In-vitro fertilisation: a simple, efficient method for obtaining sea cucumber larvae year round

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Abstract

Obtaining eggs and larvae in large quantities is a critical point for the economic viability of sea cucumber aquaculture. In this paper, spawning induction methods and in-vitro fertilisation (IVF) methods are presented and compared. The IVF technique developed in Madagascar (MH-IVF) is a simple, cost-efficient method that enables hatcheries to obtain clean, fertilised eggs of sea cucumbers year-round. MH-IVF does not require high-tech equipment and is applicable in small- and large-scale hatcheries. It ensures the best control at the very beginning of the work on the number and type of genitors (i.e. sex, length, weight, colour); the quality of the gonads (healthy versus parasitised); and the number, size and quality of spermatozoa and eggs. MH-IVF involves the sacrifice of very few genitors compared with the individuals obtained and sacrificed for production. Yet, it does not influence genetic drift any more than spawning induction methods.

Introduction

It is evident today from many surveys that the world's wild stocks of sea cucumbers are depleting fast, and almost everywhere this is due to the high demand from the Chinese market. The disappearance of sea cucumber wild stocks is not only a problem at the ecological level (these organisms being among the best bioturbators of the sediments in many marine ecosystems), but it is also a huge social problem as the sea cucumber trade ensures a livelihood for millions of humans in developing countries. One of the best answers to this worldwide problem is to develop efficient aquaculture systems where coastal villagers of developing countries can be involved in some phases of the farming. However, aquaculture is basically a business where the end product (i.e. the trepang) is sold into the Chinese market. The private companies that are involved in this process need benefits: preferably quickly and with a minimum of investments. It is thus crucial for the sustainability of sea cucumber aquaculture that the profitability of each step in the process—obtaining eggs, rearing larvae, pre-growing juveniles and growing adults—is optimised. Obtaining eggs and larvae in large quantities is thus a critical point for the economic viability of the industry.

Over the past 10 years, we have developed a new method based on in-vitro fertilisation (IVF) for obtaining sea cucumber larvae throughout the year in Madagascar. This method is used routinely by the company Madagascar Holothurie S.A. (MH.SA), which was created at the end of the research phase. We present here a description of this method and the various techniques that allow aquaculturists to obtain fertilised eggs of sea cucumbers. Some are still in the research phase but appear to show promise for the near future.

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There are basically three types of method: unforced spawnings, forced spawnings and IVF. The first method is used only on the East Pacific *Isostichopus fuscus* in Ecuador. There, and for that species only, spawning occurs monthly depending on the lunar cycle. There is no need to force the spawning but just to know the right time when genitors spawn (Mercier et al. 2007). To develop this method, Mercier et al. (2007) monitored several hundred individuals nearly every month over 4 years. *Isostichopus fuscus* displayed a lunar spawning periodicity: 0.7–34.9% of individuals consistently spawned 1–4 days after the new moon. Spawning occurred mostly within one evening; however, some gamete release was often recorded over two to four consecutive evenings. Individuals maintained in captivity for several months retained their spawning periodicity and timing with the lunar cycle. The percentage of spawning individuals was higher and a greater overlap between male and female peak spawning activity was observed during clear conditions compared with overcast conditions.

Aside from the *I. fuscus* case, fertilised eggs are obtained by either inducing the spawning of genitors or fertilising oocytes that have been extracted from gonads. Spawning induction methods are based mainly on mechanical stress inflicted on adult individuals, but can also be stimulated by chemical incubations or injections.

Spawning induction methods

In the very first attempt to artificially obtain viable gametes from sea cucumbers, a Japanese scientist attempted the stripping technique in the 1930s (Inaba 1937). The rate of fertilisation was only about 20%, and many of the larvae were malformed. Therefore, this method is no longer used for larval production. Today, the efficient mechanical stresses used are thermal shocks, and stimulation through drying and water pressure. Often a combination of these methods is used to force spawning.

Thermal shocks

Thermal shocks are the most widespread sea cucumber spawning induction technique in aquaculture. The method has been successful in Iran (Dabbagh et al. 2011), Mauritius (Laxminarayana 2005), India (James et al. 1994), Maldives (B. Giraspy, pers. comm.), the Philippines (R. Gamboa, pers. comm.), Vietnam (Pitt and Duy 2004), Australia (Morgan 2000a; Ivy and Giraspy 2006), Solomon Islands (Battaglene et al. 2002), Fiji (Hair et al. 2011), New Caledonia (Agudo 2006), Tanzania (G. Robinson, pers. comm.), and Japan and China (Shuxu and Gongehao 1981; Li 1987).

Thermal shocks involve placing genitors into baths of different temperatures, and the steps of the method vary from place to place. In Mauritius, for example, Laxminarayana (2005) induced the spawning of *Bohadschia marmorata* and *Holothuria atra* by decreasing the seawater temperature by 3–5 °C by the addition of ice. After 5 minutes the sea cucumbers were introduced into another tank filled with filtered sea water at normal temperature (3–5 °C higher than the first tank temperature). For *H. scabra*, the water temperature is raised by 3–5 °C for 1 hour, either by adding warmed sea water to the spawning tank or using aquarium heaters (Agudo 2006). The water temperatures should be kept within the range 28–32 °C. If the ambient water temperature is >30 °C, it is recommended to give a cold shock treatment for 1 hour before the heat shock. Sealed plastic bags containing ice are added to the tank to quickly lower the water temperature by 5 °C (Agudo 2006).

Thermal shock is the most commonly used method to induce spawning of *Apostichopus japonicus* in Japan and China. Most spawners release eggs or sperm when the water temperature is raised by 3–5 °C above the ambient temperature. The induction of spawning by sea cucumbers in Japanese and Chinese hatcheries is usually carried out by regulation of rearing conditions, such as temperature, water exchange and light intensity. In *A. japonicus* cultivation in Japan, wild-caught broodstock are induced to spawn in tanks of sea water about 5 °C higher than natural sea water and under dark conditions. However, this method has some drawbacks in that it is sometimes ineffective and the rate of spawning is inconsistent.

Water pressure and drying treatments

These methods are often used in combination with thermal shocks or if thermal shocks were unsuccessful. The broodstock are left to dry in a tank in the shade for about half an hour before subjecting them to a powerful jet of sea water for a few minutes (Agudo 2006). The broodstock are then returned to the spawning tank at ambient water temperature. During the drying treatment the broodstock are left in the shade, completely dry, or in a few centimetres of sea water, for 30–45 minutes. These methods are commonly used with *H. scabra* as well as

A. japonicus. For the latter species, the operation often starts at about 17:00 hours, when the water in the temporary stocking tank is drained away and the spawners are exposed to air for 30–60 minutes, after which they are jetted with water for about 5–10 minutes. After about 1.5–2.0 hours, the spawners move upwards, become restless and toss their head from side to side. The males begin to spawn first, followed by the females about half an hour later.

Chemical incubations and chemical injections

The addition of a food stimulant is sometimes used for inducing spawning in sea cucumbers. Dried algae (*Spirulina* at a rate of 30 g per 300–500 L, or Algamac 2000 at a concentration of 0.1 g/L) is added to the tank containing broodstock for 1 hour (Agudo 2006). After 1 hour of incubation, the water is removed from the tank and replaced with clean water at ambient temperature.

Mercier and Hamel (2002) demonstrated that the transfer of perivisceral coelomic fluid (PCF) can be used as a reliable tool to induce spawning in mature individuals. PCF collected from holothurians that had been in the typical spawning posture without shedding gametes for about 20 minutes triggered spawning in 71–100% of conspecifics. The individuals responded to the injection of a 2–3-mL aliquot by displaying the spawning posture within 30–62 minutes and by massive gamete broadcast. The inductive substance was found not to be sex-specific since positive responses were observed in individuals of the same or opposite sex as the donor. The PCF of a spawning donor was also active when added to the surrounding sea water, as it induced the typical posturing in 47–65% of mature individuals, and subsequent gamete release in 20–31% of them less than 85 minutes later.

Although most experiments were performed with *Bohadschia argus*, similar results were obtained with *B. marmorata*, *Holothuria leucospilota* and *H. atra*. Interspecific trials were also successful, implying that the chemical involved is not species-specific. Although this method is promising, it is still not applied in sea cucumber aquaculture as it relies totally on the observation of genitors in spawning posture, which is a real challenge in non-natural conditions. However, if the bioactive molecule present in PCF is identified, this method should have a great advantage over the stress-induced methods described above.

Kato et al. (2009) purified one small pentapeptide from the buccal tissues of *A. japonicus* that has a practical value to induce spawning in the hatchery setting (Fujiwara et al. 2010). Kato et al. (2009) named the identified native peptide 'cubifrin'. Mature *A. japonicus* injected with cubifrin during the reproductive season, from February to May, displayed sequential reproductive behaviours, which comprised climbing the side wall of the tank toward the water surface, waving the head and shedding gametes. Gamete shedding started about 60 and 80 minutes after the injection in males and females, respectively, and was completed almost simultaneously in both sexes about 2 hours after the administration. Repeated injections of cubifrin at intervals of about 10 days successfully induced multiple spawns in both males and females. Induction of spawning by cubifrin in *A. japonicus* is an effective, simple and cost-effective method requiring only the injection of cubifrin solution into the body cavity. Cubifrin injections, however, were not effective in other holothurians, and the possibility of using them on other sea cucumber species remains to be examined.

In-vitro fertilisation

In-vitro fertilisation is a process by which female germinal cells are fertilised by sperm in non-natural conditions; that is, outside the female genital tracts in organisms with internal fertilisation and in laboratory conditions for organisms with external fertilisation. The problem with IVF in sea cucumber aquaculture is that the development of oocytes (as is the case for the female germinal cells of all animals) is stopped during the meiosis at prophase I.

Oocyte maturation naturally concludes in sea cucumbers just before or during spawning, resulting in mature oocytes ready to be fertilised. Consequently, oocytes extracted from ovaries by dissection are not ready to be fertilised—they must undergo maturation first. In echinoderms the mechanism of maturation has been reviewed recently by Mercier and Hamel (2009), and is best understood in asteroids (sea stars). A gonad-stimulating substance (GSS) produced by the radial nerve cord (Kanatani 1964) acts on the ovarian follicle cells, which, in turn, produce a secondary substance, the maturation inducing substance (MIS), identified as 1-methyladenine (1-MeA) (Kanatani and Shirai 1967; Kanatani 1969). The 1-MeA acts on an oocyte membrane receptor to activate an intracellular maturation promoting factor (MPF) (Stevens 1970; Yamashita et al. 2000), which

induces oocyte maturation involving germinal vesicle breakdown (GVBD), chromosome condensation and extrusions of the polar bodies.

This oocyte maturation process is considered to be universal, with few variations among species (Kishimoto et al. 1982; Yamashita et al. 2000). It is also presumed to occur in sea cucumbers, although this assertion is untested. Maruyama (1985) demonstrated that a GSS existed in five sea cucumber species; comprising a peptide of several thousand daltons having similar characteristics to the asteroid GSS. Smiley (1988) suggested that the MIS of *Stichopus californicus* is likely to be a 2,8 di-substituted adenine. It was demonstrated, moreover, that the action of 1-MeA can be mimicked in sea stars (Kishimoto and Kanatani 1973; Kishimoto et al. 1976) and sea cucumbers (Smiley 1990) by various molecules such as L-cysteine (Kishimoto and Kanatani 1973), dithiothreitol (DTT) (Kishimoto and Kanatani 1980) or dimercaptopropanol (DMP) (Kishimoto et al. 1976). Yet, the endocrine substances involved in natural sea cucumber oocyte maturation remain unknown.

In sea cucumber aquaculture, two methods of IVF have proven to be efficient. The first method is still in the research phase and acts in activating ovarian cells that themselves induce oocyte maturation during the spawning period. This recently discovered method involves the incubation of oocytes in a gonad-stimulating substance-like (GSSL) solution before fertilisation (GSSL-IVF) (Katow et al. 2009). The second method acts directly on vitellogenic oocytes and is efficient both during and outside the spawning period (Léonet et al. 2009). This technique has been used routinely at the MH.SA hatchery in Madagascar for several years and is referred to as MH-IVF.

GSSL-IVF

Recently, Katow et al. (2009) isolated a GSSL molecule from the radial nerve of the sea cucumber *A. japonicus* (Aj-GSSL), and its partial DNA and protein sequences were characterised. The researchers incubated tubes of ovaries full of vitellogenic oocytes with various extracts during the spawning season of the sea cucumber. Radial nerve extract at 3 mg/mL induced GVBD in 85% of immature ovarian oocytes. A synthetic 43-amino acid Aj-GSSL generated from this sequence induced GVBD in 50% of immature ovarian oocytes, and an N-terminal 21-amino acid peptide of the synthetic partial Aj-GSSL (Aj-GSSL-P1) induced GVBD in 80% of immature ovarian oocytes.

MH-IVF

Léonet et al. (2009) analysed the effects of a powerful oocyte maturation inductor (OMI) used routinely by MH.SA on oocytes of the commercial species *Holothuria scabra* and on various other species of sea cucumbers. The new bioactive molecule was isolated from echinoderm extracts and identified by mass spectrometry, and the active site of the biomolecule was synthesised (international patent number: WO 2008/003691; patent title: 'Oocyte maturation method'). The new OMI induces the maturation and fertilisation of more than 90% of oocytes, while other OMIs described in the literature (i.e. 1-MeA, DTT, DMP and L-cysteine) induce between 28–90% of oocytes to mature (Figure 1). The use of the other OMIs result in fertilisation rates that never exceed 40% (Figure 1), and the resultant larvae often present developmental abnormalities.

One of the advantages of the new OMI compared with the other methods is that it is effective throughout the year, even outside the spawning season of sea cucumbers. In *H. scabra*, the difficulty of obtaining fertilised eggs throughout the year by conventional methods such as thermal shocks varies according to the geographic location, seemingly being easier when *H. scabra* populations are closer to the equator. The reproductive cycle of *H. scabra* has been well investigated over most of its geographic range—it is known in the Southern Hemisphere for populations in Indonesia (05°S; Tuwo 1999), Solomon Islands (09°S; Ramofafia et al. 2003), New Caledonia (20°S; Conand 1981) and Australia (27°S; Morgan 2000b); and in the Northern Hemisphere for populations in India (09°N; Krishnaswamy and Krishnan 1967) and the Philippines (13°N; Ong Che and Gomez 1985). The methods used during these studies were different but, globally, they strongly suggest that at least a small proportion of *H. scabra* in these populations spawn all year round (Hamel et al. 2002). However, the intensity of spawning over a year is different from one population to another: it can be continuous; it can increase once during a period of 2–3 months (i.e. an annual reproductive cycle); or it can increase twice with one of the two peaks of spawning intensity being higher than the other (i.e. a biannual reproductive cycle).

Figure 2 shows the ovarian maturation in the *H. scabra* population around Toliara (Madagascar) (Rasolofonirina et al. 2005). Each bar represents 30 females whose ovaries have been sectioned and characterised into five stages of maturity. The ovaries

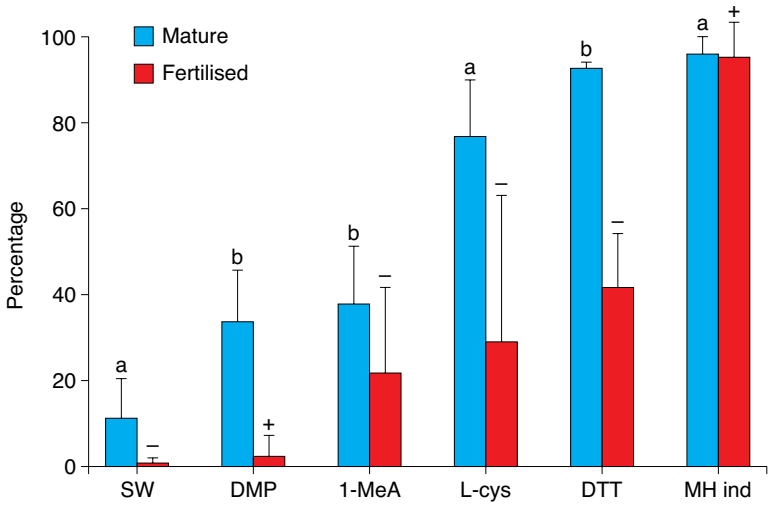

Figure 1. Comparison of the efficiency of various substances on the oocyte maturation in *Holothuria scabra* (from Léonet et al. 2009). Oocytes have been extracted from the ovaries and incubated with the following substances: SW = sea water; DMP = dimercaptopropanol; 1-MeA = 1-methyladenine; L-cyst = L-cysteine; DTT = dithiothreitol; MH ind = inductor used in Madagascar. Incubation time was 2 hours. The blue bars indicate the percentage of oocytes that were mature after incubation, and the red bars the percentage of oocytes that were fertilised after addition of spermatozoa. Small a, b, + and – indicate significant similarities or differences: two adjacent signs that are similar (e.g. b and b) indicate similarity of the effect.

of two stages, termed 'post-spawning' and 'resting', are composed mainly of oogonia and oocytes at the beginning of the vitellogenesis, and are not ready to undergo maturation. The ovaries of the three other stages, termed 'growing', 'mature' and 'spawning', include mainly oocytes at the end of the vitellogenesis, and are ready to undergo maturation under the right stimulation. The graph demonstrates how MH-IVFs are feasible monthly: it shows that batches of potentially fertilisable oocytes are present each month in ovaries of *H. scabra*. Looking at the survey results, one can observe that more than 30% of the females have batches of oocytes in their ovaries waiting to enter maturation at almost any time of the year.

Table 1 shows that the process is effective on 13 aspidochirote species tested so far (Léonet et al. 2009). The species were from the genera *Actinopyga*, *Holothuria*, *Thelenota* and *Pearsonothuria*. Interestingly, the method was successful on *H. fuscogilva*, a species of very high value. No *Stichopus* species were tested.

Figure 3 illustrates the hatchery production obtained in MH.SA from January 2009 to February 2010. During 2009, MH.SA carried out 29 IVFs, sacrificing 32 females and 24 males. The production of 4,942,876 embryos transformed into 278,486 6-day-old auricularia, then 164,545 1-day-old post-metamorphic juveniles and 48,857 1-cm-long juveniles. These juveniles were transferred into ponds for pre-growing. The average monthly production of juveniles via MH-IVF is about 4,000 1-cm-long individuals up to a maximum of 8,000 (in a hatchery where the wet room is 70 m^2).

Comparison of the methods

Spawning induction methods are not effective outside the spawning period (Table 2). The sea cucumber spawning period varies from one species to another, and seems to be extended when the population is located close to the equator. The farther the

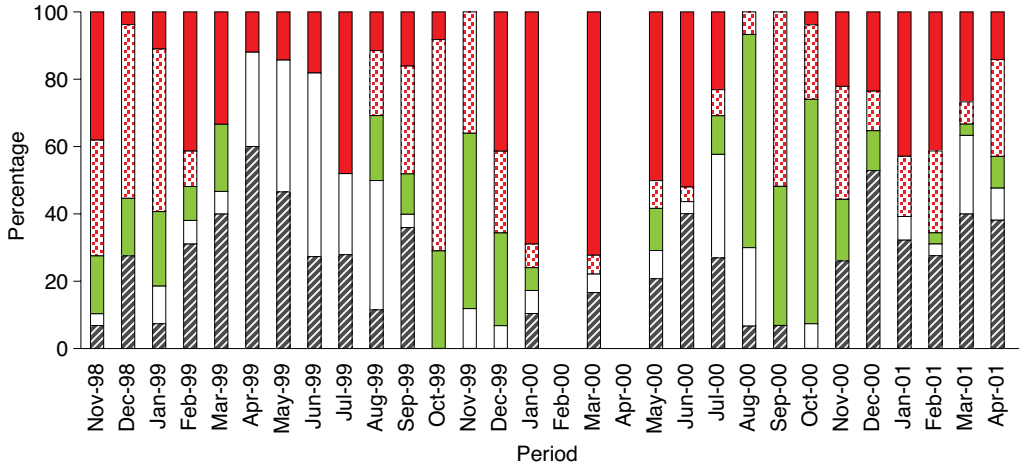

Figure 2. Ovarian maturity in the *Holothuria scabra* population of Toliara (Madagascar) (Rasolofonirina et al. 2005). At each period of the survey, the ovaries of 30 females were sectioned, analysed and characterised into five stages (post-spawning, resting, growing, mature and spawning). The ovaries of the stages post-spawning (black and white striped) and resting (white) include mainly oogonia and young oocytes. The ovaries of the stages growing (green), mature (spotted red) and spawning (plain red) include many oocytes that have completed vitellogenesis.

Table 1. Maturation rate (%) of oocytes from various sea cucumber species incubated with the MH inductor. Control is the rate of oocyte maturation in filtered sea water (n = number of individuals tested) (modified from Léonet et al. 2009)

Species	Maturation (%)	Control (%)
Actinopyga echinites (*n*=3)	81.00	31.00
Bohadschia subrubra (*n*=2)	99.00	9.00
Bohadschia vitiensis (*n*=4)	87.42	9.65
Holothuria cinerascens (*n*=3)	92.60	12.30
Holothuria edulis (*n*=2)	92.00	11.00
Holothuria forskali (*n*=2)	94.50	7.00
Holothuria fuscogilva (*n*=5)	80.00	10.00
Holothuria leucospilota (*n*=4)	70.25	6.00
Holothuria maculosa (*n*=6)	63.35	9.20
Holothuria scabra (*n*=4)	92.25	15.75
Holothuria tubulosa (*n*=4)	82.00	24.25
Pearsonothuria graeffei (*n*=3)	92.00	32.00
Thelenota ananas (*n*=3)	79.33	32.66

populations are from the equatorial line, the more difficult the spawning induction methods are to apply. For example, it is easy to obtain eggs from *H. scabra* in the Philippines with thermal shocks (R. Gamboa, pers. comm.) but hard in Toliara (Madagascar) or Mascat (Oman) with the same method. It seems also to be true for *I. fuscus*, where the spawning season extends through the year in Galapagos (Torral-Granda and Martinez 2007) but is restricted to July–September in Baja California (Herrero-Pérezrul et al. 1999).

The reliability of thermal shocks, even within the spawning period, is quite random (Table 2). The use of a substance to inject into the coelom or for incubation is much more reliable once the appropriate

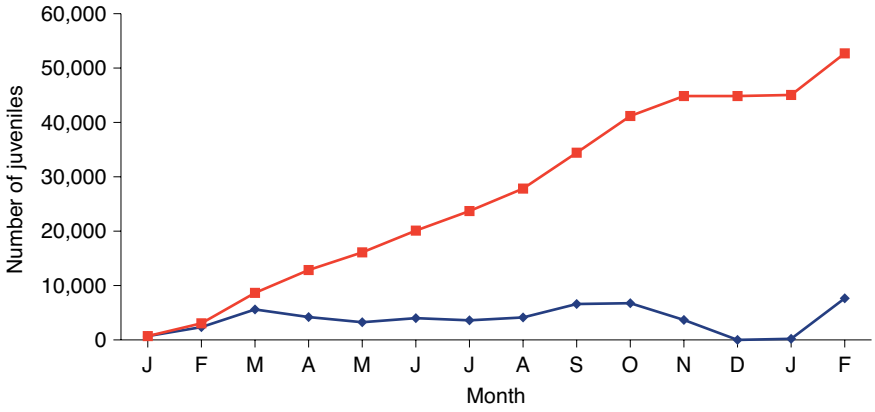

Figure 3. Production of 1-cm-long juveniles from the Madagascar Holothurie S.A. hatchery during 2009: blue line = monthly production; red line = cumulative production

concentration is known. The PCF method is actually difficult to apply in aquaculture as it requires PCF from animals in spawning posture, and that is not always easy to find. For this method to be effective in aquaculture, the bioactive molecule in the PCF needs to be identified.

The three methods of spawning induction involved genitors that are kept in a large volume of sea water (often tanks with a few hundred litres). The fertilised eggs are collected at the end of the process through the use of various filtration procedures. During filtration, all microbes (bacteria, protozoans and small metazoans such as copepods) are retained with the fertilised eggs. Non-fertilised eggs stay in the filtrates and degrade during the next hours of the process. Therefore, the risk of infestation is high with spawning induction methods and very low in IVF methods because the latter require 1–2 L of 1-µm filtered sea water at most (Table 2).

The risk of larval malformation is minimal for spawning induction methods and for both GSSL-IVF and MH-IVF. However, it is high for IVF methods that use chemicals such as DTT, dimercaptopropanol (BAL), L-cysteine or 1-MeA.

With respect to genetic issues, IVFs are no more influential on genetic drift than are the spawning induction methods. Genetic drift is a change in the frequency of alleles in a population. When populations are smaller, as is the case in aquaculture breedings, the effect of genetic drift is greater and may cause alleles to disappear completely, thus reducing genetic diversity. The reduction of genetic diversity can be a problem in the production of individuals less adapted to cope with environmental variation. In IVFs, as in spawning induction methods, genetic drift could be a problem in the future, and the only way to overcome it is to pay attention to not always using genitors from the same parental lineage. In MH.SA, IVFs are done with genitors from previous generations, but gametes are also mixed with those from wild strains: gonads of wild strains are obtained from fishermen, who usually discard them as they use only the body wall of sea cucumbers to prepare trepang.

In conclusion, IVF methods, especially MH-IVF, are simple, cost-efficient, allow the collection of fertilised eggs of sea cucumbers year round, and enable control of the basic operations in hatcheries. MH-IVF does not require high-tech equipment and is useful in both large- and small-scale hatcheries. It ensures a high degree of control at the very beginning of the work on the number and type of genitors (i.e. sex, length, weight, colour); the quality of the gonads (healthy versus parasitised); and the number, size and quality of spermatozoa and eggs. IVF necessitates the sacrifice of very few genitors compared with the individuals obtained and sacrificed for production (in 2009 the IVF sacrifices reached 0.1% of the production; the body walls of animals sacrificed for IVF were processed into trepang and entered into the production).

Table 2. Efficiency of the methods that allow sea cucumber aquaculturists to obtain fertilised eggs

Methods	Details	Useful outside spawning period	Reliability inside spawning period	Risk of infestation	Risk of larval malformations	Tested species	References
Spawning induction							
Thermal shocks	Transfer into tanks of various temperatures	–	–	+	–	Various species including *Apostichopus japonicus, Holothuria scabra* and *Isostichopus fuscus*	Hamel et al. (2002)
PCF	Injection into coelomic cavity	–	–	+	–	*Bohadschia argus* *B. marmorata* *Holothuria leucospilota* *H. atra*	Mercier and Hamel (2002)
Cubifrin	Injection into coelomic cavity	–	+	+	–	*A. japonicus*	Fujiwara et al. (2010) Kato et al. (2009)
In-vitro fertilisation							
GSSL-IVF	Incubation of gonadal tubules	–	+	–	–	*A. japonicus*	Katow et al. (2009)
MH-IVH	Incubation of oocytes	+	+	–	–	13 species (see Table 1)	Léonet et al. (2009)
DTT-IVF	Incubation of oocytes	+	+	–	+	*H. scabra* *A. japonicus* *H. leucospilota* *H. pardalis*	Léonet et al. (2009) Karaseva and Khotimchenko (1995) Maruyama (1980)
Other inductors (BAL, L-cystéine, 1-MeA)	Incubation of oocytes	–	–	–	+	*H. scabra* *H. leucospilota* *H. pardalis*	Léonet et al. (2009) Maruyama (1980)

PCF = perivisceral coelomic fluid; GSSL-IVF = gonad-stimulating substance-like in-vitro fertilisation; MH-IVH = Madagascar Holothurie S.A. in-vitro fertilisation; DTT-IVF = dithiothreitol in-vitro fertilisation; BAL = dimercaptopropanol; 1-MeA = 1-methyladenine

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