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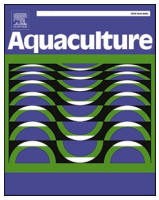

Yields of embryos and larvae produced in a "large-scale" hatchery of *Holothuria scabra* comparing thermal shock and *in vitro* fertilization methods

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ABSTRACT

Worldwide, there exist fewer than 10 large-scale hatcheries dedicated to the production of the sea cucumber *Holothuria scabra*. Most of the existing hatcheries operate at a pilot scale or receive financial support from research programs. Large-scale hatcheries are defined as private industries that annually produce a minimum of 500,000 juveniles and engage in the exportation of several tons of dried product. Here, we present, for the first time, an analysis of the embryo and larval productions from a large-scale hatchery that annually exports over 5 tons of *H. scabra* trepang. Utilizing this data, we assessed the impact of isolating broodstock in tanks with elevated temperatures for 10 days, referred to as "maturation tanks," on ovarian maturation. Furthermore, we conducted a comparative analysis to assess the effectiveness of thermal shock and *in vitro* fertilization (IVF) in obtaining embryos and larvae. The analyzed data originated from *Indian Ocean Trepang*, a private aquaculture company located in Madagascar. Between August 2017 and December 2018, a total of 291 fertilization trials were conducted, involving 6154 females and 2173 males. Over these 17 months, the total number of embryos obtained was 225 million through IVF and 147 million through thermal shock. The number of 3-day-old auricularia larvae obtained at 11 months was 95 million for IVF trials and 51 million for thermal shock trials. The incubation of broodstock in maturation tanks has been found to enhance ovarian maturity, resulting in a notable 1.3 to 1.5-fold increase in the number of ootids. The combined implementation of IVF and thermal shock methods demonstrated a substantial increase in the productivity of the *H. scabra* hatchery. Thermal shock is comparatively simpler to implement than IVF and yields higher embryo production per trial. However, it is characterized by greater unpredictability and leads to a higher degree of variability in outcomes. IVF offers superior control over fertilization parameters and furnishes valuable insights into the sexual maturity of the broodstock. During challenging periods, such as the cold season or copepod infestations, the implementation of *in vitro* fertilizations becomes essential for ensuring hatchery profitability.

1. Introduction

Holothuria scabra, commonly referred to as sandfish, is one of the two species of sea cucumber (holothurians), alongside *Apostichopus japonicus*, for which the entire development process is successfully controlled in aquaculture. *H. scabra* has a wide geographical distribution ranging from the eastern coast of Africa to the western Pacific, covering over 50 countries within the tropical regions. As of the latest information available, *H. scabra* aquaculture is predominantly in the research or pilot stage in most countries, with only a handful of established large-scale, profitable private companies (Hamel et al., 2022).

The breeding of *H. scabra* involves three phases: *Hatchery, Nursery,* and *Grow-out*. The details of these phases have been recently reviewed by Hamel et al. (2022). In the hatchery phase, the smooth development of larval growth, metamorphosis, and juvenile growth to reach a transferable size in tanks is ensured. In the nursery phase, young juveniles are maintained in high density in small tanks or ponds until they reach sufficient size to be transferred to larger areas. The final phase, the

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grow-out, involves rearing the individuals in large ponds or at sea until they reach the desired marketable size. The optimization of parameters specific to these phases is crucial for ensuring the efficiency and profitability of aquaculture operations.

It is essential for the economic prosperity of this industry that the hatchery has maximum productivity and therefore that it operates throughout the year. In managing the broodstock, it is crucial to exert comprehensive control over two key processes in the physiology of holothurian reproduction: (i) the maturation of ovaries (while testicle maturation is of lesser concern in hatcheries due to the consistent availability of viable spermatozoa) and (ii) the maturation of oocytes. The maturation of ovaries is the ovarian development until reaching stage V, *i.e.*, the *spawning* stage. *H. scabra* is a gonochoric species and, its gonads become macroscopically visible when the individuals reach a length of 3 cm, typically at the age of 4 months (Demeuldre and Eeckhaut, 2012). The first germ cells are only distinguishable from the somatic cells in individuals measuring >10 cm in length, typically around 8 to 9 months old (Demeuldre and Eeckhaut, 2012). Five stages are defined during the maturation of ovaries: the *spent* stage (Stage I) where the ovary is empty, indicating that the individual has recently laid eggs, the *recovery* stage (Stage II) where the ovary lumen is still empty, and oogonia are distinguishable at the level of the epithelium bordering this lumen, the *growing* stage (Stage III) and the *maturation* stage (Stage IV) where the ovary lumen becomes filled with oocytes in previtellogenesis and vitellogenesis and the *spawning* stage (Stage V) where the number of female germ cells close to maturity is maximum (Rasolofonirina et al., 2005 for *H. scabra* in Madagascar; Hamel et al., 2022 for review). Stage V corresponds to females ready to spawn. In sea cucumbers, most of the large oocytes in Stage V are arrested in prophase I of meiosis. The oocytes at this stage are immature and must complete their meiosis to be fertile. Naturally, this meiotic release takes place at the time of spawning through an unknown process, likely hormonal in nature (see Léonet et al., 2009, 2019; Léonet, 2010; Eeckhaut et al., 2012; Delroisse et al., 2021). Maturation of oocytes is the development of oocytes during meiosis which ends with the emergence of ootids that are, in sea cucumbers, the female germ cells ready to be fertilized.

Studies on the natural maturation of *H. scabra* ovaries have been conducted in various regions, as reviewed by Hamel et al. (2022). In many geographic regions, the maturity level of ovaries in female *H. scabra* exhibits seasonal variations. Therefore, it is crucial to ascertain that individuals selected for hatchery purposes have ovaries at their peak maturity. Typically, populations near the equator display more consistent ovary maturity, facilitating continuous reproduction. In contrast, populations closer to the tropics exhibit seasonal variation in ovarian maturity due to the varying environmental conditions throughout the year. *H. scabra* may experience annual, biannual, seasonal, or irregular cycles of ovary maturation (Hamel et al., 2022).

In most research projects focusing on *H. scabra* aquaculture, genitors are either collected from the wild or purchased from dealers, and they are immediately induced to spawn. Although this method might be effective on a limited scale, it is not suitable for achieving economic profitability and lacks ecological sustainability. The state of maturity of wild-caught ovaries can be highly variable, leading to inconsistent results in spawning and fertilization trials. Several studies in the scientific literature explore the conditioning of the broodstock before laying and fertilization trials (reviewed by Hamel et al., 2022). However, the quantification of the positive effects of broodstock conditioning on ovary maturation has yet to be documented. In instances highlighted in the literature, broodstock conditioning is carried out in maturation tanks, commonly made of concrete or fiberglass. These tanks are situated either indoors with shade or outdoors with sun protection. The tanks contain a 10–15 cm layer of clean, dried sand and are supplied with clean seawater. The broodstock comprises adult sandfish weighing >350 g, obtained either from the wild or from the nursery. The sediment may be enriched with various food supplements, including prawn waste, soya bean powder, rice bran, or seagrass powder. Additionally, in some cases, additives such as vitamin E are incorporated (Agudo, 2006; Duy, 2012; Sembiring and Hutapea, 2019).

Ootids, the fertilizable germ cells, are obtained in hatcheries through three methods: natural spawning, induced spawning or *in vitro* fertilization (IVF) (Eeckhaut et al., 2012). The first approach, natural spawning, presents challenges with *H. scabra* due to its efficacy being dependent on species exhibiting spawning events coordinated with the lunar cycle. This coordination allows for predictable timing of ootid collection within the reproductive period, a characteristic not consistently observed in *H. scabra* (*e.g.*, Mercier et al., 2007). Thermal shock stands out as the most commonly employed technique for inducing spawning in sea cucumber aquaculture (Hamel et al., 2022, for review). This method involves changing the temperature of the water in which the genitors are placed, with the temperature decrease typically ranging from 3 to 5 °C for 1 h. While thermal shock is a straightforward method to implement, it has certain drawbacks, as its success is inconsistent and unpredictable throughout the entire year. IVF, which requires the dissection of sea cucumbers to extract their gonads and gametes, was initially employed in India (James, 2004; James et al., 1994). However, the low fertilization rate obtained did not encourage the broader use of this method. Over time, various oocyte maturation inducers have been tested (Léonet et al., 2009, Hamel et al., 2022 for review). Among these, Trx-REES, a thioredoxin, isolated from sea urchin ovaries (REES for "Rough Extract of Echinoid Spawn") is the sole inducer currently used in certain companies and research teams (Léonet et al., 2009, 2019; Léonet, 2010; Eeckhaut et al., 2012; Al-Rashdi et al., 2012, 2019). The method for maturing oocytes into ootids that precedes IVF was patented in 2007 (Eeckhaut, 2021). The patent protected the method internationally. The protection granted by this patent is currently only valid in Madagascar. Outside Madagascar, IVF is used in Tanzania, Sri Lanka, and the Sultanate of Oman to produce *H. scabra*. It's noteworthy that there is currently no scientific literature providing data comparing the efficiency of thermal shock and IVF for this purpose.

The first objective of the present work is to provide, for the first time, comprehensive data on the productivity of embryos and larvae of *H. scabra* within a large-scale hatchery. Currently, the available data in the scientific literature only comes from a few fertilization results obtained from research projects. The data presented here include the fertilization results obtained from an industrial hatchery over 17 months. The second objective is to evaluate the efficacy of maturation tanks in inducing ovarian maturation. While this method is well mentioned in the literature, there is currently a lack of data allowing for the assessment of its efficiency. The third and final objective is to compare the effectiveness of thermal shock and *in vitro* fertilization (IVF) in the context of *H. scabra* hatcheries. Collectively, these results are aimed at improving the productibility of hatcheries of *H. scabra*.

2. Material and methods

2.1. Biological material

Experiments were carried out in collaboration with the Research and Development department of the Indian Ocean Trepang (IOT), a private company based in Toliara (Madagascar). IOT produces *H. scabra* in aquaculture and it includes a hatchery, a nursery and 200 ha of sea pens for adult growth.

Most data from the IOT hatchery were documented between August 1, 2017, and December 14, 2018. During this period, 291 fertilization trials (*i.e.*, spawning induced by thermal shock or *in vitro* fertilization after oocyte maturation with REES) were carried out using 6154 females and 2173 males. Individuals took part either in a spawning trial forced by thermal shock ($n = 82$) or in an *in vitro* fertilization trial ($n = 209$).

2.2. Induction of breeding with thermal shock and IVF

A thermal shock trial consisted of placing multiple females along

with males in tanks containing 50 to 300 l of filtered seawater. Baths with reduced water temperature, lowering it by 5 °C, were applied for one hour. Individuals were left overnight, a period during which they could potentially release their spermatozoa and eggs. In the morning, during the spawning event, all eggs, whether fertilized or not, and the dividing embryos were collected by filtration through a 125 μm sieve.

During an IVF trial, ovaries from multiple females were obtained through dissection. For each female, the ovary was extracted, thoroughly rinsed with seawater, and finely sectioned for 10 min to release germ cells (oogonia, oocytes and ootids) from the walls of the gonad tubules. The structure of the ovaries of *H. scabra* is detailed in Kumara and Dissanayake (2017). The germ cells were then washed from the tubular waste by filtering through a 125 μm sieve. Following this, they underwent a subsequent filtration through a 63 μm sieve to eliminate germ cells with a size <63 μm. Germ cells with a diameter >63 μm consisted of oocytes (whose maturation is arrested in prophase I of meiosis). In parallel, the testes were removed, sectioned, and placed in 500 ml of filtered seawater. The mobility of the spermatozoa was briefly checked under a microscope: if the spermatozoa were immobile or not very mobile, the sperm was discarded for IVFs.

The maturation of oocytes (from germ cells >63 μm) was induced by incubating them in a solution of 2 ‰ of REES in filtered seawater (see Léonet et al., 2009). The molecule causing oocyte maturation in REES is the thioredoxin and the maturation of sea cucumber oocytes extracted from ovaries can be induced by the active molecule Trx-REES, by its active site (WCGPCK) or by the crude product (REES) (Léonet et al., 2019). Considering an economic point of view, REES is much less expensive than the thioredoxin or its active site (both need to be synthetized) since it is a raw extract from the ovaries of sea urchins. In the present experiments, the REES was obtained by collecting *Tripneustes gratilla* sea urchins in low tide conditions, on the Great Barrier Reef of Toliara. Upon arrival at the laboratory, sea urchin ovaries were collected by dissections and were then freeze-dried. The REES can then be stored at 4 °C for 6 months. For oocyte maturation, the oocytes, rinsed with filtered seawater, were incubated in the REES solution for 2 h and then rinsed abundantly. The rate of maturation (number of ootids/number of germinal cells >63 μm) induced by REES was estimated at each trial by measuring 5 times (*i.e.*, on 5 samples taken with a Pasteur pipette) the maturation rate on 100 germinal cells kept 2 h in REES.

For fertilization, after oocyte maturation, the germ cells >63 μm (ootids and the rest of unmatured oocytes; Fig. 1a, b) were rinsed and placed in seawater for 1 h (concentration: 100 germinal cells ml^{-1}) in the presence of spermatozoa (concentration: 1 droplet per 400 ml). The germ cells were then flushed out of excess spermatozoa and put to rest overnight (Fig. 1a, b).

2.3. Efficiency assessment of in vitro fertilization and thermal shock methods

Three parameters were recorded during each trial and compared globally and monthly between *in vitro* fertilization and thermal shock methods:

(i) the number of embryos (Fig. 1c)

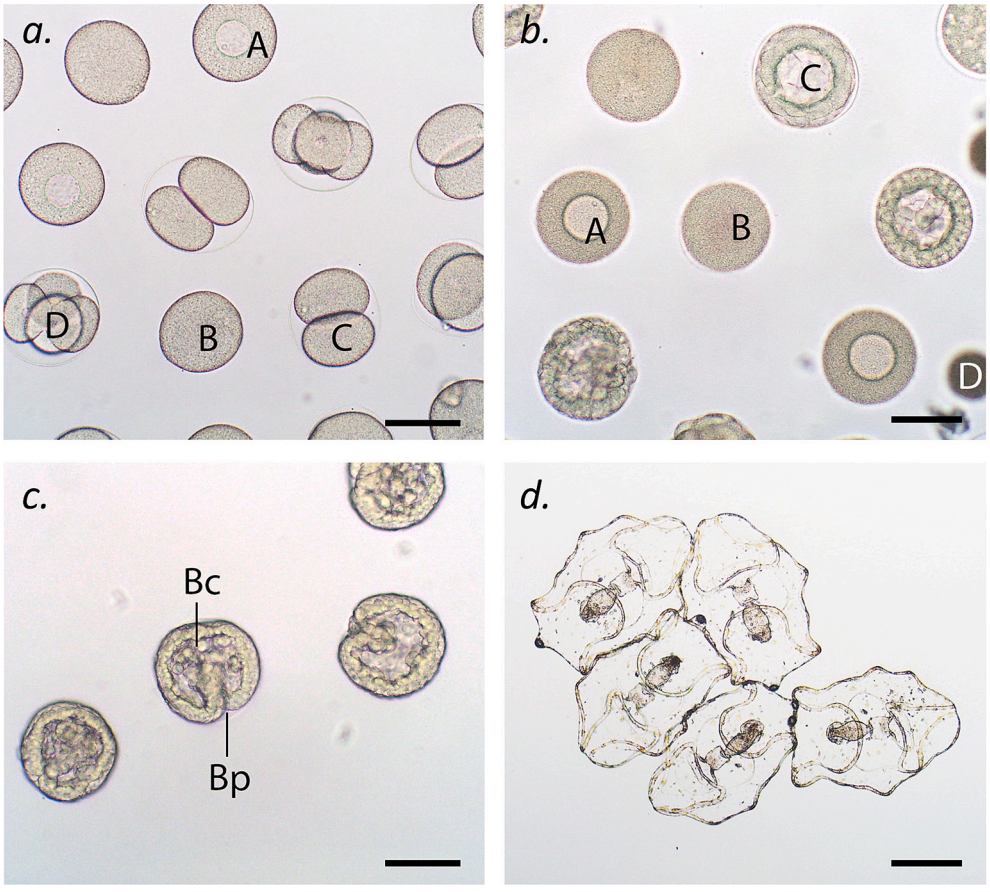

Fig. 1. (a) Microscopic view of germ cells that have been matured with thioredoxin for 3 h and then placed with spermatozoa for 1 h. The sample shows unmatured oocytes (A), ootids (B) and embryos with 2 (C) and 4 (D) blastomeres. Scale: 150 μm. (b) Microscopic view of a sample 6 h after fertilization. Spermatozoa have been flushed out of the sample. The sample still shows some unmatured oocytes (A) and unfertilized ootids (B) that will degrade and some embryos at the blastula stage (C). Some small degrading oocytes are also present (D). Scale: 150 μm. (c) Microscopic view of gastrulae. Bc: blastocoel with mesenchymal cells coming from the top of the archenteron; Bp: blastopore. Scale: 150 μm. (d) Microscopic view of early Auricularia. Scale: 150 μm.

(ii) the number of larvae (Fig. 1d)
(iii) the ratio larvae/embryos

The numbers of embryos obtained through *in vitro* fertilization and with thermal shock were calculated from 18 to 24 h after fertilization. Following the homogenization of embryos through gentle mixing in 1 l of seawater, the embryos present in 10 samples of 2 ml each were recorded. The average was then calculated and extrapolated to the total quantity of seawater. Most embryos at this time are in a young gastrula stage (Fig. 1c). The blastopore is present, the archenteron is growing, and mesenchymal cells detach from the blind end of the archenteron into the blastocoel (Fig. 1c). These gastrulae are mobile and move within the water.

The counts of larvae resulting from *in vitro* fertilization and thermal shock were computed 96 h after fertilization, beginning on February 9, 2018, and spanning the 175 trials conducted over the subsequent 11 months. After 96 h, the water containing the larvae was first filtered through a sieve with a mesh size of 125 μm. After homogenization in 1 l of seawater, the larvae present in 10 samples of 2 ml were counted, and the average value was calculated and extrapolated to the total volume of seawater. Larvae at this stage are young auricularia where the body folds begin to differentiate (Fig. 1d). A ratio of larvae to embryos can then be calculated, representing the number of living larvae compared to the number of embryos obtained (see Fig. 1c, d).

With the *in vitro* fertilization method, only, four other parameters can be recorded:

(i) the number of adult individuals without gonads. The observation of the presence/absence of the gonads was performed visually.
(ii) the number of large (> 63 μm) and small (< 63 μm) germ cells.
(iii) the number of ootids after induction with REES. A batch of germ cells >63 μm from ovaries was incubated with REES. After 2 h, induced (induction by REES) maturations were recorded by counting the ootids out of 100 germ cells.
(iv) a "fertilization index", represented as a proportion, calculated by dividing the number of embryos obtained from ootids (*i.e.*, number of embryos after 24 h) by the number of germ cells >63 μm. This index provides insight into the quality of the fertilized oocytes, with higher values indicating better fertilized eggs conducive to proper cell division.

2.4. Efficiency assessment of maturation tanks on ovarian maturation

Ovarian maturation is supposed to be induced when broodstock is kept in maturation tanks. To test this hypothesis, we carried out a comparison between individuals taken from the wild between November 14 and December 7, 2016, with some kept in maturation tanks for 10 days. Data was collected from 71 *in vitro* fertilization trials on 126 females, divided into two sub-samples. The first sub-sample consisted of 67 females used in 34 trials of one to six females. The females were collected at low tide in Belaza and transported in tanks to the R&D department. At the Belaza grow-out site, 20 g individuals were raised in 10-ha pens. Juveniles weighing 20 g came from nursery ponds and were transferred to the grow-out site at the ages of four and six months, where they grew for a minimum of 8 months (minimum size 350 g) before being harvested and processed into trepang. The site is in natural seagrass meadows dominated by *Thalassia hemprichrii* and *Syringodium isoetifolium* and is mostly submerged between 0.1 and 1.5 m below the surface throughout the year. The sediment at the site was previously analyzed (Plotieau et al., 2014).

During transportation, individuals were kept moist using towels saturated with seawater. Upon their arrival, the individuals were dissected, oocytes were collected and *in vitro* fertilized. The second subsample consisted of 59 females distributed into 37 trials of one to five individuals. For the second subsample, individuals were kept in maturation tanks for 10 days. The maturation tanks are indoor ponds of about 36 m^2 covered on their bottom with a 15 cm-thick sediment layer. In November–December, the temperature of the waters of Belaza varied between 23 and 36 °C and the waters of the maturation tank were from 33 to 36 °C (daily temperature cycle). Females observed directly after sampling or placed for 10 days in a maturation tank were dissected, their oocytes removed and *in vitro* fertilized.

Furthermore, we repeated the same experiment, at a smaller scale, during the cold season between July 25 and August 4, 2017. One *in vitro* fertilization trial was performed and analyzed. The trial was made on 13 females collected on July 25, 2017. Seven females were dissected directly and six were placed in maturation tanks until August 4, 2017, before dissection. At the end of July, and beginning of August, the temperature in Belaza varied between 17 and 24 °C and the waters of the maturation tank were from 25 to 32 °C (daily temperature cycle).

For each season, we compared three parameters on the two batches of females:

(i) the proportion of germ cells >63 μm, in size, was counted on each dissected female; each proportion was calculated on a sample of 100 germ cells coming from each ovary.
(ii) the induced rate of maturation that is proportion of ootids observed after 2 h of incubation in a REES solution calculated on a sample of 100 germ cells coming from each ovary.
(iii) the "fertilization index".

2.5. Data analyses and manuscript edition

Permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2008; Anderson, 2014) was used to examine the measured parameters in both the analysis of ovarian maturation and the efficiency assessment of fertilization methods (*i.e.*, *in vitro* fertilization *versus* thermal shock). In the case of the former, a two-way PERMANOVA based on Euclidean distances was employed to assess the impact of maturation tanks on various parameters, including the proportion of germinal cells <63 μm, the rate of maturation before and after induction with REES, the number of embryos per female, and the "fertilization index." This analysis incorporated the factors of maturation, season, and their interactions. To evaluate differences in the number of embryos per female and the ratio of larvae to embryos among fertilization methods and months, a two-way PERMANOVA was conducted. Regarding the number of embryos per female, a monthly comparison between fertilization methods was feasible for 11 out of the 17 months. This was due to the unavailability of data for thermal shock in five months (September and November 2017; April, June, and December 2018), with only one trial replication available in October 2018. For the ratio of larvae/embryos, the monthly comparison was possible for 7 months out of 11 since no data was available for the thermal shock in April, June, and December 2018 and only one trial replication was available in October 2018. We performed a one-way PERMANOVA to test the effect of the month on all other measured parameters. The PERMANOVA analyses variances in univariate or multivariate data caused by a set of explanatory factors that are based on Euclidean distances, so that effects linked to each factor, and interactions between factors, can be tested. PERMANOVA can analyze an unbalanced design with no limit on the number of factors that can be used (Anderson, 2008). When PERMANOVA is used with univariate data, *p*-values are obtained by permutation, thus avoiding the assumption of normality (Anderson, 2014). Here, while the factor 'month' was treated as random, the factors 'fertilization method', 'maturation' and 'season' were treated as fixed, and 9999 residual random permutations were run in a reduced model. Post-hoc tests (pairwise comparisons) were conducted to investigate significant interactions. If too few permutations were available for a given test (*i.e.*, 100 or less), the Monte Carlo p-values were preferred over the permutation p-values (Anderson, 2008). Statistical significance was determined with an alpha value of 0.05. All statistical analyses were conducted using PRIMER 6 (version 6.1.13) and PERMANOVA+

(version 1.0.3) and R (version 4.0.2).

During the preparation of the present manuscript, the authors used the webtool ChatGPT 3.5 to correct potential English or grammatical errors. After using this tool, the authors carefully reviewed and edited the content as needed and take full responsibility for the content of the publication.

3. Results

3.1. Parameters recorded with IVF

3.1.1. Monthly variation of H. scabra females without gonad

The first interesting parameter that can be extracted only from IVF data is the number of dissected individuals without gonads. Throughout the 17 months of observations, gonads were not detected in 2441 out of the 8305 dissected individuals. The results also revealed a monthly variation in the occurrence of individuals without gonads, ranging from

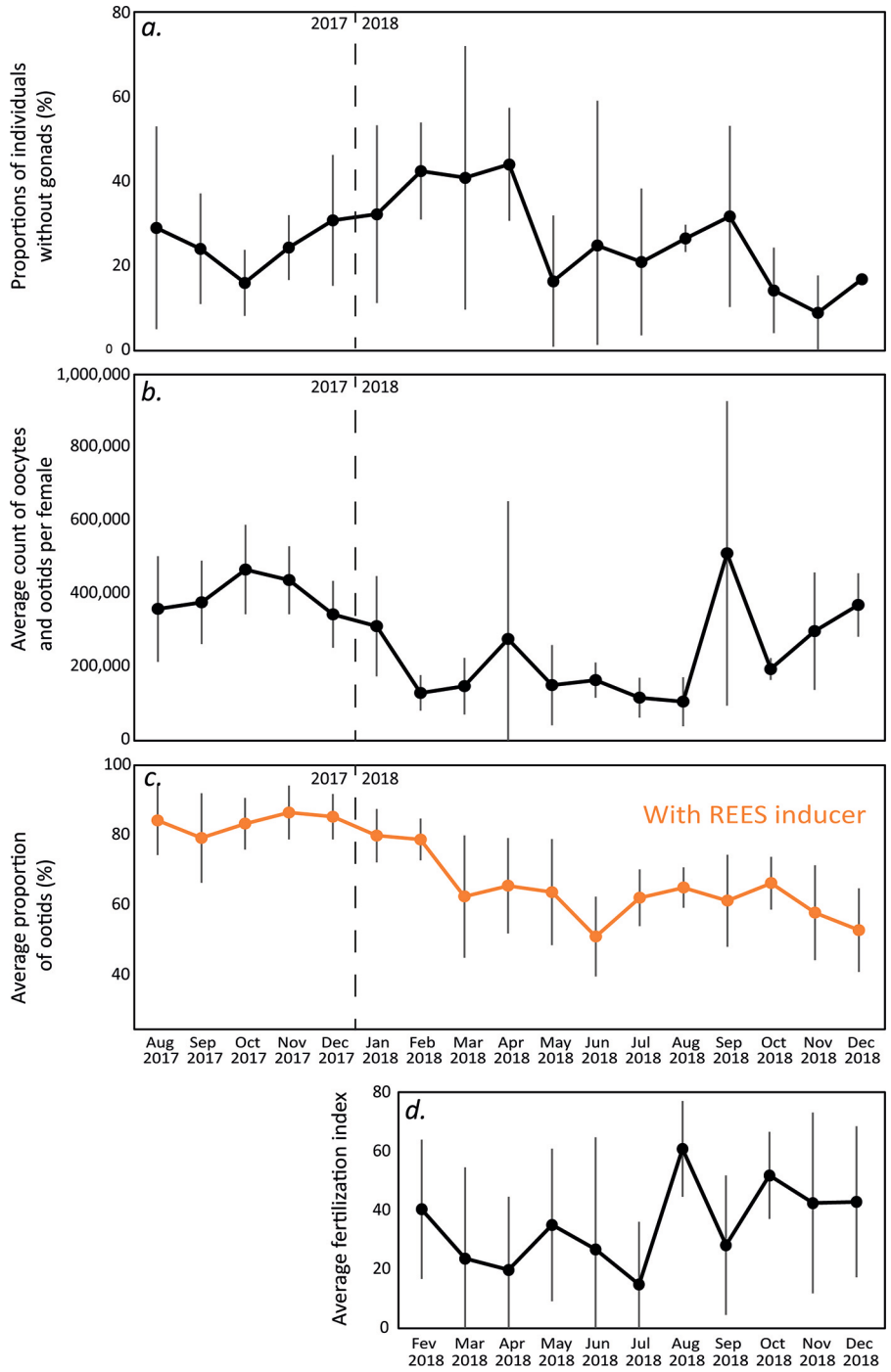

Fig. 2. (a) Monthly proportions (%) of individuals without gonads (± standard deviation) observed during trials representing standard deviations (August 2017 to December 2018). (b) Average monthly count of oocytes and ootids per female with standard deviation (August 2017 to December 2018). (c) Monthly average of the proportion of ootids (± standard deviation) with the control group represented in blue and the REES inducer-treated group represented in orange (August 2017 to December 2018). (d) Monthly average of the fertilization index (± standard deviation) (from August 2017 to December 2018).

9% in November 2018 to 44% in April 2018, with notable differences among months ($p < 0.001$). The number of individuals without gonads increased regularly from October 2017 to April 2018, then had a sharp decrease in May, before increasing again up to September and decreasing again up to November. The significant presence of individuals without gonads observed between February and April corresponds to individuals with gonads in stage 1 (post-spawning) while the abrupt decrease observed between April and May aligns with the emergence of individuals with gonads at stages 2 (recovery) and 3 (growing).

3.1.2. Monthly variation of oocytes in H. scabra females

Another interesting parameter that can be obtained by carrying out *in vitro* fertilization is the monthly variation in the number of oocytes extracted from one ovary. This parameter cannot be obtained with thermal shock as data on ovaries are not accessible when females are placed with males in tanks for breeding. The average monthly number of oocytes per female shows significant variability, with the highest average recorded in September 2018 at 511,310 and the lowest in August 2018 at 106,363 (Fig. 2b). In practical terms, for the operational efficiency of a hatchery, this implies a need for variation in the number of females used in IVF from month to month. More females can be used in periods when this average number is low, facilitating adaptability to fluctuations in gonadal development. For example, the trials with the highest average number of oocytes obtained per female were conducted on April 28, 2018, using 15 females, and on September 4, 2018, using 7 females, resulting in 1,650,000 and 1,085,714 oocytes per female respectively. On the other hand, the least successful trial was performed on May 2, 2018, yielding an average of 30,000 oocytes per female, using 80 females. Additionally, with these observed variations, there is a discernible variability in the monthly production of oocytes in the hatchery (Table 2): the number of oocytes extracted from ovaries over 17 months was 879 million, reaching a low point in February 2018 with 6 million oocytes and a peak in November 2017 with 122 million.

3.1.3. Monthly variations of oocyte maturation and the fertilization index

On the total of 879 million germ cells obtained from dissections and filtrations, 658 million ootids were obtained after incubation in REES (Table 2). From 51 to 85% of oocytes matured each month for an average of 75% (Table 2, Fig. 2c). Out of the 658 million ootids obtained, not all will progress into embryos, as evidenced by the acquisition of only 225 million embryos through IVF, constituting 34%. A final parameter that can be recorded with IVF unlike thermal shock is the fertilization index (FI) offering insights into the number of embryos obtained compared to the number of ootids. It is observed that the FI exhibits considerable variation from one month to another, highlighting that a high number of oocytes/ootids does not necessarily translate to a proportional increase in embryos. Indeed, a monthly fluctuation of the FI was recorded, with the highest value of 61% observed in August 2018 and the lowest value of 14.8% seen in July 2018 (Fig. 2d). Also, as an example, in November 2018, 104 million ootids were obtained after oocyte maturation but only 18 million embryos, the FI being 15.9%. Conversely, 14 million ootids were obtained in August 2018, but they gave 11 million embryos with an FI of 56% (Table 2). The average FI was 25.6%.

3.2. Comparison between the efficiency of in vitro fertilization and thermal shock

The first difference that exists between thermal shock and IVF is that the previously discussed parameters cannot be obtained with thermal shock and the first information obtained by the last method concerns the obtained embryos. The total number of embryos obtained during the 17 months by thermal shock was 147 million, and by IVFs, 225 million for a total of 372 million (Table 2). To obtain these embryos, the number of males and females manipulated during these two methods were different: the numbers of females and males used for the *in vitro* fertilizations were 4197 and 1701, respectively and the numbers of females and males used in thermal shock trials were 1957 and 472, respectively (Table 1). The future of the manipulated animals is also different: the males and females used in IVFs are directly sacrificed and their body walls are sent to the factory to be transformed into trepang, while the individuals used for thermal shocks are placed back in enclosures or maturation tanks. They can be reused later for other thermal shocks (individuals can lay eggs 2 to 3 times over a season), participate in IVF or be processed into trepang. In the end, all individuals are processed into trepang.

The success of thermal shock is more sporadic compared to performing IVF. IVF demonstrated success consistently each month over the 17 months, whereas thermal shock trials resulted in embryos only during 11 of the 17 months (Fig. 3). The effectiveness of thermal shock was particularly notable during the period of rising water temperatures between September and November 2018, as well as in the hot season from December to February (Fig. 3). The implementation of these two methods differed both in terms of successful trials per month and the number of individuals used per trial. In a given month, the number of successful thermal shock trials ranged from 1 to 11, while successful IVF trials varied between 4 and 35 per month (Table 2). For thermal shock, from 2 to 10 males were placed with 2 to 70 females during a successful trial. We did not observe any significant correlation between the success of thermal shock (characterized by the number of embryos collected) and the number of males or the number of females used (non-parametric Spearman correlation, Supplementary Fig. 1). For IVF, from 1 to 43 testicles were extracted for a single trial, and droplets of the mixed sperm were used to fertilize eggs extracted from 1 to 100 ovaries per trial. Notably, there was no significant correlation observed between the success of IVF (characterized by the number of embryos collected) and the number of males. However, a weak but significant correlation (0.29) was identified between the number of females and the produced embryos.

While the thermal shock method may be more random in terms of obtaining embryos, when it proves successful, the number of embryos obtained tends to be greater. Indeed, in a thermal shock trial, the lowest number of embryos obtained was 13,800 (on August 21, 2017), while the highest number reached 20,000,000 (on November 26, 2018). In comparison, the lowest number of embryos obtained during an *in vitro* fertilization trial was 10,000 (September 13, 2017) and the highest number was 6,968,000 (December 2, 2018). On average, the number of

Table 1
Summary of fertilization trials carried out each month from August 1st, 2017 and December 14th, 2018 in Indian Ocean Trepang hatchery.

Month	Thermal shock			*In vitro* fertilization		
	N° trials	N° males	N° females	N° trials	N° males	N° females
Aug-17	1	4	30	21	130	277
Sep-17	0	0	0	18	224	260
Oct-17	2	4	20	12	131	182
Nov-17	0	0	0	15	161	276
Dec-17	8	20	84	15	148	244
Jan-18	11	52	152	13	164	234
Feb-18	9	47	135	6	24	77
Mar-18	10	46	167	12	62	175
Apr-18	3	34	56	16	185	317
May-18	7	55	191	11	93	278
Jun-18	3	33	100	5	40	85
Jul-18	5	25	129	35	166	942
Aug-18	6	44	230	4	20	250
Sep-18	3	15	72	4	27	59
Oct-18	1	10	24	4	33	142
Nov-18	9	48	372	12	69	279
Dec-18	4	35	195	6	24	120
Total	82	472	1957	209	1701	4197

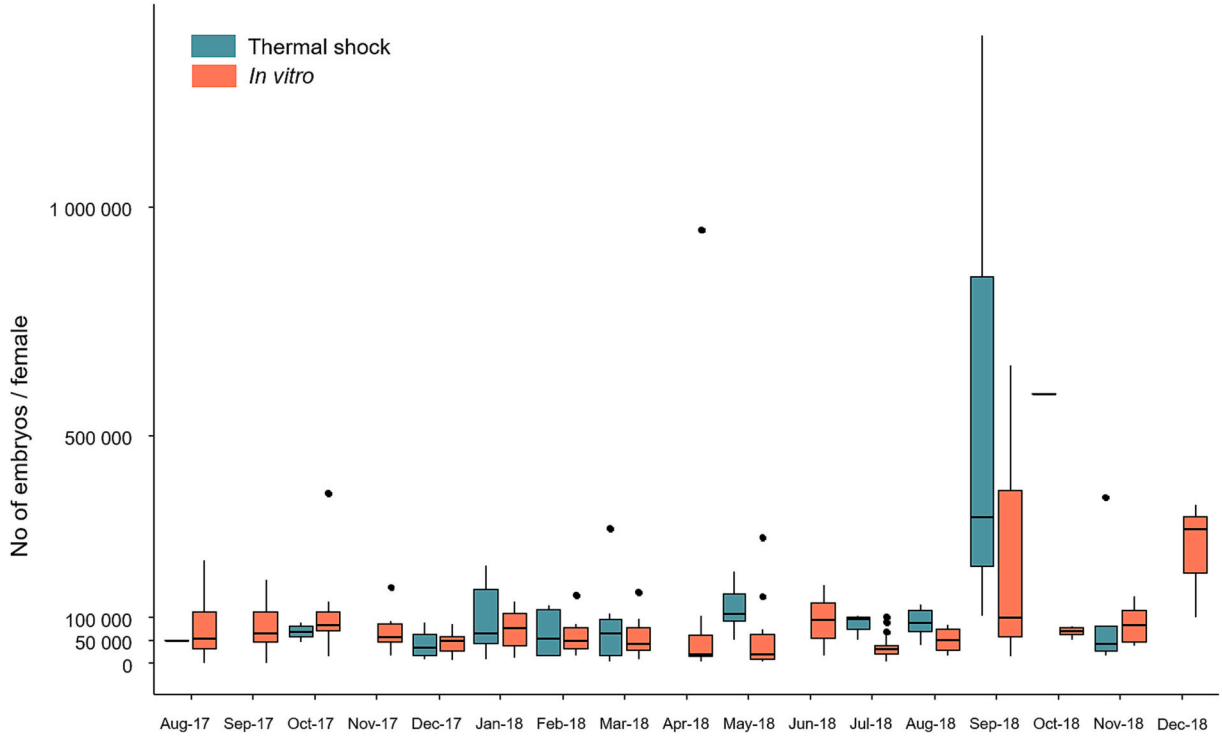

Fig. 3. Monthly comparison of the median number of obtained embryos per used female to compare *in vitro* fertilization and thermal shock. The thick central horizontal line in the box indicates the median. The two ends of the box are the 1st and 3rd quartiles (*i.e.*, 50% of the observations). The ends of the vertical line represent 1.5 times the interquartile space (±1.5 IQR). Dots are atypical ('outlier') values (> 1.5 IQR). Only the values for July 2018 are significantly different ($p = 0.001$), the others are not ($p > 0.05$) with a PERMANOVA analysis.

Table 2
Characteristics of monthly production at the Indian Ocean Trepang hatchery (August 1, 2017 to December 14, 2018).

Month	Total number oocytes	Fraction of ootids after maturation (%)	Total number of ootids (with REES)	Total number of embryos (IV)	Total number of embryos (TC)	Fertilization index (%)
Aug 2017	96,200,000	84	80,808,000	19,748,000	1,513,800	19.8
Sep 2017	95,900,000	79	75,761,000	20,707,000	0	21.3
Oct 2017	81,100,000	83	67,313,000	19,540,000	1,370,000	21.8
Nov 2017	122,000,000	86	104,920,000	18,703,000	0	15.9
Dec 2017	82,100,000	85	69,785,000	10,968,000	3,346,000	12.8
Jan 2018	61,440,000	80	49,152,000	17,676,000	12,316,000	26.7
Feb 2018	6,043,000	79	4,773,970	2,024,000	7,462,000	33.9
Mar 2018	22,310,000	62	13,832,200	9,330,000	7,032,000	47.4
Apr 2018	39,974,000	66	26,382,840	10,309,000	0	22.2
May 2018	26,358,000	64	16,869,120	8,362,000	22,555,000	29.3
Jun 2018	4,900,000	51	2,499,000	1,298,000	0	41.6
Jul 2018	106,072,000	62	65,764,640	31,403,000	5,964,000	31.8
Aug 2018	21,500,000	65	13,975,000	11,095,000	21,373,000	56.0
Sep 2018	16,400,000	61	10,004,000	6,564,576	24,563,000	36.8
Oct 2018	18,800,000	66	12,408,000	7,124,000	14,170,000	37.4
Nov 2018	54,780,000	58	31,772,400	16,108,000	24,871,000	34.2
Dec 2018	23,500,000	53	12,455,000	14,278,000	910,000	59.4
TOTAL	879,377,000	75	658,475,170	225,237,576	147,445,800	25.6

embryos by trial was 2,268,397 for thermal shock and 1,209,293 for IVF. Also, the average number of embryos per female was 88,466 for thermal shock and 68,347 for *in vitro* fertilization ($p = 0.008$; Fig. 4). The medians of the number of embryos per female obtained each month during the 17 months are illustrated in Fig. 3. Except for July 2018, the Permutational multivariate analysis of variance (PERMANOVA) indicates non-significant different values between the number of embryos per female obtained by *in vitro* fertilization and thermal shock per month

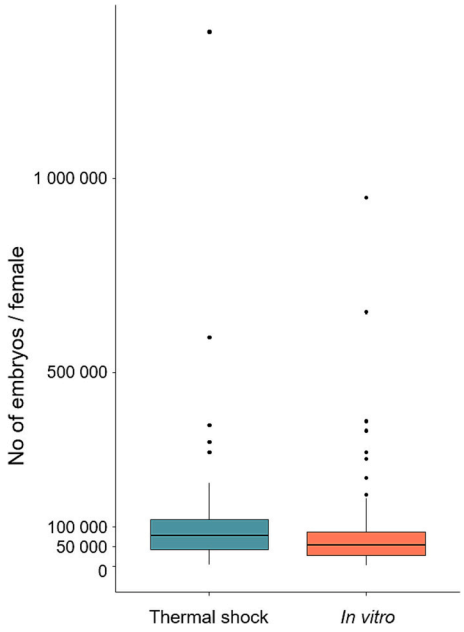

Fig. 4. Global comparison of the median number of obtained embryos per female to compare *in vitro* fertilization and thermal shock. The thick central horizontal line in the box indicates the median. The two ends of the box are the 1st and 3rd quartiles (*i.e.*, 50% of the observations). The ends of the vertical line represent 1.5 times the interquartile space (±1.5 IQR). Dots are atypical ('outlier') values (> 1.5 IQR).

($p > 0.05$).

The number of larvae obtained 72 h after fertilization for the 11 months of experimentation, from February to December 2018 is 51,687,000 for thermal shock, 43,104,000 for *in vitro* fertilization, for a total of 94,791,000. The monthly average of the larvae/embryos ratio is 38.8% for thermal shock and 26.5% for *in vitro* fertilization ($p = 0.626$) (Fig. 5). When this ratio is compared monthly, the values are significantly different in July and September 2018 ($p < 0.05$) but not in the other months (Fig. 6). This ratio varies from 28.7 to 61.5% for thermal shock and from 9.2 to 56.1% for *in vitro* fertilization.

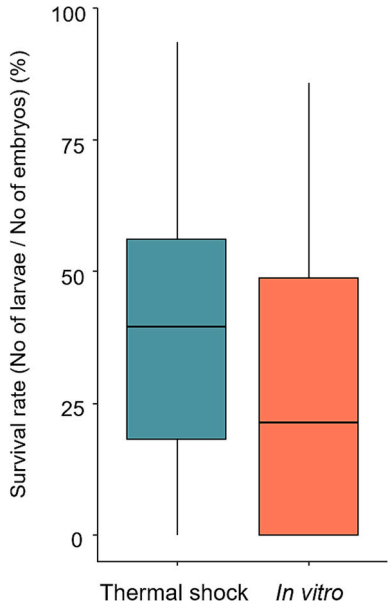

Fig. 5. Global comparison of the ratio larvae/embryos to compare *in vitro* fertilization and thermal shock. The thick central horizontal line in the box indicates the median. The two ends of the box are the 1st and 3rd quartiles (*i.e.*, 50% of the observations). The ends of the vertical line represent 1.5 times the interquartile space (±1.5 IQR). Dots are atypical ('outlier') values (> 1.5 IQR).

3.3. Efficiency of maturation tanks

The positive effect of a maturation tank is evident for the 3 tested parameters. First, the proportion of germ cells <63 μm (*i.e.,* oogonia and small oocytes) decreased significantly when the broodstock was placed in maturation tanks for the cold and warm periods ($p = 0.011$; Fig. 7). These proportions were also significantly different depending on the season ($p = 0.015$). When the broodstock is placed in maturation tanks for 10 days, the induced rate of maturation increases to 96% in the cold season and 86% in the hot season ($p = 0.021$) (Fig. 8). These proportions are also significantly different depending on the season ($p = 0.002$). The fertilization index significantly increased when the broodstock was placed in maturation tanks ($p = 0.023$) (Fig. 9). It is not significantly different depending on the season ($p = 0.944$). When the parents are placed in maturation tanks for 10 days, the fertilization index increases to 32% in the cold season and 28% in the hot season.

4. Discussion

There is limited information in the scientific literature regarding the performance of large-scale hatcheries, primarily because they are associated with the private sector. Purcell et al. (2012) conducted a survey on the number of hatchery-produced juveniles of *H. scabra* worldwide, involving the participation of 11 countries in production. At that time, hatcheries in the Maldives, Australia, and Madagascar were among the few producing approximately 500,000 juveniles annually. In their review, Hamel et al. (2022) noted the existence of 10 large-scale hatcheries producing juveniles of *H. scabra*. The known yields on the development of *H. scabra* only come from research programs or pilot-scale hatcheries. These yields are based on very few trials, often <10 at well-defined periods of the year. The number of eggs obtained during thermal shock trials in these small-scale hatcheries varies from 0.02 to 22 million and the survival of embryos to juveniles transferred to the nursery varies from 0.009 to 2.2% (reviewed in Hamel et al., 2022). Also, Kummara and Dissanyake (2017) reported a survival ranging from 0.2 to 5.2% of embryos to young auricularia.

The Indian Ocean Trepang aquaculture company successfully exported in 2017 5 tons of trepang. Knowing that most of the animals caught in farms were around 350 g and that drying reduces these animals to 15 g trepang sticks (4–5% yield), the 5 tons of trepang correspond to approximately 330,000 individuals. During that period, they produced a total of 377,211,376 embryos obtained over 17 months. Based on this, it can be estimated that a company aiming to produce 5 tons of trepang should have a hatchery that produces an average of 22 million embryos per month. From these embryos, 5.8 to 8.5 million young auricularia (26.5 to 38.8%) may arise. If thermal shock represents the best-known technique, the use of IVF makes it possible to at least double the quantity of larvae obtained. The two techniques have however their benefits and drawbacks. In terms of ease of implementation, thermal shock is more straightforward, requiring only broodstock, aquariums, sea water at varying temperatures, and patience. On the other hand, IVF can be performed on animals destined to be transformed into trepang and is much more controllable than thermal shock. IVF however requires a higher level of expertise with the organisms, as it involves removing the ovaries, extracting the oocytes, filtering, and incubating them in the REES maturation inducer. Obtaining REES requires collecting regular sea urchins and preserving their ovaries, if possible, through freeze-drying to preserve the metabolites including thioredoxin.

Both techniques can be implemented in the entire *H. scabra* geographical distribution area. However, as noted by Hamel et al. (2022), the farther from the equator, the more challenging it becomes to implement thermal shock. Discussions with other holothurian

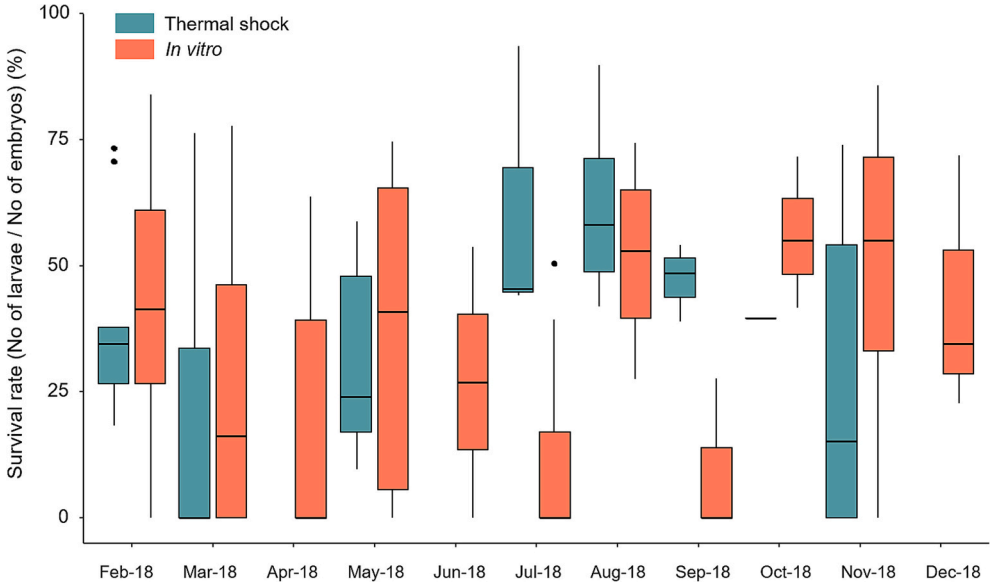

Fig. 6. Monthly comparison of the ratio larvae/embryos to compare *in vitro* fertilization and thermal shock. The thick central horizontal line in the box indicates the median. The two ends of the box are the 1st and 3rd quartiles (*i.e.*, 50% of the observations). The ends of the vertical line represent 1.5 times the interquartile space (±1.5 IQR). Dots are atypical ('outlier') values (> 1.5 IQR).

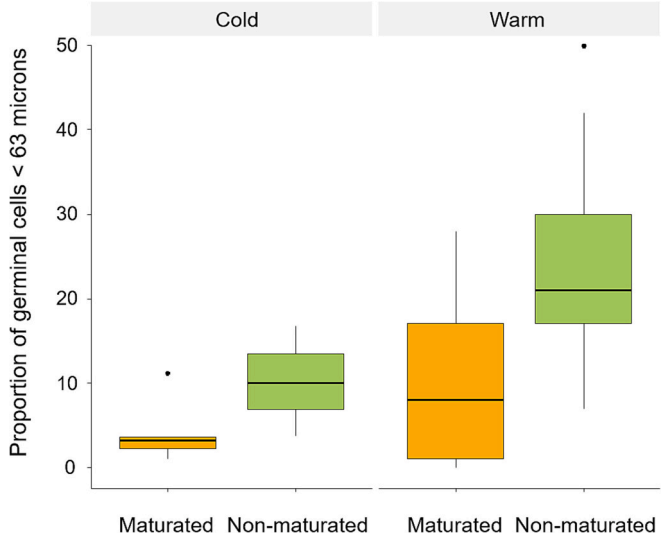

Fig. 7. The proportion of germinal cells <63 μm as a parameter to estimate the efficiency of broodstock maturation tanks. The thick central horizontal line in the box indicates the median. The two ends of the box are the 1st and 3rd quartiles (*i.e.*, 50% of the observations). The ends of the vertical line represent 1.5 times the interquartile space (±1.5 IQR). Dots are atypical ('outlier') values (> 1.5 IQR).

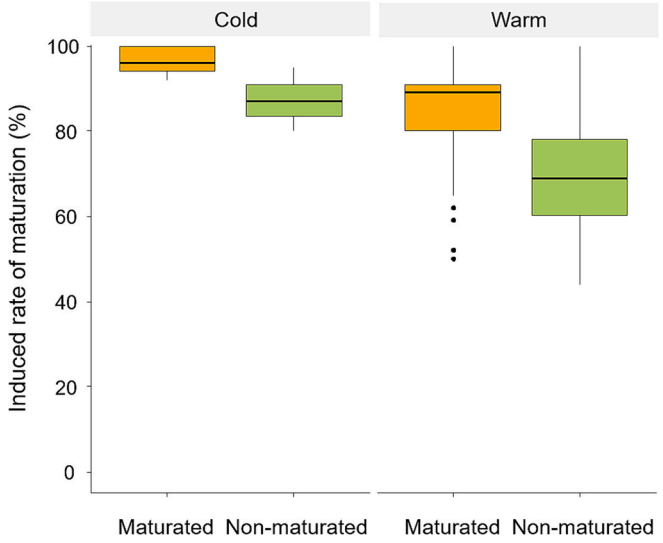

Fig. 8. The induced rate of maturation as a parameter to estimate the efficiency of broodstock maturation tanks. The thick central horizontal line in the box indicates the median. The two ends of the box are the 1st and 3rd quartiles (*i.e.*, 50% of the observations). The ends of the vertical line represent 1.5 times the interquartile space (±1.5 IQR). Dots are atypical ('outlier') values (> 1.5 IQR).

aquaculturists confirm that thermal shock tends to be more effective in areas where *H. scabra* reproduction is continuous (*i.e.,* closer to the equator; personal communication). Thermal shock stimulates egg laying, *i.e.,* the expulsion of eggs from the ovaries. The spawning is caused by the contraction of muscles specific to the ovaries and/or by the contraction of the muscles of the body wall which causes an increase in pressure in the general cavity. These muscular contractions are most probably dependent on hormonal factors which would be stimulated by the thermal shock.

In contrast, IVF bypasses the egg-laying stage, and oocytes are directly taken from the ovary. This technique has the advantage of being effective everywhere, even in places where reproduction is not continuous. In these latitudes, a fraction of the germ cells is found at an advanced stage of development, even if the ovary is not ready to emit its gametes. IVF provides greater control over reproduction and the selection of parents, as experimenters can decide on the number of parents and exclude certain individuals if desired. IVF also provide a cleaner environment for ootid fertilization, whereas broodstock placed in thermal shock tanks can be infested with ectocommensals/parasites/predators, such as copepods, which can compromise future larval cultures. IVF is performed in a sterile environment using small volumes of water, ensuring optimal conditions for the development of the fertilized eggs. This contrasts with the process of harvesting fertilized eggs from the water column after thermal shock, which may include copepod eggs due to their similar size to sea cucumber eggs (Eeckhaut pers. obs.).

The average number of embryos per female obtained through

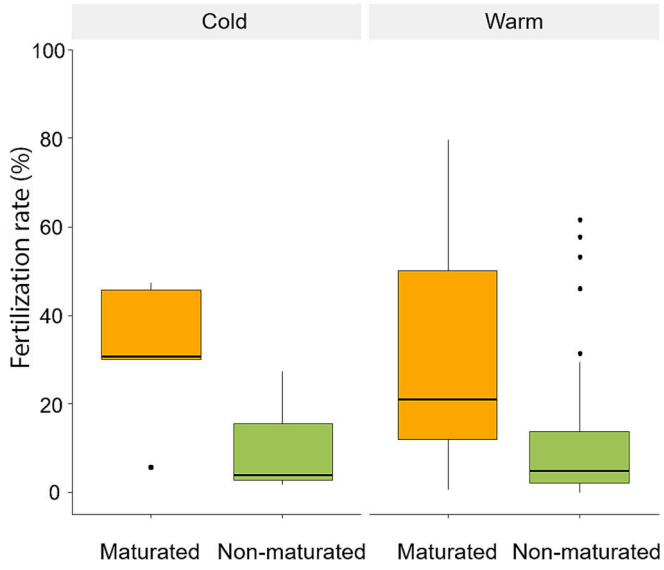

Fig. 9. The fertilization rate (or index) as a parameter to estimate the efficiency of broodstock maturation tanks. The thick central horizontal line in the box indicates the median. The two ends of the box are the 1st and 3rd quartiles (*i.e.*, 50% of the observations). The ends of the vertical line represent 1.5 times the interquartile space (±1.5 IQR). Dots are atypical ('outlier') values (> 1.5 IQR).

thermal shock is higher than through IVF. Therefore, we typically obtain more embryos and larvae with thermal shock for an equivalent number of females used. For both methods, we analyzed the larvae/embryos ratio, which is indicative of the quality of the embryos to transform into auricularia. The data obtained show that the quality of the embryos obtained is similar for both techniques.

Another advantageous aspect of IVF is the acquisition of information on the reproductive state of the population, providing insights into its evolution over time. Thus, important parameters can be obtained thanks to IVF as the proportion of individuals without gonads, the number of oocytes, the number of ootids after incubation in REES and the fertilization index. These parameters are useful to assess the health of the population and to make predictions on economic profitability.

Individuals without gonads refer to individuals that are either in stage I of ovarian maturation or are potentially congenitally sterile. Our data showed that the proportion of individuals without gonads varied significantly by month, with the highest percentage (> 40%) observed in February, March, and April, which precede the coldest water temperature period. This annual cycle supports the idea that these individuals are in stage I, consistent with the findings of Rasolofonirina et al. (2005), who studied the reproductive cycle of the same population from November 1998 to April 2001. However, while the authors reported that the average length of ovaries in 91 stage I individuals was 6.1 cm, our observations of individuals without gonads may indicate that the regression of ovarian tubules is complete in many individuals.

Incubating *H. scabra* broodstock in maturation tanks at relatively high temperatures leads to improved ovaries in aquaculture. The number of germ cells below 63 μm decreases by 2.5 times, indicating the cells are rapidly advancing towards later stages such as vitellogenic oocytes and ootids. The proportion of ootids increases after incubation and reaches over 85% when oocytes are further incubated in REES during hot or cold periods. The fertilization index, which measures the quality of fertilized ootids, increases from 1.75 to 2.9 times (varying by season) after incubation in maturation tanks. These tanks allow rapid 10-day development of female germ cells which accumulate the necessary substances for embryogenesis. While a deeper understanding is needed to understand the physiological response scaffolding these observations, the method appears practically effective in the aquaculture context. Other parameters may also have an impact on the maturation such as the decreased illumination caused by the shading, the sediment enrichment, or the higher density of individuals in the maturation tanks (around 3 individuals/m^2 against 1 individual/m^2 in normal aquaculture conditions).

In conclusion, to optimize hatcheries of *H. scabra*, we recommend using both thermal shock and IVF techniques, as each has its advantages and disadvantages. Thermal shock is preferable for ease of execution and higher yields of embryos when they work, while IVF becomes crucial during challenging times such as the cold season or when larval development is impeded by copepod infestations to ensure hatchery profitability. We also recommend incubating broodstock in maturation tanks, as this produces more suitable ovaries.

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CRediT authorship contribution statement

Igor Eeckhaut: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Nicolas Sturaro:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Christophe Andriantsilonina:** Writing – review & editing. **Richard Rasolofonirina:** Writing – review & editing. **Guillaume Caulier:** Writing – review & editing. **Jérôme Delroisse:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Data availability

Data will be made available on request.

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References

Agudo, N., 2006. Sandfish Hatchery Techniques. The WorldFish Center, Secretariat of the Pacific Community and Australian Centre for. International Agricultural Research, Noumea, New Caledonia.

Al-Rashdi, K.M., Eeckhaut, I., Claereboudt, M.R., 2012. A Manual on Hatchery of Sea Cucumber *Holothuria scabra* in the Sultanate of Oman. Ministry of Agriculture and Fisheries Wealth, Aquaculture Centre, Muscat, Sultanate of Oman.

Al-Rashdi, K.M., Claereboudt, M.R., Eeckhaut, I., 2019. Preliminary trials on hatchery and larval development of the sea cucumber, *Holothuria scabra* (jaeger 1883), in the Sultanate of Oman. Asian J. Fish. Aquat. Res. 2 (3), 1–9.

Anderson, M., 2008. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. Primer-E Limited.
Anderson, M.J., 2014. Permutational multivariate analysis of variance (PERMANOVA). Wiley Statsref: Statistics Reference Online 1–15.
Delroisse, J., Léonet, A., Alexandre, H., Eeckhaut, I., 2021. Intracellular pathways of holothuroid oocyte maturation induced by the thioredoxin Trx-REES. Antioxidants 10, 1–16.
Demeuldre, M., Eeckhaut, I., 2012. Gonad development in the sea cucumber *Holothuria scabra* jaeger, 1833. SPC Beche-de-mer Inf. Bull. 2, 15–23.
Duy, N.D.Q., 2012. Large-scale sandfish production from pond culture in Vietnam. In: Hair, C.A., Pickering, T.D., Mills, D.J. (Eds.), Asia-Pacific Tropical Sea Cucumber Aquaculture. Proceedings of an International Symposium Held in Noumea, New Caledonia. ACIAR Proceedings, pp. 34–39. Citeseer.
Eeckhaut, 2021. From fundamental to applied research: the history of the Indian Ocean Trepang. SPC Beche-de-mer Inf. Bull. 41, 40–47.
Eeckhaut, I., Lavitra, T., Léonet, A., Jangoux, M., Rasolofonirina, R., 2012. In-vitro fertilisation: a simple, efficient method for obtaining sea cucumber larvae year round. In: Hair, C.A., Pickering, T.D., Mills, D.J. (Eds.), Asia-Pacific Tropical Sea Cucumber Aquaculture. Proceedings of an International Symposium Held in Noumea, New Caledonia. ACIAR Proceedings, pp. 40–49.
Hamel, J.-F., Eeckhaut, I., Conand, C., Sun, J., Caulier, G., Mercier, A., 2022. Global knowledge on the commercial sea cucumber *Holothuria Scabra*. In: Mercier, A., Hamel, J.-F. (Eds.), Advances in Marine Biology, Vol 91. Academic Press, London, UK, pp. 1–286.
James, D.B., 2004. Captive breeding of the sea cucumber, Holothuria scabra, from India. FAO Fisheries Technical Paper (463), 385–395.
James, D.B., Gandhi, A.D., Palaniswamy, N., Rodrigo, J.X., 1994. Hatchery techniques and culture of the sea-cucumber Holothuria scabra. CMFRI Special Publication 57, 1–40.
Kumara, A., Dissanayake, C., 2017. Preliminary study on broodstock rearing, induced breeding and grow-out culture of the sea cucumber Holothuria scabra in Sri Lanka. Aquac. Res. 48 (3), 1058–1069.
Léonet, A., 2010. Caractérisation d'un agent induisant la maturation ovocytaire chez *Holothuria scabra* (Holothuroidea), espèce à haute valeur commerciale. PhD Thesis, University of Mons, Belgium.
Léonet, A., Rasolofonirina, R., Wattiez, R., Jangoux, M., Eeckhaut, I., 2009. A new method to induce oocyte maturation in holothuroids (Echinodermata). Invertebr. Reprod. Dev. 53, 13–21.
Léonet, A., Delroisse, J., Schuddinck, C., Wattiez, R., Jangoux, M., Eeckhaut, I., 2019. Thioredoxins induce oocyte maturation in holothuroids (Echinodermata). Aquaculture 510, 293–301.
Mercier, A., Ycaza, R.H., Hamel, J.-F., 2007. Long-term study of gamete release in a broadcast-spawning holothurian: predictable lunar and diel periodicities. Mar. Ecol. Prog. Ser. 329, 179–189.
Plotieau, T., Lepoint, G., Lavitra, T., Eeckhaut, I., 2014. Isotopic tracing of sediment components assimilated by epibiontic juveniles of Holothuria scabra (Holothuroidea). J. Mar. Biol. Assoc. U. K. 94 (7), 1485–1490.
Purcell, S.W., Hair, C.A., Mills, D.J., 2012. Sea cucumber culture, farming and sea ranching in the tropics: progress, problems and opportunities. Aquaculture 368, 68–81.
Rasolofonirina, R., Vaïtilingon, D., Eeckhaut, I., Jangoux, M., 2005. Reproductive cycle of edible echinoderms from the southwestern Indian Ocean. II the sandfish *Holothuria scabra* (Jaeger, 1833). West. Indian Ocean J. Mar. Sci. 4, 61–75.
Sembiring, S.B.M., Hutapea, J.H., 2019. Gonad development of sandfish (*Holothuria scabra*) fed with vitamin E-supplemented diet. Indones. Aquac. J. 14, 47–54.