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NEW METHODS AND STRATEGIES TO IMPROVE THE PERFORMANCE OF *HOLOTHURIA SCABRA* HATCHERIES

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Hatchery-produced juveniles of the tropical sandfish, *Holothuria scabra*, in Sri Lanka ©FAO/L. Walpita.

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NEW METHODS AND STRATEGIES TO IMPROVE THE PERFORMANCE OF *HOLOTHURIA SCABRA* HATCHERIES

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PREPARATION OF THIS DOCUMENT

The tropical sea cucumber *Holothuria scabra*, commonly known as sandfish, is an important commercial species in the Indo-Pacific region. In Sri Lanka, where it is locally known as "*Jaffna attaya*", this species is widely distributed in seagrass meadow along inshore areas and lagoons. Back in the 1970s a lucrative fishery started in and around the Puttalam lagoon but collapsed in the mid-1980s due to overexploitation and lack of proper management measures. Similar declines were observed in the northern provinces, impacting the livelihoods of numerous fishing communities.

In response, various initiatives were launched to manage and restore sandfish populations. One such initiative involved the National Aquaculture Development Authority (NAQDA) introducing the grow-out culture of wild-collected sandfish, a practice that gained traction in Kalpitiaya, Mannar and Jaffna areas. Unfortunately, conflicts among fishers led to the ban on fattening small sandfish in 2011.

To address the shortage in seed supply, Sri Lanka initiated a sea cucumber aquaculture programme over a decade ago. While breeding efforts by organizations like the National Aquatic Resources Research and Development Agency (NARA) and NAQDA, along with some private hatcheries, have seen success, production still falls short of meeting demand. As a result, both hatchery-reared juveniles and wild-caught sub-adults are stocked into grow-out pens set up in lagoons and shallow coastal waters.

To ensure sustainable expansion, the industry must secure a consistent supply of viable juveniles. This necessitates advancements in seed production technologies. Recognizing this need, the Food and Agriculture Organization (FAO), in collaboration with Sri Lanka's national authorities, has conducted training sessions for both public and private technicians. These sessions focused on conventional breeding methods like thermal shock spawning and innovative approaches like *in vitro* fertilization (IVF), a technique developed in Madagascar in 2007 and generously shared with hatchery operators in Sri Lanka.

This publication is tailored for hatchery operators, providing comprehensive guidance on both thermal shock spawning and *in vitro* fertilization processes. Subsequent chapters delve into embryonic and larval rearing, early juvenile production, and offer practical insights into water management, feeding regimes, and disease control measures. The spawning and rearing techniques described in this guide are applicable in hatcheries established across the geographical distribution of the sandfish.

ABSTRACT

Comparing the current aquaculture development status of the tropical sandfish, *Holothuria scabra*, with that of thirty years ago, reveals significant advancements. However, despite advancements, the development of profitable commercial aquaculture ventures originating from these research projects remains limited. This suggests that the farming of *H. scabra* has yet to fully meet the expectations set three decades ago, unlike the remarkable success witnessed with the temperate species, *Apostichopus japonicus*, in China. Several factors contribute to this moderate success, including the intrinsic biological characteristics of *H. scabra*, which may only become apparent during the transition from pilot-scale hatcheries to large-scale production. For instance, the gregarious behaviour of adults significantly affects breeding densities, thereby impacting growth rates. For example, the average density of one individual/m^2 in a 1-hectare enclosure does not accurately represent reality, where patches with densities of up to 100 individuals/m^2 can form. Additionally, all stages of upscaling pose greater challenges in control. For instance, the production of two million juveniles/year demands a higher level of precision compared to producing 100 000 juveniles. Similarly, the daily transformation of 10 000 individuals into trepang necessitates a certain level of automation in the handling processes. Additionally, challenges such as low hatchery yields, and a lack of breeding expertise generally hinder the profitability of many hatchery operations.

The present document serves as a comprehensive guide for hatchery operations, introducing novel methods and strategies aimed at enhancing financial profitability. It details the step-by-step process of *in vitro* fertilization (IVF), a proven technique validated in Madagascar and Sri Lanka. IVF is presented as a complementary approach to spawning through thermal shock treatments, widely employed in sea cucumber hatcheries worldwide, to bolster hatchery seed output. Moreover, the IVF method can be effectively applied using gonads collected from sea cucumber specimens delivered to processing plants for transformation into the dried trepang.

The manual also explains the use of greenhouses to advance ovarian maturation in low-temperature conditions, thereby increasing overall productivity of a hatchery operation. Comprehensive guidance is provided on optimizing productivity levels of embryos, larvae and juveniles to achieve financial profitability. The productivity benchmarks described sprung from extensive commercial trials conducted across dozens of hatcheries in Madagascar, ensuring practical applicability and effectiveness.

CONTENTS

Preparation of this document ... iii
Abstract ... iv
Abbreviations ... vi
Acknowledgements ... vii

Chapter 1: BIOLOGY OF *HOLOTHURIA SCABRA* (SANDFISH) ... 1

Chapter 2: GENERAL SCHEME FOR PRODUCING *HOLOTHURIA SCABRA* ... 3

Chapter 3: HATCHERY AND NURSERY INFRASTRUCTURES ... 4

Chapter 4: BROODSTOCK MANAGEMENT ... 7

Chapter 5: SPAWNING STRATEGIES ... 8

Some notions on ovarian and oocyte maturation ... 8
Thermal shock spawning method ... 9
In vitro fertilization method ... 11
In vitro fertilization using individuals from processing factories ... 13

Chapter 6: CONDITIONING BROODSTOCK IN A GREENHOUSE ... 15

The maturation greenhouse: why, how and when it is useful? ... 15

Chapter 7: EMBRYONIC AND LARVAL REARING ... 16

Chapter 8: WATER MANAGEMENT ... 18

Chapter 9: FOOD AND SETTLEMENT MANAGEMENTS ... 20

Microalgae culture for larval feeding ... 20
Larval feeding ... 21
Preparation of settlement plates ... 21
Sargassum juice preparation for juveniles 1 (0.7–1.2 cm) ... 21
Preparation of indoor tanks and feeding of juveniles 2 (1.2–2 cm) ... 21
Preparation of outdoor tanks for juveniles 3 (>2 cm) ... 22

Chapter 10: DISEASE IDENTIFICATION AND MANAGEMENT ... 23

Chapter 11: COUNT MANAGEMENT ... 25

Chapter 12: PRODUCTION MANAGEMENT ... 28

How should the production process unfold across various developmental stages leading up to the appearance of type-1 juveniles? ... 28
Problems and potential solutions with embryos and larvae production ... 30
Projected growth and survival rates of the different juvenile types ... 31
The problems and the potential solutions with juveniles ... 33
Key benchmarks for achieving adequate production levels in a sandfish hatchery ... 34

SELECTED REFERENCES ... 35

ANNEX 1. Selected laboratory equipment typically used in a sea cucumber hatchery ... 37

ANNEX 2. How to count the germ cells/embryos ... 41

ANNEX 3. Steps for the processing *Holothuria scabra* into beche-de-mer ... 42

ABBREVIATIONS

EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
IVF	*in vitro* fertilization
MPF	maturation promoting factor
OMI	oocyte maturation inducer
PPM	parts per million
RPM	revolutions per minute
SKUD	skin ulceration disease
UV	ultraviolet

>	greater than
<	less than
≤	less or equal than
°C	degree Celsius
%	percent
‰ or **ppt**	parts per thousand
µl	microlitre
ml	millilitre
L	litre
µm	micrometre
mm	millimetre
cm	centimetre
m	metre
g	gram
kg	kilogram
min	minute
hr	hour
Ø	diameter

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Thanks are also due to Prof Michel Jangoux, former Director of the UMONS Laboratory, who launched the research activities in Madagascar that lead to the development of the *in vitro* fertilization (IVF) procedure for the tropical Indo-Pacific sandfish, *Holothuria scabra*. Prof Chantal Conand is also acknowledged for her valuable advisory role throughout this research. Thanks are also expressed to Nicolas Fohy, Christophe Andriantsialonina, Rijaniaina Ratsimbazafy, Hanitriniala Marie Jeanne Mahavory, Pascal Manohitsara, Lahiru Walpita, Didulanga Auchith and Amirthalingam Anojan for their technical work without which the spawning techniques would not have been improved.

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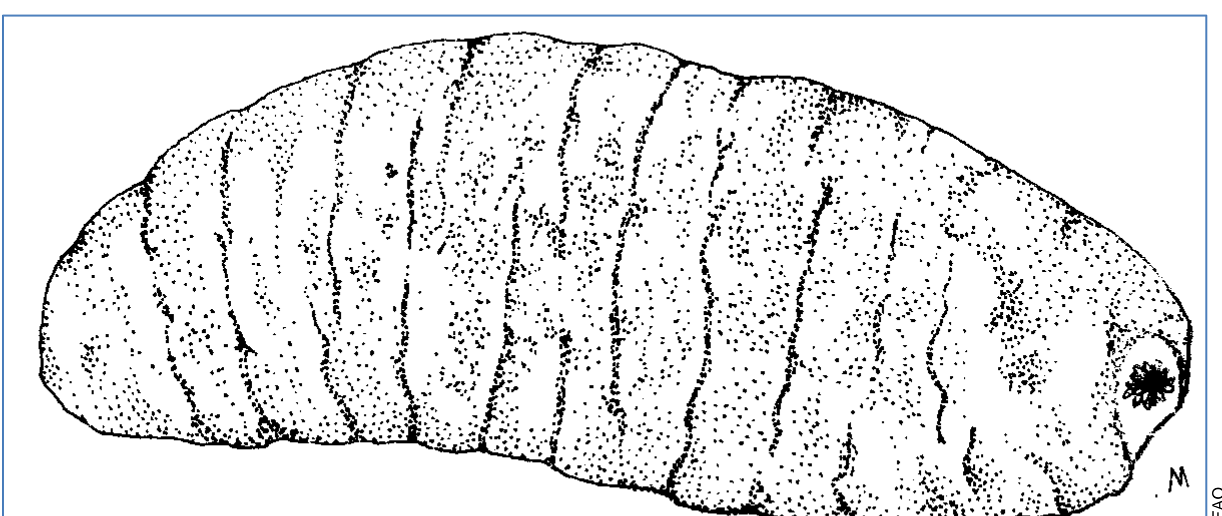
Chapter 1: BIOLOGY OF *HOLOTHURIA SCABRA* (SANDFISH)

The sandfish or *Holothuria* (*Metriatyla*) *scabra* Jaeger, 1933 belongs to the class Holothuroidea in the phylum Echinodermata.

The body of the sandfish is elongated, cylindrical and stout (Figure 1). The dorsal body surface is relatively smooth and has small papillae (i.e. sensory tube feet) with black dots. The colour varies from grey to black with dark and deep transverse wrinkles. The ventral surface of the body is moderately flattened and is generally whitish in colour. The oval-shaped mouth is positioned ventrally at the anterior end of the body with 20 short peltate greyish tentacles. The anus is terminal with no teeth.

This tropical sea cucumber species is easily recognizable from the other species except from the closely related Golden sandfish, *Holothuria* (*Metriatyla*) *lessoni* (previously known as *Holothuria scabra versicolor*) whose anatomy is similar, but dorsally more brownish in colouration.

Figure 1. Illustration of an adult sandfish, *Holothuria scabra*, specimen



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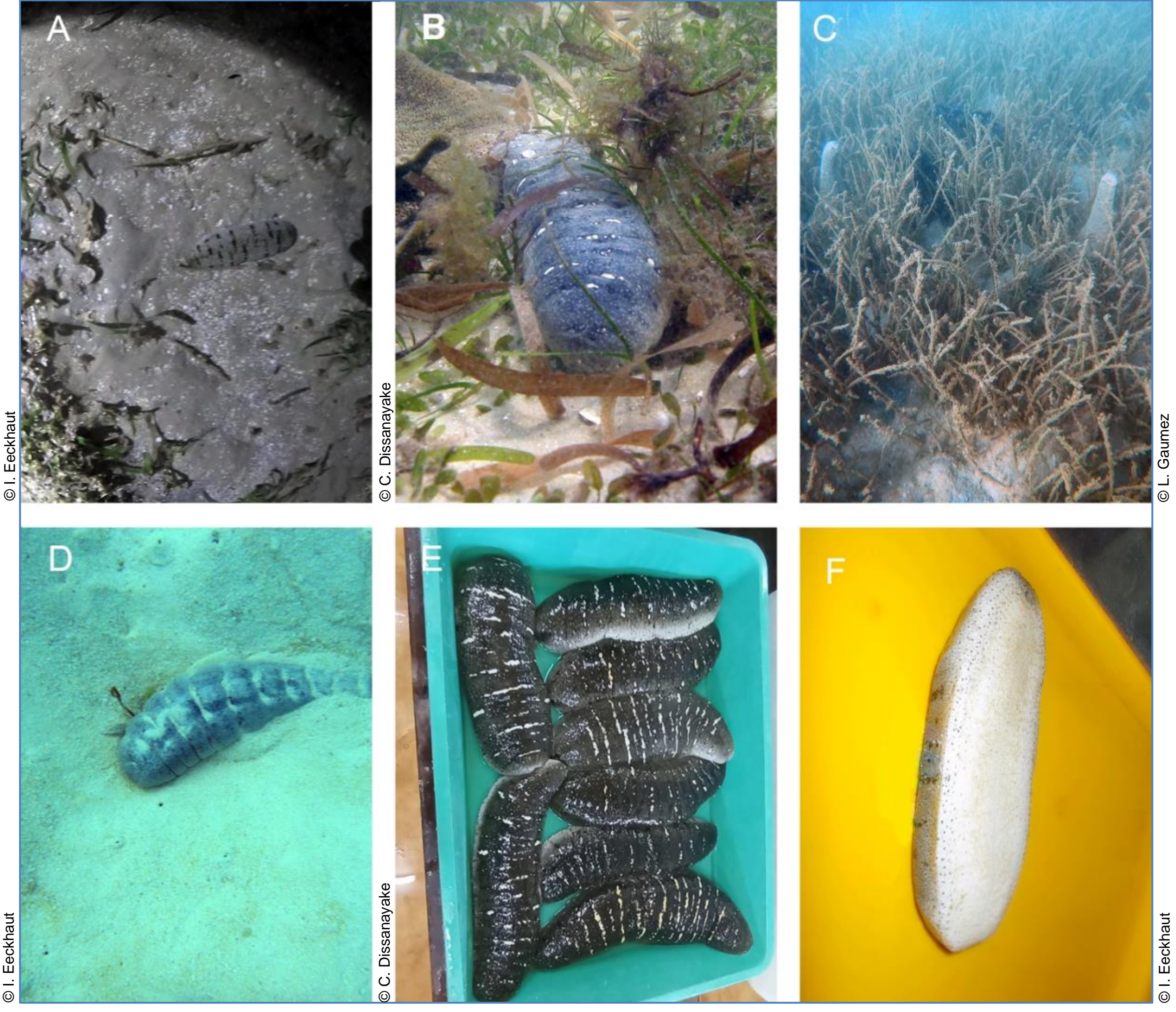
Where and how to find sandfish specimens

This species of sea cucumber is known from locations throughout the Indo-Pacific, roughly between latitudes 30°N and 30°S. It occurs mainly in low-energy environments behind fringing reefs or within protected bays and shores. Numerous reports indicate that *H. scabra* is often found in areas of low salinity and some research findings demonstrated the ability of the sandfish to tolerate salinity levels down to 20 ppt by burrowing deep into the sediment, which could explain why this species can sometimes be found near estuaries. The sandfish is one of the few tropical species that prefers inner coastal sandy flats to coral reefs inhabiting (Figure 2). Studies indicate that *H. scabra* seem to favour sandy or muddy-sand habitats. According to laboratory experiments and field studies, juveniles show a clear preference for medium-sized grains around 0.4 mm Ø or finer muddy sand, whereas coarse sand and crushed coral are generally avoided. Overall, there is a clear relationship between seagrass cover and the abundance of *H. scabra*. The habitat of *H. scabra* is optimally characterized by depth ranges between 0.5 and 5 m (it is found up to depths of 30 m) and a temperature around 28 °C. Recorded abundances of *H. scabra* are quite variable throughout the Pacific and Indian Oceans with average values ranging between 0.1 and 0.6 ind./m^2. The average length of this sea cucumber species is 30 cm (max. length recorded 46 cm) averaging a fresh weight between 1 100 to 1 400 g.

Juveniles of *H. scabra* had rarely been documented in the wild and information on their distribution and habitat preferences is scarce. To date, reports and research investigations of juveniles in the field remain limited. Field studies conducted in the Solomon Islands showed that small individuals of *H. scabra* (<10 mm) occurred on leaves of the large flowering marine plant *Enhalus acoroides* and the Pacific turtlegrass, *Thalassia hemprichii*, but not on other substrata.

The sandfish generally moves in a sluggish manner and often remains partly or totally buried in the sediment. In general, *H. scabra* emerges from the substrata at sunset and remains active until the first hours of light the following morning.

Figure 2. Live *in situ* specimens of the sandfish, *Holothuria* (*Metriatyla*) *scabra*, from different geographical regions



***Note*:** Individuals observed *in situ* (A to D) and in a hatchery (E and F). Specimens from Thailand (A), Sri Lanka (B and E), Madagascar (C), where individuals are in the spawning position, and Seychelles (D).

Chapter 2: GENERAL SCHEME FOR PRODUCING *HOLOTHURIA SCABRA*

There are several ways to artificially produce sandfish seed for on-growing or for restocking programmes and the production of the processed/dried beche-de-mer or trepang for human consumption. All go through these four stages: hatchery, nursery, grow-out and post-harvest processing (Figure 3).

The hatchery is the stage where reproduction, larval rearing and the initial stage of juvenile rearing take place. The embryos are obtained by applying thermal shocks to mature wild or farmed sea cucumbers or via *in vitro* fertilization using wild individuals or gonads from freshly gutted specimens obtained from a processing plant. The development of sandfish in a hatchery goes from the egg stage to juveniles of about 20 mm. This process generally takes about 3 months. Larval development is preferably carried out in cylindrical tanks typically of fiberglass with water volume capacities ranging from 250 to 3 000 L. Larval metamorphosis, settlement and early juvenile development also take place in these tanks.

As sea cucumber juveniles of 20 mm are still too small to avoid predation at sea, they are preferably pre-grown in a nursery facility to a body length of 60–80 mm. This nursery phase may have a duration spanning from 2 to 3 months depending on the environmental conditions and food levels in the medium where they are grown. The nursery may consist of tanks and/or ponds of different sizes ranging from 30 to 1 000 m^2. In some parts of the world, hapa nets are used for pre-fattening small sea cucumbers. These hapas are typically placed in large ponds, lagoons or shallow coastal areas.

The grow-out of sea cucumbers to commercial size is done at sea or in large earthen ponds. Two socio-economic farming models are currently practiced: 1) village or community farming, and 2) individual company farming. In the former practice, coastal villagers are engaged to monitor the growth of the juveniles they receive and sell the final product to exporting companies which often own processing facilities to handle the fresh product received. On the other hand, in a company farm, the grow-out is taken care of by dedicated staff employed by the company. The target of the grow-out phase is the production of individuals with a body length >20 cm and weighing >350 g. The duration of the grow-out varies from place to place depending on the biotic and abiotic parameters characterizing the farm site. To reach the average weight indicated above this culture phase generally lasts around eight months.

The processing factory transforms the harvested adults into trepang, i.e. the dried and exportable product. Three products are obtained from the farmed individuals: viscera, gonads and body wall. Only the latter is transformed into trepang. The gonads can be used for *in vitro* fertilization while the viscera are eliminated or used as a by-product. The processing of the body wall into trepang takes about 1 week.

Figure 3. General scheme for the farming and production of beche-de-mer from *Holothuria scabra*

Chapter 3: HATCHERY AND NURSERY INFRASTRUCTURES

The infrastructure used for juvenile production, which are then moved at sea for the grow-out, includes the hatchery and the nursery. Besides the administrative office space and living quarters for the technicians operating the hatchery, this facility consists of several functional and service areas most of which should have access to freshwater and seawater outlets (Figure 4). The main physical structures consist in:

- A conventional laboratory for biological manipulations including microscope, binocular microscope, precision balance and equipped with standard laboratory tools (see Annex 1).
- An *in vitro* fertilization room where the broodstock specimens can be dissected and the gonads handled appropriately. The conventional laboratory can also be used as an *in vitro* fertilization (IVF) room but must be further equipped with a centrifuge (5 000 rpm) and a filtration system to obtain filtered seawater down to 1 µm.
- A wet room housing the cylindrical fiberglass tanks for larval and type-1 juveniles (0.5 to 1 cm) development as well as the rectangular tanks in plastic, fiberglass or concrete, for the development of type-2 juveniles (1 to 2 cm). The number and volume of the cylindrical fiberglass tanks will depend on the production numbers to be achieved. Typically, the volume of the cylindrical tanks is 1 000 L with a white internal surface. The recommended maximum culture density is 0.5 larvae/ml. The culture density in the rectangular tanks is around 100 ind./m^2. The wet room should also include tanks to induce spawning through thermal shocks and embryo incubators. The latter are cylindrical fiberglass tanks generally of smaller size (200–300 L) where the embryos are kept for 2–3 days until the appearance of the auricularia stage (see *Embryonic and Larval Rearing* chapter). The wet room should be maintained at a temperature of 28–32 °C.
- An algal room where the desired microalgae strains are stored to produce the food volumes required for the feeding of the growing larvae. This room should be kept at a lower temperature of around 25 °C (depending on the algal species grown).
- A “juvenile food room” (i.e. both type-1 and type-2) to receive fresh macroalgae (e.g. *Sargassum*), their cleaning, grinding and conservation. This room can be a part of the wet room.
- A maturation greenhouse where the broodstock individuals, intended for thermal shock or *in vitro* fertilization, are retained and conditioned for 10 days. The facility is fitted with large capacity tanks (3 000 L or more). The bottom of each tank should be covered with a layer of marine sediment 10 cm deep. The room temperature of this facility must be maintained at around 35 °C.
- A nursery that consists of at least ten outdoor ponds measuring 1 000 m^2 each. These ponds can be of concrete or earthen ponds covered with a thick liner used in aquaculture. In both cases, they should always be covered with a layer of marine sediment 2 cm thick. Type-3 sea cucumber juveniles (2–6 cm) are grown in the outdoor facilities at a maximum density of 20 ind./m^2.
- A workshop to house and store the equipment and tools for repairs and general maintenance of all hatchery facilities.

The entire infrastructure must be supplied with clean filtered seawater except for the outdoor nursery ponds which can be fed with unfiltered water directly from the sea. Different filtration systems are used in hatcheries. In the Mannar hatchery in northern Sri Lanka the following system is used. Seawater is pumped from an intake placed 15 m beyond the low tide mark where the water never drops below 1 m. The water is initially stored in a 500 m^3 sedimentation basin and from here through a sand filter system and into a chlorination tank of 40 000 L. This tank contains 500 g of mixed sodium hypochlorite. The chlorine concentration is monitored by a colorimetric test (if there is too much chlorine, 10 g of thiosulphate is added). The chlorinated water is then sent to an upper storage tank. Finally, this water

goes through a 1 µm filtration and UV system before being distributed and used in the hatchery. Figure 5 depicts the infrastructure layout required for producing 300 000 sandfish juveniles annually.

Figure 4. Photos illustrating some of the key infrastructures required to produce *Holothuria scabra* juveniles

Note: (A) Outdoor tanks for the production of type-3 juvenile (>2 cm in length); (B) a wet room with 1 000 L cylindrical fiberglass tanks for larval and type-1 juvenile rearing and rectangular tanks for type-2 juveniles (<2 cm in length); (C) a laboratory for *in vitro* fertilization; (D) a greenhouse for the maturation of the breeders including tanks filled with marine sediment; and (F) a filtration/sterilization and (G) pumping/water storage system.

Figure 5. Example of infrastructure needed for a minimum production of 300 000 juveniles/year

***Source*:** Authors' own elaboration (Igor Eeckhaut)

The hatchery buildings include an administration office, a place to house residents and a canteen. A technical workshop is necessary for the maintenance and repair of the entire infrastructure. A seawater pumping system fills a settling pond and from here the water is transferred to a treatment unit. The water from this unit is filtered down to 1 µm and used in the hatchery.

The water from the settling basin also goes to the greenhouse where the sea cucumber broodstock are conditioned and allowed to mature.

The hatchery building includes a laboratory where IVF is carried out, an algal room to produce microalgae, and a wet room where thermal shocks and rearing of the larvae and early juveniles takes place.

To produce a minimum of 300 000 juveniles/year, the wet room must be equipped with at least 8 embryo-rearing incubators, 10 cylindrical rearing tanks (for larvae and type-1 juveniles) and 15 rectangular tanks (for type-2 juveniles).

A second pumping system at sea fills the outdoor ponds which serve as the nursery facility to produce type-3 juveniles. At least 5 outdoor ponds with an area of 1 000 m^2 each are required.

Chapter 4: BROODSTOCK MANAGEMENT

To obtain gametes from sexually mature sea cucumbers, broodstock specimens will either be forced to spawn using thermal shock treatments or individuals will be sacrificed and dissected to obtain eggs and spermatozoa for *in vitro* fertilization (IVF).

In most hatcheries, the first broodstock generation is obtained from adults collected from wild populations. The next generation of broodstock may also come from the wild but most often from the offspring of the first set of breeders. In a commercial operation a small population of breeders is maintained in protected and monitored sea pens or in dedicated conditioning and maturation tanks placed in the greenhouse facility located within the grounds of the hatchery complex (see Chapter 6).

A broodstock is generally chosen from large individuals >500 g and eventually transferred to the appropriate holding structures. The transport of broodstock from the capture site to the holding facilities must be as rapid as possible to avoid unnecessary stress including the spontaneous and undesirable spawning of an adult specimen. Collected individuals can be transported in relatively small containers either filled with clean seawater (maximum density of 20 ind./m^2) or with the sea cucumbers simply covered with fresh seaweed.

When the collected adult sea cucumbers are kept in sea pens, between 50 to 100 individuals would constitute the right number of broodstock required for hatchery use with ideally a male to female ratio of 1:1.

When collecting and selecting the broodstock, three key physical characteristics should be recorded for every individual (one sheet/specimen): external dorsal pattern features, overall health status and body weight.

- *External dorsal anatomy*: take a picture of the distinct striped bands located on the dorsal surface of each sea cucumber. This visual “fingerprint” will help identify the individual specimen and record its performance history within the hatchery.
- *Health status*: record obvious and unusual visual characteristics/abnormalities and discard seemingly diseased individuals or individuals heavily infested with copepods.
- *Body weight*: record the wet body weight of the single specimen 5 minutes after it has been removed from the water (individual would have discharged seawater from the respiratory tree).

Sea pens (or lagoon pens) for holding broodstock should be located as close as possible to the hatchery so they can be easily and regularly monitored (Figure 6). Overcrowding of a single pen should be avoided and a desirable stocking density should be around 100 g/m^2. At this stocking density additional feed will most likely not be required to maintain a healthy population.

Figure 6. A sea pen off the coast of Jaffna, Northern Province, Sri Lanka, for the grow-out of wild and hatchery-produced sandfish, *Holothuria scabra*, juveniles

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Chapter 5: SPAWNING STRATEGIES

There are essentially three methods used to obtain fertilized oocytes in sea cucumber aquaculture: 1) through natural spawning events, 2) artificially induced spawning, and 3) via *in vitro* fertilization (IVF). In the case of the sandfish, *Holothuria scabra*, the first method is rather complicated as the natural spawning cycle of this species is not easily determined. Natural spawning is efficient and the preferred method for species where the spawning events occur in synchrony with the lunar cycle. With these species, fertilized oocytes can be regularly obtained on predictable days within the reproductive period.

The thermal shock method is the most widespread spawning induction technique used in *H. scabra* aquaculture. When possible, commercial hatcheries should attempt this technique using individuals with body weight >500 g. Thermal shocks are frequently combined with supplementary methods such as Spirulina baths and dry treatments (detailed below).

Some notions on ovarian and oocyte maturation

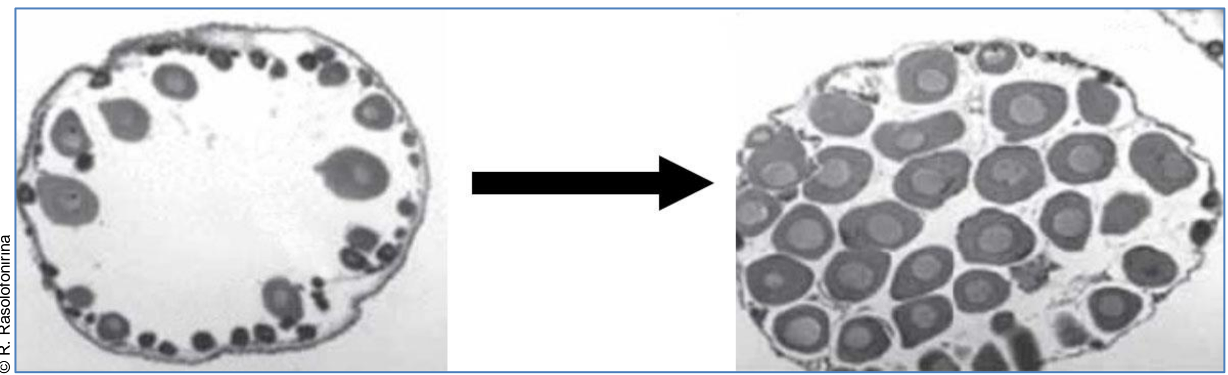
It is obviously important for the economic profitability of sandfish aquaculture that the hatchery maximizes its seed production output and remains in full operation throughout the year. For what concerns the broodstock itself, it is most important to control as far as possible the following two physiological processes: (i) the maturation of the ovaries (testicle maturation is less important in hatcheries as good spermatozoa are very often available year round), and (ii) the maturation of oocytes.

The sandfish is a gonochoric organism (i.e. sexes are separated). The gonads become macroscopically visible in individuals of 3 cm body length, at the age of 4 months, but the first germ cells are evident from the somatic cells only in individuals >10 cm in length or aged 8 to 9 months.

In adult specimens, five gonad development stages occur during the maturation of the ovaries: the "spent" stage (or stage I) where the lumen of the ovary is empty, as the adult individual has just released the mature eggs. stage II is the "recovery" stage where the lumen is still empty and where oogonia (renewing stem germ cell) are distinguished at the level of the epithelium bordering the lumen. The "growing" stage (or stage III) and the "maturation" stage (or stage IV) where the lumen gets filled with oocytes in previtellogenesis and vitellogenesis, and finally the "spawning" stage (or stage V) where the number of female germ cells close to maturity is maximum. Stage V corresponds to females ready to spawn (Figure 7).

The maturation of the ovaries (or "ovarian maturation") is the development of the ovaries up to stage V or when the "spawning" stage is reached (Figure 7). Ovarian maturation can be stimulated and optimized by placing adult females in the greenhouse maturation tanks (see Chapter 6).

Figure 7. Ovarian maturation in an adult sandfish



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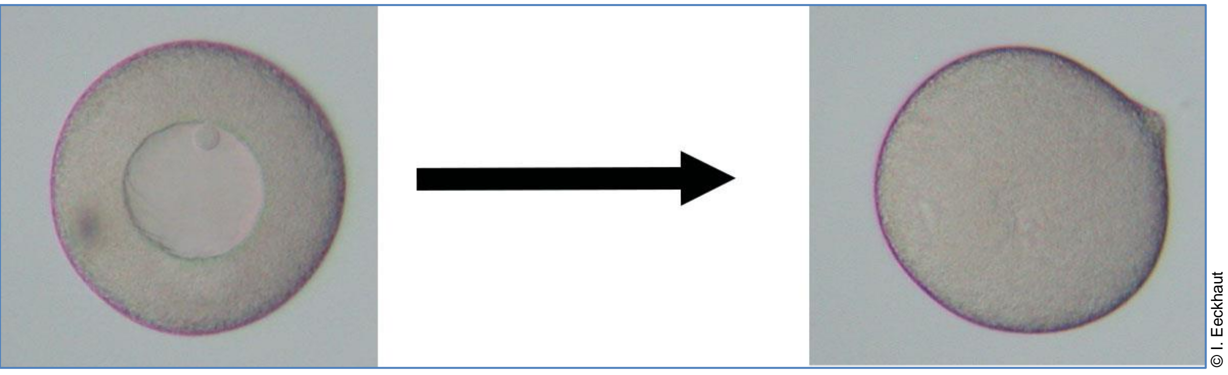
Note: The development of the ovary lumen from spent to stage V. Ovary maturation occurs naturally but can be positively stimulated through the provision of the right external stimuli in the greenhouse maturation tanks.

However, in sea cucumbers with stage V ovaries, oocytes are blocked in their meiosis in prophase I. Immature oocytes must complete their meiosis to become fertile. In nature, this meiotic release takes place during spawning by an unknown process, probably hormonally induced.

Maturation of oocytes is the development of oocytes during meiosis which ends with the appearance of ootids that are, in sea cucumbers, the female germ cells ready to be fertilized. Oocyte maturation can be induced by oocyte maturation inducers (e.g. during *in vitro* fertilizations). An ootid is recognizable by the lack of germinal vesicle (i.e. the nucleus and the presence of two polar bodies outside) (Figure 8).

Figure 8. Oocyte maturation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Note: The development of an oocyte to an ootid (or egg cell that results from the second meiotic division of an oocyte and that develops into a mature egg) occurs naturally during or just before spawning or when using a maturation inducer.

Thermal shock spawning method

- Select broodstock individuals preferably with a body weight >500 g and clean them thoroughly with a jet of seawater to remove any particulate matter attached to the body tissue (Figure 9).
- Keep the treated broodstock individuals outside the water for 40 minutes.
- Prepare one tank with a volume capacity of about 500 L with filtered hatchery seawater cooled with ice placed in plastic bags (the water should be around 25 °C. **Not below this value**).
- Place ~20–30 individuals in the cold tank for 40 minutes (preferably in the afternoon).
- Move them to a different tank for 40 minutes where the water temperature is 35 °C.
- Place the treated broodstock individuals in a large bucket with powdered spirulina (concentration: 2 g/L) for 30 minutes.
- Place the treated broodstock in a spawning tank of 500 L with the water at ambient temperature. Observe the behaviour of each specimen. Pre-spawning behaviour typically involves rolling movements, rhythmic contractions, lifting and swaying of the front end of the body and climbing the tank walls. After 2 hours, check for sperm and/or eggs in the water. Males usually spawn before females (water appears milky-dirty) and spawning usually lasts between 30–60 minutes.
- If only males release their gametes (sperm) wait for the female to spawn overnight.
- When the eggs are released, take a sample of water every 30 minutes and check if fertilization has occurred (presence of 2 blastomere stage and advanced stages). When embryonic division has occurred, collect the embryos and unfertilized eggs by siphoning them through a 80 µm mesh. The water level of the collecting bucket must be above the mesh of the sieve so that the eggs are not pressed against the mesh. Siphoning must be smooth. The water temperature in the collecting bucket must be 28–30 °C.
- Individuals that have spawned must be returned to the broodstock holding facility. If none of the individual have responded to the thermal shock treatment overnight, they must also be returned to the broodstock holding facility.

Figure 9. Thermal shock treatment of adult sandfish, *Holothuria scabra*

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Note: The process consists in a dry treatment (A) followed by a spirulina bath (B), a cold shock at 25 °C (C) and a warm treatment at 35 °C (D). The density of individuals is always high, around 20-30 breeders are involved. After about 1 h, spawning behaviour may occur (E, G). When females and males have spawned, eggs and embryos are collected through a 80 µm sieve (F).

When spawning is successful, proceed as follows:

- Transfer the collected eggs/embryos to a 20 L bucket filled with 1 µm-filtered seawater (temperature at 28–30 °C).
- Take 5 droplets to count the eggs under a microscope and estimate the total number of eggs (see Annex 2).
- After 2 hrs, check fertilization success by confirming the presence of more advance embryonic stages such as 4-cell stage or other.
- Place the embryos and the rest of unfertilized eggs in gently aerated embryonic incubators (tanks of about 300 L) filled with 1 µm-filtered seawater at 28–30 °C. The maximum stocking density of embryos should preferably be 2 embryos/ml.

In vitro fertilization method

Embryo production by thermal shock treatment is often insufficient to ensure the economic profitability of the hatchery as spawning occurs randomly and is often influenced by various environmental parameters, the reproduction and/or lunar cycle (thermal shocks are mostly successful during new and full moons). *In vitro* fertilization (IVF) uses an oocyte maturation inducer (OMI) before proceeding with oocyte fertilization using sperm. The OMI method was developed by the Belgium Belaza Marine Station (Igor Eeckhaut, University of Mons) in collaboration with the Fisheries and Marine Sciences Institute (Richard Rasolofonirina, University of Toliara) in Madagascar. The OMI can be requested by sending an email to: oceanic.ingeneering@gmail.com.

IVF has the advantage that it can be carried out at any time, many times during a month with a great level of success. To correctly practice this method, a hatchery technician needs to have relevant practical experience (biological skills) and the correct laboratory and hatchery tools and equipment.

The active molecule in the OMI is a thioredoxin which acts on the redox potential of the oocytes. All thioredoxins have an active site made of WCNPCK amino acids which will act on the redox potential of certain surface proteins of oocytes. This action leads to an intracytoplasmic enzymatic cascade which activates the maturation promoting factor (MPF) responsible for meiotic unblocking. Oocytes become ootids ready to be fertilized using the collected sperm.

An IVF requires the following steps:

- Perform all operations using seawater filtered at 1 µm (filtration through a Millipore filter or equivalent is preferred) and maintained at 29–30 °C. Prepare around 15 L of filtered seawater.
- Collect and use 5–10 adult sea cucumber weighing >350 g.
- With the use of a sharp dissecting scalpel cut a ~1 cm opening on the ventral-anterior part of the animal and gently squeeze out the viscera and the gonad (Figure 10).
- Using forceps, remove the gonad by separating it at the height of the gonoduct. Remove visceral waste remaining on the gonad using a wash bottle. The body wall can be sent to a factory for trepang processing.
- Rinse the gonad thoroughly with filtered seawater to get rid of as many saponins as possible.
- Sex the gonad, weigh it and determine its degree of maturity. Ripe ovaries have typically a pinky-orange colouration, while testicles are white. Gonads which are mostly transparent, small, with thin and light tubules are poorly matured.
- When an individual is female, take a gonadal tubule and check the maturity level using a microscope. A mature ovary has tubules filled with oocytes >80 µm in diameter. When the individual is a male, the testicular tubules release highly motile sperm into the seawater.
- After rinsing, place each ovary and testis in separate 250 ml beakers containing filtered seawater.
- Save the testicles for later. Cut each ovary with a scissor. Continue cutting for 5 minutes to extract the oocytes from tubules.
- Take 5 droplets of the milky solution to count the germ cells under a microscope and estimate the total number of eggs (see Annex 2).
- Filter the contents of the beakers through a double sieve. A top 120 µm mesh size sieve for the collection of any ovarian tubule debris whilst allowing passage of the germ cells and a bottom 80 µm mesh size sieve for the collection of the oocytes while allowing passage of the oogonia.
- Invert the 80 µm sieve and using a wash bottle, filled with filtered seawater, collect the oocytes in a clean beaker.

Figure 10. *In vitro* fertilization of sandfish oocytes

***Note*:** The female ovary (A), and male testicle (B) extracted from mature sea cucumber specimens. Testicles extracted from different males at different level of maturity (C). Quality ovaries that can each provided ~1 million oocytes (D). A selection of poor to medium quality ovaries (E). Microscopic views of gonadal tubules showing poor quality tubules (F) from which no oocyte can be obtained and ovaries of a high (G) and medium quality (H). Microscopic view of germinal cells following completion of the IVF procedure (I). One oocyte (with germinal vesicle) is visible, few unfertilized ootids (single cells without germinal vesicle) and oocytes in the 2 to 4 blastomere stage.

- Collect and mix all the oocytes in 900 ml of seawater. Take 5 droplets to count, using a microscope, and estimate the total number of oocytes following filtration (see Annex 2).
- Prepare the OMI solution. Add 2 g of the OMI powder in 100 ml of seawater filtered (1 µm) and stir vigorously using a magnetic stirrer for 10 minutes.
- Place the solution obtained in two Falcon conical centrifuge tubes of 50 ml each and centrifuge for 5 minutes at 5 000 rpm.
- Take the OMI supernatant solution and add it (approx. 100 ml) to the 900 ml filtered seawater beaker containing the oocytes to induce maturation. Incubate for about 3 hrs.

- Check for oocyte maturation every 30 minutes by examining samples under a microscope: the oocytes should transform into ootids. For most of the oocytes (>60%) ensure that the germinal vesicle has disappeared (germinal vesicle breakdown is effectively achieved) and that both polar bodies have been expelled.
- When more than 60 percent of the oocytes have transformed into ootids, stop the maturation process. To do this, filter the oocytes through an 80 µm sieve (do not use the 120 µm sieve anymore) and rinse thoroughly. Recover the ootids from the sieve using a wash bottle.
- Place the ootids in 10 L container filled with filtered seawater.
- Section the testicles at the rate of 1 testicle in 250 ml of water. Check sperm activity under microscopes: they must be very active.
- Place 2–4 drops of sperm from this solution in the 10 L container with the ootids.
- Every 30 minutes, check the conditions of the ootids which must be surrounded by a few active spermatozoa; when fertilized, the fertilization membrane is clearly visible.
- When many 2-blastomere stage embryos are visible (>30%), filter the fertilized ootids through an 80 µm sieve, rinse thoroughly and collect the embryos.
- Check the development of the embryos, different stages appear during the next few hours.
- Place the embryos overnight in at least 10 L of seawater and aerate gently.
- Count the embryos the next morning by observing 5 droplets under a microscope (Annex 2).
- Place the embryos in the embryo incubators (fiberglass tanks) at a density of 2 larvae/ml.

In vitro fertilization using individuals from processing factories

Another advantage of IVF is that it can be performed using gonads collected from sea cucumbers delivered to plants for evisceration and processing (Figure 11). Indeed, only the body wall of the individuals arriving to the factories are used, the rest, including the gonads, is generally discarded. As several hundred individuals arrive daily to the processing facilities, the gonads from these individuals can be used in IVF if collected and handled correctly.

The correct method to collect, store and transport these gonads is as follows:

- Using a sharp knife open the sea cucumber antero-ventrally for a length of 1–2 cm starting from the mouth cavity.
- Squeeze the specimen so that all the viscera are dislodged from the body. Generally, the digestive tract and respiratory trees come out first, followed by the gonads. To fully remove the gonads press hard on the body wall starting from the anus and moving forward to the mouth where the incision has been made.
- After extracting the gonad, place it as cleanly as possible on a clean Petri dish.
- Sex the gonad visually and weigh it. Retain only the gonads that are well developed and mature.
- Depending on the planned production, collect the required number of ovaries and testicles (generally a 40 g ovary has a distinct orange colouration and will yield around 500 000 oocytes after filtration through a 80 µm sieve).
- Rinse thoroughly the gonads with filtered seawater to eliminate as much of the saponins they contain, bearing attention to the ovaries. During all the following steps rinsing is very important.
- Collect the ovaries and testicles separately in clean 10 L containers filled with filtered seawater (1 µm). As many as 10 fully mature gonads can be transported in a 10 L container at 30 °C for a period lasting 3 hrs.
- Return to the sea cucumber hatchery and initiate the IVF process. Initiate by eliminating as much as possible the small oocytes as they easily deteriorate when unfertilized and may further inhibit fertilization and embryonic development. To do this, do not hesitate to filter the collected oocytes twice through the 80 µm sieve.

Figure 11. *In vitro* fertilization using gonads from factory-processed sea cucumbers

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Note: Large sea cucumber specimens reaching a processing plant in Madagascar (A). Gutting of the individuals in plants in Madagascar (B) and Sri Lanka (C). The discarded gonads are collected for IVF. Gonads are thoroughly rinsed with filtered seawater (D); the ovaries (E) are separated from testicles (F) and transported back to the sea cucumber hatchery. The ovaries are cut and oocytes are collected on sieves (G). The OMI solution is prepared (H) and oocytes are incubated for 3 hrs (I). Ootids are collected on a sieve (J). They are ready to be fertilized with sperm.

Chapter 6: CONDITIONING BROODSTOCK IN A GREENHOUSE

The maturation greenhouse: why, how and when it is useful?

In many regions across the world, *H. scabra* spawn mainly during the hot season or when the seawater temperature reaches its maximum value. Temperature, within the tolerance range, is a key parameter influencing ovarian maturation either directly and/or indirectly (e.g. influencing the diet they feed on). A maturation greenhouse will be useful when spawning using wild individuals is basically impossible.

To build a maturation greenhouse, proceed as follows:

- Place large tanks with a surface area of about 20–25 m^2 in a greenhouse and control the ambient and seawater temperature at around 35 °C.
- Place a layer of sediments at the bottom of each tank. This layer consists of fine sand and muddy substrate taken from the nearby sea location (preferably known to be inhabited *H. scabra*).
- Sieve the sediment using a sieve fitted with a mesh of 1 mm mesh. The sediment layer should have a height of around 15 cm to allow the sandfish to completely bury itself.
- Wash the sediments with freshwater and allow it to soak for 2–3 hrs to kill potential predators.
- Fill the tank with seawater and leave for a week, before introducing the sea cucumbers, to allow the formation of a bacteria microlayer over the sediment particles.
- Oxygenate the incubator thoroughly and regularly check the oxygen concentration level at the bottom of the water column just above the sediment layer (it should be >3 mg/L).
- Introduce around 20–25 adult sea cucumbers in each incubator and leave for 10 days.
- Change 10–20 percent of the tank water daily and record main parameters (temperature and salinity).
- Feed the broodstock with a mix of 2:1 seagrass Sargassum/Algamac, at a rate of 1–2 percent total wet biomass of sea cucumber in the tank.
- For the next 10 days, check the health of all the sea cucumbers daily. Individuals with signs of disease or other symptoms of concern (e.g. evisceration, skin ulcerations) should be discarded.
- Following this period, the sea cucumbers are ready for thermal shocks or to be used for IVF.

Maturation greenhouses must be cleaned and the tank sediments preferably changed after a few weeks. To check the efficiency of the ovarian incubator, two female batches of the same age (or weight) must be compared, one batch observed directly after sampling from the wild, the other placed for 10 days in a gonad maturation incubator. At least 2–3 females must be sacrificed and analysed.

To check the efficiency of the maturation greenhouse, proceed as follows:

- A female specimen is dissected, the ovary removed, washed in seawater and oocytes extracted.
- To extract the oocytes, the ovaries are finely cut up with the help of dissecting scissors.
- Germinal cells (i.e. oogonia, oocytes, ootids) with fragments of ovarian tubules are collected in 200 ml seawater.
- Sieve the organic matter through a 120 µm mesh to eliminate unwanted particles.
- The three germ cell categories – oogonia, oocytes, ootids – must be recorded for at 2–3 females. For this, after standardizing the germ cells in the 200 ml by gently mixing, 3 × 1 ml are sampled and the proportion of each germinal cell is calculated on a sample of 100 germ cells. Means and standard deviations are calculated for both batches of females (wild and incubated).
- If the mean values are similar, change the substrate and clean the tanks in the maturation greenhouse: it works when the proportion of oogonia coming from wild individuals is higher than the proportion of oogonia coming from incubated individuals.

Chapter 7: EMBRYONIC AND LARVAL REARING

Described below are the different embryonic and larval development stages of the sandfish *H. scabra* indicating the time it takes to move from one larval stage to the next and reaching the juvenile stage under normal environmental conditions (Figure 12).

Early stages Fertilized ootids undergo first cleavage to produce embryos with two blastomeres generally after ~2 hrs following fertilization. Subsequent cleavages will lead to the formation of the **blastula**, generally occurring after 10 hrs, while the **gastrula** only appears 12–24 hrs post-fertilization. The gastrula is characterized by the presence of a blastopore and a developing archenteron (primitive digestive tract). It is ciliated and able to move. The gastrula measures 150 µm.

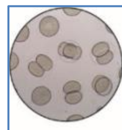
Auricularia The majority appear between the 2nd and 3rd day post-fertilization. The auricularia is fully formed after 5 or 6 days under normal environmental conditions. Three sub-stages of auricularia are often reported and named early, mid and late auricularia. The early auricularia measures ~500 µm while a fully developed late auricularia measures ~1 100 µm. The auricularia are ear-shaped, transparent, possess a preoral loop anteriorly and an anal loop posteriorly. The digestive tract consists of a mouth, an elongated pharynx, a sacciform stomach and an anus, the whole enabling the larvae to feed on planktonic matter. Various types of calcareous ossicles appear in the auricularia of *H. scabra*. The late auricularia is the larval stage that is physiologically ready to undergo metamorphosis if adequately stimulated. Distinct hyaline spheres appear at this stage. These provide a good and visible indication that the larvae are competent and ready to metamorphose. Metamorphosis does not occur at a fixed time post-fertilization. The process can be delayed if the larvae do not find adequate stimulation or simply because their development is anomalous.

Doliolaria This stage develops from the 11th to 18th day following fertilization. The doliolaria is a barrel-shaped larva with five ciliated bands across the body and five tentacles present in a vestibular cavity (i.e. visible through the transparent body, but not extended outside of the body). This stage measures between 460–620 µm in length. The doliolaria spends less time swimming, intermittently touching the bottom (or the bottom of the rearing facility when hatchery-produced) to find an adequate substrate where to settle.

Pentactula The pentactula appear from the 12th to the 22nd day post-fertilization. This is the stage that clings to the substrate with the help of five tentacles (that have become fully expendable) and one to two primary podia (also called "terminal tentacle"). The pentactula is 600–700 µm long. In the pentactula stage, the young individual has 5 faint ciliary bands still visible while an older individual has cilia disposed in regressing tufts on the body coming from the ciliary bands. The colour of the body is greenish brown. The pentactula becomes a true juvenile following the appearance of a third podium and/or other tentacles. A sea cucumber juvenile is considered as such only when the settlement phase is fully completed.

Juveniles Sea cucumber early juveniles appear from the 13th to the 30th day after fertilization. They gradually gain more weight and size, hence must be reared in three different structures. Type-1 juveniles are 0.6 to 1.2 mm long and are reared on the walls of indoor fiberglass tanks. As soon as they reach this length, type-2 juveniles are transferred to rectangular indoor tanks where they can grow to a length of 2 to 2.5 cm. From this size, type-3 juveniles are transferred to outdoor ponds where they are grown to a length of 4 to 8 cm, as required. They are then transferred at sea.

Figure 12. Development of the sandfish embryo to early juvenile stage



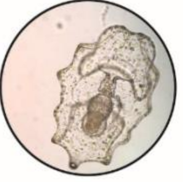
***1 hr** after fertilization*: 2 and 4 blastomere stages (**120 µm**)

*After **10 hrs***: spherical blastula stage (**120 µm**)

*After **24 hrs***: rounded gastrula stage (**150 µm**)

*After **48 hrs***: oval gastrula stage with archenteron (**300 µm**)

*After **72 hrs***: young feeding auricularia stage (**500 µm**)



*After **5 days***: mid-auricularia with well-formed lateral lobes (**900 µm**)

*After **8 days***: late competent auricularia stage with well-formed hyaline spheres in the lateral lobes (**1 100 µm**)

*After **11 days***: non-feeding doliolaria stage (**500 µm**)

*After **12 days***: first pentactula stage (**700 µm**)

*After **13 days***: early juvenile stage (**800 µm**)

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Chapter 8: WATER MANAGEMENT

The culture seawater used in rearing the sea cucumber larvae needs to be regularly replaced to ensure the healthy development of the larvae. Below the recommended tanks volumes to be used, culture densities and water volume change frequencies (Figure 13).

For embryos

- Embryos are retained for 2–3 days in the embryonic incubation tanks (i.e. fiberglass tanks of 500 L) without any water change until the early auricularia stage.

For auricularia

- Following the first 2–3 days, the auricularia are transferred to fibreglass tanks preferably with a volume capacity of 1 000 L at a maximum density of 500 000 units (average density: 300 000).
- The bottom of the culture tank must be siphoned every two days with a plastic pipe fitted at its fore end with a 120 µm sieve to retain the larvae in the tank.
- Change 30 to 50 percent of the seawater every 2 days.

For doliolaria and pentactula (first days)

- The doliolaria stage appears on day 11. When 50 percent of larvae have reached this stage, the bottom of the culture tank should no longer be siphoned nor conduct any seawater changes.

For pentactula and juveniles 1 (indoor cylindrical tanks)

- The pentactula stage appears on day 12. When most of the larvae have settled, replace 50 percent of the culture medium with new filtered seawater up until day-21.
- From day-21, siphon the rearing tanks and replace 50 percent of the seawater daily.

For juveniles 2 (indoor rectangular tanks)

- Carry out a water change (50%) once a week.

For juveniles 3 (outdoor ponds)

- Weekly water change (from 20 to 50%) until the juvenile sea cucumbers are transferred to the sea pens.

Figure 13. Sandfish juvenile management and rearing

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***Note*:** Collection of type-1 sea cucumber juveniles (A, B). Type-1 and type-2 juveniles collected from a rearing fiberglass tank (C, D). Larger type-2 juveniles are selected and transferred to indoor rectangular tanks (E, F). Two specimens of type-2 juveniles (G) at the end of their indoor growth cycle and ready to be transferred to an outdoor concrete pond in plastic bags (H). Type-3 juveniles after three (I) and five (J) weeks rearing in outdoor ponds.

Chapter 9: FOOD AND SETTLEMENT MANAGEMENTS

Microalgae culture for larval feeding

Many microalgae species are used for feeding the auricularia of *H. scabra*. These have been reviewed by Hamel *et al*. (2023). Below is the procedure to culture *Chaetoceros* sp. strains widely used in hatcheries.

Prepare the algae culture medium (Guillard's Modified F/2 Medium)

Nutrient medium preparation in 1 L of distilled water

A. $NaNO_3$ 84.148g + $NaH_2PO_4.H_2O$ (10.0 g)
B. $Na_2SiO_3.9H_2O$ (30.0 g)
C. $FeCl_3.6H_2O$ (3.2 g)
D. Na_2 EDTA (10.0 g)
E. Trace metal solution

Trace metal solution = 1 ml of each of the following solutions (a, b, c and d).

a) $CuSO_4.5H_2O$ 1.96g + $ZnSO_4.7H_2O$ 4.40g in 100 ml of distilled water.
b) $NaMoO_4.2H_2O$ 1.26g + $(NH_4)MO_7O_{24}.4H_2O$ 6.43g in 100 ml of distilled water.
c) $MnCl_2.4H_2O$ 36.0g in 100 ml of distilled water.
d) $CoCL_2.6H_2O$ 2.0g in 100 ml of distilled water.

Vitamin solution = 1 ml of each of the following solutions (e, f and g)

e) B1: 0.2g dissolved in 100 ml of distilled water.
f) B12: 0.1g dissolved in 100 ml of distilled water.
g) Biotin stock: 0.1g dissolved in 100 ml of distilled water.

For the final algal medium:

- Add 1 ml of each Solution A, B, C, D, E to 1 L of 25 ‰ salinity seawater.
- Autoclave at 121 °C for 20 minutes.
- Add 3 × 1 ml of vitamin solution to the 1 L solution here above.

Prepare stock cultures

- Add 120 ml of the algal medium and 30 ml of pure algal culture.
- Set room temperature at 25 °C with enough light (6 000 lux LED).
- Maintain two series of stock cultures to reduce risk of contamination.
- Stir the stock cultures manually every day to maintain the algae in suspension.
- Subculture the stock cultures every week to increase the volume of microalgae up to the desired volume:
  - For 1 L: 850 ml of nutrient medium: 150 ml of algae medium
  - For 4 L: 3 L of nutrient medium: 1 L of algae culture
  - For 20 L: 16 L of nutrient medium: 4 L of algae culture

When preparing 20 L containers

- Wash with detergent, rinse and leave to dry.
- Wash with 5 ppm HCl and leave containers for 10 minutes.
- Wash with 5 ppm chlorine, rinse and leave to dry.
- Prepare 25 ‰ salinity UV treated, 1 µm filtered seawater.
- Add 5 ppm chlorine to sea water and leave one day

- Conduct a chlorine test and, if any residue, add sodium thiosulphate (5 g/100 L seawater).

Larval feeding

- Starting from the embryos to 2–3 days old auricularia, no food is added into the incubators.
- For auricularia (now transferred into 1 000 L fiberglass tanks), add daily 10 L of microalgae solution from day-2 or day-3 up to the appearance of the doliolaria (approx. day-11) and reaching a concentration of 20 000 algal cells/ml (in the larval tank). Deliver the 10 L microalgae solution in two equal volumes in the morning and in the evening.
- From the start of the doliolaria stage until the first appearance of pentactula (approx. day-14), decrease gradually the microalgae concentration in the larval tanks from 20 000 to 5 000 cells/ml.
- Stop larval feeding when no pelagic larvae (auricularia/doliolaria) are visible (approx. day-16).

Preparation of settlement plates

- Prepare settlement plates measuring 30 × 60 cm and 60 × 60 cm and place 30 units in each 1 000 L fiberglass tank (Figure 14).
- Disinfect the settlement plates in chlorinated water for one full day (50 ppm).
- Following chlorination, wash the plates thoroughly with soap and water, rinse with freshwater and finally with UV-treated seawater.
- Air dry for one full day.
- Coat the plates with a spread of spirulina; the spirulina paste is prepared by mixing the blue-green algae powder with UV-treated seawater (10 g of spirulina powder dissolved in 50 ml of clean seawater).
- Air dry for one full day.
- When 50 percent of doliolaria are present in the cultures, add the treated settlement plates.

Sargassum juice preparation for juveniles 1 (0.7–1.2 cm)

- Collect fresh *Sargassum* seaweed daily from the seashore.
- Wash the collected seaweed using freshwater to remove sand and other unwanted particles.
- Wash the seaweed with filtered seawater.
- Cut the seaweed into small pieces and crush with the use of a grinder.
- Filtrate the seaweed paste through a 120 µm mesh sieve.
- Collect and use the filtered *Sargassum* juice. Surplus juice can be frozen and retained for up to four days.
- Stop aeration in the culture tanks when the food is added.
- Add 200 ml of *Sargassum* juice in one 1 000 L tank (once daily) up until day-30.

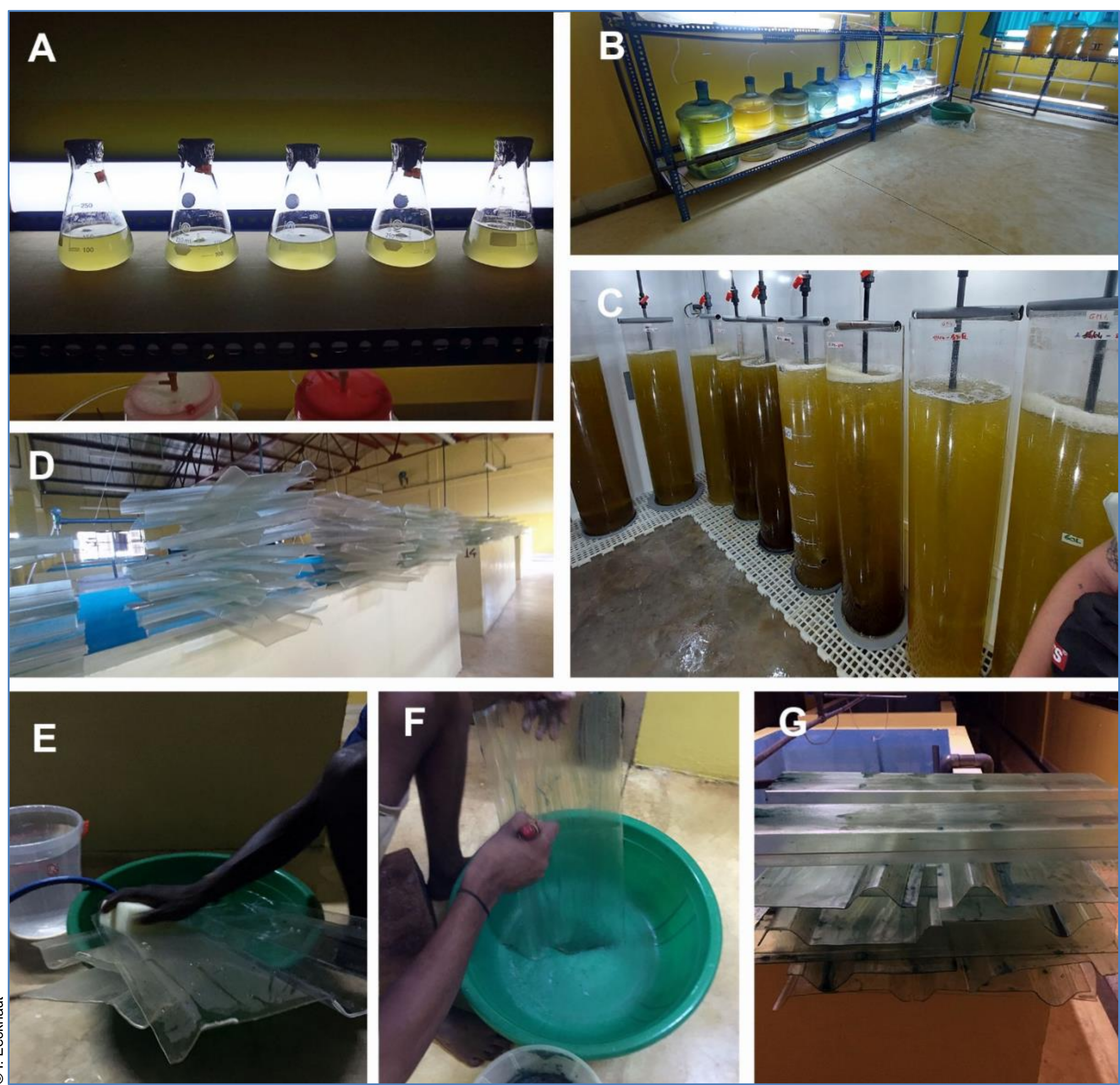
Preparation of indoor tanks and feeding of juveniles 2 (1.2–2 cm)

- Collect the sandy/muddy substrate from an appropriate site (sediments from existing seagrass beds or close to mangrove forests).
- Place the collected substratum in freshwater for one full day to kill parasites and predators.
- Place the substratum in filtered seawater for one full day to get the right salinity.
- Place the treated substratum in the rectangular indoor tanks (1 cm layer).
- Distribute the juveniles randomly in the tanks (100 ind./m^2).
- Stop the aeration in the tanks.
- *Sargassum* juice or similar food sources can be added to boost the growth of the juveniles.
- Change the bottom substratum after a few trials when growth rate or survival decreases.
- Transfer the juveniles to the outdoor tanks after two weeks (approx. 40-days old juveniles).

Preparation of outdoor tanks for juveniles 3 (>2 cm)

- Collect sandy/muddy substrate from appropriate locations (seagrass beds/close to mangrove).
- Place the substratum in the outdoor tanks (1 cm layer).
- Wash with freshwater (5 cm high) for one full day to kill parasites and predators.
- Fill the outdoor tanks (1–1.5 m high) with of non-filtered seawater.
- Distribute the juveniles randomly in the tanks (10–20 ind./m^2).
- Leave the juvenile sea cucumbers to grow to the desired length/weight (no more than 2 months).
- Change the seawater in the outdoor tanks weekly (50 percent of the volume).
- Change the bottom substratum after a few culture trials when there is clear evidence of a reduced growth and/or survival rate of the cultured juveniles.

Figure 14. Algal culture for auricularia and settlement plates for pentactula



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Note: Microalgae stock cultures in 250 ml conical flask (A). Algal culture in 20 L (B – Sri Lanka) and 100 L (C – Madagascar) containers. Preparation of the larval settlement plates (D). Soap washing of the settlement plates after chlorination (E). Spirulina coating of the settlement plates (F). Air drying of the settlement and algae coated plates (G).

Chapter 10: DISEASE IDENTIFICATION AND MANAGEMENT

Diseases are alterations in the normal health status of an organism due to multiple reasons. Such alterations can have inheritance causes or externally induced by either biotic or abiotic factors. The known diseases affecting the sandfish *H. scabra* are reviewed in Hamel *et al*. (2023).

Larvae and juveniles of *H. scabra* are generally affected by inadequate salinity levels in the water. Juveniles placed in a water body with a salinity level of ≤20 ‰ show clear behavioural changes: they tend to remain burrowed in the sediment throughout the day inclusive of the night hours when they usually move to the surface for active feeding. The body tends to swell and develop blisters. However, when placed at normal salinity the diseased specimens recover within 96 hrs. After 9 days at a salinity of 20 ‰ the juveniles became weak, shrink in size and developed a fragile skin. After 17 days, the epidermis largely disappears from parts of the body; juveniles eviscerate after 22 days and die shortly thereafter.

Larvae as well as juveniles of *H. scabra* are also affected by inadequate temperature levels. The optimal temperature range in the larvae tanks should be between 28 and 30 °C. Below this level, the growth rate slows significantly while at temperatures above this level the survival rate decreases. The temperature tolerance of the juveniles is between 22 and 35 °C. Below 22 °C the juveniles simply maintain their basic metabolism, hardly move or feed. Furthermore, their immune system becomes less efficient and the juveniles are more vulnerable to infectious attacks from external organisms.

In *H. scabra* larvae, no bacterial, viral or protozoal agent have been identified so far. Among the metazoans affecting larval development in *H. scabra*, copepods are suspected to be among the most important as they appear in most marine habitats across all oceans. Their effects on sea cucumber larvae have not yet been thoroughly studied. They are likely to compete for food available in the culture tanks.

Skin ulceration disease (SKUD) or skin ulceration syndrome (SUS) may cause severe skin ulcerations of the sea cucumbers (particularly when farmed), and these do occur in *H. scabra*. In some cases, the infection can spread rapidly from diseased individuals to healthy ones making it difficult to control. In other instances, SKUDs are not transmitted by contact. The cause of SKUDs can be multiple such as a drop or rise in seawater temperature, abnormal drop in the water oxygen level, significant variations in water salinity, incorrect density of the cultured individuals or due to a skin lesion caused by a predator (e.g. crab). A sea cucumber with skin lesions is further prone to infections and can easily become infected by opportunistic bacteria such as *Vibrio* spp.

- Managing diseases of sea cucumber larvae is almost impossible and prevention is better than treatment. If a detectable infection appears in tanks and/or if the larvae show an anachronic (abnormal) development, it is preferable to discard the larvae batch from the infected tanks and have these thoroughly cleaned and disinfected. On the other hand, if many of the culture tanks are infected, it is recommended to discard the entire larval production and verify the quality of the seawater, microalgae used and carefully review the procedures and protocols concerning water renewal and feeding.
- Managing diseases of juveniles is somewhat possible but time consuming. If some juveniles are infected, they should be placed in quarantine tanks with clean, filtered and chlorinated seawater. If the great majority of the juveniles are affected, it is also advisable to get rid of them and adequately dispose of the tank sediment where they have been reared. Following this, tanks and ponds should be thoroughly washed, and the sediment replaced with a fresh and clean layer.

Table 1. Pathogens and/or food competitors of *Holothuria scabra* sea cucumber larvae and juveniles (reviewed in Hamel *et al.*, 2023).

Organism	Species
Bacteria	*Arcobacter bivalviorum* *Pseudoalteromonas citrea* *Vibrio azureus* *Vibrio fortis* *Vibrio owensii* *Vibrio parahaemolyticus* *Vibrio rotiferianus* *Vibrio tubiashiand* *Vibrio natriegens* *Vibrio harveyi* *Vibrio alginolyticus* *Vibrio variabilis* *Vibrio* sp. Bacterium from the CFB group *Roseobacter* sp.
Ciliates	Unknown species
Nematodes	Unknown species
Polychaetes	Unknown species
Crustaceans	Unknown Copepods *Cymodoce* sp. (Isopod)
Insects	Larvae of dipteran flies

Chapter 11: COUNT MANAGEMENT

Data collection and analysis is crucial for better understanding problems that may occur in the hatchery and to increase the overall productivity of the facility. For each IVF and thermal shock trial, data sheets such as those illustrated in Tables 2 and 3 must be prepared and diligently completed. When performing IVF, the number of eggs (oocytes + ootids) must be recorded along with the number of embryos obtained. Based on the development cycle of the fertilized egg, it might be more convenient to perform IVF in the afternoon and collect the number of embryos the following morning.

On the other hand, when a thermal treatment shock is performed, only the number of embryos harvested is known. Three days after fertilization, the auricularia are counted and distributed in one or more tanks. The distribution of the larvae must be carefully recorded, for example: 2 000 000 auricularia distributed in batches of 500 000 in tanks # 1, 2, 3 and 4. For each tank receiving auricularia larvae a separate data sheet (i.e. Table 3) must be maintained.

Data must be collected daily for each tank. An example is shown in Table 3. The following data are required:

- Date
- Age
- Stage: **Au**: auricularia
  - **Au/Do**: auricularia / doliolaria (record any pelagic pentactula, if present)
  - **Pe/Ju**: pentactula / juveniles
  - **Ju**: juveniles
- Number (in K; 1 000 = 1 K)
- Water exchange (in %)
- Phytoplankton (quantity in L)
- Type of phytoplankton (taxonomic name)
- Water T °C (a.m.)
- Water T °C (p.m.)
- *Sargassum* (quantity of juice added in ml)
- Observations/comments

Table 2. An example of a hatchery data collection sheet that includes information on the number of eggs and embryos collected in any given batch, along with the numbers of auricularia and juveniles transferred to one or more larval tanks and indoor ponds, respectively.

EGGS & EMBRYOS		**TRANSFER TO TANK 1**					**TRANSFER TO INDOOR PONDS**				**Observation**
Eggs	**Embryos**	**Date**	**No. trial**	**Origine**	**Number**	**Stage**	**Date**	**Number**	**Wt/length**	**Destination**	
Indicate the number of the eggs collected. **Example:** 1 100 000	Indicate the number of the embryos collected. **Example:** 500 150	Indicate the transfer date. **Example:** 21.12.2021	Indicate the code of the culture trial. **Example:** Dec3/IVF	Indicate if the eggs come from an IVF or thermal shock.	Indicate the number of auricularia. **Example:** 248 000	Should normally be auricularia 3-days old.	Indicate the transfer date. **Example:** 12.01.2022	Indicate the number of juveniles transferred. **Example:** 1 688	Indicate average weight and/or length. **Example:** >0.4 cm	Indicate the code of the indoor pond. **Example:** Pond ID #1	All auricularia from Dec3/IVF transferred in tank 1

Table 3. Example of hatchery sheet and data/information that must be collected daily from each culture tank.

Date	Age	Stage	Number (K)	Water change (%)	Phytoplankton (L)	Type	T °C (a.m.)	T °C (p.m.)	Sargassum (ml)	OBSERVATIONS
21/10	3	Au	248	100	20	Iso			--	Young auricularia that have just been sorted.
22/10	4	Au	210	100	20	Iso	28	28,2	--	Clean bottom, large auricularia coarse (98%); digestive tube with food on 1/2 the larvae.
23/10	5	Au			20	Iso	28,4	28,3	--	--
24/10	6	Au			20	Iso	28	28,1	--	Clean background, big and competent auricularia (around 98%), some small, digestive tube OK, lots of rotifers.
25/10	7	Au			20	Iso	28	28,1	--	Slightly dirty bottom, auricularia large and active at the surface, some small at the bottom, rotifers & vorticella present.
26/10	8	Au	126	100	20	Iso	28	28,1	--	--
27/10	9	Au			20	Iso	27,8	27,9	--	Bottom not very clean, auricularia large and competent 100%, digestive tube OK, lots of rotifers.
28/10	10	Au		50	20	Iso	27,6		--	..
29/10	11	Au/Do	100		20	Iso	28	28,1	--	Dirty bottom, auricularia large and competent (100%), digestive tube full, lots of rotifers and vorticella, dead larvae present, lots of doliolaria/pentactula.
30/10	12	Au/Do		100	20	Iso	28	28,2	--	Very dirty bottom, remaining auricularia 100% competent, digestive tube full, bulk of pentactula, worms, rotifers, bacteria at the bottom, many dead.
31/10	13	Au/Do			20	Iso	28		--	Bottom clean, remaining auricularia 100% competent, digestive track full, vorticella, ciliates and rotifers observed, and dead larvae.
1/11	14	Au/Do			20	Iso				--
2/11	15	Pe/Ju	10	100				29,2	50	Separation larvae from benthic stages. Remaining larvae 30 000 discarded. Estimated benthic stages 10 000.
3/11	16	Pe/Ju					28,6	28	50	
4/11	17	Pe/Ju		50			28,6		50	Bottom not very clean, juveniles still small, slow food consumption, slow growth.
5/11	18	Pe/Ju					28,2	28,2	50	--
6/11	19	Pe/Ju		50			28,7		50	--
7/11	20	Pe/Ju					28,8		50	Dirty bottom juveniles still small, slow consumption, slow growth.
8/11	21	Pe/Ju		100					50	--
9/11	22	Pe/Ju					27,8	27,5	50	Dirty bottom, juveniles still small, decrease in number observed, slow growth.
10/11	23	Pe/Ju		50			27,9	27,5	50	Slightly dirty bottom, healthy juveniles, average consumption, average growth.
11/11	24	Pe/Ju					27,1	27	50	--
12/11	25	Ju	1,688	100						Tank emptied: 1 688 juveniles transferred to nursery Pond#1.

Chapter 12: PRODUCTION MANAGEMENT

Hatchery juvenile production numbers depend on many factors, the most important of which is the maximum rearing volume capacity of the hatchery. Knowing that the maximum larvae culture density is 0.5 larvae/ml, a 1 000 L tank will generate a maximum of 500 000 larvae.

How should the production process unfold across various developmental stages leading up to the appearance of type-1 juveniles?

- The success of any reproduction trial in obtaining viable sea cucumber embryos is extremely variable. For example, in one large and established sandfish hatchery in Madagascar, the lowest number of embryos obtained through a thermal shock trial was 13 800 whilst the highest number was 20 000 000; the lowest number of embryos obtained during an IVF trial was 10 000 whilst the highest number was around 7 000 000 (see Eeckhaut *et al*. 2024, for detailed information on *H. scabra* juvenile production on a large-scale).
- Normal survival rate during reproduction trials is generally between 15–72 percent for embryos (after 12 h) and between 6–45 percent for 3-day old auricularia. In normal reproductions trials, the number of auricularia/pentactula around day-13 typically decrease by 40–60 percent (100% representing the number of 3-day old auricularia). The number of pentactula/juveniles present around day-20 is between 5–14 percent. The number of juveniles transferable to indoor ponds varies from 1–5 percent after 30 days from fertilization. Approaching the bottom line becomes critical and indicates that there is probably a problem in the culture that must be solved (see below some potential solutions to the problems), while reaching the top line must be the goal to achieve the economic viability of the hatchery. In other words, below the bottom line, the production is poor, above the top line, it is excellent (Figure 15).

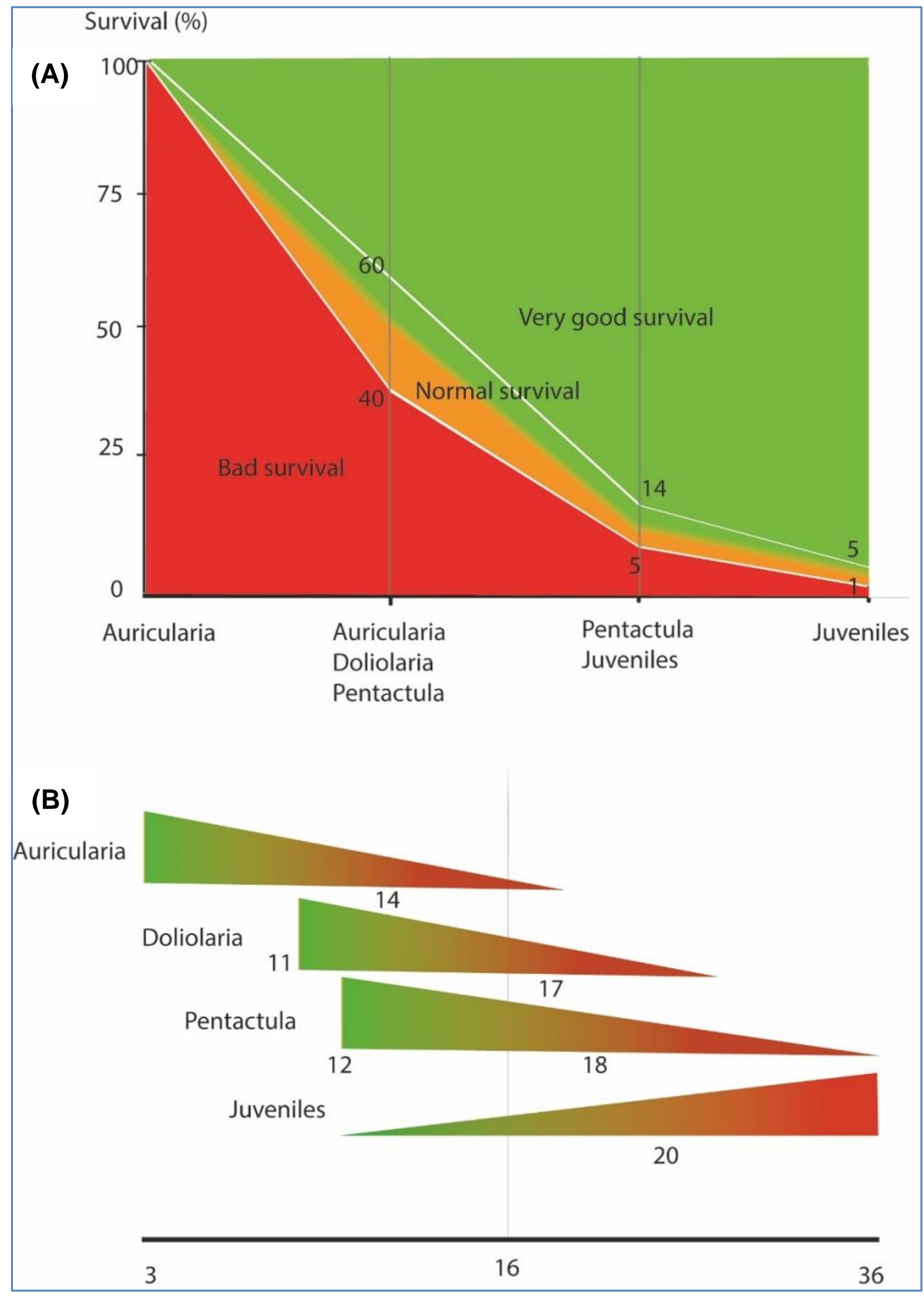
Figure 15. Survival of embryos and 3-day old auricularia during a typical reproduction trial

Note: In a hatchery the survival of sandfish embryos typically ranges from 15–72% and between 6–45% for 3-day old larvae. Survival levels below the minimum values become critical, while those above the upper values must be reached in a commercial operation.

Source: Authors' own elaboration (Igor Eeckhaut)

- In a successful reproduction trial, the times of appearance of the different stages of development are as follows (Figure 16A and B): the auricularia stage appears on day-3 and disappears around day-19; the doliolaria stage appears around day-11 and disappears around day-22; the pentactula stage appears around day-12 and disappears around day-34; and the juvenile stage appears around day-13. As the time for the appearance of each stage prolongs and nears the red zone (specifically day 14, 17, 18, and 20 for the respective stages), it serves as a potential issue within the culture.

Figure 16. Survival of different stages during the early ontogeny of *Holothuria scabra* in hatchery



Note: Figure A - In the hatchery, monitoring the development of individuals occurs at various intervals. Typically, around day-11, doliolaria emerge alongside auricularia and are counted together. A few days later, pentactula and benthic juveniles also appear, and they are counted collectively. In standard production trials, the count of auricularia/doliolaria around day-13

typically decreases from 40–60 percent (with 100% representing the count of 3-day old auricularia). By the day-20, the count of pentactula/juveniles ranges between 5–14 percent. The number of juveniles transferable to indoor ponds after 30 days varies from 1–5 percent. It is crucial to note that approaching or falling below the critical lines indicates a poor production performance, while above these indicates excellent results. Figure B - In a successful reproduction trial, the developmental stages typically follow these timelines: the auricularia stage emerges on day-3 and concludes around the day-19; the doliolaria stage arises around day-11 and concludes around day-22; the pentactula stage emerges around day-12 and concludes around day-34; while type-1 juvenile stage typically appears around day-13.

***Source*:** Authors' own elaboration (Igor Eeckhaut)

- A larval rearing tank of 1 000 L containing 300 000 3-day old auricularia will therefore typically produce between 3 000 (or 1%) to 15 000 (or 5%) juveniles. Ten tanks will produce 30 000 to 150 000 type-1 juveniles in 30 days. Considering both the frequency and duration of tank cleaning activities throughout the year, a hatchery with 10 tanks can produce annually from 300 000 to 1 500 000 juveniles ready to be transferred to the indoor on-growing tanks.

Problems and potential solutions with embryos and larvae production

IVFs does not work well – not enough ootids or embryos are obtained

1) It may be a seasonal-related issue. This issue becomes particularly crucial when seawater temperatures exhibit significant variations between seasons. Consequently, ovaries extracted during these times may be either immature or spent (post-spawning). Addressing this challenge involves primarily two strategies. Firstly, increase the number of females from which ovaries are extracted (>30 individuals) as there are likely to be individuals with mature gonads among them. This approach increases the likelihood of obtaining mature ovaries. Secondly, an alternative solution involves conditioning the broodstock prior to IVF (see Chapter 6).
2) It may be an IVF procedural-related issue. When using the maturation inducer, the number of viable ootids should generally exceed 60 percent. If this is not the case, the following actions should be considered:
   (a) Consider substituting the inducer, as it may have degraded over time. Freshly produced/purchased inducer can be stored in the fridge for a maximum of six months.
   (b) Check the quality of the seawater used, ensuring it has been filtered to 1 µm.
   (c) Examine the condition of the sieves and review the procedures associated with their use.

Thermal shock does work well – but not enough embryos are obtained

1) It may be a seasonal-related issue. Spawning following thermal shocks is highly variable, leading to frustration when multiple attempts yield negative results. To address this challenge, consider the following solutions:
   (a) Check the ovarian maturity of the female brooders. Used different individuals if the ovaries are poorly developed.
   (b) Patiently repeat the thermal shock procedure with the same broodstock individuals.
   (c) Increase the seawater temperature differences between the hot and cold shocks and adjust the immersion duration of the brooders.
   (d) Prior to initiating the thermal shock procedure, prepare the broodstock by subjecting them to a few days of enhanced conditioning (see Chapter 6).

The number of auricularia obtained is ≤6 percent

1) The seawater quality in which the embryos develop is poor. Verify the parameters of the culture seawater, including pH, temperature, nitrate/phosphate values and microbial load.
2) The quality of the oocytes from which the embryos develop is poor. Change the broodstock or condition them further to further enhance ovarian maturity.

The count of auricularia/doliolaria larvae after 12 days of culture is <40 percent

1) The quality of the water in which the auricularia are reared is poor. Consider the following solutions:
   (a) Check and adjust the parameters of the culture seawater, including pH, temperature, nitrate/phosphate values and microbial load.
   (b) Check parasite load in the culture medium (e.g. nematodes, copepods or other organisms) and mitigate through adjustments to the water renewal regime.
   (c) Check water quality following the addition of feed and follow proper water renewal procedures.
2) The food amount provided is inadequate. Consider the following solutions:
   (a) Increase the concentration of microalgae supplied. The concentration should range from 5 000 to 40 000 cells/ml.
   (b) Change the species of microalgae used as feed.
   (c) Feed the larvae with a mix of several species of microalgae.

   Note: As populations of *H. scabra* can differ from one another, the appropriate food mixture may also vary depending on the locality.
3) Metamorphosis into doliolaria may be compromised. Verify the timing of doliolaria stage appearance: nearing day-22, the more likely a development issue exists. Confirm if the auricularia have reached the competent stage, identifiable by the presence of hyaline spheres in their body lobes. An effective intervention to stimulate metamorphosis initiation involves injecting filtered seawater collected from areas inhabited by marine phanerogams (e.g. *Thalassia empichrii*). The juveniles of *H. scabra* have only been observed in nature on phanerogams, particularly on the leaves of *T. empichrii*. While lacking empirical confirmation, there is an observed correlation suggesting that the introduction of filtered water previously inhabited by marine phanerogams may stimulate metamorphosis. This process potentially triggers a signal indicating to the auricularia that it has arrived at an environment conducive to its development.

The count of pentactula/juveniles after 25 days of culture is <5 percent

1) Larval settlement is poor. Inspect the settlement plates to ensure proper spirulina coating. If insufficient, enhance the coating by increasing the paste application over the plates. Also, consider replacing the spirulina source, if necessary. Settlement plates can also be enriched with the benthic diatom, e.g. *Navicula*, by leaving them in unfiltered seawater for several days.

Projected growth and survival rates of the different juvenile types

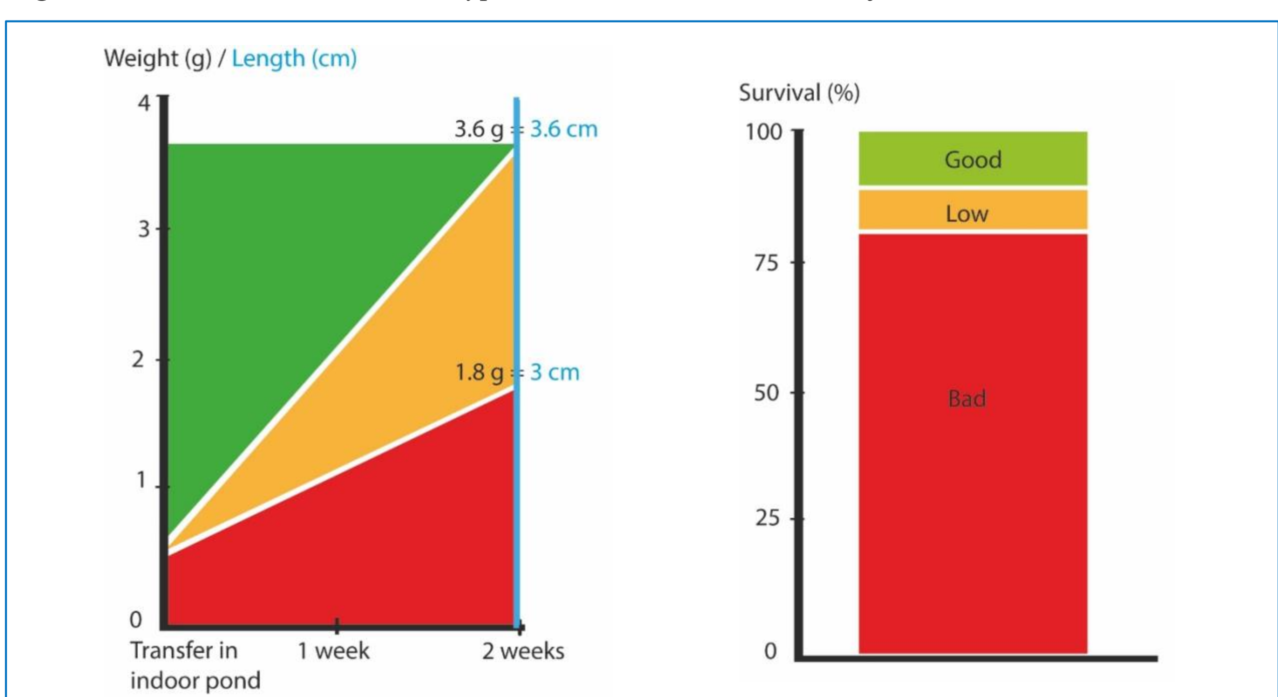
- **Type-1 juveniles** range in length from 0.7 to 1.2 cm. They are fragile and reared in fiberglass tanks at a density of 100 ind./m^2 for several weeks before being transferred to the indoor rectangular tanks previously prepared with an adequate bottom substrate. Six weeks after settlement, more than 50 percent of the juveniles should have attained a length of 1.2 cm. The survival rate at this stage is generally around 70 percent. See Figure 17.

Figure 17. Growth and survival of type-1 sandfish, *Holothuria scabra*, juveniles

Source: Authors' own elaboration (Igor Eeckhaut)

- **Type-2 juveniles** measure in length from 1.2 to 2 cm. They are raised in indoor rectangular tanks on a marine substrate at a density of 100 ind./m^2. During this phase, the juveniles acclimate to the substrate allowing the hatchery operator to supplement their diet to enhance both growth and their immune system. They remain in this controlled environment for approximately two weeks, during which they can attain a length of 3.6 cm, reaching readiness for transfer to outdoor ponds upon reaching 2 cm. The survival rate during this period stands at around 90 percent. See Figure 18.

Figure 18. Growth and survival of type-2 sandfish *Holothuria scabra* juveniles



Source: Authors' own elaboration (Igor Eeckhaut)

- **Type-3 juveniles** range in length from 2 to 6 cm. They are raised in outdoor ponds on a marine substrate at a density of 10–20 ind./m^2. As a result of this culture phase, they become more resilient and better equipped to evade predation prevalent in the marine environment as they progress through the grow-out phase. They will reach the length of 6 cm (15 g) in about six weeks. The survival in these basins is usually around 80 percent. See Figure 19.

Figure 19. Growth and survival of type-3 sandfish, *Holothuria scabra*, juveniles

Source: Authors' own elaboration (Igor Eeckhaut)

The problems and the potential solutions with juveniles

The count and growth of juveniles are in the red zone (see Figures 16–19).

Juvenile mortality and suboptimal growth levels predominantly stem from three primary factors:

1) The amount of food is insufficient. The solution is to increase the amount of food (*Sargassum* juice for juveniles of type-1 and type-2; in the case of juveniles of type-3, no food is added). Such increase may however affect water quality (especially in fiberglass tanks) which can lead to the death of the juveniles. There is therefore a balance to be found between the quantity of food given and the quality of the culture environment.

2) The quality of food is inadequate. The solution is to change the food. The entry of *Sargassum* juice and the decrease in water turnover for juveniles of types-1 and type-2 can lead to the development of parasites and/or predators which increase the mortality of juveniles. Check the quality of the benthos under the microscope. If the growth rate of juveniles of type-2 and type-3 is not adequate, change the substrate.

3) The rearing density of the juveniles is too high. The solution is to decrease the density in the culture vessels. The density of juveniles of type-1 and type-2 should not exceed 100 ind./m^2. The density of juveniles of type-3 should not exceed 20 ind./m^2 and is much better when there are no more than 10 ind./m^2.

Key benchmarks for achieving adequate production levels in a sandfish hatchery

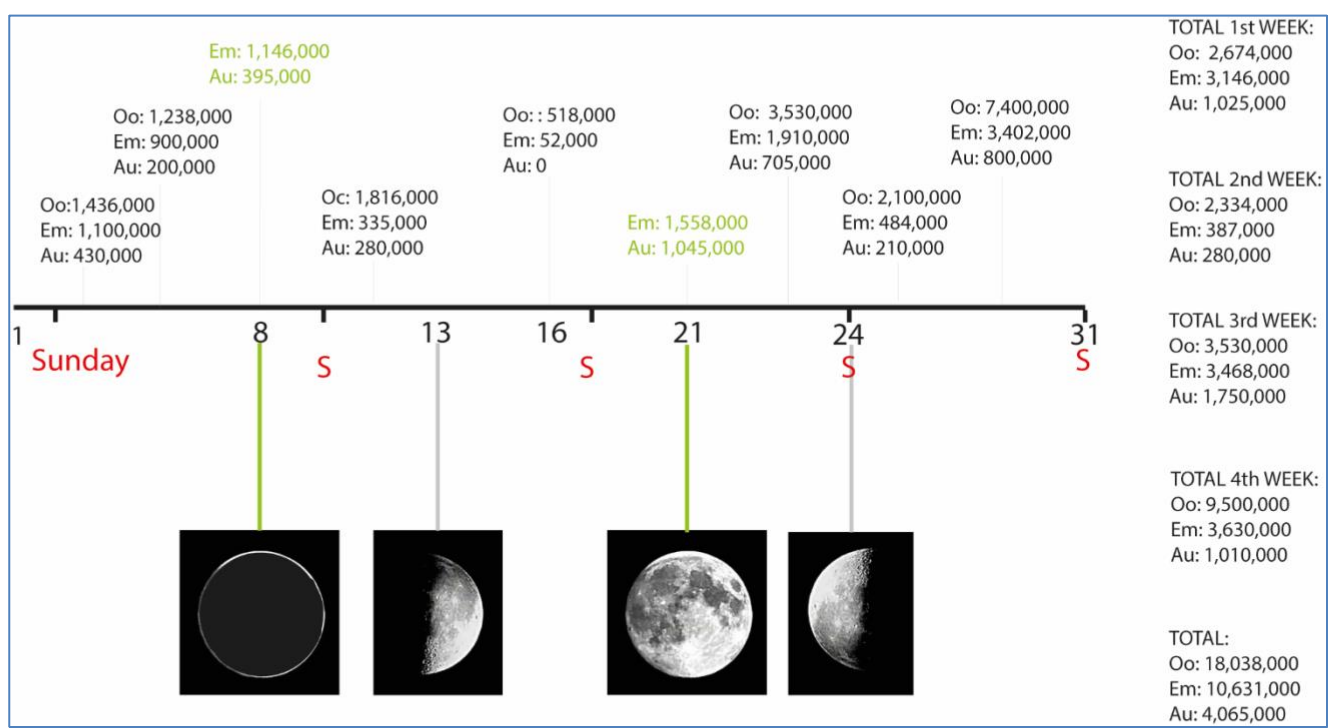
The number of males and females used during IVFs as well as the level of production obtained can vary greatly from one trial to the next. Table 4 shows an example of such hatchery output variations.

Table 4. Examples of numbers of males and females used during IFV and the number of eggs, embryos and auricularia obtained.

Males	Females	Oocytes/Ootids obtained with IVF	Embryos	3-day old auricularia
6	18	1 199 000	804 000	510 000
3	16	2 412 000	840 000	705 000
4	8	992 000	240 000	130 000
2	25	2 800 000	1 078 000	525 000
4	14	3 200 000	768 000	520 000
3	15	2 000 000	1 456 000	640 000
3	30	2 500 000	618 000	480 000
4	30	3 000 000	1 822 000	485 000
19	10	2 400 000	542 000	285 000
15	4	6 600 000	3 805 000	2 085 000

Figure 20 shows the production outputs from a sandfish commercial hatchery in March 2019. The new moon and full moon were on March 8 and March 21, respectively. During this month, seven IVF and two thermal shock trials were carried out. The thermal shocks were conducted during the new and full moon while the IVF trials were carried out between the two lunar phases. Two IVF trials were performed each week. The trials produced 10 631 000 embryos from which 4 065 000 3-day old auricularia were obtained.

Figure 20. An example of hatchery production over a 1-month period



***Note*:** The figure shows the lunar phases, the week, and the production with IVF (**in black**) or thermal shocks (**in green**). The numbers shown in the right column illustrates the weekly production. Thermal shocks were conducted during the new and full moon.

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ANNEX 1. Selected laboratory equipment typically used in a sea cucumber hatchery

The equipment items listed below are essential when working in a sea cucumber hatchery. Typical technical specifications are provided to guide purchase of the items. For some items specification will change depending on the size of the facility and volumes processed.

Distilled water plant

TYPE	Automatic – Cabinet water still (glass/quartz)
Capacity	2 L/h
Output	2 L/h
pH	5.5 to 6.0
Cooling required water	1.5 L/min.
Conductivity	1 to 2
Power	1.6KW / 230V

Autoclave

TYPE	Vertical laboratory autoclave with top load
Range	50 L
Pressure	0.22 MPa
Temperature range	0 to 134 °C
Time range	0 to 90 min.
Dimension	340 × 550 mm
Power	3KW / 220V

Electronic top-pan balance

TYPE	Top loading balance
Range	0.01 to 3 200 g
Readability	0.01 g
Calibration type	Internal
Language	English
Power	120V

Electronic analytical balance

TYPE	Vertical laboratory autoclave with top load
Capacity	0.001 to 320 g
Minimum value	0.001 g
Calibration type	Automatic
Repeatability	0.001 g
Linearity	0.002 g
Power	adapter 12V/1AH / Adapter 230V
Pan size	90 / 120 mm

Salinity meter – portable/digital

TYPE	Portable refractometer seawater dual scale
Range	0–100 ‰ or 100 ppt
Resolution	1 ‰ or 1 ppt
Temperature range	10 to 50 °C
Specific gravity	1.0 to 1.070

Centrifuge

TYPE	BEST PRP CENTRIFUGES
Max speed	4 000 rpm
Max RCF	2 200 g
Max capacity	8 × 15 ml
Temperature range	0 to 134 °C
Time range	0 to 99 min
Dimension	320 × 260 × 230 mm
Power	50W AC 220V 50HZ 2A

Laminar flow cabinet

Clean grade	Grade 100 (209 E US Federal)
Number of bacteria	≤0.5 per utensil/hour (90 mm utensil)
Noise	≤62 dB
Power	230V / 50HZ
wind speed	0.25–0.45 m/s (3 speeds: slow, medium & fast)
illumination	≥300 LX
Working area dimension	1140 × 615 × 580 mm (W×D×H)
Overall dimension	1295 × 765 × 1765 mm (W×D×H)
HEPA filter size and quantity	1135 × 600 × 38mm (3pc)
Fluorescent/UV lamp specifications and quantity	30W (1pc) / 30W (3pc)

Micropipettes

Digital

TYPE	Micropipettes digital-5
Capacity range	5 to 100 µl
Accuracy	±3% for 100 ml, ±1% for 500 ml, ±0.6% for 1 000 ml
Autoclavable	Yes
Number of channels	1
Tip ejector	Yes
Speed	Five essential modes: forward, reverse, multi, mixing & diluting
Battery	Rechargeable lithium ion

Non-digital

TYPE	Micropipettes-5
Capacity range	100 to 1 000 ul
Accuracy	0.60 / 0.70 / 2.00 %/ µl
Repeatability/precision	0.20 / 0.25 / 0.70 %/ µl
Autoclavable	Yes
Number of channels	1
Tip ejector	Yes
Speed	Five essential modes: forward, reverse, multi, mixing & diluting

Cell culture incubator

TYPE	CO_2 incubator / Cell culture incubator
Temperature	
Control and range	±0.1 °C, 5 °C above ambient to 50 °C
Heating method	Air jacket
Uniformity	±0.3 °C @ 37 °C
Alarm setting	±5 °C
Sensor	Platinum thermistor
Controller	PID
Readability & adjustability	0.1 °C
Sterilization method	140 °C dry heat sterilization
CO_2 concentration	
Control and range	Better than ±0.1% ; 0–20%
Sensor	Heat-resistant infrared (IR) sensor
Inlet pressure	1.0 bar (recommended)
Uniformity	±0.3%
Readability & adjustability	0.10%
Alarm setting	±1%
Humidity	
Humidity Fan Volume	~3.8 L
Display	1% RH
Shelves	
Dimensions	47.0 × 47.0 cm
Surface area (standard/max)	214. 6 / 220.7 cm^2
Number (standard/max)	3 / 16
Size and weight	
Interior volume	175 L
Interior dimensions	51.0 × 54.7 × 67.5 cm (W×D×H)
Exterior dimensions	66.0 × 67.0 × 97.0 cm (W×D×H)
Net weight	110 kg

Defreezer

TYPE	Defreezer
Capacity	200 L
Doors	01
Temperature range	0 to 134 °C
Display and control	Compressor activation and power / Temperature control
Dimension	946 × 839 × 556 mm
Power	AC 100–240V, 50–60Hz

Seawater UV filter system stainless steel covered single tube and sand filter system

TYPE	Non-corrosive PEHD UV systems aquaculture seawater
Capacity	20 m^3/hr
Pressure	0.22 MPa
Input size & output size	2”
UV lamps	3
Maximum pressure	150 PSI
Build	Non-corrosive 304 stainless steel
Power	165W 110–240V, 50/60Hz, Single phase, 2W+GND
Flanges size	2”
Panel	Digital control panel

ANNEX 2. How to count the germ cells/embryos

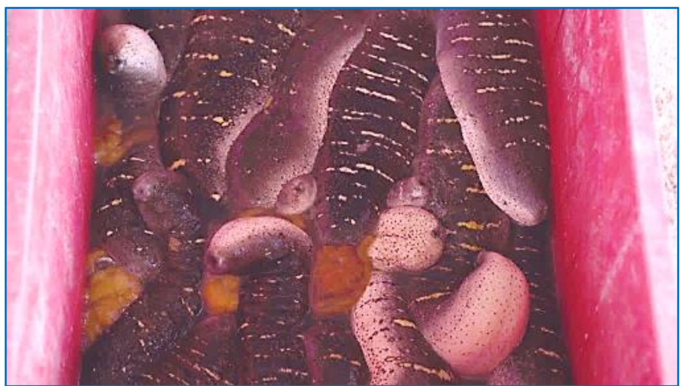
It will be necessary to count germ cells and/or embryos resulting from fertilizations during the performance of thermal shocks and IVF. The steps to follow are described below:

- Mix the seawater volume containing the germ cells/embryos for 2 minutes to homogenize the suspension.
- Take 5 drops with the aid of a Pasteur pipette from the mixed suspension for counting.
- Place the 5 drops on a microscope slide (each drop has a volume of 0.050 ml or 1/20 of a ml). Alternatively, for better accuracy, take 50 µl with a precision micropipette.
- Count under a low magnification optical microscope (25× or 50×) the number of germ cells/embryos in each drop. According to the time or the sample checked (e.g. water from thermal shocks; sectioned ovary for IVF; fertilized eggs from IVF), the samples will include various proportions of unfertilized eggs, fertilized eggs and/or embryos typically at stages 2 to 8 blastomeres (see Figure 10 I).
- Average the number of germ cells/embryos per drop.
- Multiply the average by the right number to get the number of germ cells/embryos in the starting volume. For example, for a volume of 20 L (20 000 ml = 40 000 drops), the number of germ cells/embryos = the average number x 40 000.

ANNEX 3. Steps for the processing *Holothuria scabra* into beche-de-mer

This concise description outlines the steps involved in processing sandfish (*Holothuria scabra*) into beche-de-mer, from harvesting live sea cucumbers to producing the dried products. It emphasizes the critical importance of diligently following each step to ensure a quality product suitable for the export market. Proper handling and adherence to these steps will minimize losses and ensure the production of top-grade beche-de-mer.

The annex was drafted by Vishwa Dulanji Samaraweera, PhD Candidate, University of Sri Jayewardenepura, and kindly revised by R.K. Viraj Lahiru Rathnayake, Research and Development Manager and E. Sharujan, Quality Manager at Suganth Sea Farm (Pvt) Ltd; P.A.D. Ajith Kumara, Senior Scientist at the National Aquatic Resources Research and Development Agency (NARA); Chamari Dissanayake from the University of Sri Jayewardenepura; and Alessandro Lovatelli from the FAO Fisheries and Aquaculture Division.

Step 1: Collecting/harvesting sandfish	
▪ *Collect only large individuals (>300 g).* **Why:** processed large individuals attain a better market price. ▪ *Always use hands to collect sandfish.* **Why:** to avoid/minimize damage to sea cucumbers. ▪ *Do not destroy coral reefs or seagrass beds.* **Why:** as these are natural habitats of sea cucumbers.	© FAO/J. Liyanage
Step 2: Post-harvest handling at sea	
▪ *Store the harvest in plastic containers or polythene bags.* **Why:** to minimize post-harvest losses. ▪ *Always keep the catch immersed in seawater (cold water is preferred).* **Why:** to keep them alive to avoid skin damage and evisceration. **Tip:** Cold water minimizes stress due to sudden temperature fluctuations. ▪ *Avoid exposure to direct sunlight.* **Why:** prolonged exposure to direct sunlight damages the skin of the collected individuals and will lower the value once the sea cucumber is processed. ▪ *Transport to the processing plant.* **Why:** minimize post-harvest losses due to the onset of the self-autolysis process. **Tip:** within 3–5 hours.	 © FAO/J. Liyanage

Step 3:	Cleaning and grading

- *At the processing site, wash with clean seawater.*
  Why: to remove sand and slime.
  Tip: wash gently using seawater for 3–5 minutes.

- *Grade according to their size.*
  Why: to ensure high-quality beche-de-mer as boiling time varies with the size of individuals even within the same species.

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Step 4:	Gutting

- *Make a small cut (1–5 cm) near the anus.*
  Tip: 1–2 cm away from the anus tip.
  Why: for evisceration.

- *Gently press the body to remove internal organs (gut, respiratory tree, and gonads).*
  Why: smooth pressing ensures no / minimum damages to individuals.

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Step 5:	Washing

- *Wash eviscerated individuals with seawater.*
  Tip: 2–3 minutes.
  Why: to remove all traces of internal organs.

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Step 6:	First boiling

- *Boil the eviscerated sea cucumbers in salt water.*
  Tip: 4 kg of salt per 100 L of water at 100 °C for 30–35 minutes. Can also use clean seawater, if needed.

- *Gently stir the sea cucumbers every 5 minutes.*
  Why: to minimize the damage to the body wall of fresh individuals.
  Tip: obtain perfectly cylindrical products with a rubber-like texture at the end of the first boiling.

- *Remove the sea cucumbers from the boiling container, place them on a drying rack, and leave till they cool down to room temperature.*
  Tip: 2–3 hours.

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Step 7:	First salting (wet salting)

- *Dip the boiled sea cucumbers in a concentrated salt solution.*
  Tip: 8–10 kg of salt for 25 L of water.
- *Keep for 24 hours (01 day).*
  Why: to facilitate preservation by removing water.

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Step 8:	Second boiling

- *Boil the salted products again in a similar boiling medium used in first boiling for 10 to 15 minutes.*
- *Stir the sea cucumbers at a moderate speed every 5 minutes.*
  Tip: to remove excess salt.
- *Remove the sea cucumbers from the boiling container, place them on a drying rack, and leave till they are cool down to room temperature.*
  Tip: 2–3 hours.

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Step 9:	Soaking

- *Introduce boiled sea cucumber into a freshwater bath at ambient temperature for 12 to 18 hours.*
  Tip: to loosen the chalky structures ($CaCO_3$) in the body wall.

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Step 10:	Third boiling

- *Add the washed sea cucumbers to a boiling container containing freshwater at room (ambient) temperature.*
- *Heat the water slowly until it reaches the boiling point.*
  Tip: 1–3 hours.
  Why: To remove chalky materials ($CaCO_3$).
- *Remove the sea cucumbers from the boiling container, place them on a drying rack, and leave them to cool down to room temperature.*
  Tip: 2–3 hours.

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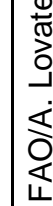
Step 11: Brushing

- *Brush the body wall of the sandfish with a stiff plastic brush.*
  Why: to remove $CaCO_3$ deposits.

- *Wash with freshwater.*
  Why: to clean the animals.



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Step 12: Second salting (wet salting)

- *Dip the brushed and cleaned sea cucumbers in a concentrated salt solution.*
  Tip: 8–10 kg of salt for 25 L of water.

- *Keep for 24 hours (01 day).*
  Why: to facilitate preservation by removing water.

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Step 13: Fourth boiling

- *Boil the salted sea cucumbers again in fresh water for about 10 minutes at 100 °C with continuous rapid stirring.*
  Tip: to get the golden colour in the ventral surface.

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Step 14: Sun drying	
▪ *The processed sea cucumbers (now known as beche-de-mer) are sun dried for 3 days.* **Tip:** Mats, wooden planks, or polycarbonate storage facilities can be used to dry the sea cucumbers.	© FAO/J. Liyanage
Step 15: Grading	
▪ *Grade the beche-de-mer according to the size and quality of the final product.*	© FAO/J. Liyanage ©Suganth International Pvt Ltd

Notes and observations

The manual is a comprehensive guide for hatchery operations, introducing innovative methods and strategies to boost financial viability. It provides a detailed, step-by-step explanation of *in vitro* fertilization (IVF), a proven and validated technique. IVF is proposed as a complementary approach to spawning, utilizing thermal shock treatments commonly seen in sea cucumber hatcheries globally to enhance seed output. In addition, IVF can be applied using gonads harvested from sea cucumber specimens destined for processing into dried trepang. The manual also describes the utilization of greenhouses to enhance ovarian maturation in colder climates, thereby improving overall hatchery productivity. It offers comprehensive advice on optimizing productivity levels of embryos, larvae, and juveniles to attain financial profitability.

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