refill with new benthic diatoms (100L mas culture each) and 1/20th strength of nutrient media. After a couple of days for drying, the settlement plates were sprayed (or painted) with Spirulina (30g/L solution) and keep drying for a day or two (Figs. 22-23). On day 10, a day prior to transfer larvae and post-larvae to settlement tanks, return the Spirulina-sprayed plates in the settlement tanks. During the work at COM's hatchery in 2017, three different sizes of corrugated plates were used for settlement (refer to the table 2 of plate's dimensions). In a 2,500 L settlement tank, 300 - 400 plates of the type-M are mainly deployed. The plates were washed by detergent, disinfected by chlorine and dried for a couple of days before growing benthic diatoms on the plates. For larval rearing with a 2,000L tank, two of 2,500 L rectangular tanks (raceways) were used for settlement of doliolaria to pentactula, approximately up to 100,000 pentactula in each settlement tank (Fig. 24). On day 11, about 200,000 swimming stages, which consist of feeding and non-feeding stage of late auricularia, post-late auricularia and doliolaria, are expected in each 2,000L rearing tank out of 600,000 larvae on day 1.

5-2. Transferring swimming and settled stages to settlement tank.

Onset of the settlement (on day 11) is called "the 1st phase nursery culture", which continues for about 2 - 3 months until the juveniles of 1- 2 g body weight (BW in wet weight) in average are transfer further to a down-weller "juvenile habitat simulator" tank or bag net "hapa" for a pre-growout or "the 2nd phase nursery culture".

Living individuals on day 11 are usually consisted of the settled pentactula stage (10 - 15%), transitional doliolaria stage (20 - 25%), metamorphosing late auricularia = post-late auricularia stage (30 - 40%), fully-developed late auricularia (20 - 30%) and late auricularia stage (10 - 20%). It is better to transfer procedure when the proportion of settling or settled animals (50% < post + dolio + penta) occupy more than a half in the larval rearing tank. The larval rearing tank is drained to collect all the animals onto 80 µm and 100 µm sieve. Collected specimens should be poured over the plates in the settlement tanks.

Figure 22. Spraying *Spirulina*.

Figure 23. Drying plates after spray.

Figure 24. Settlement tank.

Toward day 11, all the settlement substrates (plates and tank surface) should be covered with light brown -colored benthic diatoms. If sunlight is too strong, the settlement tanks will be dominated by green alga (e.g. *Enteromorpha* spp.). In that case, the tanks should be covered by shade-cloths (75-80% shading rate). If pink- colored organisms (pathogenic bacteria *Pseudomonas* spp.) began visible after a week of benthic diatom culture in the settlement tank, those plates, tank surface and fittings such as air stones, air tubing, PVC pipes or ropes must be removed from the tank and disinfected by chlorine or discarded all or part of them to avoid further infection of the pathogenic bacteria. Such infection is always caused by inexperienced hatchery technician's careless setting of the settlement tank and plates, poor skill of culturing microalgae and larval rearing.

After day 11, the larval rearing continues in a static water condition because there are still swimming and feeding stages of auricularia larvae (late auricularia, fully developed late auricularia and metamorphosing post-late auricularia). Therefore, it is necessary to provide microalga *C. muelleri* until around day14. From day 15, the rearing water in the settlement tank should be switched from static to flow-through, continuously flowing in from filter bag and flowing out to overflow-drain pipe at 100 % water exchange rate over 24 hours. 100 μm screen is used to cover the drain outlet for minimize loss of swimming stages (Fig. 25).

5-3. Examples of juvenile productions using the settlement plates.

The measurements of the settlement tank (ST), settlement plates (LA, LB, M), sample plates showing surface areas (in square meters) and estimated number of juveniles were given in the following tables 3 and 4: 30 small sample plates, 0.1m x 0.1m corrugated plastic, were also made for estimating settlement on the tank surface sides and bottom. Total of 79,180 one-month-old juveniles (on day 28, J1W1) were estimated to settle in three tanks (Figs. 26-27).

6. Pentactula and Juvenile Rearing

Pentactula and the early juveniles settled on the plates or tank surface are given additional food daily with a mixture of Spirulina, fishmeal, seaweed and mud. The mixtures of these food are homogenized and passed through 100 μm mesh screen before feeding. The juveniles on the plates and tank surface are kept in the settlement tank for two or three months and then the juveniles are transferred to "the 2nd phase nursery culture" either in the down-wellers (or habitat simulators) for juveniles or hapas bag net. The 2nd nursery culture continues further two or three months before being transferred to grow-out farm/ ponds/ enclosure. In Australia, a large-scale private farming enterprise does not conduct "the 2nd phase nursery culture", except for selective breeding programs, but those grown juveniles of average 2 - 5 g BW from the settlement tanks are also transferred to ocean grow-out sites (Fig. 28-29).

Culture techniques of pentactula and early juvenile stages in the settlement tank (the 1st phase nursery culture) from day-11 to day-56 or 8 weeks after spawning (approximately 2-month-old juveniles of 10 – 20mm, average 1g size) include selection and collection of food, preparation of food (e.g. drying, sieving and storing) and calculation of amount of food based on the number and weight of juveniles. As the feeding begins with mixture of three food (fish meal, seaweed and mud), rearing method for the 1st nursery phase switches from a static water method to a flow-through method with 100 % water exchange rate over 24 hours. 1 μm filter bag is attached to the inlet of seawater and a 100 μm screen is attached to the drain outlet, the latter is for catching any swimming stages such as doliolaria and auricularia which

Table 3. Surface areas of settlement tank and plates used at COM's hatchery in September - October 2017 in Pohnpei, FSM.

Surface Area (square meters)	bottom	sides	up-down	total surface area
2,500L mark =	5.00	5.00	1.00	11.00
	Surface Area - both sides (square meters)	No. of plates used in Tank #1	No. of plates used in Tank #3	No. of plates used in Tank #2
plate-LA (0.65 x 0.44)	0.57	32	7	0
plate-LB (0.44 x 0.44)	0.39	0	142	0
plate-M (0.39 x0.33)	0.26	0	92	212
	Total surface areas of plates in ST#1-3	18.24	83.29	55.12
	Total surface area ratio (tank vs. plates)	1.66	7.57	5.01

Table 4. The estimated number of the early juveniles on day 28 (1-month after spawning) during the juvenile production work in September - October 2017 at COM's hatchery in Pohnpei, FSM.

	Tank #1	Tank #3	Tank #2
Results of Sampling on Day 28 (J1W1) plate-LA	498 juveniles on 6 out of 30 plates	906 juveniles on 4 out 15 plates	
Estimated number of juvenile on the plates	2,485	3,398	
Results of Sampling on Day 28 (J1W1) plate-LB		5,279 juveniles on 20 out of 215 plates	
Estimated number of juvenile on the plates		53,372	
Results of Sampling on Day 28 (J1W1) plate-M	161 on 2 out of 2 M (side) converted as 1/2 of LA (side)		355 juveniles on 33 out of 212 plates
Estimated number of juvenile on the plates	161		2,503
Results of Sampling on Day 28 (J1W1) sample plates (0.1mx0.1mx30plates)			68 juveniles on 30 out of 30 plates
total number of juveniles (on plates)	**2,646**	**56,770**	**2,503**
total number of juveniles (tank surface)	**3,179**	**11,589**	**2,493**
total number of juveniles (plates + tank)	**5,825**	**68,359**	**4,996**

Table 5. Examples of daily feeding amount after settlement (if total 200,000 settled) for the subsequent two weeks between day 15 and day 27.

	ratio against 100,000 juveniles =	**2.00**	**(If total 200,000 juveniles)**	
	Spirulina (g)	**Fishmeal (g)**	**Seaweed (g)**	**Mud (g)**
d14	**N/A**	**10.00**	**10.00**	**25.0**
weekly	**N/A**	**70.0**	**70.0**	**175.0**
d21	**N/A**	**20.0**	**20.0**	**50.0**
weekly	**N/A**	**140.0**	**140.0**	**350.0**

Table 6. Feeding schedule for the sandfish juvenile grow-out for the COM's hatchery work in September – October 2017 in Pohnpei, FSM.

No. of Juveniles	***feeding amount changes from 0.25% - 0.25% - 0.25% - 0.5% - 0.5% - 1% of the average body weight in each month**							
26,458 (estimated number of pentactula on day 15)	daily amount of feed (g) = **smaller size**				daily amount of feed (g) = **larger size**			
survival rate to 1M = 19%	Spirulina	Fishmeal	Seaweed	**Mud (g) = 3-feed total**	Spirulina	Fishmeal	Seaweed	**Mud (g)=3-feed total**
1-2M (0.01-0.5g) (ratio) 0.25% BW	1	2	2	3-feed total (g)	1	2	2	3-feed daily total (g)
***estimated number on day 28 in ST2 (2,500L tank)**								
4,996	0.02	0.05	0.05	0.12	1.2	2.5	2.5	6.2
4,996	0.14	0.29	0.29	0.72	2.2	4.4	4.4	10.9
4,996	0.26	0.52	0.52	1.31	3.1	6.2	6.2	15.6
4,996	0.38	0.76	0.76	1.90	4.1	8.1	8.1	20.3
***2M transferred from ST#2 to JHS#3: 4 weeks total feed=**	**5.68**	**11.37**	**11.37**	**28.42**	**74.3**	**148.6**	**148.6**	**371.6**
2-3M (0.2-2g) (ratio) 0.25% BW	1	2.5	2.5	3-feed daily total (g)	1	2.5	2.5	3-feed daily total (g)
5,000	0.4	1.0	1.0	2.5	4.2	10.4	10.4	25.0
5,000	0.8	2.1	2.1	5.0	5.7	14.3	14.3	34.4
5,000	1.3	3.1	3.1	7.5	7.3	18.2	18.2	43.8
5,000	1.7	4.2	4.2	10.0	8.9	22.1	22.1	53.1
survival rate to 3M = 100% 4 weeks total feed =	**29.2**	**72.9**	**72.9**	**175.0**	**182.3**	**455.7**	**455.7**	**1225.0**
3-4M (1-5g) (ratio) 0,25% BW	1	5	5	3-feed daily total (g)	1	5	5	3-feed daily total (g)
5,000	1.1	5.7	5.7	12.5	5.7	28.4	28.4	63
5,000	2.6	13.0	13.0	28.6	7.1	35.5	35.5	78
5,000	3.1	15.6	15.6	34.4	8.5	42.6	42.6	94
5,000	3.6	18.2	18.2	40.1	9.9	49.7	49.7	109
survival rate to 4M = 100% 4 weeks total feed=	**73.6**	**367.9**	**367.9**	**809.4**	**218.8**	**1093.8**	**1093.8**	**2406.3**
4-5M (2-10g) (ratio) 0.5% BW	1	10	10	3-feed daily total (g)	1	10	10	3-feed daily total (g)
5,000	2.4	23.8	23.8	50.0	11.9	119.0	119.0	250
5,000	3.3	32.7	32.7	68.8	14.9	148.8	148.8	313
5,000	4.2	41.7	41.7	87.5	17.9	178.6	178.6	375
5,000	5.1	50.6	50.6	106.3	20.8	208.3	208.3	438
survival rate 5M = 100% *4M transfer to farm/pond 4 weeks total feed=	**104.2**	**1041.7**	**1041.7**	**2187.5**	**458.3**	**4583.3**	**4583.3**	**6926.0**
5-6M (5-20g) (ratio) 0.5% BW	1	20	10	3-feed daily total (g)	1	20	10	3-feed daily total (g)
5,000	4.0	80.6	40.3	125.0	16.1	322.6	161.3	500
5,000	5.0	100.8	50.4	156.3	20.2	403.2	201.6	625
5,000	6.0	121.0	60.5	187.5	24.2	483.9	241.9	700
5,000	7.1	141.1	70.6	218.8	28.2	564.5	282.3	875
survival rate to 6M = 100% *5M transfer to farm/pond 4 weeks total feed=	**155.2**	**3104.8**	**1552.4**	**4812.5**	**621.0**	**12419.4**	**6209.7**	**19250.0**

are returned to the settlement tank. Depending on presence of the swimming stages i.e. late auricularia and post-late auricularia, feeding with *C. muelleri* ends on around day 14. The juvenile foods are prepared as follows:

- after collecting from the wild, seaweed (Fig.30) and mud (Fig. 31) are sundried (Fig. 32);
- mud is sieved through 200 or 250 µm screen (Fig 33) and the seaweed is chopped in fine pieces (Fig. 34);
- after weighing, seaweed and fish meal are dipped in 1 µm filtered seawater for a several hours or overnight (Fig. 35)
- Spirulina is dipped in freshwater (rainwater) for several hours or overnight;
- Use blender to make the softened-foods finer pieces;
- sieve through 100 µm before feeding the animals

The protein content is the most important element in feeding pentactula and juveniles. Low fat content (crude fat around 5%) is desirable to maintain better water quality during the larval rearing and the 1st phase nursery culture in the settlement tanks. The author uses dried alga Spirulina instead of Algamac® (*Algamac ProteinPlus®) because Spirulina has much higher protein with low fat contents and the latter has higher fat contents. The author's choice of fishmeal is for milkfish (herbivore fish) farming because of less crude fat contents (about 3 %) compared to other fishmeals (Fig. 36). The author also uses the seaweed Sargassum spp. and Gracilaria sp., which contains about 10% crude protein with other essential nutrients, for free feed from the wild supplementing Spirulina and fishmeal. Mud or silt can be collected from the tidal flat area of the mangrove shore. The author has been using to mix with other feed (i.e. seaweed, fishmeal and Spirulina) for the juvenile grow-out as well as broodstock conditioning. The mud


Figure 24. Settlement tank.


Figure 25. Screen-covered drain outlet.

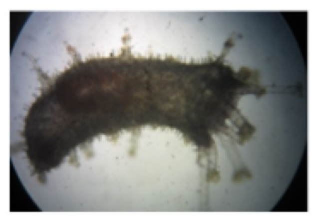


Figure 26. Day 28 juvenile.


Fig 27. Day 28 settled juveniles.


Figure 28. 5 g size 3-mo juveniles


Figure 29. Juveniles 3-mo in ocean grow-out site.


Figure 30. Seaweed *Sargassum* sp.


Figure 31. Mud from mangrove tidal flat.


Figure 32. Drying seaweed and mud.


Figure 33. Dried and sieved mud.


Figure 34. Dried seaweed.


Figure 35. Preparation for feeding.

Figure 36. Fishmeal.

Figure 37. Down-weller “habitat simulators” for juvenile grow-out.

contains detritus with organic substances and symbiotic heterotrophic micro-organisms in the mangrove forest (i.e. Schizochtrium spp.) which are known to have high contents of poly-unsaturated fatty acid (ω3 fatty acid). This heterotrophic (propagating without using sunlight) organisms live in the mangrove shore. *Algamac® and Algamac ProteinPlus® are commercial products from Aquafauna BioMarine, USA.

Figure 38. Estimating settled 2-mo juveniles by detaching from the plates.

From day 28 or one month after spawning to six-month-old juveniles, an example of feeding schedule was given in the following table 6: juveniles from 2-month-old (Settlement Tank #2 = ST2 with 4,996 J2W1) to 3-month-old in the 2,500L down-weller (Juvenile Habitat Simulator #3 = JHS3 with 5,000 J3W1) where the juveniles were produced from the spawning on September 8, 2017.

Juveniles attached on the plates are transferred on around day 56 (two-month-old after spawning) to the down-weller “habitat simulators for juveniles” (Figs. 37-38). Although the larger the animals, it is the easier for counting and estimating the standing crop, the juveniles were large enough (10 - 20 mm, 0.2 - 2g) to estimate the number of individuals by a naked eye.

Between 2 - 3 months old (1 - 5 g) after spawning, down-wellers “habitat simulators for juveniles” are stocked with juveniles either being attached on or being detached from the settlement plates. Stocking density of each tank was approximately 1,500 juveniles per square meter. Those remaining juveniles in the settlement tanks need to be fed weekly but are to be transferred in earlier occasions to hapas or ocean grow-out sites. The juveniles in the habitat simulators will be fed weekly for subsequent two or three months before transferring to grow-out phase (farming) in the ocean sites, earthen ponds or restocking sites.

7. Grow-out Culture

At around four or five months after spawning, the juveniles should reach to average 5 - 10 g. So, they are ready for farming. There is no published information available on the feeding techniques or feeding amount for both settled pentactula, early juveniles and juveniles up to six months after spawning. Also, there is no information about feeding techniques after six months old to harvesting size to export. The

Table 7. The results of counting of two-month-old juveniles attached on the settlement plates and estimate of those attached on the tank surface during the hatchery work in September - October 2017 at COM's hatchery in Pohnpei, FSM.

day 49 (Oct. 27-29, 2017)	**Settlement Tank #1 (from LR2-1)**	**Settlement Tank #2 (from LR1)**	**Settlement Tank #2 (from LR2-2)**
counted total number of juveniles (on plates)	**2,154**	**2,975**	**17,067**
estimated total number of juveniles (tank surface)	**3,670**	**2,021**	**51,304**
estimated total number of juveniles (plates + tank)	**5,824**	**4,996**	**68,371**

Table 8. Restocking JHS tanks with 2-mo juveniles.

2.5t Habitat Simulator Tank for Juveniles (with plates)	**No. of Juveniles Counted**
JHS#1 =	**5,985**
JHS#2 =	**4,642**
JHS#3 =	**5,129**
JHS$4 =	**4,569**
JHS#5 =	**1,871**
total =	**22,196**

Table 9. Number of 2-mo juveniles in the settlement tanks.

2.5t Settlement Tank after Transfer (without plate)	**No. of Juveniles Estimated**
ST#1 =	**3,670**
ST#2 =	**2,021**
ST#3 =	**51,304**
total =	**56,995**

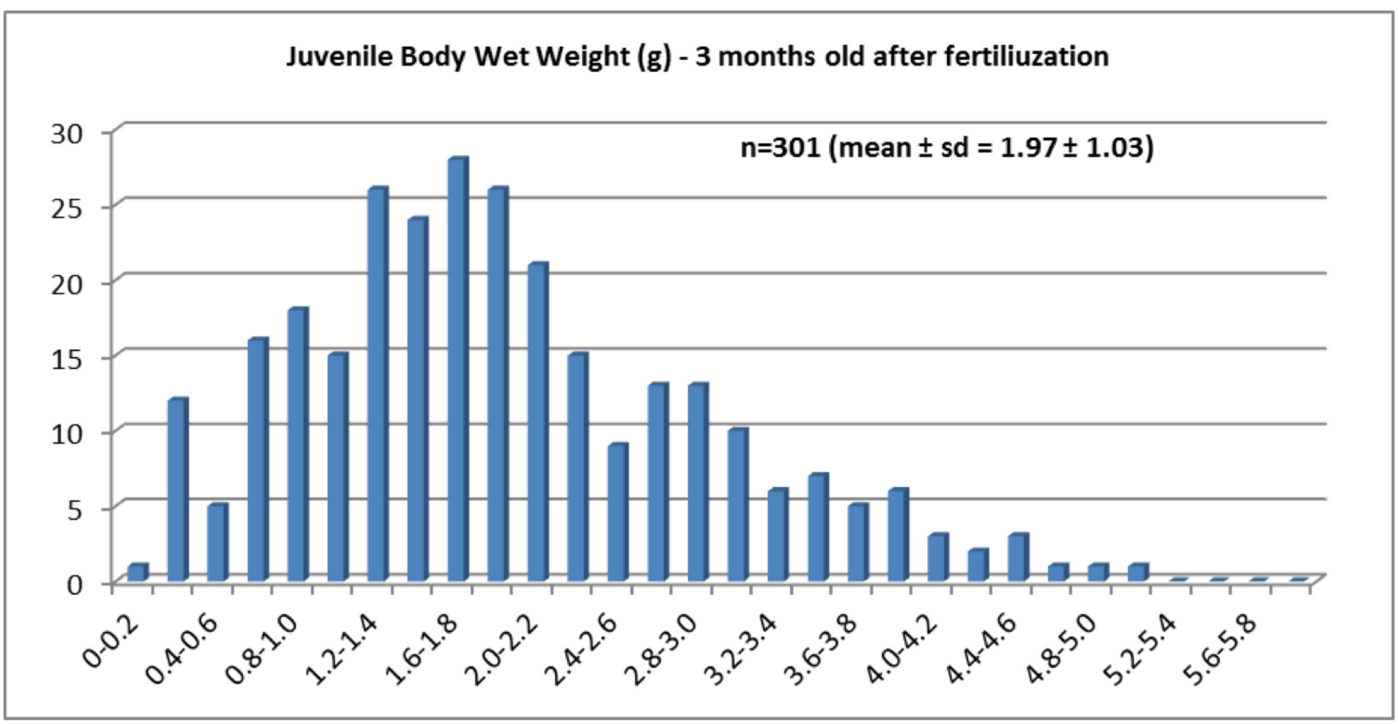
Table 10. Feeding table for 6-month-old juveniles in the downweller habitat simulator for juveniles.

	daily amount of feed (g) = **smaller size**				daily amount of feed (g) = **larger size**			
	Spirulina	Fishmeal	Seaweed	Mud (g) = 3-feed total	Spirulina	Fishmeal	Seaweed	Mud (g) = 3-feed total
6M (10-40g) (ratio) 1% BW	1	20	10	3-feed daily total (g)	1	20	10	3-feed daily total (g)
4,750	NA	306.5	153.2	475.0	NA	1225.8	612.9	1900
4,750	NA	536.3	268.1	831.3	NA	1379.0	689.5	2138
4,750	NA	766.1	383.1	1187.5	NA	1532.3	766.1	2375
4,750	NA	990.0	498.0	1543.0	NA	1685.5	842.7	2613
survival rate to 12M = 95% - 4 weeks total feed=	NA	18233.9	9116.9	28262.5	NA	40758.1	20379.0	63175.0

feeding table (Table 10) was developed by the author for private farming enterprise in Australia. Note that the feeding tables should be used only if actual or estimate of number of juveniles are not available. Feeding the juveniles and/or young adults are generally based on and calculated by the average body weight (wet weight). When they reach preferably to 10 - 20 g (average 5 - 10g) size after 3 - 5 months from spawning, they are ready for grow-out in the farm (ocean enclosures and/or ponds). Stocking density for the grow-out farming could be at 2 - 3 individuals per square meter (m2). In a hapa method elsewhere, 1- 2 g size about 2 or 3-month-old juveniles are stocked at 200 individuals per m2. On the other hand in a down-weller juvenile habitat simulator (e.g. 10,000L in Australia) with tank floor area of approximately 10 m2, initial stocking density is about 1,000 juveniles per m2). It is recommended to reduce the stocking density to at least half or preferably to 1/4 on around 5-month-old.

The following pages describe how to estimate feeding amount for the juveniles of 3-month-old. Also, explaining how accurate these feeding tables and how to use these tables developed by the author. For rearing early juveniles, it is recommended to feed the animals with 1/4 of adult grow-out amount = (1% body weight) x ¼ = 0.25% daily, and then increase the amount gradually to 0.5% and later around 5-month-old to 1%. The hatchery and farmhands must monitor the animals daily or at least weekly, such as measuring body weight by sampling monthly to adjust (increase) amount of weekly feeding amount. The following is an example of 300 juveniles of 3-month-old collected from a hapa elsewhere and measured body weight (mean ± sd = 1.97 ± 1.03 g). The histogram of these 301 (say 300) showed that they were smaller and closer to minimum weight group (1g group), indicating that they had not been fed well.

The following is how to estimate the weekly feeding amount of the 300 juveniles of 3-month-old, when they are required daily with 1% of body weight of food. Also, how-to estimate the feeding amount by using the juvenile feeding table when actual body-weight measurements were not done onsite.



(EXAMPLE) Histogram of the 3-month-old juvenile body weight obtained from hapa in a pond.

"How-to-Compute" weekly feeding amount (1% Body Weight) for 3-month-old "300" juveniles from hapa.

1.97g x 1% BW per day = 1.97g x 0.01 = 0.0197g per day per juvenile
0.0197 x 300 juveniles per day = 5.91g per day = 5.91 x 7days = 41.37g per week in total
*Spirulina vs. Fishmeal vs. Seaweed = 1 : 5 : 5 for the 3-month-old juveniles
therefore,

Spirulina = 41.37 x 1/(1+5+5) = 3.76g per week (0.54g per day)
Fishmeal = 41.37 x 5/(1+5+5) = 18.8g per week (2.69g per day)
Seaweed = 41.37 x 5/(1+5+5) = 18.8g per week (2.69g per day)

* If number of juvenile is 300, then the feeding amount is 300/10,000 of the figures given in the following Feeding Table.

daily amount of 3-feed total = 25 x 300/10,000 = 0.75g when given 0.25% BW

If given 1% BW,
daily amount = 0.75g x 1/0.25 = 3g and weekly amount = 3g x7days = 21g
Spirulina = 21 x 1/(1+5+5) = 1.91g
Fishmeal = 21 x 5/(1+5+5) = 9.55g
Seaweed = 21 x 5/(1+5+5) = 9.55g
However, the amount is based on the Smaller Group (1g).

If the juvenile's average body wet weight is 1.97g (= 2g), the feeding amount should be increased

and then, re-calculated for the "300" juveniles.

i.e. Each 1 g increment is (875-1,75)/4 = 1,75 per week

1g weight group = 175; 2g weight group = 175 + 175 = 3,50g; 3g weight group = 350 + 175 = 525g

Feeding Table for the 10,000 Juveniles (3 M): Smaller Group (1g) and Larger Group (5g)								
	Smaller Group (1g)			Larger Group (5g)				
10,000 3M juveniles	Spirulina	Fishmeal	Seaweed		Spirulina	Fishmeal	Seaweed	
3-4M (ratio) 0,25%BW	1	5	5	3-feed daily total (g)	1	5	5	3-feed daily total (g)
wk-1 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-2 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-3 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-4 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
Weekly Total (g)	15.9	79.5	79.5	175.0	79.5	397.7	397.7	875.0

4g weight group = 525 + 175 = 700g; 5g weight group = 700 + 175 = 875g

therefore,

weekly amount of the 2g weight group could be 350g per "10,000" juveniles

weekly amount of the 2g weight group of the "300" juveniles = 350g x 300/10,000 = 10.5g when given 0.25% BW

therefore,

Spirulina = 10.5 x 1/11 = 0.95g

Fishmeal = 10.5 x 5/11 = 4.77g

Seaweed = 10.5 x 5/11 = 4.77g

If given 1% BW, weekly feeding amount of 2g weight group = 10.5 x 4 = 42.0g

therefore,

Spirulina = 0.95 x 1/0.25 = 3.8g

Fishmeal = 4.77 x 1/0.25 = 19.1g

Seaweed = 4.77 x 1/0.25 = 19.1g

The estimated weekly feeding amount from BW measurements (1.97g ± 1.03g) was total 41.37g = Spirulina 3.76g + Fishmeal 18.8g + Seaweed 18.8g

Thus,

the above “Feeding Table” is very accurate (+0.04g < > +0.67g), almost the same feeding amount obtained from actual BW measurements.

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Hatchery Manual for Sea Cucumber Aquaculture in the U.S. Affiliated Pacific Islands

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Masahiro Ito

Hatchery-Based Sea Cucumber Farming

It is common knowledge that aquaculture farms will result in employment opportunities for island communities and provide potential source for exports. A hatchery-based sea cucumber production is to make available for stock enhancement program and for aquaculture-based farming enterprises. The author (Masahiro Ito) is an independent sea cucumber hatchery consultant and a former director of aquaculture research and extension of COM. He has propriety technology in possession which can significantly boost the sea cucumber juvenile production in the hatchery. He has agreed to write this manual to contribute to the benefit of the U.S.-Affiliated Pacific Islands and its future industry development. The main objective is to provide the advanced methodologies and to improve the hatchery technology for the holothurian sea cucumbers, particularly the sandfish (*Holothuria scabra*) in the U.S.-affiliated Pacific islands.

Status of World Sea Cucumber Trading

The sandfish sea cucumber business was once prosperous and has been a valuable source of income for decades in the tropical and subtropical coastal communities, but it was based on “boom and bust” business resulting over-fishing to the level of extinction of this high-valued species. Similar phenomenon on almost all sea cucumber fishery have occurred worldwide. Despite of these facts, a sustained demand for bêche-de-mer (processed sea cucumbers) from China and other Asian sea food markets has pushed up the price of this favored *holothurian* sea cucumber species. Most of the sandfish product which has been regarded as one of the most valuable tropical sea cucumber is traded and sold in the dried form in the Asian market mainly in Hong Kong where the products are distributed into mainland China. Dried sea cucumbers are brought from all over the world to be bought and sold in Hong Kong. Traders and wholesalers are located along Nam Pak Hong Street in the Sheung Wan area in the north-west of Hong Kong Island. Hong Kong and Guangzhou in Guangdong province, China, have been tightly connected since the birth of Hong Kong in the 19th century. Through this channel, most of the dried marine products imported into Hong Kong are re-exported to Guangdong, from where they are traded throughout China. Currently, retail prices of the sandfish in Hong Kong are from around US$50 for the low quality with small sized products to US$300 per kg for high quality with larger size and the highest quality sandfish fetches between US$500 and US$800 per kg. The “Australian” or “Australian-made” sandfish have always been regarded as the highest quality and price in Hong Kong wholesale and retail markets.

This hatchery manual includes the following topics; i.e. broodstock management and juvenile production work of the sea cucumber sandfish, notes on microalgae culture, complete larval development as well as descriptions of post-larvae and juveniles of the sandfish and the black teatfish (*Holothuria whitmaei*):

1) quarantine culture of the broodstock, recovering them from spawning stress and conditioning for spawning induction by using down-weller “Habitat Simulator” system;
2) microalgae culture of benthic diatoms and knowledge of heterotrophic algae (micro-organisms) for feeding the settled pentactula and early juvenile stages;
3) spawning induction methods with disinfection of spawners, fertilization, collection and incubation of eggs;
4) larvae rearing including specific knowledge of feeding capability of larval stages and combination of feed, calculating amount of larval feed mix, controlling algal cell suspension, adjusting feeding amount and rearing water volume, and knowledge of optimal larval development by expecting

proportions of larval and post-larval stages between day-1 and day-11;

5) settlement techniques including preparations of settlement plates and tanks, maintenance of benthic diatom culture and water quality, nutrient media preparation and culture techniques of benthic diatoms and/or naturally occurring epiphytes, knowledge on the types of benthic diatoms (*Navicula* sp. & *Cocconeis* sp.) and symbiotic heterotrophic micro-organisms in the mangrove ecosystem for feeding pentactula and early juveniles;
6) culture of pentactula and early juvenile stages in the settlement tank (the 1st phase nursery culture) from day-11 to day-56 or 8 weeks after spawning (approximately 2-month-old juveniles of 6 – 15 mm, 0.2 – 1g size), including calculation of feeding mix amount for the juvenile culture and preparation of feed mix;
7) grow-out culture using the down-weller "habitat simulator" tanks from day-56 or 8 weeks (onset of the 2nd phase nursery culture) until 5-6 months old (approximately 20 – 50mm, 5 – 20g size).

Broodstock Management

Elsewhere, the sandfish broodstock are usually held either in FRP (fiber-reinforced plastic) raceways, concrete tanks or earthen ponds for spawning work (Figs. 1a-c). The COM's hatchery in Pohnpei, Federated States of Micronesia, uses freshly caught sandfish broodstock from nearby its hatchery a day prior to the spawning induction work without doing any conditioning work.


Figure 1a. Habitat simulators.


Figure 1b. Concrete tanks.


Figure 1c. Earthen ponds.

The sandfish habitat is characterized by a seagrass bed of the tidal flat along the mangrove-covered shoreline from low-tide line to 10 – 20m deep in subtidal zone with soft muddy or sandy substrate. Seagrass bed is characterized by turtle-grass such as *Thalassia* spp. or by eel-grass such as *Zostera* spp. in the Indo-Pacific region (Fig. 2a-b). It is said that stocking density of the sandfish grow-out in a pond is one or two animals per square meter and the broodstock may be stocked at 3 – 5 per m-2 in a tank for conditioning if they are provided good aeration, water flow (water exchange rate at 400% per day), ample feeding with periodic tank cleaning at least once a fortnightly or renewal of tank with fresh muddy sand substrate (Duy, 2011; Purcell et al., 2012).

A key technological innovation developed by the author is a land-based broodstock culture system with a "down-weller" or "habitat simulator" tank system. The system uses a combination of closed recirculating seawater and partial flow-through method, which enables a long-term holding and domestication of healthy broodstock for selective breeding programs rather than relying on wild-caught parents on each hatchery operation. The tank system holds 5 - 10 broodstock per m-2 by providing with good air, water circulation (100% daily water exchange) and enabled to feed without periodic tank cleaning or renewal of

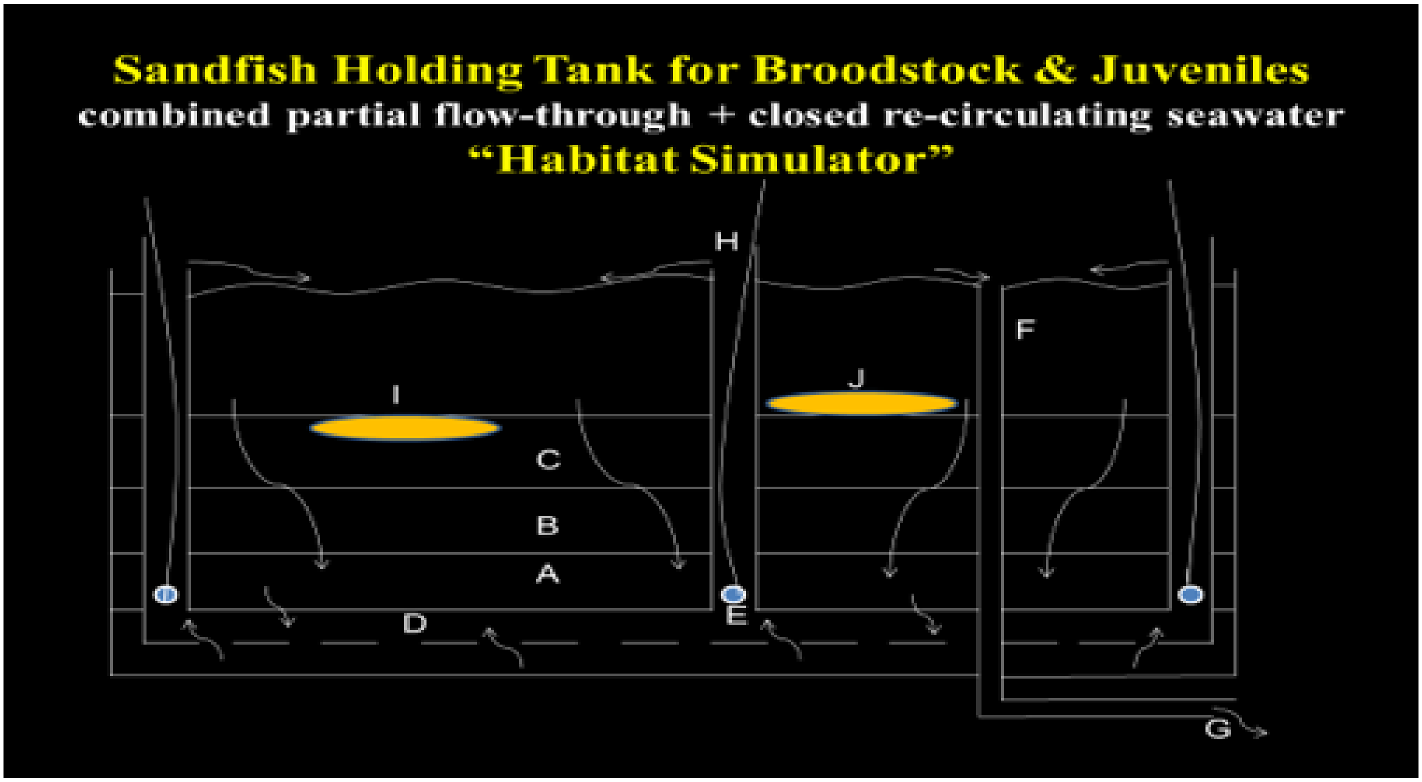


Figure 3. Diagram of down-weller "Habitat Simulator" tank. A: coral rock, B: coral gravel & sand, C: fine sand & silt, D: perforated pipe, E: air stone, F: overflow standpipe, G drain, H: airlift pipe, I & J: sandfish

tank. The down-weller was consisted of two or three layers of substrates to form a false-bottom structure; sand and mud, which are collected from tidal flat areas in the mangrove shore. Water re-circulates through the surface muddy and sand layer by air-lift pump which also maintains aerobic condition of the tank bottom and substrates (Fig. 3).

For quarantine purpose, an Australian private hatchery uses this tank system to prevent spreading potential disease among the wild-caught or domesticated broodstock (Fig. 4). Furthermore, this system has been used for recovering the spawners which had been injured or stressed during the transportation and/or spawning induction work. In Pohnpei of the Federated States of Micronesia, the COM built 2,500L rectangular tanks with down-weller system were made for broodstock recovering (Fig. 5) and juvenile grow-out (Fig. 6). Routine maintenance of the down-weller system for the broodstock is to: 1) avoid the macro-green algae (e.g. *Enteromorpha* spp.) over-grown on the tank surface; and 2) adjust aeration so as not to give strong air to drag too much seawater into the perforated piping system. To prevent green algae over-grown, use shade-screen to cut too much sunlight onto the tank. Continuously strong air-lift causes hardening the sandy substrates from strong downward water movement.

Figure 2a. Seagrass beds of turtlegrass (*Thalassira* species).

Figure 2b. Seagrass beds of eelgrass (*Zostera* species).

Figure 4. Down-wellers for commercial

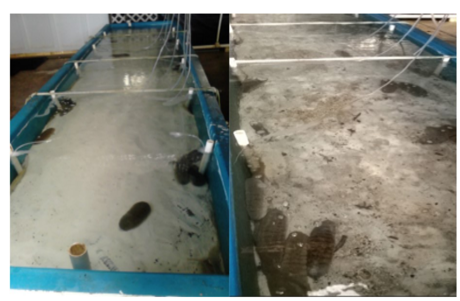


Figure 5. Quarantine and recovery for broodstock in Pohnpei, Micronesia.

Figure 6. Down-wellers for juvenile grow-out in Pohnpei, Micronesia.

Figure 7a. Transporting in bags.

Figure 7b. Selecting broodstock for spawning.

1-1. Feeding broodstock

The broodstock in the “down-weller” tanks are fed daily with 1~2 % BW (body wet weight) per individual depending on the purposes; i.e. grow-out or quarantine at 1%, fattening or recovery at 1.5~2%. For practical reason, it is recommended to feed them weekly. The feed consists of dried alga Spirulina, fishmeal and seaweed of which ratio varies depending on the sizes and conditions of the animals; approximately 1 vs. 20 vs. 10. Mud/silt collected from tidal flat zone of the mangrove-covered shore is also included as an important food for both broodstock and juveniles. The amount of mud is equivalent to a total weight of other three foods. The amount of food should be adjusted by increasing or decreasing according to their average body weight. Therefore, the body weight needs to be measured at least monthly or bi-monthly. Feeding the broodstock in the Habitat Simulator can be done without a renewal of sandy and/or muddy substrates because this down-weller system itself contains organic matters such as seaweed and detritus. Currently with no mortality has been recorded by using with a combination of dried seaweed, fishmeal and mangrove-silt.

1-2. Transporting broodstock

When transporting broodstock for a long distance (4 hours or more) from wild habitat to the hatchery, the animals should be packed individually in a plastic zip-bag in a polystyrene box or ice-chest (Fig. 7a-b). It is better using ambient seawater the same water collected at the habitat and better inserting ice-gel pack (s) in the box to keep temperature at lower than 25 °C during the transportation. If the animals are found eviscerated (vomited the gut/internal organ), they should be removed from the spawners and held in the recovery-fattening tank for at least six months period for the next spawning work.

2. Microalgae Culture Management

For microalgae culture of benthic diatoms refer to "Trainer's manual for hatchery-based pearl farming" (Ito, 2005), "Development of pearl aquaculture and expertise in Micronesia" (Ito, 2006) or "A hatchery operations manual for rearing sandfish, *Holothuria scabra*, in Tarawa, Republic of Kiribati." (SPC, 2015). Detailed descriptions of the microalgae culture management and techniques had been written by the author during the COM Land Grant Program's pearl project in 2001-2013, which offered basic but practical knowledge on the microalgae culture in the tropical conditions.

2-1. Precaution for microalgae culture

- soak in freshwater and wash with detergent, brushing off dirt/wastes. Although it is not always necessary, hydrochloric acid (5 - 10% HCl solution) can be used for cleaning flasks by soaking when the dirt is difficult to clean off. Collect the used hydrochloric acid in a glass bottle for re-use.
- rinse with freshwater 5 - 10 repeats, completely wash off residue of detergent or chemicals.
- dry flasks upside down and avoid air-born dirt inside the flask.
- spray alcohol (isopropyl-alcohol or ethanol 75 % solution), rinse with distilled /filtered rainwater, and wait for dry upside down.
- put the lid on (aluminum foil) or place them in a dust-free cabinet for longer storage.
- rinse with filtered (0.2 µm or 1µm) seawater and, if available, UV-sterilized seawater before use.
- make sure washing your hand, particularly dirty finger nails and oily fingers, with soap and rinse off any residue of soap/chemicals, and then spray alcohol before commencing work.
- spray alcohol on the surface of culture flasks/containers/fittings/ working bench when entering the room.
- keep the floor and bench clean and dry and, if necessary, clean a floor with chlorinated freshwater.
- soak your feet in the chlorine bath before stepping into the room.
- periodically check and clean air filter/air outlet of air pump, air-conditioner and ventilator.

- always keep the room door/windows closed and avoid unnecessary entry into the room.

The hatchery staff tend forget general precautions for the microalgae culture work and how to properly operate the autoclave. Usually, hatchery operation elsewhere uses 121 °C for 45-60 minutes for larger flasks such as 3~5L high density culture, and small 100~250mL flasks for stock culture are sterilized for 10-15 minutes at 121°C. Periodical maintenance of autoclave is also necessary by changing or refreshing water in the chamber.

2-2. Culture methods for the sea cucumber hatchery

Sea cucumber hatchery work involves microalgae culture of planktonic and benthic diatoms. The author also uses mud/silt collected for the tidal flat zone of the mangrove shore for feeding the settled pentactula and early juvenile stages as well as broodstock. This kind of mud contains nutrient rich, particularly Omega-3 (ω3) fatty acids, derived from heterotrophic algae (micro-organisms).

Live microalgae are not required for feeding broodstock (adults) of the sea cucumbers. During the larval and post-larval rearing, however, live and/or dried microalgae are used toward settlement stage (pentactula stage) and after settlement to juveniles. The author simplified feeding methods for the larval rearing to reduce workload of culturing live microalgae. With higher survival rate at 30 - 40 % from day-1 to the settlement stage, the author has been using a single live planktonic diatom species of *Chaetoceros muelleri* together with dried form of microalga, Spirulina sp. For settlement phase and post-settlement rearing of juveniles, the author developed to use two kinds of live benthic diatoms (*Navicula* sp. and *Coconeis* sp.) by combining with dried Spirulina during the pentactula and early juvenile stages and during the juvenile stage by combining fishmeal, seaweed, Spirulina and tidal flat mud. Note that there are eight types of benthic diatoms and *N. ramosissima* (Type-A benthic diatom) and *C. scutellum* (Type-B benthic diatom) are commonly used at abalone hatchery for post-settlement juvenile culture in Japan (Kawamura, 1998). The author has been using two types as live epiphytes on the settlement substrates for the sea cucumbers, such as *N. jeffreyii* for type-A and *Coconeis* sp. for type-B. Master stock culture of these benthic diatoms can be purchased commercially such from Commonwealth Scientific Industrial Organization (CSIRO) in Australia or elsewhere. Culture media of these benthic diatoms or naturally occurring epiphytes are same as planktonic diatom such as *C. muelleri* with nutrient media strength varies from 1/100th to 1/10th. Starter high density (3L - 5L flasks) & mass culture (20Lcarboys - 100L polycarbonate tanks) are used for the above three diatom species. For these benthic diatom culture techniques and work plan, refer to Chapter 4 (Larval Rearing) and 5 (Settlement Techniques).

For specific knowledge of heterotrophic algae, refer to some of many publications such as "*Schizochytrium limacinum* sp. nov., a new thraustochytrid from a mangrove area in the west Pacific Ocean" (Honda et al., 1998), "Fatty acid composition and squalene content of the marine microalga *Schizochytrium mangrovei*" (Jiang et al., 2004), "Effects of dried algae *Schizochytrium* sp., a rich source of docosahexaenoic acid, on growth, fatty acid composition, and sensory quality of channel catfish *Ictalurus punctatus*" (Li et al.,2009), and "Heterotrophic cultivation of microalgae as a source of docosahexaenoic acid for aquaculture" (Taberna, 2008).

Figure 8a. Cold water treatment using ice cubes.

Figure 8b. Cold water treatment in algae room.

Figure 9a. 1ppm iodine bath.

Figure 9b. Disinfecting (1 min.)

Figure 9c. Rinse with freshwater.

Figure 10a. Thermal shock.

Figure 10b. Gently stirring.

Figure 10c. Siphoning droppings.

Figure 11a. Sp*irulina* bath (12g/60L seawater)

Figure 11b. Monitoring water temp.

Figure 12a. Spawning.

Figure 13a. Collecting eggs.

Figure 13b. Sampling for counting eggs.

Figure 13c. Cutting broodstock.

Figure 13d. Stripping gonads.

Figure 14. Incubating eggs in 1,000L

3. Spawning Induction

Spawning induction work involves; conditioning and disinfection of spawners; inducing by stimulations or stressing such as exposing to the air, changing water temperature, water pressure and/or salinity, and chemical or food; fertilization and washing of eggs; and collection, sampling, counting and incubation of eggs (see Figs 8-14). When using a 2,500L tank for a small-scale juvenile production work, about 50 - 60 broodstock (spawners) are used for single larval run. Prior to spawning induction work before transferring from cold water treatment to spawning tank, all the spawners are disinfected by iodine, in which the animals were immerged in 1 ppm iodine bath (freshwater) for 1 minute. Spawning induction are usually done by: 1) stress by handling with exposure to the air, 2) thermal shock from cold (20-22 °C) to warm water (32-34 °C), 3) chemical stimulation by dried microalga Spirulina (20g/100L) in seawater for 30 minutes, 4) changing water pressure (decreasing/increasing water level), and/or changing salinity (decreasing salinity to about 30 ppt.

Collection of the spawned eggs are usually two-step approaches; 1) the first batch by scooping the spawned eggs by beakers and 2) the second batch by draining spawning induction tank. For a small-scale work, the former method is better to obtain cleaner with enough number of eggs. This also requires careful and continuous observation of female spawning posture. Noticeable change is observed in gonophore shape by swelling outwardly. Therefore, swift and timely scooping actions to collect eggs are required. If the latter method is used, collection of eggs should be commenced soon after several females spawned before the spawning tank becoming cloudy from too many sperms.

For incubating the fertilized eggs, stocking density should be less than 10 eggs per mL. Seawater is filtered to 1 µm by using filter-bags or cartridge filters, which does not necessarily required sterilization by in-line UV sterilizer unless virus infection or other disease has been reported from the surrounding environment. A combination of plankton screen (50 µm and 80 µm or 90 µm) is essential for collecting eggs.

3-1. Spawning procedures

The following describes timeline of spawning induction work which is based on a combination of 1) physical stress; 2) exposure to air, 3) thermal shock from cold to warm water, 4) chemical (dried algae Spirulina-bath) and/or 5) changing water pressure.

- Start preparing boiled seawater in deep pan (20-40L) using firewood (or use immersion heaters in the spawning tank) to maintain the induction tank water temperature at 33-34 °C.
- Cleaning off dirt from the body surface, measuring body weight (BW) and selecting spawners to be at least 200g, so the smaller ones should be returned to the broodstock holding tank. Before transfer to a cold water, quickly rinse with filtered seawater.
- Prepare cold seawater (1 μm filtered) beforehand, in the preceding day by placing it in the algae room. Transfer spawners to 100L cold treatment tank at about 20-22 °C and keep them for at least 2~3 hours, preferably for overnight.
- Transfer spawners to iodine (freshwater) bath at 1ppm of iodine (or 100ppm of Betadine®*) for 1minute (= 60 seconds). *Betadine® contains 1%W/V iodine. Therefore, 1g Betadine contains 0.01g iodine. To make 1ppm iodine solution (or 0.1g iodine per 100L), add 10g Betadine® per 100L to make 1ppm iodine (freshwater) bath.
- Start spawning induction work immediately after the iodine bath, rinse off iodine with filtered seawater and transfer to the spawning tank (2,500L raceway with approximately 1,000L water volume) at 33-34°C.
- Wait spawning (male and female) for at least an hour and keep cleaning droppings on the tank floor by siphoning. Use a plunger to stir gently spawning tank water and keep mixing the warm water.
- If the spawners dose not respond to the above thermal shock, transfer them to "Spirulina bath" for 30 minutes at 12g of Spirulina in 60L of filtered seawater. Spirulina is dissolved faster and better in freshwater (or rainwater), so prepare 12g Spirulina in about 500mL rainwater before making 60L seawater solution.
- Rinse off the Spirulina with filtered seawater and introduce them again to the spawning tank.
- Wait for the spawning. Males usually spawn before females release eggs.
- Observe spawning posture of female(s) and scoop the eggs with beakers when the female releases the eggs. If excess eggs are needed after confirming the females finished spawning, drain the spawning tank to collect remaining eggs inside the tank. Use a combination of 50 and 80 μm-pore size mesh screen to collect and wash the eggs. If no female responded after two hours, return all the spawners to broodstock holding tank.
- After washing/rinsing off sperms for 10-20 minutes, transfer the eggs into a 20L bucket to make 15L volume of 1 μm filtered seawater.
- Take at least two samples of 2mL volume while stirring the bucket by a plunger.
- Count the eggs under microscope with an aide of Rafter Counting Chamber and estimate total number of eggs obtained. While counting the eggs, check the fertilization by confirming the 1st polar body or more advance embryonic development such as 2-cell stage, 4-cell stage, and so on.
- Stock the eggs in incubator tanks, maximum stocking density of the eggs being 10 eggs per mL.

3-2. In vitro Fertilization (Gonad Stripping Method)

At present, a method using thermal shock with or without Spirulina bath treatment has been effective, but it has not always resulted in 100% success rate of the sea cucumber spawning induction work. Sooner or later, it is inevitable to develop an effective method for spawning both males and females. A Japanese group of scientists (Kato et al., 2009) found that neuronal peptides induced oocyte (ovum) maturation and gamete spawning of the Japanese sea cucumber *Apostichopus japonicus*. They extracted the neuronal peptides and so synthesized it chemically, which was effective to mature 150 μm diameter or larger eggs. They also experimented to inject this synthesized hormone into the body cavity of the sea cucumbers, resulting the male and female spawned 60 minutes and 80 minutes later, respectively. Unfortunately, they did not describe how far the eggs developed as embryos and whether their hatchery work went through to settlement as pentactula stage. Therefore, no information was available from their study for fertilization and hatching rates as well as survival rates during the larval and post-larval rearing works. They also stated that this chemical did not work effectively on immature ova and, thus, they concluded that the maturation mechanisms and process of ova/spermatozoa still needed further studies. Synthesizing and producing such a neuro-hormone commercially could be very expensive and won't be available for the Indo-Pacific region in foreseeable future. The important fact is that developing techniques of artificial maturation of oocytes (ova/spermatozoa) and activation of gametes (sperms) are the keys to success in obtaining the fertilized eggs. In this end, a gonad stripping method* could be an alternative to spawning induction work near future, either using synthesize neuronal hormone or other chemicals such as ammonia-seawater which has been used for commercial pearl oyster hatcheries in Japan, or just use of natural seawater.

**Note that the gonads are removed from the parent animal by cutting a small portion of the body and the gametes are obtained by stripping/squeezing the gonads (Fig. 13c-d). This is called "gonad stripping method".*

Although no one has been successful for in vitro fertilization of the sea cucumbers, the author thought that it was worthwhile for the hatchery technicians to understand principle and procedures of this method. During the hatchery training workshop in May 2015 at the Fijian Government's hatchery in Galoa, the author used filtered seawater (1 μm nominal pore) without using any other chemicals for the gonad stripping method for the sandfish (Ito, 2015). As a result, fertilized eggs subsequently underwent embryonic and larval development to the settlement as pentactula stage. Although the number of eggs and resultant pentactula were very small, several hundred, and low survival rate at less than 10 % to day 11, this method may be economical and could be the first step towards future improvements for a large-scale juvenile production on a regular basis.

4. Larval Rearing

Larval rearing of the sandfish sea cucumber requires knowledge of feeding capability of larval stages, suitable combination of food, calculating amount of feed mix, controlling algal cell (feed mix) suspension, adjusting feeding amount and rearing water volume, water quality control, and knowledge of optimal larval development in changing proportions of larval and post-larval stages; i.e. from hatching as auricularia stage to settlement as pentactula stage. Approximately 18-24 hours after spawning depending on water temperature, larval rearing work commences by draining the incubator to collect gastrula and/or auricularia larvae. For collecting larvae and post-larvae, a combination of 80 μm and 100 μm mesh screen is to be used. Larval rearing tanks should be completely drained every other day, on days 1, 3, 5, 7, 9 and 11. The larval specimens need to be kept alive but immobilized/anesthetized by isopropyl alcohol for counting, measurements and microscopic photographs. For longer term preservation, use formalin (10% seawater formalin). Water temperature in the larval rearing could be better between 27-30 °C.

Hatchery facility should be maintained good conditions in terms of hygiene and efficiency for larval and post-larval rearing work:

- animals such as cats need to be kept away in- and out-side the hatchery, around the indoor and under-cover tanks and indoor storage areas;
- microalgae culture room should not be a sort of storage room with scattered lab supplies and equipment on the culture benches, dusty air-conditioners and air pumps without filter maintenances;
- air supply system needs to be functioning effectively, with sufficient air pressure in the under-cover areas as well as in the microalgae culture room;
- in-use or used tools should not be scattered on the floors, e.g. filter cartridges, filter-bags, hoses, pipes, buckets, air-stones, airlines, pipe-fittings, nets, plumbing machines; air leaks from many outlets with unnecessary accessories and fittings;
- sea water and freshwater supply piping are better to be simple and do not need unnecessary connections, diversions and outlets fittings;
- the hatchery staff understand general hygiene procedure before and during the hatchery operation.

4-1. Precautions before, during and after working at hatchery

For precautions of the onsite hatchery work, refer to "Trainer's manual for hatchery-based pearl farming" (Ito, 2005) or "A hatchery operations manual for rearing sandfish, *Holothuria scabra*, in Tarawa, Republic of Kiribati." (SPC, 2015). The following were also described in those manuals.

- make sure your hands are clean. Wash your hand with soap, particularly dirty finger nails, before starting work.
- Soak your feet in "chlorine-bath" before entering in the larval and/or microalgae room.
- don't work with you own shoes. Always ware designated boots or work with barefoot.
- always rinse again the cleaned and dried equipment with filtered rainwater (1 µm) before use.
- for the used equipment/tools, wash first with chlorinated (public) water to wash out the waste/dirt.
- second-wash by using detergent and wash-off the dirt thoroughly with a soft sponge or brush.
- rinse with 1 µm-filtered rainwater and completely wash out residual soap/detergent.
- soak in chlorine-batch (a diluted Sodium Hypo-chloride, NaHClO) for overnight. Don't mix with soap or this may release Cl2 (chloride gas).
- rinse completely with filtered rainwater (1 µm). Make sure "no residual chlorine".

Figure 15. Seawater filters and UV sterilizer.

Figure 16a. Larval rearing (2,000L).

Figure 16b. Larval rearing (5,000L).

Figure 17. Sieves for collecting larvae.

- always hang and dry the equipment after being cleaned. Do not leave them on the floor or dirty bench.
- if necessary, use isopropyl-alcohol spray (75 % solution) and wait for it to dry. *Note that the use of methanol (methyl-alcohol) will become a health hazard in a small microalgae culture room.
- don't touch inside of the cleaned surface of equipment and tools such as bucket/ container/tank/tub/flask, etc.
- wash filter bags, cartridges and housings after every use. Wash out the dirt with pressurized freshwater, filtered rainwater (1µm), soak in chlorine-bath, rinse with filtered rainwater (1 µm) and dry them on a designated bench. Keep the filter bags, cartridges in sealable plastic bags each with alcohol-sprayed inside. For the filter housings, spray alcohol inside and store them upside-down on the bench.
- make sure always clean the floor; wash with freshwater (chlorinated town-water or rainwater). It is the best that the floor is a "dry" condition when you start working in the following morning.
- don't disturb animal (larvae/juveniles/broodstock) and minimize giving shock or stress to the animals. Avoid unnecessary entry to the microalgae culture room and larval rearing unit.

4-2. Preparation for the larval rearing

The seawater for the larval rearing should be filtered to 1µm with a bag filter or cartridge filter. In-line UV-sterilizer is not necessarily required (Fig 15). When the day-1 larvae exceeded 0.35 larvae per mL in a rearing tank, the stocking density should be adjusted to make acceptable number of larvae in each tank: e.g. between 0.5 - 0.7 million larvae in a 2,000L tank or 1.25 - 1.75 million larvae in a 5,000L tank (Figs. 16). Gentle aeration is given throughout the larval rearing and the tank must be protected by a lid (tank cover) from debris from the ceiling. If the stocking density is less than 0.25 larvae per mL, the rearing water volume must be adjusted (reduced) to maintain required range of algal cell suspension based on *C. muelleri* culture density and number of larvae in each day. A combination of sieves is usually 80/100 µm throughout larval rearing and each sieve must be deep and wide enough (30 cm deep x 50 cm wide) to do sieving efficiently from a larger diameter drain pipe e.g. 25.4 cm (2 inch) pipe (Fig. 17).

Apart from tools and equipment, preparation of the live microalgae species (*C. muelleri, Navicula sp.* and *Cocconeis* sp.) need to be cultured at least two weeks before commencing spawning and larval run. For continuous culture and feeding the larvae, several subcultures should be made after commencing larval rearing, instead of starting from new stock culture (Figure 18). Larval rearing period with microalga *C. muelleri* feeding would finish within two weeks on day-14 after spawning. When a hatchery operation is planned a single spawning0larval run, therefore, it is not useful to start any new culture of *C. muelleri*. Generally, 7 days needed for a 2 - 5L high density starter cultures to be ready for starting 20 - 100L mass cultures, and these mass culture needs further 4 - 5 days to use for feeding the larvae. The author uses dried microalga Spirulina to mix with live diatom *C. muelleri* for larval and post-larval rearing. *Navicula* sp. and *Cocconeis* sp. are used for feeding settled pentactula and early juvenile stages up to two months old in settlement tanks. If live microalgae are used for sea cucumber hatchery, therefore, it is necessary to culture diatoms.

Figure 18. Microalgae (diatoms) for feeding larvae and post-larvae.

4-3. Feeding methods for larval rearing

Mixture of *C. muelleri* and Spirulina are given for feeding the larvae twice a day. Feeding is maintained by estimating algal cell suspension in each larval tank, starting from approximately 1,000 up to 20,000 cells per mL during the two-weeks larval rearing. Total cell suspension in each day is attained by combining these two feeds and expressed as the number of *C. muelleri* cells, where amount of Spirulina is computed and expressed as the number of *C. muelleri* cells. Feeding ratio of *C. muelleri* and Spirulina is approximately 80 % and 20 %, respectively. Feeding is divided into two; one in the morning between 8 and 10 AM, and the other in the late afternoon between 4 and 6 PM. The author developed daily feeding tables with simplified data-inputting methods, therefore, the hatchery staff are usually trained to use such feeding tables.

A hatchery protocol with daily work schedule for larval rearing was summarized and shown in Table 1. The hatchery staff needs to count the number of living larvae and post-larvae after tank draining every two days and *C. muelleri* to input daily culture density (million cells per mL). The feeding amount of both *C. muelleri* and Spirulina are obtained instantly in those feeding tables. All feeding amount were converted and expressed as *C. muelleri* cells. For the Spirulina, the hatchery staff simply measures the dry-weight according to the feeding table, prepare for AM or PM feeding amount, dissolve in the freshwater (about 500 mL) and wait for an hour before adding to the rearing tanks. It is advised the stocking density of larvae from onset of larval rearing on “day 1” to be between 0.25 - 0.35 larvae per mL.

Table 1. Hatchery protocols for juvenile production of the sandfish sea cucumber.

HATCHERY PROTOCOL **(WORK SCHEDULE)** **days of run (size.; µm)** **Algal cell suspension** **(as *C. muelleri* cells mL^{-1})**	***larval & post-larval development based on the water temperature at 29 ± 1 °C** ***larval rearing tank (LR) = 1 x 5,000L** ***settlement tank (ST) = 4 x 5,000L (bottom area = 5,000 m^2); settlement plates=50cm x 50cm x 800~1,000 plates per 5,000L tank** ***plates = corrugated plastic plates** ***juvenile down-weller tank (JHS) = 4 x 10,000L (bottom area = 10,000 m^2); sandy bottom covered with mangrove mud (dried mud sieved through 100 µm screen)**
-3d before spawning	Start conditioning settlement tanks and plates; add benthic diatoms (i.e. *Navicula sp.* & *Cocconeis sp.* 200L each in 5,000L settlement tank) & nutrient (100% strength) to culture in the settlement tanks
0 (egg: 150-160)	Collecting (sieves 50 & 80 µm mesh screen), washing, counting & incubating eggs (up to 50 million eggs per 5,000L incubator)
1 (420 x 320) 900-1,500 cells/mL	Draining incubator & collecting gastrula & early auricularia (sieve 80 & 100 µm) approx. 20 hours after fertilization; sampling, counting & stocking to larval rearing tanks (1.5 million larvae in 5,000L tank); start feeding larvae with live microalga *Chaetoceros muelleri* and dried microalga *Spirulina* sp. as soon as stocking larvae.
2 - 4,000-7,500 cells/mL	
3 - 7,000-12,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% Water Exchange); early auricularia (20%) + mid-auricularia (80%)
4 - 8,000-14,000 cells/mL	
5 (800-1000) - 8,500-15,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); mid-auricularia (20%) + late-auricularia (8
6 - 9,000-17,000 cells/mL	
7 (800-1200 x50-80) 9,500-18,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); late-auricularia + fully developed late auricularia; remove settlement plates to dry; drain settlement tanks & refill the settlement tanks with new benthic diatoms (200L each in 5,000L tank) & nutrient (1/20th strength)
8 - 10,000-20,000 cells/mL	Spray *Spirulina* on the settlement plates (*Spirulina* 30g per liter freshwater solution) & dry the plates
9 - 8,000-18,000 cells/mL	Larval tank draining (80 & 100 µm) & tank change (100% WE); late-auricularia + fully developed late auricularia + post-late auricularia (= metamorphosing to doliolaria) + doliolaria (~ 5%)
10 - 6,000-16,000 cells/mL	Return the plates to the settlement tanks
11 (400-1200 x 60-80) 4,000-13,000 cells/mL	Larval tank draining (80 & 100 µm), collect larvae & post-larvae & transfer them to the settlement tanks; late-auricularia + fully developed late auricularia + post-late auricularia + doliolaria + pentactula; *survival rate from day-1 = ave. 30% (approx. 0.5 million each in 5,000L settlement tanks)
12 - 2,000-6,000 cells/mL	*feeding only with *C. muelleri* for the swimming stages (no need of using *Spirulina*)
13 - 500-2,000 cells/mL	*feeding only with *C. muelleri* for the swimming stages (no need of using *Spirulina*)
14 - 200-1,000 cells/mL	Finish using *C. muelleri* & *Spirulina* for feeding larvae; fully developed late auricularia + post-late auricularia + doliolaria + pentactula
15 (~ 1mm)	Start flow-through over 24 hrs. 100% WE; (inlet water = 1 µm filter & outlet water = 100 µm screen); post-larvae; Start feeding daily with 3 foods (fishmeal 5g + seaweed 5g + mud 12.5g) per 100,000 post-larvae; *Sieve mud, fishmeal & seaweed with 100 µm screen for feeding; *no need of giving *Spirulina* as the settlement plates still covered with *Spirulina*
21 (1 ~ 2mm)	flow-through 24 hrs.100% WE; (inlet = 1 µm filter & outlet = no screen); continue daily feeding at 0.25% of BW with 3 foods (fishmeal 10g + seaweed 10g + mud 25g) per 100,000 early juveniles; *sieve feed-mix with 100 µm screen for feeding
28 (1-mo) (2~5 mm, 0.01~0.1g)	flow-through 24 hrs.100% WE; start daily feeding at 0.25% of BW with 4 foods (*Spirulina* : fishmeal : seaweed : mud = 1 : 2 : 2 : 5); *start giving *Spirulina*; *settlement success rate from day-11 to 1-mo = 20-50%
30	Start weekly feeding at 0.25% of BW with 4 foods (*Spirulina*, fishmeal, seaweed & mud)
Day **35**	Continue weekly feeding
Day **42**	Continue weekly feeding
Day **49**	Start transferring juveniles (2-mo = ave. 1g BW, 10-20mm BL) from settlement tank (ST) to down-weller “habitat simulator” for juveniles (JHS);
Day **56** (2-mo = J2) (0.2~2g)	*increase proportion of fishmeal and seaweed (Spirulina, Fishmeal & Seaweed = 1 : 2.5 : 2.5); *stocking density of JHS = approx. 10,000~1,500 x J2 per m^2 (= 10,000~15,000 xJ2 in a 10,000L JHS tank);

(Example 1)

If the rearing tank volume is 2,000 L (=2,000,000 mL),
C. muelleri culture density is 3.0 million cells/mL, and
today's total amount of *C. muelleri* required is 2,000 mL

"total *C. muelleri* cells in the rearing tank" =
"culture density of *C. muelleri* " x " today's required amount of *C. muelleri* "
(= 3 million x 2,000 = 6.0 billion cells)

Therefore, today's " *C. muelleri* cell suspension " in the 2,000L tank
= 6 billion cells / 2,000,000 mL = 3,000 cells/mL

(Example 2)

If today's required total feeding amount of larva is 12,000 cells/larva/day,
number of larvae in 2000L tank is 500,000,
today's required algal cell suspension is 3,000 cell/mL, and
today's algal culture density is 3 million cells/mL

"required total algal cells in the 2,000 L rearing tank"
= 12,000 (cells/larva/day) x 500,000 (larvae) = 6 billion cells
*algal cell suspension in this 2000L tank = 6 billion cells / 2 million mL = 3,000 cells/mL

Therefore, today's "total amount of algae" = 6 billion cells / 3 million cells/mL = 2,000 mL

For feeding the larvae, the hatchery staff must take algal samples (= *C. muelleri*) and counts the culture density; input each counting results into the designated cell in the MS Excel spreadsheet ("HIROITO CONSULTING's Sandfish Feeding Tables"), of which table automatically calculates the daily (in the morning-AM and afternoon-PM) feeding amount of both *C. muelleri* and Spirulina. "estimated available food per larva as CM" is based on the past results of larval feeding experiments of the sandfish and other sea cucumber species by the author and others in Japan and elsewhere, which ranges from 3,000 to 60,000 cells/larva/day.

Note that a commercially available dried alga Spirulina contains certain amount of dried form of cells (e.g. 2.6 billion cells per 1 g). The author found about three different sizes (e.g. 10 µm x 15 - 50 µm) and shapes (e.g. rectangular). Average size of *C. muelleri* is about 10 x 5 µm. 1 g of Spirulina cells are approximately to 42.8 billion *C. muelleri* cells. Therefore, it is necessary to estimate approximate volumes of those different sizes of Spirulina cells and to convert to the number of cells to *C. muelleri.* This is because total feeding amount is expressed as the number of *C. muelleri* cells and the feeding ratio (based on the number of cells) of *C. muelleri* vs. Spirulina is 80 % vs. 20 %.

4-4. Example of two spawning-larval runs in September 2017 at COM's Nett Point Hatchery in Pohnpei, FSM.

During the work, 1.4 million eggs (trial 1) and 5.3 million eggs (trial 2) were stocked in the 1,000L incubators. Approximately 20 hours after spawning, each yielded 0.42 million larvae (trial 1) and 2.5 million larvae (trial 2). As the two 2,000L round tanks were needed each with approximately 600,000 larvae for the trial 2. The followings were the results of larval runs between day 1 and day 11:

- trial 1 at survival rate of 10.9 % with 63,750 swimming stages on day 11 including late auricularia, doliolaria and pentactula from 416,250 larvae on day 1;
- trial 2-1 with 67,500 from 620,625 at survival rate of 15.3%;
- trial 2-2 with 221,250 from 620,625 at survival rate of 35.6%

Average survival rate of the three larval runs was 20.6% from day 1 to day 11. Total 353,500 larvae and post-larvae were transferred to three 2,500L settlement tanks.

4-5. Larval Development

Detailed morphological descriptions of the complete larval and post-larval development for both sandfish (*H. scabra*) and black teatfish (*H. whitmaei*) were given in Appendix 1 with notes on larval growth and durations in Appendix 2. Comparisons of morphological features of larval and post-larval stages of these two holothurian sea cucumbers were also given in Appendix 3.

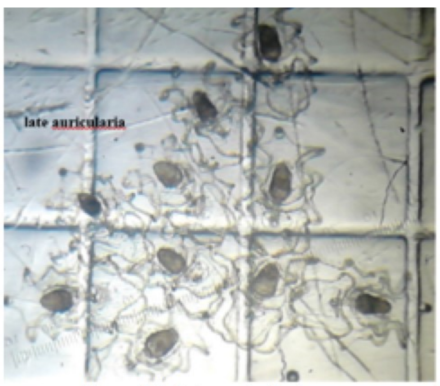


Figure 19. Late auricularia.

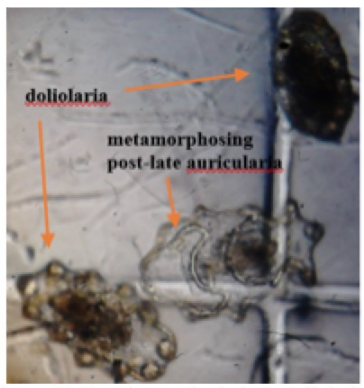


Figure 20. Post-late auricularia & doliolaria.

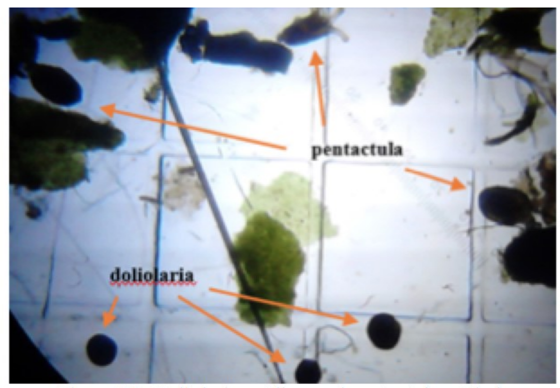


Figure 21. Doliolaria settling and pentactula settled.

5. Settlement Techniques

Settlement techniques involves conditioning of settlement plates and tanks, culturing benthic diatoms. The pentactula generally settles on the sandy sea grass bed such as *Talassira* spp. (for sandfish) in tidal flat zone, coralline gravels and rocks in the intertidal or littoral zone (e.g. black teatfish, greenfish). In the hatchery, the pentactula settle anywhere in the tank and so corrugated plastic plates are usually used as settlement substrates. Surface of settlement substrates such as plastic plates and inside the tank are conditioned with dried alga Spirulina and/or with epiphytes either naturally occurring or cultivated. The author uses two species of benthic live diatoms (*Navicula* sp. & *Cocconeis* sp.) as epiphytes on the settlement substrates. On day 11, larval rearing tank was drained and all swimming stages including fully developed late auricularia, metamorphosing post-late auricularia and doliolaria were collected onto a 100 µm screen (Figs. 19-20). A small percentage of pentactula up around 5% (Fig. 21) might have also settled on this day, so soft brushes were used to collect those settled individuals. Samples were taken for counting to estimate number of living individuals of all stages. Then, they were transferred to settlement tanks.

Table 2. Dimension of settlement plates used at COM's Hatchery in Pohnpei, FSM.

Settlement Plates (type)	Length (m)	Width (m)	Surface Area - both sides (square meters)
Large A (LA) =	0.65	0.44	0.57
Large B (LB) =	0.44	0.44	0.39
Medium (M) =	0.39	0.33	0.26

5-1. Conditioning settlement substrates.

Conditioning settlement plates and tanks should commence at least two weeks prior to settlement. To start culturing benthic diatoms *Cocconeis* sp. and *Navicula* sp. in the settlement tank, 2,500L tank for example, 100L each of mass-cultured those diatoms and a full strength of nutrient media (i.e. medium F2 in Australia and Fiji, OFCF/JICA's medium in Kiribati, MI medium and Kent's F2 media at COM in FSM) together with sodium meta-silicate 15 - 30 g were added to the 2,500L tank. About 10 days after the initial diatoms culture, the settlement plates were removed for drying and the tanks were drained to renew the culture water (1 µm filtered seawater) and to refill with new benthic diatoms (100L mas culture each) and 1/20th strength of nutrient media. After a couple of days for drying, the settlement plates were sprayed (or painted) with Spirulina (30g/L solution) and keep drying for a day or two (Figs. 22-23). On day 10, a day prior to transfer larvae and post-larvae to settlement tanks, return the Spirulina-sprayed plates in the settlement tanks. During the work at COM's hatchery in 2017, three different sizes of corrugated plates were used for settlement (refer to the table 2 of plate's dimensions). In a 2,500 L settlement tank, 300 - 400 plates of the type-M are mainly deployed. The plates were washed by detergent, disinfected by chlorine and dried for a couple of days before growing benthic diatoms on the plates. For larval rearing with a 2,000L tank, two of 2,500 L rectangular tanks (raceways) were used for settlement of doliolaria to pentactula, approximately up to 100,000 pentactula in each settlement tank (Fig. 24). On day 11, about 200,000 swimming stages, which consist of feeding and non-feeding stage of late auricularia, post-late auricularia and doliolaria, are expected in each 2,000L rearing tank out of 600,000 larvae on day 1.

5-2. Transferring swimming and settled stages to settlement tank.

Onset of the settlement (on day 11) is called "the 1st phase nursery culture", which continues for about 2 - 3 months until the juveniles of 1- 2 g body weight (BW in wet weight) in average are transfer further to a down-weller "juvenile habitat simulator" tank or bag net "hapa" for a pre-growout or "the 2nd phase nursery culture".

Living individuals on day 11 are usually consisted of the settled pentactula stage (10 - 15%), transitional doliolaria stage (20 - 25%), metamorphosing late auricularia = post-late auricularia stage (30 - 40%), fully-developed late auricularia (20 - 30%) and late auricularia stage (10 - 20%). It is better to transfer procedure when the proportion of settling or settled animals (50% < post + dolio + penta) occupy more than a half in the larval rearing tank. The larval rearing tank is drained to collect all the animals onto 80 µm and 100 µm sieve. Collected specimens should be poured over the plates in the settlement tanks.


Figure 22. Spraying *Spirulina*.


Figure 23. Drying plates after spray.


Figure 24. Settlement tank.

Toward day 11, all the settlement substrates (plates and tank surface) should be covered with light brown -colored benthic diatoms. If sunlight is too strong, the settlement tanks will be dominated by green alga (e.g. *Enteromorpha* spp.). In that case, the tanks should be covered by shade-cloths (75-80% shading rate). If pink- colored organisms (pathogenic bacteria *Pseudomonas* spp.) began visible after a week of benthic diatom culture in the settlement tank, those plates, tank surface and fittings such as air stones, air tubing, PVC pipes or ropes must be removed from the tank and disinfected by chlorine or discarded all or part of them to avoid further infection of the pathogenic bacteria. Such infection is always caused by inexperienced hatchery technician's careless setting of the settlement tank and plates, poor skill of culturing microalgae and larval rearing.

After day 11, the larval rearing continues in a static water condition because there are still swimming and feeding stages of auricularia larvae (late auricularia, fully developed late auricularia and metamorphosing post-late auricularia). Therefore, it is necessary to provide microalga *C. muelleri* until around day14. From day 15, the rearing water in the settlement tank should be switched from static to flow-through, continuously flowing in from filter bag and flowing out to overflow-drain pipe at 100 % water exchange rate over 24 hours. 100 μm screen is used to cover the drain outlet for minimize loss of swimming stages (Fig. 25).

5-3. Examples of juvenile productions using the settlement plates.

The measurements of the settlement tank (ST), settlement plates (LA, LB, M), sample plates showing surface areas (in square meters) and estimated number of juveniles were given in the following tables 3 and 4: 30 small sample plates, 0.1m x 0.1m corrugated plastic, were also made for estimating settlement on the tank surface sides and bottom. Total of 79,180 one-month-old juveniles (on day 28, J1W1) were estimated to settle in three tanks (Figs. 26-27).

6. Pentactula and Juvenile Rearing

Pentactula and the early juveniles settled on the plates or tank surface are given additional food daily with a mixture of Spirulina, fishmeal, seaweed and mud. The mixtures of these food are homogenized and passed through 100 μm mesh screen before feeding. The juveniles on the plates and tank surface are kept in the settlement tank for two or three months and then the juveniles are transferred to "the 2nd phase nursery culture" either in the down-wellers (or habitat simulators) for juveniles or hapas bag net. The 2nd nursery culture continues further two or three months before being transferred to grow-out farm/ ponds/ enclosure. In Australia, a large-scale private farming enterprise does not conduct "the 2nd phase nursery culture", except for selective breeding programs, but those grown juveniles of average 2 - 5 g BW from the settlement tanks are also transferred to ocean grow-out sites (Fig. 28-29).

Culture techniques of pentactula and early juvenile stages in the settlement tank (the 1st phase nursery culture) from day-11 to day-56 or 8 weeks after spawning (approximately 2-month-old juveniles of 10 – 20mm, average 1g size) include selection and collection of food, preparation of food (e.g. drying, sieving and storing) and calculation of amount of food based on the number and weight of juveniles. As the feeding begins with mixture of three food (fish meal, seaweed and mud), rearing method for the 1st nursery phase switches from a static water method to a flow-through method with 100 % water exchange rate over 24 hours. 1 μm filter bag is attached to the inlet of seawater and a 100 μm screen is attached to the drain outlet, the latter is for catching any swimming stages such as doliolaria and auricularia which

Table 3. Surface areas of settlement tank and plates used at COM's hatchery in September - October 2017 in Pohnpei, FSM.

Surface Area (square meters)	bottom	sides	up-down	total surface area
2,500L mark =	5.00	5.00	1.00	11.00
	Surface Area - both sides (square meters)	No. of plates used in Tank #1	No. of plates used in Tank #3	No. of plates used in Tank #2
plate-LA (0.65 x 0.44)	0.57	32	7	0
plate-LB (0.44 x 0.44)	0.39	0	142	0
plate-M (0.39 x0.33)	0.26	0	92	212
	Total surface areas of plates in ST#1-3	18.24	83.29	55.12
	Total surface area ratio (tank vs. plates)	1.66	7.57	5.01

Table 4. The estimated number of the early juveniles on day 28 (1-month after spawning) during the juvenile production work in September - October 2017 at COM's hatchery in Pohnpei, FSM.

	Tank #1	Tank #3	Tank #2
Results of Sampling on Day 28 (J1W1) plate-LA	498 juveniles on 6 out of 30 plates	906 juveniles on 4 out 15 plates	
Estimated number of juvenile on the plates	2,485	3,398	
Results of Sampling on Day 28 (J1W1) plate-LB		5,279 juveniles on 20 out of 215 plates	
Estimated number of juvenile on the plates		53,372	
Results of Sampling on Day 28 (J1W1) plate-M	161 on 2 out of 2 M (side) converted as 1/2 of LA (side)		355 juveniles on 33 out of 212 plates
Estimated number of juvenile on the plates	161		2,503
Results of Sampling on Day 28 (J1W1) sample plates (0.1mx0.1mx30plates)			68 juveniles on 30 out of 30 plates
total number of juveniles (on plates)	**2,646**	**56,770**	**2,503**
total number of juveniles (tank surface)	**3,179**	**11,589**	**2,493**
total number of juveniles (plates + tank)	**5,825**	**68,359**	**4,996**

Table 5. Examples of daily feeding amount after settlement (if total 200,000 settled) for the subsequent two weeks between day 15 and day 27.

	ratio against 100,000 juveniles =	**2.00**	**(If total 200,000 juveniles)**	
	Spirulina (g)	**Fishmeal (g)**	**Seaweed (g)**	**Mud (g)**
d14	**N/A**	**10.00**	**10.00**	**25.0**
weekly	**N/A**	**70.0**	**70.0**	**175.0**
d21	**N/A**	**20.0**	**20.0**	**50.0**
weekly	**N/A**	**140.0**	**140.0**	**350.0**

Table 6. Feeding schedule for the sandfish juvenile grow-out for the COM's hatchery work in September – October 2017 in Pohnpei, FSM.

No. of Juveniles	***feeding amount changes from 0.25% - 0.25% - 0.25% - 0.5% - 0.5% - 1% of the average body weight in each month**							
26,458 (estimated number of pentactula on day 15)	daily amount of feed (g) = **smaller size**				daily amount of feed (g) = **larger size**			
survival rate to 1M = 19%	Spirulina	Fishmeal	Seaweed	**Mud (g) = 3-feed total**	Spirulina	Fishmeal	Seaweed	**Mud (g)=3-feed total**
1-2M (0.01-0.5g) (ratio) 0.25% BW	1	2	2	3-feed total (g)	1	2	2	3-feed daily total (g)
***estimated number on day 28 in ST2 (2,500L tank)**								
4,996	0.02	0.05	0.05	0.12	1.2	2.5	2.5	6.2
4,996	0.14	0.29	0.29	0.72	2.2	4.4	4.4	10.9
4,996	0.26	0.52	0.52	1.31	3.1	6.2	6.2	15.6
4,996	0.38	0.76	0.76	1.90	4.1	8.1	8.1	20.3
***2M transferred from ST#2 to JHS#3: 4 weeks total feed=**	**5.68**	**11.37**	**11.37**	**28.42**	**74.3**	**148.6**	**148.6**	**371.6**
2-3M (0.2-2g) (ratio) 0.25% BW	1	2.5	2.5	3-feed daily total (g)	1	2.5	2.5	3-feed daily total (g)
5,000	0.4	1.0	1.0	2.5	4.2	10.4	10.4	25.0
5,000	0.8	2.1	2.1	5.0	5.7	14.3	14.3	34.4
5,000	1.3	3.1	3.1	7.5	7.3	18.2	18.2	43.8
5,000	1.7	4.2	4.2	10.0	8.9	22.1	22.1	53.1
survival rate to 3M = 100% 4 weeks total feed =	**29.2**	**72.9**	**72.9**	**175.0**	**182.3**	**455.7**	**455.7**	**1225.0**
3-4M (1-5g) (ratio) 0,25% BW	1	5	5	3-feed daily total (g)	1	5	5	3-feed daily total (g)
5,000	1.1	5.7	5.7	12.5	5.7	28.4	28.4	63
5,000	2.6	13.0	13.0	28.6	7.1	35.5	35.5	78
5,000	3.1	15.6	15.6	34.4	8.5	42.6	42.6	94
5,000	3.6	18.2	18.2	40.1	9.9	49.7	49.7	109
survival rate to 4M = 100% 4 weeks total feed=	**73.6**	**367.9**	**367.9**	**809.4**	**218.8**	**1093.8**	**1093.8**	**2406.3**
4-5M (2-10g) (ratio) 0.5% BW	1	10	10	3-feed daily total (g)	1	10	10	3-feed daily total (g)
5,000	2.4	23.8	23.8	50.0	11.9	119.0	119.0	250
5,000	3.3	32.7	32.7	68.8	14.9	148.8	148.8	313
5,000	4.2	41.7	41.7	87.5	17.9	178.6	178.6	375
5,000	5.1	50.6	50.6	106.3	20.8	208.3	208.3	438
survival rate 5M = 100% *4M transfer to farm/pond 4 weeks total feed=	**104.2**	**1041.7**	**1041.7**	**2187.5**	**458.3**	**4583.3**	**4583.3**	**6926.0**
5-6M (5-20g) (ratio) 0.5% BW	1	20	10	3-feed daily total (g)	1	20	10	3-feed daily total (g)
5,000	4.0	80.6	40.3	125.0	16.1	322.6	161.3	500
5,000	5.0	100.8	50.4	156.3	20.2	403.2	201.6	625
5,000	6.0	121.0	60.5	187.5	24.2	483.9	241.9	700
5,000	7.1	141.1	70.6	218.8	28.2	564.5	282.3	875
survival rate to 6M = 100% *5M transfer to farm/pond 4 weeks total feed=	**155.2**	**3104.8**	**1552.4**	**4812.5**	**621.0**	**12419.4**	**6209.7**	**19250.0**

are returned to the settlement tank. Depending on presence of the swimming stages i.e. late auricularia and post-late auricularia, feeding with *C. muelleri* ends on around day 14. The juvenile foods are prepared as follows:

- after collecting from the wild, seaweed (Fig.30) and mud (Fig. 31) are sundried (Fig. 32);
- mud is sieved through 200 or 250 µm screen (Fig 33) and the seaweed is chopped in fine pieces (Fig. 34);
- after weighing, seaweed and fish meal are dipped in 1 µm filtered seawater for a several hours or overnight (Fig. 35)
- Spirulina is dipped in freshwater (rainwater) for several hours or overnight;
- Use blender to make the softened-foods finer pieces;
- sieve through 100 µm before feeding the animals

The protein content is the most important element in feeding pentactula and juveniles. Low fat content (crude fat around 5%) is desirable to maintain better water quality during the larval rearing and the 1st phase nursery culture in the settlement tanks. The author uses dried alga Spirulina instead of Algamac® (*Algamac ProteinPlus®) because Spirulina has much higher protein with low fat contents and the latter has higher fat contents. The author's choice of fishmeal is for milkfish (herbivore fish) farming because of less crude fat contents (about 3 %) compared to other fishmeals (Fig. 36). The author also uses the seaweed Sargassum spp. and Gracilaria sp., which contains about 10% crude protein with other essential nutrients, for free feed from the wild supplementing Spirulina and fishmeal. Mud or silt can be collected from the tidal flat area of the mangrove shore. The author has been using to mix with other feed (i.e. seaweed, fishmeal and Spirulina) for the juvenile grow-out as well as broodstock conditioning. The mud


Figure 24. Settlement tank.

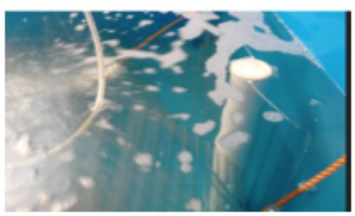


Figure 25. Screen-covered drain outlet.

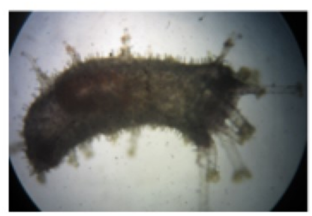


Figure 26. Day 28 juvenile.


Fig 27. Day 28 settled juveniles.


Figure 28. 5 g size 3-mo juveniles


Figure 29. Juveniles 3-mo in ocean grow-out site.


Figure 30. Seaweed *Sargassum* sp.


Figure 31. Mud from mangrove tidal flat.


Figure 32. Drying seaweed and mud.


Figure 33. Dried and sieved mud.


Figure 34. Dried seaweed.


Figure 35. Preparation for feeding.

Figure 36. Fishmeal.

Figure 37. Down-weller "habitat simulators" for juvenile grow-out.

contains detritus with organic substances and symbiotic heterotrophic micro-organisms in the mangrove forest (i.e. Schizochtrium spp.) which are known to have high contents of poly-unsaturated fatty acid (ω3 fatty acid). This heterotrophic (propagating without using sunlight) organisms live in the mangrove shore. *Algamac® and Algamac ProteinPlus® are commercial products from Aquafauna BioMarine, USA.

From day 28 or one month after spawning to six-month-old juveniles, an example of feeding schedule was given in the following table 6: juveniles from 2-month-old (Settlement Tank #2 = ST2 with 4,996 J2W1) to 3-month-old in the 2,500L down-weller (Juvenile Habitat Simulator #3 = JHS3 with 5,000 J3W1) where the juveniles were produced from the spawning on September 8, 2017.

Figure 38. Estimating settled 2-mo juveniles by detaching from the plates.

Juveniles attached on the plates are transferred on around day 56 (two-month-old after spawning) to the down-weller "habitat simulators for juveniles" (Figs. 37-38). Although the larger the animals, it is the easier for counting and estimating the standing crop, the juveniles were large enough (10 - 20 mm, 0.2 - 2g) to estimate the number of individuals by a naked eye.

Between 2 - 3 months old (1 - 5 g) after spawning, down-wellers "habitat simulators for juveniles" are stocked with juveniles either being attached on or being detached from the settlement plates. Stocking density of each tank was approximately 1,500 juveniles per square meter. Those remaining juveniles in the settlement tanks need to be fed weekly but are to be transferred in earlier occasions to hapas or ocean grow-out sites. The juveniles in the habitat simulators will be fed weekly for subsequent two or three months before transferring to grow-out phase (farming) in the ocean sites, earthen ponds or restocking sites.

7. Grow-out Culture

At around four or five months after spawning, the juveniles should reach to average 5 - 10 g. So, they are ready for farming. There is no published information available on the feeding techniques or feeding amount for both settled pentactula, early juveniles and juveniles up to six months after spawning. Also, there is no information about feeding techniques after six months old to harvesting size to export. The

Table 7. The results of counting of two-month-old juveniles attached on the settlement plates and estimate of those attached on the tank surface during the hatchery work in September - October 2017 at COM's hatchery in Pohnpei, FSM.

day 49 (Oct. 27-29, 2017)	**Settlement Tank #1 (from LR2-1)**	**Settlement Tank #2 (from LR1)**	**Settlement Tank #2 (from LR2-2)**
counted total number of juveniles (on plates)	**2,154**	**2,975**	**17,067**
estimated total number of juveniles (tank surface)	**3,670**	**2,021**	**51,304**
estimated total number of juveniles (plates + tank)	**5,824**	**4,996**	**68,371**

Table 8. Restocking JHS tanks with 2-mo juveniles.

2.5t Habitat Simulator Tank for Juveniles (with plates)	**No. of Juveniles Counted**
JHS#1 =	**5,985**
JHS#2 =	**4,642**
JHS#3 =	**5,129**
JHS$4 =	**4,569**
JHS#5 =	**1,871**
total =	**22,196**

Table 9. Number of 2-mo juveniles in the settlement tanks.

2.5t Settlement Tank after Transfer (without plate)	**No. of Juveniles Estimated**
ST#1 =	**3,670**
ST#2 =	**2,021**
ST#3 =	**51,304**
total =	**56,995**

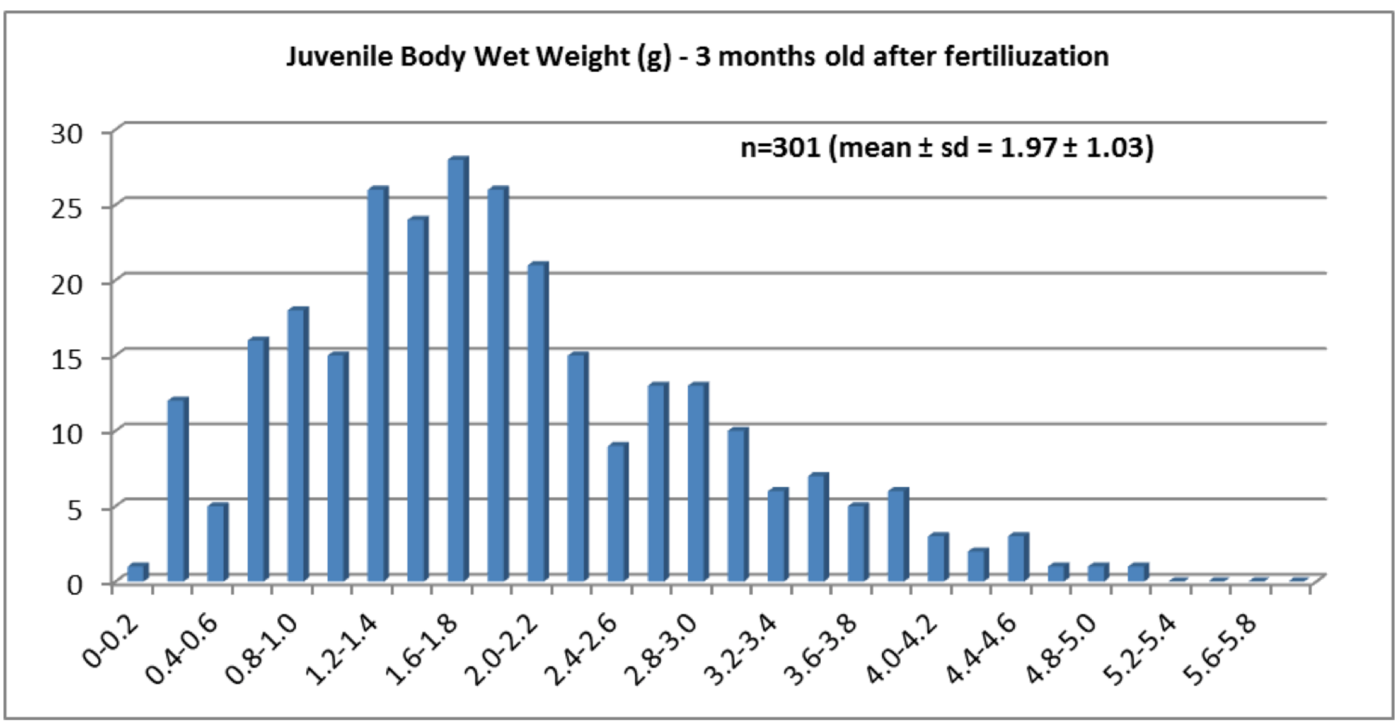
Table 10. Feeding table for 6-month-old juveniles in the downweller habitat simulator for juveniles.

	daily amount of feed (g) = **smaller size**				daily amount of feed (g) = **larger size**			
	Spirulina	Fishmeal	Seaweed	Mud (g) = 3-feed total	Spirulina	Fishmeal	Seaweed	Mud (g) = 3-feed total
6M (10-40g) (ratio) 1% BW	1	20	10	3-feed daily total (g)	1	20	10	3-feed daily total (g)
4,750	NA	306.5	153.2	475.0	NA	1225.8	612.9	1900
4,750	NA	536.3	268.1	831.3	NA	1379.0	689.5	2138
4,750	NA	766.1	383.1	1187.5	NA	1532.3	766.1	2375
4,750	NA	990.0	498.0	1543.0	NA	1685.5	842.7	2613
survival rate to 12M = 95% - 4 weeks total feed=	NA	18233.9	9116.9	28262.5	NA	40758.1	20379.0	63175.0

feeding table (Table 10) was developed by the author for private farming enterprise in Australia. Note that the feeding tables should be used only if actual or estimate of number of juveniles are not available. Feeding the juveniles and/or young adults are generally based on and calculated by the average body weight (wet weight). When they reach preferably to 10 - 20 g (average 5 - 10g) size after 3 - 5 months from spawning, they are ready for grow-out in the farm (ocean enclosures and/or ponds). Stocking density for the grow-out farming could be at 2 - 3 individuals per square meter (m2). In a hapa method elsewhere, 1- 2 g size about 2 or 3-month-old juveniles are stocked at 200 individuals per m2. On the other hand in a down-weller juvenile habitat simulator (e.g. 10,000L in Australia) with tank floor area of approximately 10 m2, initial stocking density is about 1,000 juveniles per m2). It is recommended to reduce the stocking density to at least half or preferably to 1/4 on around 5-month-old.

The following pages describe how to estimate feeding amount for the juveniles of 3-month-old. Also, explaining how accurate these feeding tables and how to use these tables developed by the author. For rearing early juveniles, it is recommended to feed the animals with 1/4 of adult grow-out amount = (1% body weight) x ¼ = 0.25% daily, and then increase the amount gradually to 0.5% and later around 5-month-old to 1%. The hatchery and farmhands must monitor the animals daily or at least weekly, such as measuring body weight by sampling monthly to adjust (increase) amount of weekly feeding amount. The following is an example of 300 juveniles of 3-month-old collected from a hapa elsewhere and measured body weight (mean ± sd = 1.97 ± 1.03 g). The histogram of these 301 (say 300) showed that they were smaller and closer to minimum weight group (1g group), indicating that they had not been fed well.

The following is how to estimate the weekly feeding amount of the 300 juveniles of 3-month-old, when they are required daily with 1% of body weight of food. Also, how-to estimate the feeding amount by using the juvenile feeding table when actual body-weight measurements were not done onsite.



(EXAMPLE) Histogram of the 3-month-old juvenile body weight obtained from hapa in a pond.

"How-to-Compute" weekly feeding amount (1% Body Weight) for 3-month-old "300" juveniles from hapa.

1.97g x 1% BW per day = 1.97g x 0.01 = 0.0197g per day per juvenile
0.0197 x 300 juveniles per day = 5.91g per day = 5.91 x 7days = 41.37g per week in total
*Spirulina vs. Fishmeal vs. Seaweed = 1 : 5 : 5 for the 3-month-old juveniles
therefore,

Spirulina = 41.37 x 1/(1+5+5) = 3.76g per week (0.54g per day)
Fishmeal = 41.37 x 5/(1+5+5) = 18.8g per week (2.69g per day)
Seaweed = 41.37 x 5/(1+5+5) = 18.8g per week (2.69g per day)

* If number of juvenile is 300, then the feeding amount is 300/10,000 of the figures given in the following Feeding Table.

daily amount of 3-feed total = 25 x 300/10,000 = 0.75g when given 0.25% BW

If given 1% BW,
daily amount = 0.75g x 1/0.25 = 3g and weekly amount = 3g x7days = 21g
Spirulina = 21 x 1/(1+5+5) = 1.91g
Fishmeal = 21 x 5/(1+5+5) = 9.55g
Seaweed = 21 x 5/(1+5+5) = 9.55g
However, the amount is based on the Smaller Group (1g).

If the juvenile's average body wet weight is 1.97g (= 2g), the feeding amount should be increased

and then, re-calculated for the "300" juveniles.

i.e. Each 1 g increment is (875-1,75)/4 = 1,75 per week

1g weight group = 175; 2g weight group = 175 + 175 = 3,50g; 3g weight group = 350 + 175 = 525g

Feeding Table for the 10,000 Juveniles (3 M): Smaller Group (1g) and Larger Group (5g)								
	Smaller Group (1g)			Larger Group (5g)				
10,000 3M juveniles	Spirulina	Fishmeal	Seaweed		Spirulina	Fishmeal	Seaweed	
3-4M (ratio) 0,25%BW	1	5	5	3-feed daily total (g)	1	5	5	3-feed daily total (g)
wk-1 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-2 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-3 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
wk-4 daily amount	2.3	11.4	11.4	25.0	11.4	56.8	56.8	125.0
Weekly Total (g)	15.9	79.5	79.5	175.0	79.5	397.7	397.7	875.0

4g weight group = 525 + 175 = 700g; 5g weight group = 700 + 175 = 875g

therefore,

weekly amount of the 2g weight group could be 350g per "10,000" juveniles

weekly amount of the 2g weight group of the "300" juveniles = 350g x 300/10,000 = 10.5g when given 0.25% BW

therefore,

Spirulina = 10.5 x 1/11 = 0.95g

Fishmeal = 10.5 x 5/11 = 4.77g

Seaweed = 10.5 x 5/11 = 4.77g

If given 1% BW, weekly feeding amount of 2g weight group = 10.5 x 4 = 42.0g

therefore,

Spirulina = 0.95 x 1/0.25 = 3.8g

Fishmeal = 4.77 x 1/0.25 = 19.1g

Seaweed = 4.77 x 1/0.25 = 19.1g

The estimated weekly feeding amount from BW measurements (1.97g ± 1.03g) was total 41.37g = Spirulina 3.76g + Fishmeal 18.8g + Seaweed 18.8g

Thus,

the above “Feeding Table” is very accurate (+0.04g < > +0.67g), almost the same feeding amount obtained from actual BW measurements.

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Culture of Sea Cucumbers in Korea: A Guide to Korean Methods and the Local Sea Cucumber in the Northeast U.S.

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In an effort to develop suitable culture techniques for sea cucumber (*Cucumaria frondosa*) in the Northeast, this guide reviews the current knowledge of *C. frondosa* biology and reports on techniques for the hatchery culture of the Japanese sea cucumber *Apostichopus japonicus* learned during a research exchange between the United States (NOAA Sea Grant) and South Korea (National Fisheries Research and Development Institute). The final portion of the guide discusses the potential adoption of the culture techniques for *A. japonicus* for use with *C. frondosa*.

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Introduction

Sea cucumbers are a valuable fishery around the globe. The United Nations Food and Agriculture Organization (FAO) estimated the global production from both aquaculture and capture fisheries to be 153,183 metric tonnes in 2011 (FAO 2013a). Of that production, roughly 85% is the Japanese sea cucumber *Apostichopus japonicus* (**1**). Sea cucumbers are developed into a variety of products including dried muscle, fermented guts, and dried gonads (FAO 2013b). Wild stocks have been heavily fished in many areas around the world. As a result of overfishing, sea cucumber culture techniques have been developed for variety of species. Hatchery production has centered in the Pacific Rim and is used both as a source of juveniles for various culture activities and for stock enhancement and restoration efforts. Currently hatchery production exists for approximately a dozen different tropical and temperate species (Purcell et al. 2012, Table 1).

Integrated Multi-trophic Aquaculture (IMTA) efforts in northeastern North America have focused on various combinations of fish, shellfish, and algae. Sea cucumbers have been proposed as one potential species that could be added as a consumer of benthic deposits. Within the Gulf of Maine, the predominate sea cucumber species, *Cucumaria frondosa* (**2**), has been fished commercially in Maine since 1990. Landings peaked in 2004 with a harvest of just over 10 million pounds (ME DMR 2013). The value of the fishery in Maine fluctuated between $66,000 and $562,000 from 1994 until the peak in 2004. The price per pound has more than tripled since the peak harvest in 2004. The economic value for the Maine fishery is based on the boat price paid to fishermen, and does not represent the significant increase in value that occurs with processing into various final consumer products.

Sea cucumbers from the Maine fishery traditionally have gone to the Asian food market, however the potential for various nutraceuticals may represent a higher value use. For example, trials are underway to examine the potential for Frondoside A, a compound extracted from *C. frondosa*, to treat cancer (Attoub et al. 2013). At least one company in the region is processing sea cucumbers for the extraction of various nutraceuticals.

A recent review of the potential for culture of *C. frondosa* in the Northeast (Nelson et al. 2012a) highlighted advantages and challenges for commercial culture of this species, in particular under IMTA. Strengths include an existing market for the product with good potential for increased nutraceutical use, a well-understood reproductive biology (especially for populations in the St. Laurence River area), and evidence that *C. frondosa* would be a suitable species for integration in IMTA farms as a benthic filter feeder. Culture of this species faces several challenges. These include a relatively low market price, even when the fishery was at its peak, due to the thinner muscle wall, a long grow-out period, and a lack of published techniques for culturing this species.

Apostichopus japonicus

Michael Pietrak

Cucumaria frondosa

Hamel & Mercier (SEVE)

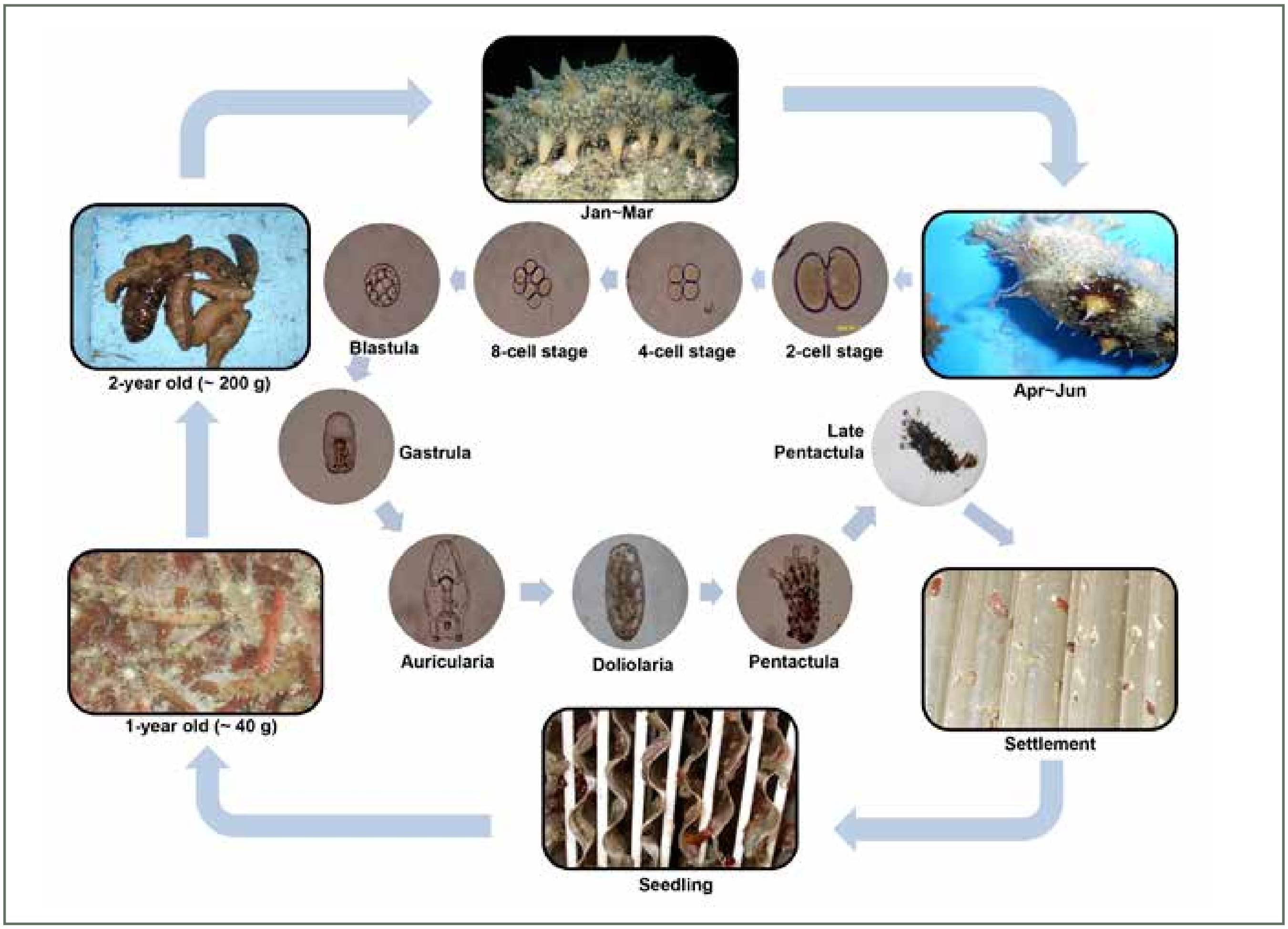
Table 1: Global hatchery production of sea cucumber juveniles. Modified from Table 1 in Purcell et al. 2012.

Country	Species cultured	Annual production of 1 g juveniles	Use of juveniles	Start year to end year
Australia (Northern Territory)	*Holothuria scabra*	62,000+	Sea ranching; pond farming	2004-ongoing
Australia (Queensland)	*H. scabra*	500,000	Sea ranching	2003-2009
Australia (Queensland)	*H. lessoni*	330,000	Sea ranching	2004-2009
Australia (Queensland)	*H. scabra*	1,000	Experimental	2004-2007
Canada	*Parastichopus californi-cus*	n/a	Pond farming	2009-ongoing
China	*Apostichopus japonicus*	>6 billion	Sea ranching; pond farming	1990-ongoing
Ecuador	*Isostichopus fuscus*	n/a	Experimental	2002-2008
Fiji	*H. scabra*	500	Experimental	2008-2010
FSM (Pohnpei)	*H. scabra*	10,000	Experimental	2009-ongoing
FSM (Yap)	*Actinopyga sp.*	n/a	Stock enhancement	2007
India (Tuticorn)	*H. scabra*	3000	Experimental	1988-2006
India (Tuticorn)	*H. spinifera*	n/a	Experimental	2001-2006
Iran	*H. scabra*	n/a	Experimental	2011
Japan	*A. japonicus*	>3 million	Stock enhancement	1977-ongoing
Kiribati	*H. fuscogilva*	500-8,000	Stock enhancement	1997-2009
Madagascar	*H. scabra*	200,000	Sea farming (pens)	2007-ongoing
Maldives	*H. scabra*	5 million	Sea ranching	1997-ongoing
Mexico	*I. fuscus*	300,000	Pond farming	2008-ongoing
New Caledonia	*H. scabra*	18,000	Experimental	2000-2006
New Caledonia	*H. scabra*	450,000+	Sea ranching; pond farming	2011-ongoing
New Zealand	*Australostichopus mollis*	n/a	Experimental	2007-ongoing
Palau	*Actinopyga mauritiana*	500,000	Stock enhancement	2009-2011
Palau	*Actinopyga miliaris*	50,000	Stock enhancement	2009-2011
Philippines (Bolinao)	*H. scabra*	32,000	Sea ranching	2001-ongoing
Philippines (Mindanao)	*H. scabra*	15,000	Sea ranching; pond farming	2009-ongoing
Philippines (Bolinao)	*Stichopus horrens*	500	Experimental	2009-ongoing
Philippines (Dagupan)	*H. scabra*	20,000	Experimental	2009-2011
Philippines (Iloilo)	*H. scabra*	11,000	Experimental	2010-ongoing
Saudia Arabia	*H. scabra*	n/a	Sea ranching	n/a
Solomon Islands	*H. scabra*	n/a	Experimental	1996-2000
USA (Alaska)	*P. californicus*	n/a	Experimental	2010-ongoing
Vietnam	H. scabra	200,000+	Pond farming	2001-ongoing

Life history and biology of *Cucumaria frondosa*

Mature adult sea cucumbers "broadcast spawn" their gametes. Fertilized embryos develop into pentacula, the five-tentacled larval stage in the life history of echinoderms. After a period of time in the plankton, the larvae settle to the sea floor and metamorphose into juveniles. *C. frondosa* average 20 cm in length, although they are capable of growing up to about 50 cm, and live in temperatures between -1.8 °C and 8 °C. Levin and Gudimova (2000) provide a good description of *C. frondosa*'s morphology.

Adult animals reach sexual maturity in about five years and around 80-100 mm in length. Adult animals display sexual dimorphism and can be easily distinguished when the feeding tentacles are extended. Females display a gonopore approximately 4 mm in diameter and a papilla approximately 7 mm in length with a single genital pore. Males possess a larger gonopore approximately 8-10 mm in diameter surrounded by between five and 22 papillae (Hamel and Mecier 1996).



Life history of sea cucumbers and milestones for their culture in Korea. Figure from NFRDI.

Spawning typically occurs in the St. Lawrence around mid-June (Hamel and Mercier 1996), while populations near Mount Dessert Island in Maine spawned between the end of March and the end of May (Ross 2011). Day length is likely an important cue to start the process of producing gametes (Hamel and Mercier 1996, 1999), however cues in the mucus appear to be more important in synchronizing the spawning of animals in a given locality (Hamel and Mercier 1999). Following fertilization, the embryos develop into pentacula larvae. According to Hamel and Mercier (1996) this took nine days at 12 °C and 26 psu (practical salinity units; Table 2). Ross (2011) does not report embryo development timing beyond the 64-cell stage, which could take up to 48 hours. Ross did report that in one sampling season "elongated embryos" were found in the early plankton tows, and that lab observations showed that this stage was reached after four to seven days. Larvae prefer to settle on rock or gravel substrate after approximately 48 days after fertilization (Hamel and Mercier 1996).

Few have looked at growth rates, but Hamel and Mercier (1996) reported that the mean growth rate in the wild was 2 mm a month, while the maximal growth rate during the spring was 5-6 mm per month. Studies have also shown that the rate an individual *C. frondosa* inserts its tentacle into its mouth is related to the feeding rate of the animal (Holtz and MacDonald 2009). It has also been shown that *C. frondosa* are capable of feeding on high organic content materials, such as feces and fish feeds, from IMTA salmon farms (Nelson et al. 2012a). The absorption rate increased with increasing organic content, suggesting that *C. frondosa* might be cultured on salmon-mussel-algae IMTA farms.

Table 2: The time and size evolution of selected developmental stages of *Cucumaria frondosa* up to young sea cucumbers, under natural environmental conditions reproduced in the laboratory. Table modified from Hamel and Mercier 1996.

Stage of development	Time	Size (mm)
Fertilized oocytes	0 min	0.90±0.2
64-cell	24.0±2.0 h	1.40±0.5
Blastula	48.0±3.6 h	1.35±0.3
Hatching	62.0±8.0 h	1.30±0.4
Young gastrula	72.0±8.5 h	1.30±0.3
Late gastrula	4.3±0.5 d	1.40±0.5
Elongation	5.5±0.5 d	1.60±0.5
Vitellaria (uniformly ciliated	8.0±1.0 d	1.55±0.3
Young pentactula (5 tentacles)	11.0±1.5 d	1.30±0.3
Appearance of the anal pore	13.0±1.0 d	1.35±0.4
Late pentactula (with pair of ambulacral podia)	17.0±1.4 d	1.40±0.5
Settlement (cilia loss)	46.0±2.0 d	1.40±0.4
Young sea cucumber with 2 pairs of ambulacral podia	3.0±0.2 months	1.70±0.5
Young sea cucumber with 5 pairs of ambulacral podia	4.0±0.2 months	1.90±0.5

Note: The temperature recorded throughout the development varied from 0 °C to 13 °C as reported in Figure 1 of Hamel and Mercier 1996. A new stage was considered attained when 50-60% of the embryos reached it.

Korean hatchery culture techniques

Hatchery culture systems

Culture facilities

In March 2013, a NOAA Sea Grant-sponsored delegation from the United States visited three different sea cucumber hatcheries near Gangneung in Gangwon Province, South Korea.

One of the facilities was a private hatchery that used discharged cooling water from a nearby coal-fired power plant as a heat source. This water was mixed approximately 50-50 with local seawater and sand filtered prior to use in the facility.

The second hatchery visited was a provincial government hatchery that reared various species for stock enhancement. A main seawater line supplied storage tanks for the entire facility. Incoming water was filtered and heated as required in the individual species culture buildings. Neither the private nor the provincial facility sterilized incoming water.

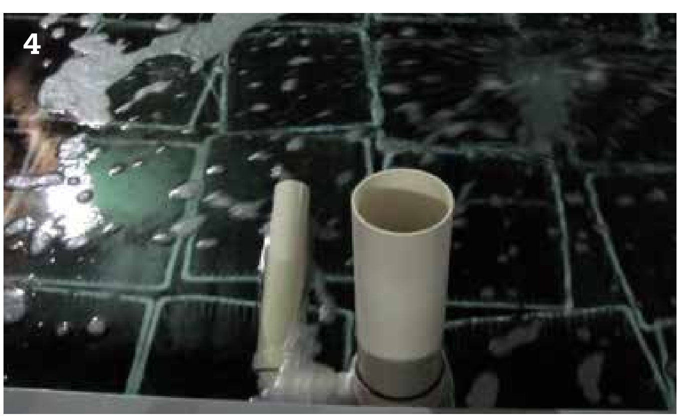
The final hatchery visited was an NFRDI research hatchery.

Culture tanks

All three hatcheries used similar rectangular culture tanks approximately five meters long and three meters wide, with a water depth of about one meter (**3**). A water inlet was located at the far side of the tanks; the bottom of each tank sloped slightly toward a drain at one end (nearest the walkway) to facilitate regular cleaning.

An internal standpipe of four-inch PVC acts as an emergency drain (**4**). The private hatchery attached a two- to four-inch "T" just below the water level in the tank with an unglued two-inch elbow that actually set the water level by having the pipe angled up, shown here. To partially drain the tank for the twice-daily water changes, the pipe is turned down, submerging it, draining the tank in a limited fashion.

A similar drain was installed between the tanks so that when moving the animals to the adjacent tank in order to clean the bottom, some of the already heated water could be saved. It is important to take the surface water, which is relatively free of sediments, and not the bottom water. At all locations the culture tanks were operated as static systems with partial water replacement twice a day, although the provincial hatchery had access to flowing seawater and recirculation systems.



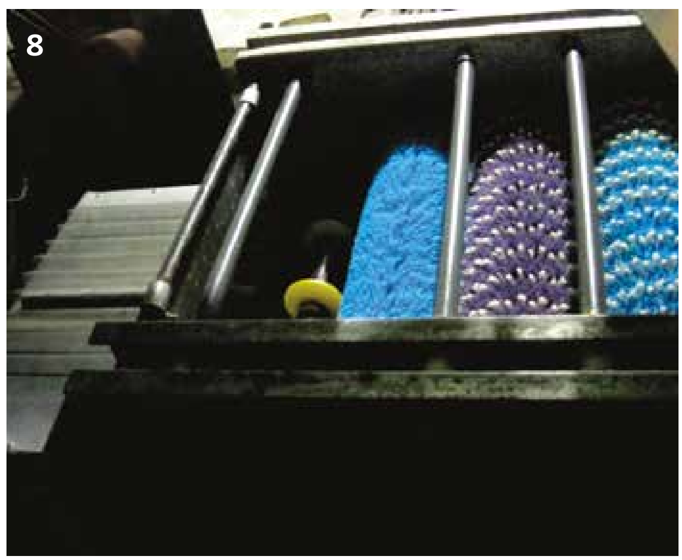
4

Culture equipment

Very little equipment is required for spawning and rearing sea cucumbers in the hatchery. In addition to the culture tanks described above, it would be useful to have some smaller tanks for conditioning broodstock. The size and style of the tanks is not critical, but conditioning requires an ability to slowly raise the water temperature to 15 °C. Nets of the appropriate mesh size (200 µm for *A. japonicus* or 800 µm for *C. frondosa*) are required to transfer the fertilized eggs to the culture tanks described above, which are used for the entire culture cycle in the hatchery. Access to a dissecting microscope in order to view the development of the larvae would be advantageous, however it is likely that an experienced operator will know the larval stage based on the degree-days since fertilization.

The most important pieces of equipment are the settling plates and the juvenile cucumber shelters (**5**). The shelters consist of an open plastic crate designed to hold file folders (**6**). Settling plates made from clear corrugated PVC sheets are cut to dimensions to fit inside the crate (**7**). In one of the hatcheries, the settling plates were treated with a vitamin mix prior to the first use, but this practice was not consistent at all of the hatcheries visited.

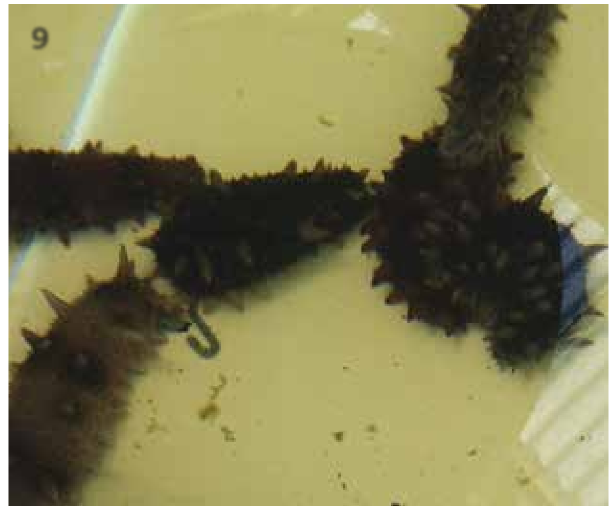
There is a need to clean the settling plates on a regular basis by hand washing/scraping or, with very dirty plates, an acid wash. One hatchery had a commercial plate washing machine that consisted of internal rollers that feed the individual panels and rotating brushes that scrub them clean (**8**).



8

Korean sea cucumber culture process

Broodstock conditioning and spawning



Adult *A. japonicus* are collected from the wild approximately 60 days in advance of the spawning season and placed into conditioning tanks at ambient seawater temperatures (**9**). The temperature is increased 1 °C per day until it reaches 15-18 °C. The animals are held at 15 °C for 800 degree days, at which point they should be ready to spawn. They can be fed a commercial diet during this time, which typically consists of powdered algae such as *Spirulina* spp. or *Dunaliela* spp. Feeding must be stopped the day before spawning to avoid contamination by sea cucumber feces.

Spawning is initiated through temperature shock: conditioned animals are moved from the holding tank directly into water that is 3-5 °C warmer. After release of both sperm and eggs, fertilized eggs can be removed with a net and placed into rearing tanks that have appropriately sized mesh covering the drains. The tanks should be set up as static systems. A sample of eggs can be taken and inspected for fertilization rates.

Larval culture

Fertilized eggs are collected from the spawning tanks and placed into the culture tanks at a density of 0.25 individuals mL^{-1} (Kang et al. 2012). No specific density was provided, but the NFRDI culture tank shown here holds approximately one million larvae (**10**). Larvae are fed a diet of microalgae. For the first two days a 50-50 mix of *Isochrysis galban* and *Pavlova lutherii* is recommended. After day two they can be fed *Cheotoceros calcitrans*. Samples of larvae should be taken daily in order to monitor development. After approximately seven to eight days, larvae should be close to the pentacula stage and settlement. At this point shelters with clean settling plates can be placed in the larvae rearing tanks.

Upon settlement the new juveniles are transitioned to a feed mixture of mud or bentonite, yeast, and powdered algae (**11**).

Predation of larvae by zooplankton was reported to be a problem in at least one facility visited. This can likely be managed through good water filtration.

Juvenile culture

Juveniles are cultured on the settling plates for about a year until they are transferred to cage systems for grow-out at sea. Maintaining good husbandry practices in the hatchery is critical for preventing disease, which can be a significant issue. All of the systems visited maintained static water in the culture tanks, with twice-daily partial water changes at feeding times. In one facility, the water was changed before feeding in the morning and after feeding in the afternoon. The water temperature is maintained around 15 °C. Conservation of water heat was given as the reason for maintaining static systems. It is possible to use a flow-through system, as was observed at the NFRDI research lab, although animals were maintained under ambient water temperatures. It should be noted that the juvenile sea cucumbers are mobile and will move within and between the shelter and throughout the tank. Appropriate screening must be maintained on drains when conducting water changes or if using a flow-through system.

To maintain good water quality it is important to clean accumulated feed and feces from the bottom of the tanks approximately every five days. This is accomplished by moving the shelters to an adjacent tank that is empty of animals (**12**).

Surface water can be moved over to reduce the energy needed to heat the water. The image here shows a drain in the wall between adjacent tanks (**13**). Tilting the standpipe down allows clean, warm surface water from one tank to partially fill the adjacent tank where animals are moved while the old tank is cleaned. Mesh bags or screens should be placed on drains to capture loose animals in the tank being drained. Once drained, the tanks can be scrubbed clean and readied for the next transfer of animals. In addition to cleaning the tanks on a regular basis, the settling plates must also be changed out and cleaned about once every three months.

Culture potential for *Cucumaria frondosa* in the Northeast

Cucumaria frondosa appears to be a potential candidate for culture in the northeastern United States. In particular, *C. frondosa* will likely make a good addition to IMTA sites being developed in the region, given its ability to feed on particulate organic wastes. Nelson et al. (2012b) reviewed the potential for culturing *C. frondosa* in the Northwest Atlantic and found it to be a potentially viable candidate species. Several major hurdles are immediately apparent to the culture of this organism. First, the current market for the wild caught *C. frondosa* does not appear to be valuable enough to make aquaculture economically viable. This situation is likely to change as higher-value uses of various extracts and nutraceuticals are further developed for both humans and animals. Second, a better understanding of some aspects of the basic biology is needed, in particular growth rates under various conditions and feeding levels. Finally, culture techniques will need to be adapted and optimized for this species.

A good starting point may be provided by the techniques observed in Korea, such as use of temperature shock to induce spawning. Nelson et al. (2012b) point out that hatchery production of juvenile sea cucumbers will likely be required as fishing for mature broodstock will compete with the wild fishery and juveniles are very difficult to find and collect in the wild. The long planktonic larval stage seen in *C. frondosa* compared to species raised in Korea may also pose additional problems. Hatchery producers will need to evaluate costs and feasibility of using the same tanks for juvenile culture and larval production. The long larval phase will also pose issues associated with water quality and treatment. Using static systems for the duration of the larval phase will likely not be possible, as a regular exchange of water will be needed to maintain water quality. Prevention of larvae loss or damage will be an issue to contend with. Larval settlement onto the plate culture system used in Korea should be tried, however the spacing of the plates may need to be adjusted. The ability to grow sea cucumbers on a vertical surface will greatly increase space efficiency. As *C. frondosa* remains a planktonic feeder all of its life, their culture should not experience the transition to deposit feeders that was hinted at as a bottleneck in production in Korea, and might permit for the use of continuous flow culture tanks that will help to improve water quality and remove uneaten feed. If neutral to slightly negatively buoyant diets can be developed, flow through the tank should allow feed to be carried to all animals.

It will be necessary to develop grow-out techniques that can incorporate sea cucumbers onto IMTA sites. While the Korean design of a net pen cage that has a false bottom to separate the fish from the sea cucumbers might be feasible, other ideas should be considered. It may be more practical to culture sea cucumbers in submerged trays or cages similar to those used for shellfish culture. Most importantly, husbandry techniques will need to be developed that allow for the grow-out to occur in two and a half years or less. It would not be advisable to culture sea cucumbers for much longer than the grow-out period of the other organisms on the site, so that the entire site may be fallowed before starting the next crop of animals.

The development and optimization of culture techniques for *C. frondosa* is likely to take five to 10 years of research and grow-out experience. Because current medical trials may appear to be successful or other economic factors could change in a manner that will improve economics of sea cucumber culture, work on culture techniques should begin immediately.

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Sea Grant
Maine
at the University of Maine

Pacific White Shrimp, *Litopenaeus vannamei*, Hatchery Industry in China

by

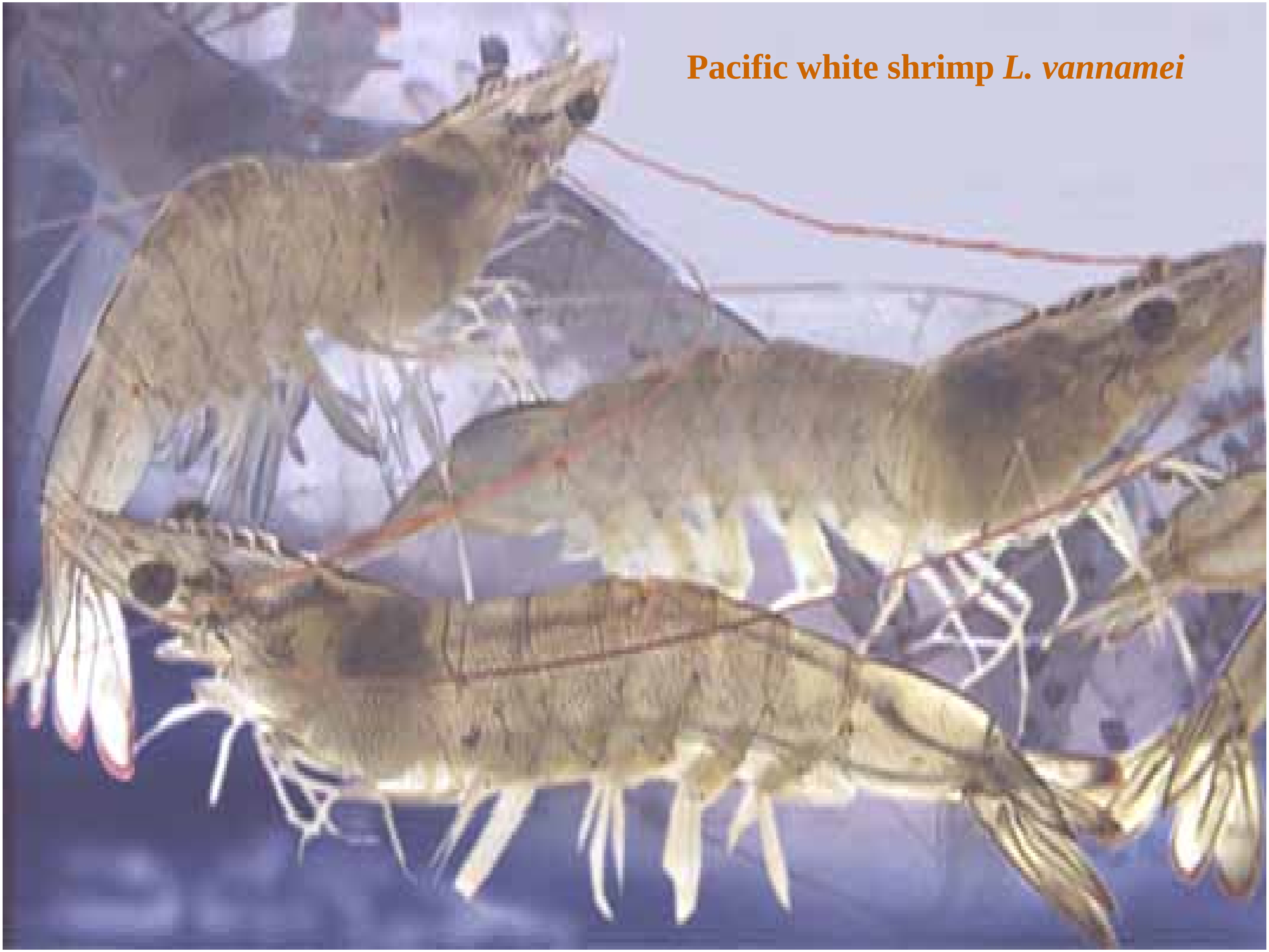
Hann-Jin Huang, Xiaoli Yang and Dan Chen

Guangdong Evergreen Group Company Ltd.

Guangdong, China

广东恒兴集团有限公司

GUANGDONG EVERGREEN GROUP COMPANY,LTD



Pacific white shrimp *L. vannamei*

A brief history of *L. vannamei* in China

1988

- ***L. vannamei*** **was first introduced into China by Professor Zhang Weiquan of the Institute of Oceanology of Chinese Academy of Sciences from University of Texas Port Aransas Marine Science Laboratory. The postlarvae were provided by Texas A&M University. Nineteen postlarvae survived upon arriving in China.**

1989

- **First succeeded in maturation and spawning in captivity (up to zoea stage)**

1992

- **Succeeded in the production of postlarvae**

1994

- **Succeeded in the mass production of postlarvae**

A brief history of *L. vannamei* in China（cont.）

1994 – 1999

- **Small scale growout production**

1999

- **Real start and acceptance of *L. vannamei* farming after the collapse of *Penaeus monodon* farming (mainly due to the epidemic white spot virus disease)**

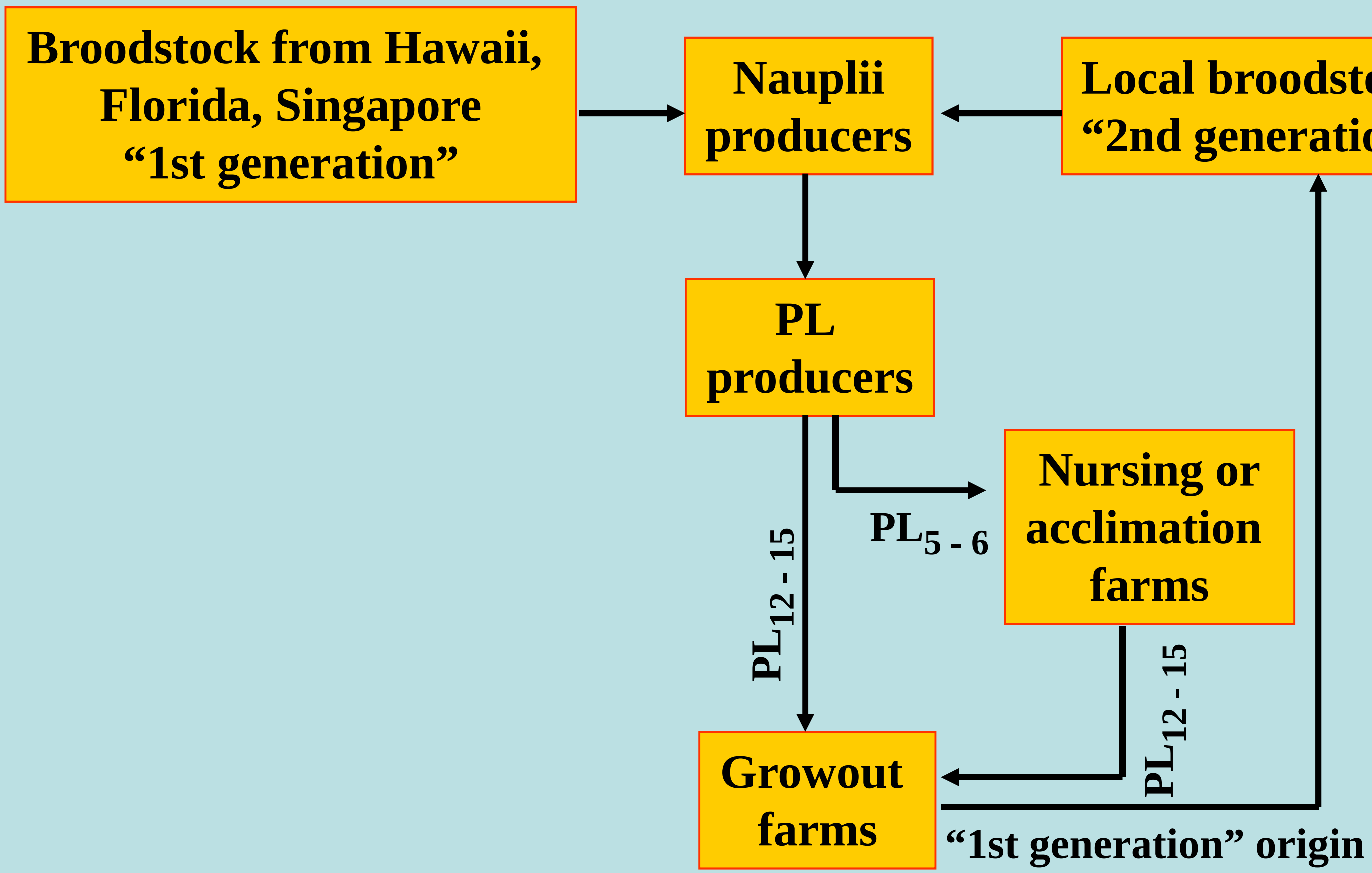
2001

- **Farming area for *L. vannamei* started to expand quickly.**

2007

- **Annual production of shrimp reached a record-high of 1.28 million metric tons among which the majority were *L. vannamei*.**

L. vannamei hatchery industry in China: diversification



L. vannamei hatcheries in China

Estimated number of hatcheries in China

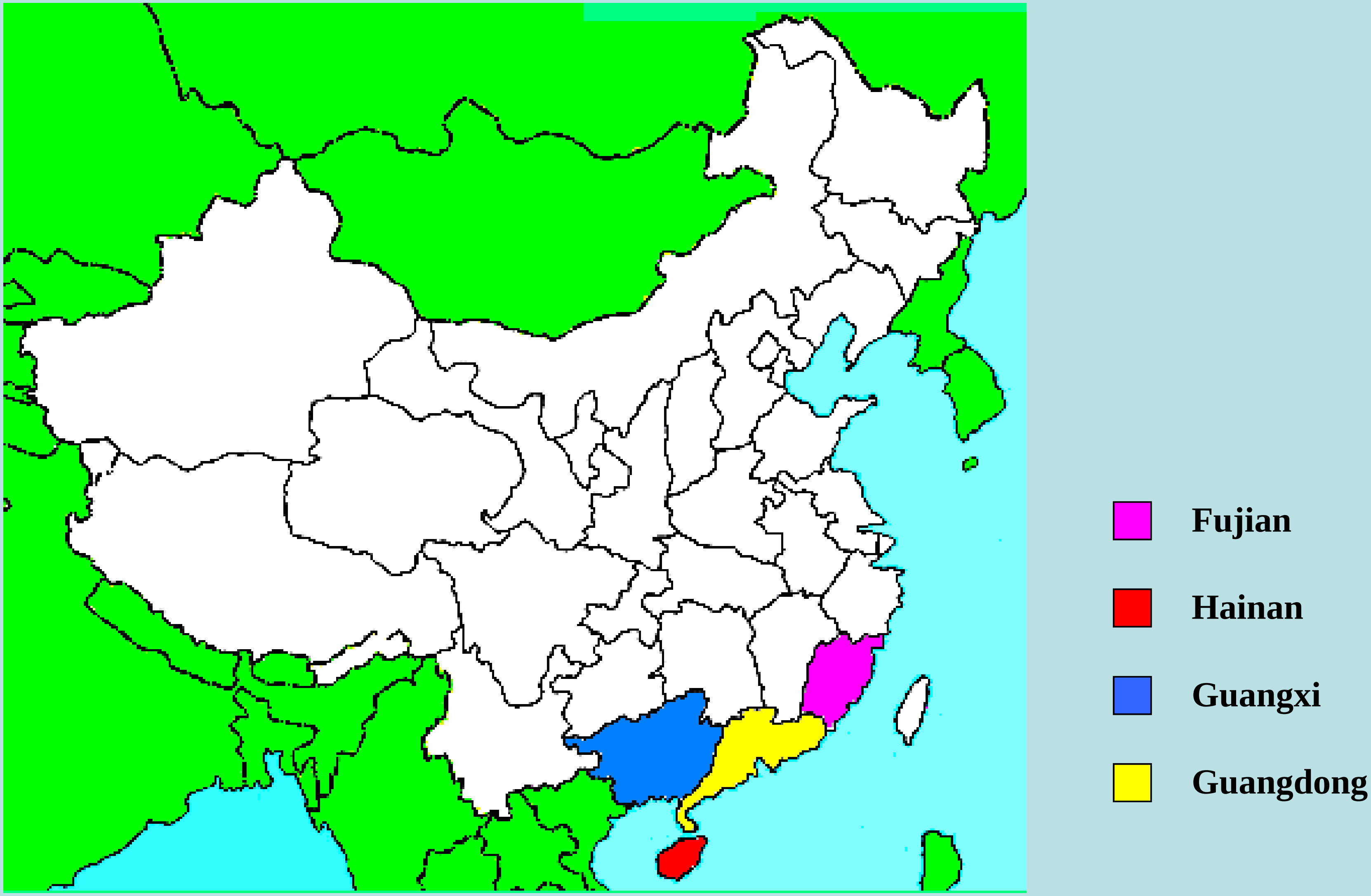
Between 2,600 and 2,700 established and 1,500 to 1,600 in production and at least 90% of them are situated in the provinces of Guangdong, Guangxi, Fujian and Hainan.

Sizes of hatcheries

From 500 m^3 to 2,000 m^3 larvae-rearing area per hatchery and production from 50 millions to 500 millions of postlarvae each year per hatchery.

Estimated total requirement of postlarvae

Between 300 billions and 400 billions each year to satisfy the growout production of 800,000 to 1 million metric tons of shrimp.



Major *L. vannamei* PL producing areas in China

Wenchang and Qionghai, the major *L. vannamei* PL producing areas in Hainan

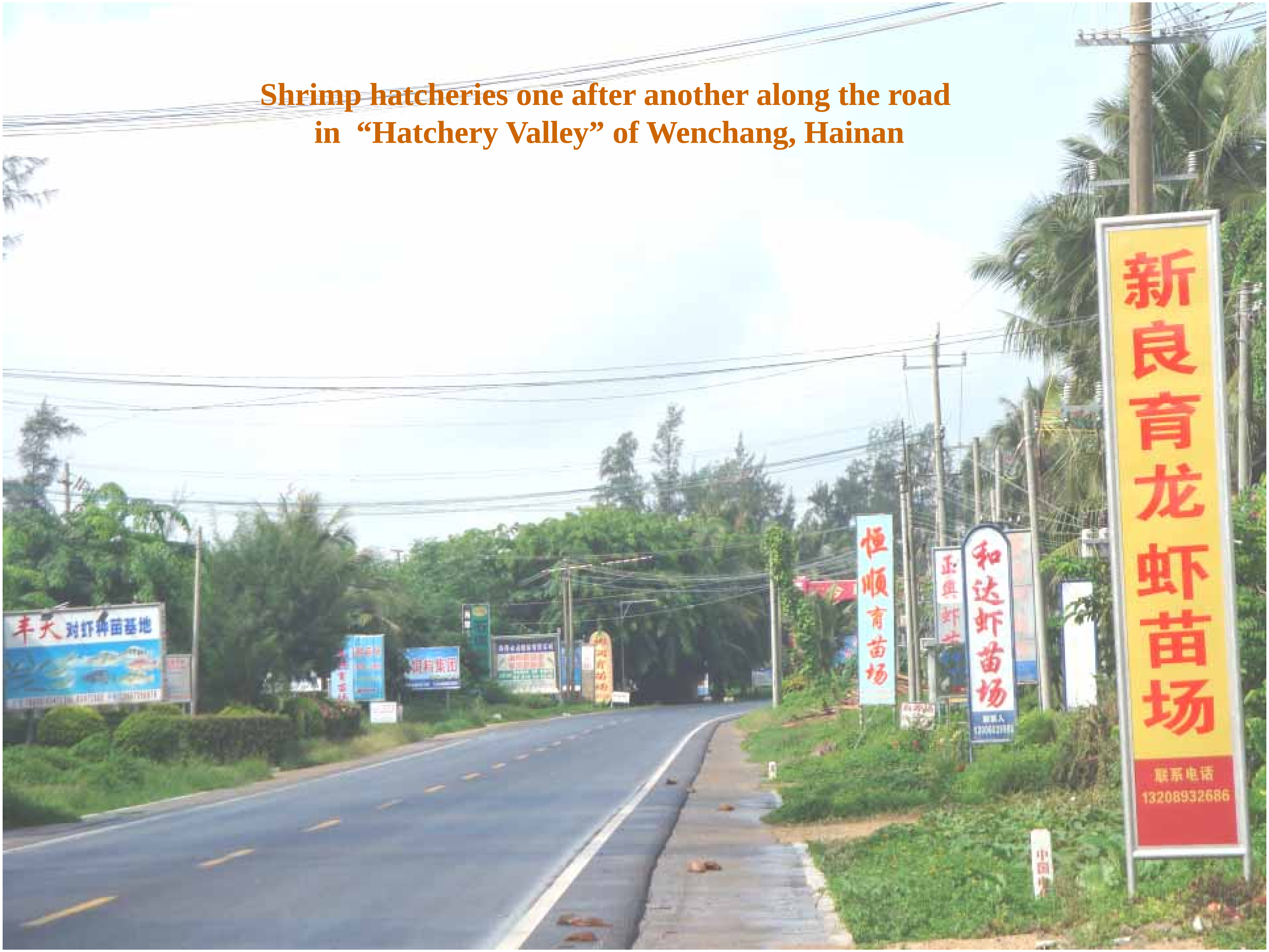
Fangcheng, Beihai and Dongxing, the major *L. vannamei* PL producing areas in Guangxi

Longhai, Zhangpu, Xiaman and Xiapu, the major *L. vannamei* PL producing areas in Fujian

Zhanjiang (Donghai Island), the major *L. vannamei* PL producing area in Guangdong

Shrimp hatcheries one after another along the road in "Hatchery Valley" of Wenchang, Hainan

Shrimp hatcheries one after another along the road in “Hatchery Valley” of Wenchang, Hainan



L. vannamei hatcheries: water treatment

Filtration

- Sand filter (most commonly used)
- Cartridge filter
- Bag filter (most commonly used)

Removal of organic matters

- Foam fractionator (protein skimmer)
- Activated carbon filter (most commonly used)

Sterilization

- Ozonation
- UV sterilization
- Chlorination (most commonly used)
- Formalin (becoming popular)

L. vannamei hatcheries: water treatment

Adjustment of alkalinity

It is a common practice to bring up the alkalinity of seawater to a minimum of 130 ppm. The effect on the survival is significant.

Application of EDTA

Constant application of EDTA at 10 - 20 ppm is generally required. Heavy metals often exist in the seawater source.

The water treatment system in Evergreen's shrimp hatchery
(Sand filters, protein skimmers and ozonators)

L. vannamei hatcheries: broodstock

Source of broodstock

1. Imported, so-called “1st generation” SPF broodstock
2. Locally-raised, so-called “2nd generation” broodstock

Size of broodstock

Males: > 40 g each

Females: > 45 g each

Age of broodstock

> 8 months and < 12 months

Cost of broodstock

1. Imported: >US$ 40 each male or female
2. Locally-raised: US$ 2 - 3 each male or female

L. vannamei hatcheries : broodstock

Density of broodstock

10 – 15 shrimp/m^2, males and females separated

Induced maturation

- Unilaterally eyestalk ablation on female
- Highly nutritive feed, mainly squid, polychaete worms, oyster and calf liver (formulated feeds, not popular)

Reproductive performance of broodstock

Expectation: 200,000 to 300,000 nauplii per spawn and a minimum of 14 spawns within 5 months after eyestalk ablation, totaling a minimum of 3 millions of nauplii per mother shrimp (imported broodstock)

L. vannamei hatcheries: nauplii to postlarvae

Capacity of larvae-rearing tank

10 - 20 m^3 per tank

Initial density of nauplii

150 – 200 per liter of water

Survival rates

Naupliar stage: 0 – 90%

Zoea stage: 0 – 70% (most critical stage)

Mysis stage: 50 – 90%

PL stage: 80 – 90%

Overall from nauplii to PL_{12-15}: 0 - 50%

Production of PL

Up to 100,000 PL_{12-15} per m^3 of water

L. vannamei hatcheries: nauplii to postlarvae

Feeding

Zoea stage:

- Live algae (mainly *Skeletonema sp.* and *Chaetoceros sp.*)
- Algal powder
- Formulated feed (in microencapsulated, flake or microbound form)
- Newly-hatched Artemia nauplii (cold or heat-shocked, starting from Zoea II or Zoea III stage)

Mysis stage:

- Formulated feed
- Live newly-hatched Artemia nauplii

PL stage:

- Formulated feed
- Live newly-hatched Artemia nauplii

L. vannamei hatcheries: quality assurance of PL

Before harvesting PL, the following steps are taken to ensure quality of PL:

1. PCR check for viral infection
2. Microscopic observation on abnormality
3. Stress test (salinity shock)

100 postlarvae from PL tank → 0 ppt salinity water (same water temp.) → 15 min. → Normal seawater and observe survival rate

1.above 98% - healthy
2.above 85% - accept
3.below 85% - reject

* Postlarvae have to be PL_{10} or older for 0 ppt salinity shock test.

L. vannamei hatcheries: costs of larvae-rearing

Nauplii (1st generation)

- **Selling price: RMB 10 – 15 (US$1.5 – 2.2) per 10,000 nauplii**
- **Cost per 10,000 PL_{12-15} based on 30% survival: RMB 33 – 50 (US$ 4.8 – 7.3)**

Feeding cost

RMB 27 - 30 (US$ 4.0 – 4.4) per 10,000 PL_{12-15}

Other cost

RMB 20 - 25 (US$ 2.9 – 3.7) per 10,000 PL_{12-15}

Total cost

RMB 80 - 100 (US$ 11.7 – 14.6) per 10,000 PL_{12-15}

Selling price

RMB 120 - 160 (US$ 17.6 – 23.4) per 10,000 PL_{12-15}

*** Note: One US dollar is equivalent to RMB 6.83**

L. vannamei hatcheries: costs of larvae-rearing

Nauplii (2nd generation)

- **Selling price: RMB 1 - 2 (US$ 0.15 – 0.30) per 10,000 nauplii**
- **Cost per 10,000 PL_{12-15} based on 30% survival: RMB 3.3 – 6.7 (US$ 0.48 – 0.98)**

Feeding cost

RMB 17 - 20 (US$ 2.49 – 2.93) per 10,000 PL_{12-15}

Other cost

RMB 10 - 15 (US$ 1.46 – 2.20) per 10,000 PL_{12-15}

Total cost

RMB 30 - 40 (US$ 4.39 – 5.86) per 10,000 PL_{12-15}

Selling price

RMB 60 - 90 (US$ 8.78 – 11.71) per 10,000 PL_{12-15}

*** Note: One US dollar is equivalent to RMB 6.83**

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Constraints

1. **Inadequate supply of quality SPF broodstock**

   While the demand for "1st generation" PL is on the rise, some imported broodstocks perform poorly (low maturity rate, low fertilization rate, poor quality nauplii). Quality SPF broodstocks are in short supply and expensive.

2. **Inferior quality of locally-raised broodstocks**

   Locally-raised broodstocks are not properly selected. The reproductive performance is poor and the growth of the offspring is slow with much size variation.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Problems

1. Zoea II syndrome often occurs, resulting in low survival.

2. Unknown causes of empty gut during mysis stage.

3. The use of formalin in water treatment, which has become popular, may impose some side effects on shrimp health and possibly cause environmental hazards.

4. The use of antibiotics still exists, though the incidents have been greatly reduced.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Problems

5. The use of probiotics, prebiotics, and immunoenhancers becomes popular. However, the effect is inconsistent. The commercially available health products are unreliable in qualities.

6. The overall survival rate from nauplii to PL is too low (< 25%). The technique of larvae-rearing needs to be further improved.

7. The contamination of pathogens especially protozoan parasites in mass culture of algae.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Problems

8. The contamination of pathogens in live feed for broodstock.

9. The majority of the hatcheries operate without bio-security setup. The importance of the bio-security has been overlooked. Potential consequence is the viral infection in the hatchery. Many incidents of the viral infection in the hatchery have been diagnosed.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

1. The extension of the duration of the hatchery operation
   Due to the establishment of the enclosure ponds in Northern Guangdong, Fujian and Zhejiang provinces, especially Yangtze River delta and Pearl River delta areas, demands for PL are now almost all year round. The hatcheries can thus operate on a non-stop basis. The operational costs are therefore significantly reduced.

2. Awareness of the importance of PL quality
   The demand for good quality "1st generation" PL is expected to increase. The hatcheries, which produce low quality PL, will be phased out and those, especially major producers with well -established brand names will prevail.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

3. **Stock improvement and selective breeding**

   China has to expedite its research and development on the stock improvement and selective breeding of *L. vannamei* in order to solve the existing problem of inadequate supply of quality broodstock .

4. **The abuse or misuse of antibiotics**

   Effective measures has to be tightened on the control of illegal use of antibiotics and other prophylactic chemicals.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

5. **Zoea II syndrome**
   The causes of Zoea II syndrome need to be identified and the problems resolved so that the overall successful rate of PL production can be ensured.

6. **The use of formalin in water treatment**
   Though it is the most effective method in sterilization, research is urgently required to define the advantages and disadvantages of using formalin in water treatment.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

7. The use of probiotics, prebiotics and immunoenhancers
   More research is required to justify the use of probiotics, prebiotics and immunoenhancers in larvae-rearing. The correct way of using those health products (eg. dosage and time of application) is yet to be defined. The governmental regulations on the production and sales of those products are anticipated.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

8. **The use of formulated feed for broodstock**

   Partial replacement of live feed with formulated feed for broodstock needs to be encouraged and promoted.

9. **Contamination in mass culture of algae**

   Method for solving the problem of the contamination of pathogens in mass culture of algae needs to be developed.

L. vannamei hatchery industry in China: constraints, problems, and perspectives

Perspectives

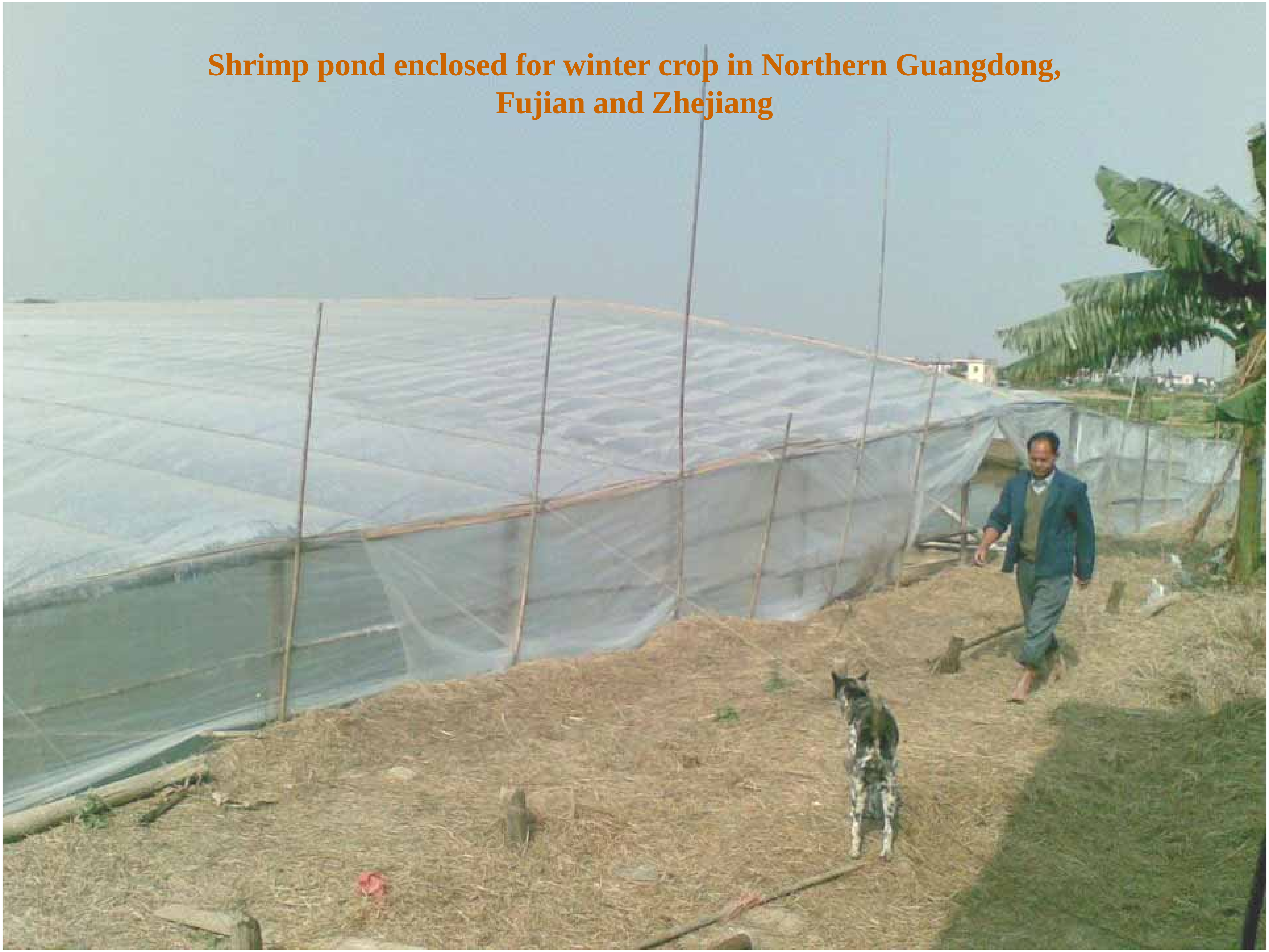
10. **Contamination in live feed for broodstock**

    Potential pathogenic contamination of live feed, such as polychaete worms and oyster, deserves more attention.

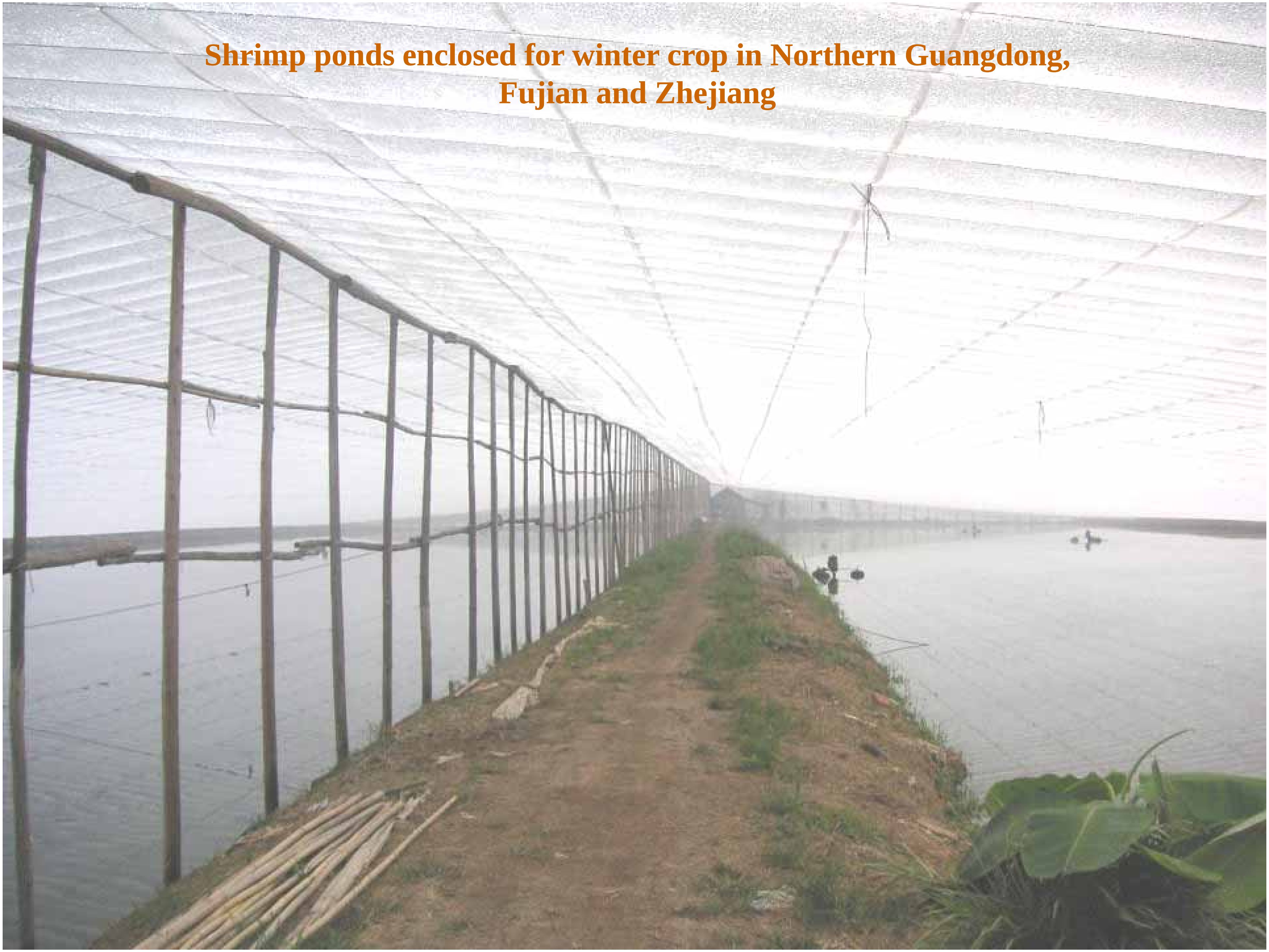
11. **The establishment of the bio-security systems**

    The importance and significance of the bio-security systems for the shrimp hatchery industry needs to be highlighted.

Typical *L. vannamei* intensive farm (Evergreen Group)

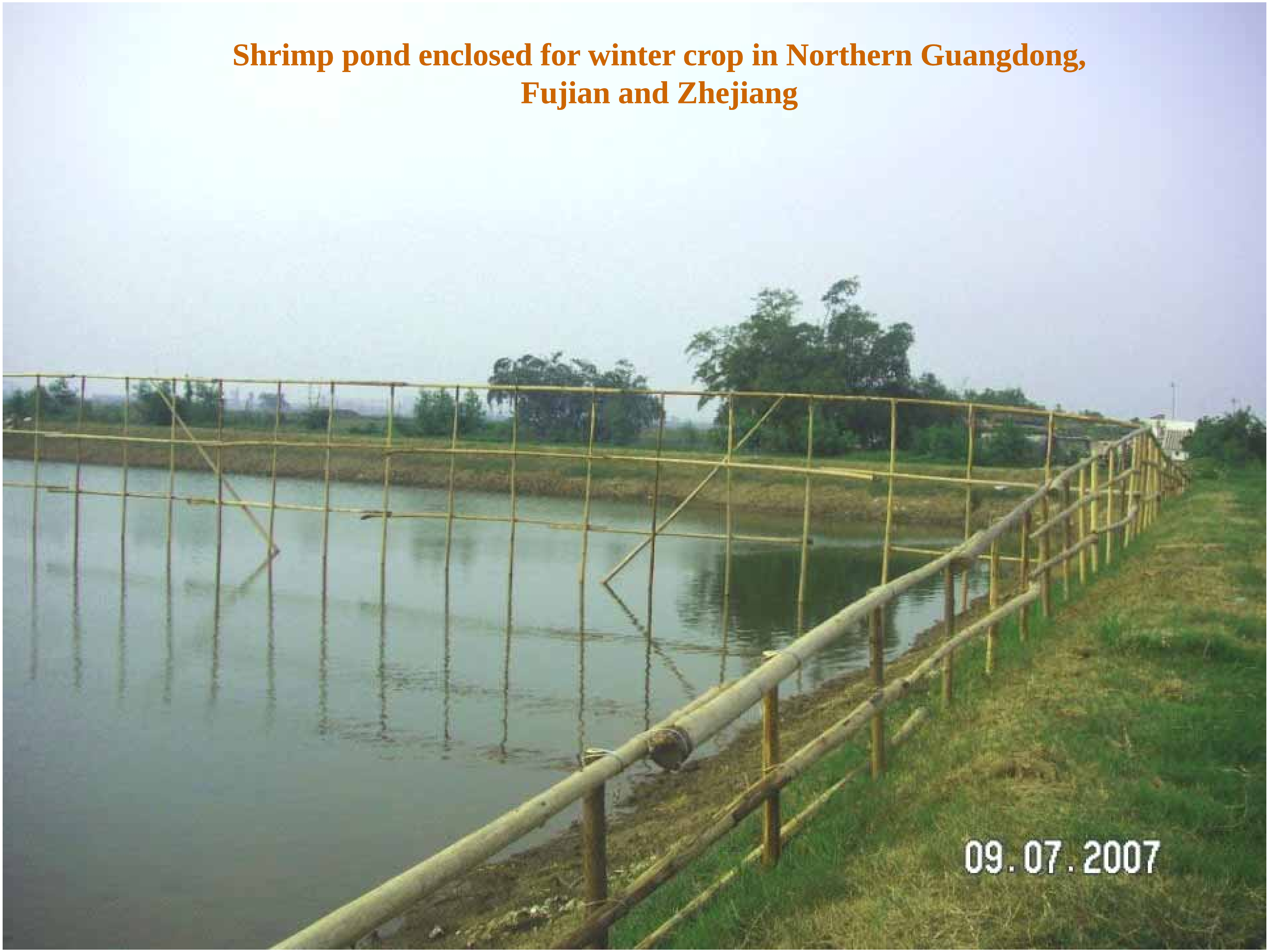


Shrimp pond enclosed for winter crop in Northern Guangdong, Fujian and Zhejiang


Shrimp ponds enclosed for winter crop in Northern Guangdong,
Fujian and Zhejiang

Shrimp pond enclosed for winter crop in Northern Guangdong, Fujian and Zhejiang

Shrimp pond enclosed for winter crop in Northern Guangdong, Fujian and Zhejiang



Shrimp pond enclosed for winter crop in Northern Guangdong, Fujian and Zhejiang


Larvae-rearing room (Evergreen Group)

Typical *L. vannamei* larvae-rearing tanks in China

Broodstock room (Evergreen Group)


L. vannamei spawners

Fully matured female *L. vannamei*

Tight bio-security: three steps in sterilization (Evergreen hatchery)

Vehicle washing basin at the gate

Foot and hand sterilization before entering larvae-rearing complex

Foot and hand sterilization before entering larvae-rearing room

Algal inocula in solid medium

Algal inocula in test tubes and flasks

Algal stock (*left:Chlorella sp.*, *right:Chaetoceros sp.*)

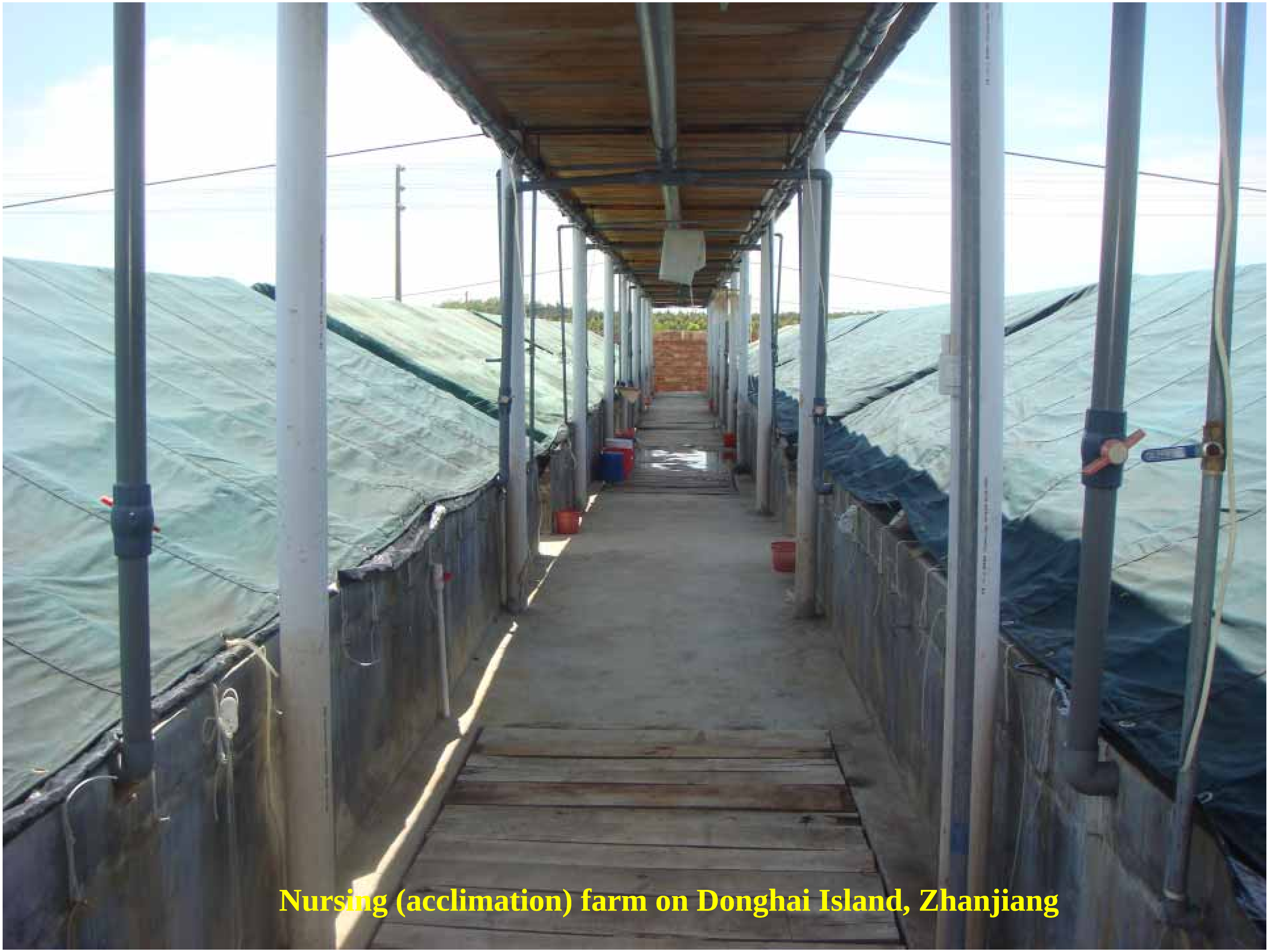
Algal stock (*Chaetoceros sp*.)

Algal stock (*Chaetoceros sp. and Chlorella sp*.)

Mass culture of algae in outdoor concrete tanks

***L. vannamei* postlarvae**

L. vannamei **postlarvae**



Nursing (acclimation) farm on Donghai Island, Zhanjiang

Nursing (acclimation) tanks equipped with heaters

Evergreen Aquaculture Research Center on Donghai Island

广东恒兴集团有限公司
GUANGDONG EVERGREEN GROUP COMPANY,LTD

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Using alternative low-cost artificial sea salt mixtures for intensive, indoor shrimp (*Litopenaeus vannamei*) production

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A B S T R A C T

Inland marine shrimp production is increasing in popularity as recirculating aquaculture systems (RAS) can provide a fresh, high-value product year-round to nearby consumer markets. However, artificial sea salt mixtures are required for inland production, which can be a substantial portion of the production costs. The purpose of this experiment was to compare marine shrimp (*L. vannamei*) production and water quality dynamics in culture systems with various mixtures of a least cost salt (LCS) formulation and a commercial, complete sea salt (CSS) formulation. Previous research using this same LCS mixture found decreased shrimp production using 100% LCS compared to ratios with 25% CSS inclusion and higher. To further investigate the decreased shrimp performance in 100% LCS, this experiment used six salt different ratios of LCS and CSS between 100% LCS and 75% LCS:25% CSS. The treatments used in this study were 100% LCS, 97.5% LCS, 95% LCS, 90% LCS, 80% LCS, and 75% LCS. The results showed that use of the LCS formulation significantly lowered the cost of salt kg^{-1} shrimp produced. The LCS formulation cost \$8.83 USD to make 1 m^3 of water at 15 salinity, compared to \$12.89 using the 75%/25% LCS/CSS mixture. Salt mixtures had a significant impact on DO, pH, salinity, and turbidity; however, these differences did not seem to impact shrimp performance, as no significant differences were found in shrimp average weight, biomass, survival, FCR, or growth rate. The results from this study indicate that using the LCS formulation reduces artificial sea salt cost significantly while having no significant impacts on shrimp production.

1. Introduction

Due to high demand and exhausted natural fisheries stock, the aquaculture sector has grown rapidly, with aquaculture products currently representing more than 50% percent of seafood production for human consumption (FAO, 2020). Although most aquaculture is pond based, indoor aquaculture is growing in popularity, especially in inland areas where fresh seafood products are often difficult to acquire. Such indoor operations primarily utilize recirculating aquaculture systems (RAS). RAS are contained production systems that provide substantial environmental control, including solids filtration, biofiltration, temperature control, and other control mechanisms based largely on animal needs and climate considerations (Timmons and Ebeling, 2010). RAS also require much less space than pond-based aquaculture through higher production densities, allowing them to be situated in a variety of building types (Martins et al., 2010).

Due to the costs of RAS, high-value species are being investigated for their suitability in RAS production (Bunting and Shpigel, 2009). One such species is the Pacific white shrimp (*Litopenaeus vannamei*), which can be marketed as a high-quality, fresh product in some niche markets (Moss and Leung, 2006; Van Wyk, 2006; Timmons and Ebeling, 2010). One limiting factor in RAS marine shrimp production is the need for salt to create marine or brackish water (Zohar et al., 2005). Most inland areas do not have direct access to saltwater; therefore, shrimp production operations must use an artificial marine salt mix (Whetstone et al., 2002; Watson and Hill, 2006). Shrimp can be grown at varying salinities from 30 g L^{-1} to 10 g L^{-1}, perhaps even lower in some cases, but require minimum levels of individual salt anions and cations, including Na, Mg, K, Ca, SO_4, CO_3, and Cl, and require specific ratios between pairs of ions like Na, Mg, K, and Ca (Saoud et al., 2003; Roy et al., 2007, 2010; Khanjani et al., 2020). Most commercial salt mixes attempt to replicate the mix of elements found in natural seawater, including trace minerals such as Fe, I, Zn, and Mn. Some of these trace elements have been shown to play a role in physiological functions when included in the shrimp diet, but the amounts of trace elements needed to meet animal requirements in water are not well documented (Ali, 2000; Lin et al.,

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2013). These commercial marine salt mixes can represent a significant portion of production costs for inland shrimp producers (Shinji et al., 2019; Maier, 2020). Reducing such costs is critical for the profitability of shrimp farms as the intensive indoor shrimp industry grows (Badiola et al., 2012; Quagrainie, 2015; Zhou and Hanson, 2017). Simplifying these salt mixes down to only essential elements may reduce costs for producers by reducing the number of ingredients and facilitating in-house made salt mixes.

A previous experiment found that there were no significant differences in shrimp production when using five salt mixtures: containing 100% commercial sea salt (CSS), 75% CSS and 25% of a least cost salt mix (LCS), 50/50% CSS/LCS, 25/75% CSS/LCS, and 100% LCS (Tierney et al., 2021). Although there were no significant differences in that study, shrimp survival in the 100% LCS treatment was low at 57%, whereas the other treatments averaged 70% survival. The LCS formula used in Tierney et al. (2021) was based on that published by Parmenter et al. (2009), who explored using the mixture in a low shrimp stocking density, outdoor setting which showed shrimp survival above 80%. The same LCS was also used by Galkanda-Arachchige et al. (2020) where it was found to be suitable for shrimp nursery settings, however the effectiveness of LCS in an intensive grow-out setting is still unclear. The purpose of the current experiment was to further examine salt mixtures in the range of 75% LCS to 100% LCS due to the low survival found in Tierney et al. (2021) and definitively determine if a LCS can result in adequate shrimp production, maintain acceptable water quality levels, and reduce salt cost in intensive, indoor shrimp aquaculture systems.

2. Materials and Methods

2.1. Experimental design and operation

This experiment took place in the Kentucky State University Sustainable Aquaculture Development Lab (SADL). The SADL is a 174 m^2 insulated and climate-controlled building used for indoor aquaculture research. Five treatments were developed for this experiment, each with 4 replicated tanks for a total of 20 tanks. Each treatment used different combinations of two salt mixes to reach the target salinity. The two salts were a commercial sea salt mixture, Crystal Sea Marine Mix (CSS) (Marine Enterprises International, Baltimore, MD, USA); and a least-cost salt mixture (LCS) made from sodium chloride (NaCl), magnesium sulfate ($MgSO_4$), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), potassium chloride (KCl), and sodium bicarbonate ($NaHCO_3$) (Table 1). The LCS formula is based on a formula from Parmenter et al. (2009) and used by Tierney et al. (2021); all ingredients were mixed prior to being added to the tank. The CSS mix is commonly used in indoor shrimp farms in the U.S. It is a proprietary product and, according to the manufacturer, is an artificially formulated complete sea salt that duplicates the major, minor, and trace elements found in natural sea water (https://www.meisalt.com/Crystal-Sea-Marinemix). The individual treatments in the experiment were 75/25% LCS/CSS, 80/20% LCS/CSS, 90/10% LCS/CSS, 95/5% LCS/CSS, 97.5/2.5% LCS/CSS, and 100% LCS. These percentages of LCS were chosen to determine if higher percentages of LCS inclusion will result in decreased shrimp performance shown in Tierney et al. (2021). The specific brands and purity of each ingredient of the LCS are listed in Table 2.

All experimental tanks had a bottom area of 1 m^2 and had a volume of 1 m^3. All tanks were HY systems and were equipped with an 18 L biofilter and an 18 L settling chamber for solids control, based on the design described by Ray et al. (2010). The biofilters were moving bed biological reactors (MBBR), and each contained 6 L of biological media (Curler Advance X-1, Aquaculture Systems Technologies, LLC. New Orleans, LA, USA.). At the start of the study, 3 L of biomedia from established shrimp production tanks was added to each MBBR to help ensure bacterial colonization in the filter. Water was pumped to the settling chamber from each tank using a 20 L min^{-1} electric water pump. Water from the settling chamber was gravity fed into the MBBR, after which the water returned to the grow-out tank. Each tank was heated with a 1000-watt submersible electric heater, three 15 cm long ceramic air diffusers provided aeration to each shrimp tank, and one such diffuser supplied air to the biofilter.

2.2. Water quality

Salinity in all systems was kept at 15 throughout the experiment. Any water loss due to evaporation was replaced with dechlorinated municipal water. When pH fell below 7.8, this was adjusted with additions of 35 g of sodium bicarbonate. Temperature, dissolved oxygen (DO), pH, and salinity were all measured twice daily at approximately 08:00 and 16:00 h using a YSI Professional Plus Multi-Meter (YSI Incorporated, Yellow Springs, OH, USA). Total ammonia nitrogen (TAN), nitrite, and turbidity were measured once weekly during this study. Turbidity was measured using a Hach 2100Q Portable Turbidimeter; TAN and nitrite were measured using Hach methods 8155 and 8507 using a Hach DR6000 spectrophotometer (Hach Company, Loveland, CA, USA).

2.3. Shrimp husbandry

The shrimp used in this study were purchased from American Mariculture, Inc. (St. James City, FL, USA). Upon arrival, the shrimp were raised in two 3.4 m^3 nursery tanks for 37 days before being stocked into the experimental tanks. The salinity in the nursery tanks was started at 30 and was lowered to 15 over the course of the nursery period. The shrimp were fed 6 different rations throughout the nursery. Four crumble diets with progressively larger sizes were fed through the first 27 days of the nursery (Raceway Plus 0, 1, 2, 3; Ziegler Brother, Inc., Gardners, PA, USA). Each of these diets contained 50% protein, 15% lipid, 1% fiber, 10% moisture, and 7.5% ash, according to the manufacturer. At 27 days the shrimp diet was transitioned to a 1.5-mm pelleted feed (Ziegler PL Raceway 40–9, 40% protein, 9% lipid, 3% fiber, 10% moisture, and 13% ash), and at 36 days transitioned to a 2.4-mm pellet (Ziegler Hyper-intensive Shrimp 35, 35% protein, 7% fat, 2%

Table 1
The formulation of the least cost salt (LCS) mixture at 15 salinity to make 1 m^{-3} of artificial seawater (Tierney et al., 2021).

Salt Formulation	
Ingredient	Weight (g)
NaCl	11,310
$MgSO_4$	1830
$MgCl_2$	855
$CaCl_2$	376
KCl	240
$NaHCO_3$	90

Table 2
Components of the LCS mixture used in this study. Table shows the specific compound used, brand, and purity. Data collected from: Cargill, Incorporated, Minneapolis, MN, United States; Giles Chemical, Waynesville, NC, United States; Nedmag B.V., Groningen, Veendam, The Netherlands; Cal-Chlor Corporation, Lafayette, LA, United States; The Mosaic Company, Tampa, FL, United States; Solvay S.A., Brussles, Belgium.

Salt	Full Compound	Brand	Purity	Grade
NaCl	NaCl	Cargill	> 99%	Food-Grade
$MgSO_4$	$MgSO_4.7\ H_2O$	Giles Chemical	> 99%	Technical-Grade
$MgCl_2$	$MgCl_2{\cdot}7\ H_2O$	Nedmag B.V.	> 99%	Feed-Grade
$CaCl_2$	$CaCl_2$	Cal-Chlor Corporation	> 94%	Industrial-Grade
KCl	KCl	The Mosaic Company	> 98%	Industrial-Grade
$NaHCO_3$	$NaHCO_3$	Solvay	> 99%	Food-Grade

fiber, 12% moisture, and 15% ash), which was also provided throughout the experiment. The shrimp were fed on 24-hour automatic belt feeders for the majority of the nursery stage to ensure continuous feed availability.

The post-nursery shrimp were stocked into the experimental tanks at 262 shrimp m^{-3} at an average individual shrimp weight of 2.9 g shrimp^{-1}. Throughout the experiment, the shrimp were fed 3 times daily at 08:00, 12:00, and 16:00 h. The amount of feed provided daily was calculated using an estimated FCR and weekly growth rate, along with water quality readings and periodic checks for uneaten feed at the bottom of the tanks. The study lasted 86 days, and at harvest, all shrimp were weighed and counted. The data gathered from the harvest was used to calculate average weight per shrimp, growth rates, FCR, survival, and total harvest weight m^{-3}. Growth rate was calculated by dividing the average total weight gain per shrimp by the number of weeks in the study and FCR was calculated by dividing the total amount of feed added to each tank by the total shrimp weight harvested from the corresponding tank.

2.4. Salt cost

The cost of salt in USD for all treatments was generated by calculating the total cost of each salt mix to reach 15 g L^{-1} salinity and the percent of each salt used in each treatment. In addition, the cost of salt m^{-3} and the shrimp production m^{-3} in each treatment were combined to calculate the cost of salt kg^{-1} of shrimp.

2.5. Data management and analysis

All statistical analyses were performed using SigmaPlot 13.0 (Systat Software Inc., San Jose, CA, USA). All data was tested using Shapiro-Wilk and Brown-Forsythe tests to check normality and variance. All harvest data from shrimp, elemental analyses, and production costs were analyzed using a one-way ANOVA. Water quality parameters measured weekly were analyzed using a one-way repeated measures ANOVA. All results were considered significant at an α-value of < 0.05, and if a significant difference was detected, a Tukey's HSD Test was applied.

3. Results

There were no significant differences in temperature, TAN, nitrite, TSS, or VSS ($p > 0.05$, Table 3). Significant differences were detected in DO, pH, salinity, and turbidity between treatments ($p < 0.05$). The DO concentration tended to increase as CSS concentration decreased, with 100% LCS and 97.5% LCS treatments having significantly higher dissolved oxygen levels than the other four treatments. The 100% LCS treatment had significantly lower overall pH than the 90% and 80% LCS treatments. Turbidity was significantly higher in the 100% LCS treatment compared to all other treatments except the 90% LCS treatment.

There were no significant differences detected between treatments in all tested shrimp production metrics, including average weight shrimp^{-1}, growth rate week^{-1}, FCR, kg of shrimp m^{-3}, and survival ($p > 0.05$, Table 4). All average shrimp weights were between 20.7 g, and 22.2 g and average growth rates were 1.4–1.6 g week^{-1}, FCRs ranged from 1.4 to 1.6:1, shrimp production ranged from 4.3 to 4.7 kg m^{-3}, and survival averaged 81% across all treatments with a range of 76.7–84.3%.

The cost of salt m^{-3} at 15 salinity was different between all treatments, and cost decreased as LCS percentage increased (Table 5). The cost of salt kg^{-1} of shrimp produced was found to be lowest on average in the 97.5% LCS tanks, and significantly lower than the 80% and 75% LCS treatments (Table 6).

4. Discussion

Total ammonia nitrogen and nitrite levels were both maintained within acceptable ranges for shrimp production throughout this experiment (Alcaraz et al., 1999; Valencia-Castañeda et al., 2018). Although there were significant differences between treatments in DO levels, pH, salinity, and turbidity, these minute differences likely had little effect on the overall performance of the shrimp and were all within acceptable ranges (Zhang et al., 2006). The reason these subtle differences were detected as significant is because of the sensitivity of the repeated measures ANOVA. This test is useful when the same measurements are made repeatedly; however, consistent but minor differences over time

Table 3
Mean ± SD water quality values over the course of the study between each treatment (treatments are based on the amount of least cost salt (LCS) included; the remainder of the salt mixture was a commercial sea salt). Different superscript letters in a row denote significant differences between treatments ($p < 0.05$).

	Treatment 100% LCS	97.5% LCS	95% LCS	90% LCS	80% LCS	75% LCS
Temperature °C	27.3 ± 0.1	27.3 ± 0.1	27.3 ± 0.1	27.3 ± 0.1	27.3 ± 0.1	27.3 ± 0.1
Dissolved Oxygen (mg L^{-1})	6.5 ± 0.0^{a}	6.5 ± 0.0^{a}	6.4 ± 0.0^{c}	6.4 ± 0.0^{b}	6.4 ± 0.1^{cd}	6.4 ± 0.1^{bd}
pH	7.9 ± 0.0^{a}	7.9 ± 0.0^{ab}	7.9 ± 0.0^{ab}	7.9 ± 0.0^{b}	7.9 ± 0.0^{b}	7.9 ± 0.0^{ab}
Salinity	15.0 ± 0.0^{b}	15.1 ± 0.0^{c}	14.8 ± 0.1^{a}	15.2 ± 0.0^{d}	15.0 ± 0.0^{b}	15.0 ± 0.0^{b}
Ammonia (mg TAN L^{-1})	0.6 ± 0.2	0.7 ± 0.1	0.8 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.1
Nitrite (mg NO_2-N L^{-1})	0.9 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.8 ± 0.2
TSS (mg L^{-1})	112.7 ± 6.0	108.1 ± 5.8	105.2 ± 5.8	112.5 ± 7.3	111.7 ± 7.0	105.4 ± 4.5
VSS (mg L^{-1})	77.1 ± 5.5	71.9 ± 3.2	70.0 ± 4.5	72.9 ± 6.1	67.5 ± 4.9	67.7 ± 2.8
Turbidity (NTU)	26.4 ± 2.7^{a}	19.9 ± 2.6^{bc}	19.2 ± 2.4^{bc}	22.7 ± 3.4^{ab}	20.2 ± 3.2^{bc}	17.7 ± 1.8^{c}

Table 4
Mean ± SD shrimp production metrics at harvest between each treatment (treatments are based on the amount of least cost salt (LCS) included; the remainder of the salt mixture was a commercial sea salt). There were no significant differences between treatments with regard to these metrics.

	Treatment 100% LCS	97.5% LCS	95% LCS	90% LCS	80% LCS	75% LCS
Average Weight (g)	21.1 ± 0.4	20.7 ± 0.3	21.9 ± 0.7	21.7 ± 0.5	22.2 ± 0.6	21.9 ± 0.4
Growth rate (g/week)	1.5 ± 0.0	1.4 ± 0.0	1.5 ± 0.1	1.5 ± 0.0	1.6 ± 0.1	1.5 ± 0.0
FCR	1.6 ± 0.1	1.5 ± 0.0	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Biomass (kg m^{-3})	4.3 ± 0.4	4.6 ± 0.2	4.6 ± 0.2	4.5 ± 0.2	4.6 ± 0.2	4.7 ± 0.2
Survival (%)	77.5 ± 6.6	84.3 ± 3.2	81.1 ± 3.9	79.2 ± 3.8	76.7 ± 5.8	82.3 ± 2.0

Table 5
The cost of each ingredient used to make the least cost salt (LCS) mixture.

Ingredient	$USD kg^{-1} Salt	$USD m^{-3} Water
NaCl	0.418	5.35
$MgSO_4$	0.946	1.95
$MgCl_2$	0.704	0.67
$CaCl_2$	0.77	0.59
KCl	0.858	0.22
$NaHCO_3$	0.726	0.06
Total	4.422	8.84

Table 6
Salt mixture cost m^{-3} of water, and kg^{-1} of shrimp produced. Treatments are based on the amount of least cost salt (LCS) included; the remainder of the salt mixture was a commercial sea salt. Different superscript letters in a column denote significant differences between treatments ($p < 0.05$).

Salt Mixture Costs		
Treatment	m^{-3} water	Kg^{-1} Shrimp
100% LCS	8.83	2.11^{ab}
97.5% LCS	9.24	2.03^{a}
95% LCS	9.64	2.09^{ab}
90% LCS	10.45	2.34^{ab}
80% LCS	12.08	2.63^{b}
75% LCS	12.89	2.76^{b}

often register as significant. There were no significant differences found between treatments in temperature, TSS, or VSS. Importantly, pH levels were maintained when using the LCS, even though a single source of alkalinity is used in the mixture (sodium bicarbonate). A complete sea salt mixture would likely include multiple buffers, such as calcium, potassium, and magnesium carbonate compounds.

The lack of significant differences in shrimp production between treatments has important implications for shrimp producers. The increased concentration of LCS used in production appears to have no detrimental impact on shrimp performance. Overall average survival was just above 80%, average FCR was 1.5 across all treatments, and the average growth rate was 1.5 g week^{-1}, all comparable to or exceeding recent shrimp studies using reduced cost salt mixtures and commercial mixtures at similar salinities (Tierney et al., 2021; Galkanda-Arachchige et al., 2020; Pinto et al., 2020). Shrimp in this study reached an average of 21.6 g individually at 86 days, a size that is within the range preferred by consumers in North America, Europe, and other regions (Wirth, 2014; Tabarestani et al., 2017). This production time scale falls within a competitive harvest schedule and the shrimp size is at the highest of the range recommended by Zhou and Hanson (2017) in their economic model, suggesting that the results of this study are commercially relevant.

These results further demonstrate the utility of this reduced-cost salt mix across several stages of shrimp growth, as the study by Galkanda-Arachchige et al. (2020) used an identical low-cost mixture and found equal performance of post-larval and juvenile shrimp between both low-cost and commercial salt mixes. Contrary to this study, Pinto et al. (2020) found severely decreased shrimp performance at 75% and 100% inclusion of low-cost salt, despite the low-cost salt mixtures having similar ingredients and levels of critical ions compared to the same commercial salt mix. There are several differences in the low-cost salt mixtures; this study used both MgCl and $MgSO_4$ for magnesium inclusion, while the mixture in Pinto et al. (2020) used exclusively $MgSO_4$. To maintain alkalinity, this study included $NaHCO_3$ in the low-cost salt mix and added small amounts of the same as needed, while Pinto et al. (2020) did not include $NaHCO_3$ but added $Ca(OH)_2$ as needed to raise pH. Pinto et al. (2020) concluded that the lack of trace minerals in the low-cost mixtures was likely the cause of decreased performance; however, this study and several others have reported adequate shrimp production without the inclusion of trace minerals (Parmenter et al., 2009; Galkanda-Arachchige et al., 2020). Another possible cause for the differences between this study and that of Pinto et al. (2020) is the sources of salts. Some salts can contain high levels of impurities, which may result in inadequate concentrations of target minerals or possibly toxic effects on shrimp. The LCS and CSS used in this study were identical to those used in Tierney et al. (2021) which found no significant difference in shrimp production between the CSS and the LCS. The results of this study found use of the LCS resulted in high survival, exceeding the results in Tierney et al. (2021) who noted shrimp jumping out of the tanks which may have resulted in the discrepancy in shrimp survival.

The similar shrimp performance between treatments, regardless of LCS concentration, influences the economics of shrimp production operations. The cost difference in salt between the 75% LCS and 100% LCS was just over $4 USD m^{-3}, which could lead to significant cost savings for shrimp producers, especially those operating at large scale. The lower salt cost also reduced the cost of production kg^{-3} of shrimp by $0.75 USD, which represents a 15% decrease in production cost over the CSS formulation. Although many shrimp production methods, including biofloc and hybrid systems, are designed to greatly reduce water use, some water is still discharged from the systems during normal operation (Avnimelech, 2015). The loss of this water (3–10% per production cycle) is usually replaced with newly mixed salt water, increasing salt use and production costs long term, which would be partially negated by replacement with the LCS mixture. The economics of pond-based shrimp farming are well studied; however, the feasibility of high intensity, indoor shrimp production is still unclear due to substantial upfront costs and variable system designs and production strategies (Moss and Leung, 2006; Badiola et al., 2012; Zhou and Hanson, 2017). Any reduction in production cost may have significant impacts on this relatively new shrimp production style.

The salts that were used to make the LCS formulation in this study were each purchased in 23 kg bags that were shipped several hundred kilometers. However, in a commercial setting it is more likely that farmers would purchase these in bulk quantities and from local vendors if possible. This commercial-scale strategy would likely reduce the cost of the mixture even further. In fact, Maier (2020) points out that scale is one of the biggest factors influencing the profitability of indoor shrimp farming. He goes on to note that using the same LCS formulation tested in this study can significantly improve profit potential for farmers.

5. Conclusion

The study shows the feasibility and cost savings of a low cost, easily made salt mixture in high-intensity indoor shrimp production. The use of the LCS mixture should be considered by shrimp producers due to the significant decrease in production costs, similar shrimp performance, and water quality compared to a commercial marine salt mixture. Further research should examine the long-term water quality dynamics associated with the LCS formula and how mineral concentrations may change over time.

Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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CRediT authorship contribution statement

Leo Fleckenstein: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Thomas Tierney:** Formal analysis, Investigation, Writing – review & editing. **Jill Fisk:** Formal analysis, Investigation, Writing – review & editing. **Andrew Ray:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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