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Hélène Laguerre, Grégory Raymond, Patrick Plan, Nadia Améziane, Xavier  
Bailly, Patrick Le Chevalier

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# 1 First description of embryonic and larval development, juvenile growth of 2 the black sea-cucumber *Holothuria forskali* (Echinodermata: Holothuroidea), a 3 new species for aquaculture in the North-Eastern Atlantic 4

5 H el ene Laguerre<sup>1,2</sup>, Gr egory Raymond<sup>2</sup>, Patrick Plan<sup>3</sup>, Nadia Am eziane<sup>4</sup>, Xavier Bailly<sup>5</sup>,  
6 Patrick Le Chevalier<sup>1\*</sup>

7  
8 1 : Univ. Brest, Univ. Bretagne Sud, Laboratoire de Biotechnologie et Chimie Marines, F-29334 Quimper, France

9 2 : Universit  Bretagne Loire (UBL), Agrocampus Ouest, UMR 985 Ecology and Ecosystems Health, Site de Beg-Meil, Fouesnant, France

10 3 : AQUA B, Lesconil, France

11 4 : UMR 7205, Institut de Syst matique, Evolution, Biodiversit  (ISYEB), Mus um national d'Histoire naturelle, CNRS, Sorbonne Universit ,  
12 EPHE, Station de Biologie Marine de Concarneau, 29900, Concarneau, France

13 5 : UPMC-CNRS, FR2424, Station Biologique de Roscoff, 29680, Roscoff, France

14  
15 \*Corresponding author : Patrick Le Chevalier, Univ. Brest, Univ. Bretagne Sud, Laboratoire de Biotechnologie et Chimie Marines, F-29334  
16 Quimper, France. [patrick.lechevalier@univ-brest.fr](mailto:patrick.lechevalier@univ-brest.fr)

## 17 18 19 ABSTRACT

20  
21 *Holothuria forskali* (Delle Chiaje, 1823), also called the “black sea cucumber or cotton spinner”, is a native species from the  
22 North East Atlantic Area and the Mediterranean Sea. This holothurian is newly targeted to supply the Asian Market. In this context,  
23 developing aquaculture is important to prevent risks of over fishing. This paper describes the first successful reproduction, larval  
24 rearing, and juvenile growth of *H. forskali* performed in captivity. Reproduction of *H. forskali* was observed in April 2018. The  
25 reproductive behavior was similar to those observed in other species. The auricularia stage hatched after 4 days and lasted more than  
26 30 days. Pentactula and metamorphosis to the benthic life was obtained after 40 days. Juveniles have been observed only after 90  
27 days and measured 8 mm long. Juvenile growth was slow (0.1 mm/day) for this first breeding. This first description of *H. forskali*  
28 breeding and rearing in hatchery is encouraging for a new innovative and sustainable aquaculture.  
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## 31 ARTICLE INFO

32 Keywords: *Holothuria forskali*, sea cucumber, aquaculture, spawning, larvae, embryonic development, metamorphosis, juvenile growth.

## 34 1. Introduction

35 Holothurians (Echinodermata: Holothuroidea), known as “sea cucumbers” or “beche-de-mer”, are popular luxury  
36 food in Asia, called “tre pang”, and represent an economic important market as high value seafood products  
37 (Battaglione et al., 1999; Chen, 2004; Conand, 2004; Purcell et al., 2012a; Purcell et al., 2013). They are also used in  
38 traditional medicine for their nutritional virtues (Chen, 2004; Vergara & Rodriguez, 2016) and in medicine,  
39 pharmacology and in cosmetics for their bioactive compounds with anti tumoral, anti-oxidant and anti-inflammatory  
40 properties actually explored and valued (Janakiram et al., 2015; Kiew & Don, 2012; Kumar et al., 2007; Mena-Bueno  
41 et al., 2015; Santos et al., 2015).

42 The increasing demand of commercialized species and the uncontrolled exploitation of wild stocks in the last  
43 decades have led to a collapse of natural resources, classifying some species in threatened species (Anderson et al.,  
44 2011; Choo, 2008; Devanadera et al., 2015; Hamel et al., 2003; Otero-Villanueva & Ut, 2007; Purcell et al., 2012b;  
45 Purcell et al., 2013; Ramofafia et al., 2003). Thereby, developing aquaculture is a sustainable way to supply the market  
46 demand and to create new economic activities in coastal areas. Moreover, sea cucumbers have an important ecological  
47 role in marine ecosystems, due to their feeding behavior as deposit feeders and detritivorous animals (Purcell et al.,  
48 2016). They are candidates for aquaculture bioremediation and for integrated multi-trophic aquaculture (Chopin et al.,  
49 2012; MacDonald et al., 2013; Paltzat et al., 2008; Slater & Carton 2007; Slater & Carton 2009; Tolon et al., 2017;  
50 Yokoyama, 2013; Zamora & Jeffs, 2013; Zhou et al., 2017).

51 Aquaculture of Indo-Pacific species have been developed since the 80s and industrial farms produce sea cucumber  
52 juveniles such as *Apostichopus japonicus* in China and Japan (Akamine, 2004; Chen, 2004; Ito & Kitamura, 1998;  
53 Soliman et al., 2013), or *Holothuria scabra* in the Indo-Pacific Region (Altamirano et al., 2017; Battaglione et al.,  
54 1999; Hamel et al., 2001; Knauer, 2011; Kumara & Dissanayake, 2017; Mazlan & Hashim, 2015; Yan et al., 2012).

55 Due to decrease in natural stocks of tropical species, European species of *Holothuroidea* are targeted by the Asian  
56 Market (Gonzalez-Wanguemert et al., 2018; Hamel & Mercier, 2008); Atlantic and Mediterranean species are now  
57 subject to an important fishing, inducing illegal fishing and effects on biometry and genetic structure  
58 (Dominguez-Godino et al., 2016; Gonzalez-Wanguemert et al., 2015; Gonzalez-Wanguemert & Godino, 2016). Only  
59 few species of European species have been described for hatchery and cultivating procedures, in a pilot-scale, *H.*  
60 *arguinensis* (Dominguez-Godino et al., 2015), *H. tubulosa* (Rakaj et al., 2018), *H. mammata*, (Dominguez-Godino and  
61 Gonzalez-Wanguemert, 2018) and *H. polii* (Rakaj et al., 2019).

62 *H. forskali* (Delle Chiaje, 1823) is a native unexploited species from Brittany Coast (France) and can be found in the  
63 North East Atlantic Area and Mediterranean Sea, in rocky sea beds, from 1 to 100 meters depth. This species is  
64 difficult to maintain in captivity because of its behavior in case of stress. It ejects - as a defensive strategy - cuvierian  
65 tubules and sometimes even several organs that can lead to death, even if *H. forskali*, like the other Holothurians and  
66 Echinoderms, are able to regenerate most of its organs. *H. forskali* is a deposit feeder as the majority of Holothurians,  
67 and ingest sediment containing fresh or detrital organic matter (Purcell et al., 2016; Massin & Jangoux, 1976; Roberts  
68 et al., 2000). This gonochoric species has an annual reproduction cycle with a synchronous spawning for male and  
69 female in spring in the Atlantic Area (Despalatovic et al. 2003; Santos et al., 2015; Tuwo & Conand 1992; Tuwo &  
70 Conand 1994). However, there is no exhaustive references on reproduction behavior, embryonic and larval  
71 development of *H. forskali*.

72 We report herein for the first time the reproductive behavior, embryonic and larval development, and juvenile  
73 growth of *H. forskali* in captivity.  
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## 75 2. Material and methods

### 76 2.1 Broodstock collection and maintenance

77 Adults of *H. forskali* (n=36, mean weight = 322.78 ± 68.27g) were collected at Brezellec (West Coast of Brittany,  
78 France: 48°4'15.15"N and 4°39'45.29"W) by scuba diving at 5 to 12 meters depth on 5th of April 2018 (fig. 1, the  
79 geographic map was obtained from the French national website [www.geoportail.gouv.fr](http://www.geoportail.gouv.fr)) and the seawater  
80 temperature was 12°C. Broodstocks were transferred directly in a 30 L box to the hatchery of the marine experimental  
81 station of Agrocampus Ouest in Beg-Meil (Fouesnant, France). Broodstocks were placed in a 250 L tank and were  
82 maintained in flowing 5 µm filtered seawater at 14°C prior to thermal induction of spawning. Animals were carefully  
83 handled to avoid expulsion of cuvierian tubules and organs such as gonads.  
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91 **Fig. 1.** Sampling location (zoom with a white box) and habitat of the sea-cucumber *H. forskali* (black arrows) in West Brittany, France  
92 (site located with a red box).

### 93 2.2 Macroscopic and microscopic examination of the gonadal tubules

94 Before induction of spawning, gonads were removed from the animals by dissection to determine the maturity  
95 stages: immature (I), resting (II), maturing (III), pre-spawning (IV) and post-spawning (V) (Ramofafia et al., 2000;  
96 Ramofafia et al., 2003; Santos et al., 2015; Tuwo & Conand, 1992). Macroscopic and microscopic observations were  
97 performed on gonadal tubules, in order to ensure the maturity stage: primary (T<sub>1</sub>), secondary (T<sub>2</sub>), growing and fecund  
98 (T<sub>3</sub>, T<sub>4</sub>), and spend tubules (T<sub>5</sub>) (Tuwo & Conand, 1992).

### 99 2.3 Induction of spawning by thermal stimulation and fertilization

100 Induction of spawning was performed by thermal stimulation on the 6<sup>th</sup> of April 2018. Water temperature was  
101 quickly raised to 19°C by continuous flow of 1µm filtered seawater, and was maintained at 19°C until spawning.  
102 Photos and videos were taken during spawning to describe spawning behavior. Fertilization in the tanks was  
103 performed and monitored.

### 104 2.4 Embryonic and larval breeding

105 When spawning occurred, fertilized eggs were removed from the tank with 80µm sieve, and washed with seawater.  
106 The seawater used for embryonic and larval rearing was sand filtered, then filtered with 1µm filter bag and sterilized  
107 by UV system. Eggs were observed and incubated at 18°C (±1°C) for 12 hours in 10 L flat bottom trays in closed-

108 water systems, before larval breeding, at a density of approximately 10 embryos/ml. Embryos were sampled and were  
109 observed every hour to describe cell divisions, and measured under a microscope equipped with a micrometric  
110 eyepiece.

111 After incubation, eggs were transferred to hatchery 35 L and 100 L aerating tanks and maintained in closed-water  
112 systems at 17°C ( $\pm 1^\circ\text{C}$ ), at a density of 5-10 larvae/ml, with natural photoperiod. Larvae were sampled and observed  
113 daily to describe the larval development (size, color, organs formation). Seawater of hatchery tanks was renewed 3  
114 times a week: twice with partial seawater change (50%) and once with full change (100%), using a 120 $\mu\text{m}$  mesh  
115 sieve. Water temperature was daily monitored, and 5 ml was collected to observe and describe larval development.  
116 Between the late Gastrula and the early Auricularia stage, larvae were fed daily with a mixture of the microalgae  
117 *Isochrysis galbana* and *Chaetoceros muelleri* (1:1), at a final concentration from  $5 \cdot 10^3$  to  $10^4$  cell.mL<sup>-1</sup>. From the late  
118 Auricularia stage until Doliolaria stage, they were daily fed with *I. galbana* and *Skeletonema costatum* (1:1).  
119 Microalgae were cultivated in Conway Growth Medium, successively in 500 mL, 2 L, 10 L Erlenmeyers and 310 L  
120 cylinder, using the method of the batch culture of microalgae. Salinity was maintained at 35 PSU.

## 121 2.5 Metamorphosis and juvenile breeding

122 At Pentactula stage, larvae were transferred in a micro-nursery with airlift systems, in 300 mm diameters sievers  
123 containing Plexiglas plates, previously striped with a wire brush and colonized by a fouling of periphytic microalgae.  
124 Plates of slightly roughened Plexiglas, which were vertically stacked in the water column with each sheet separated by  
125 a spacer. Tanks were placed in black during metamorphosis – settlement at ambient temperature, 18°C ( $\pm 1^\circ\text{C}$ ).  
126 Microalgae *S. costatum* and *C. muelleri* (2:1) were daily added 2 days after settlement. When juveniles were observed  
127 in tanks, fish micropellets (Carp fish feed from Alltech Coppens, The Netherlands) were distributed once a week.  
128 Approximate measurements were made with a ruler under the binocular magnifier.

## 129 3. Results

### 130 3.1 Gonadal morphology in mature broodstock

131 Gonads of mature broodstock appeared as a well-developed tree constituted by a multitude of branched tubules,  
132 converging towards the gonadal basis and attached to the body wall. The gonadal basis was divided into primary  
133 tubules, and then bifurcated in secondary ramifications and then fecund tubules during the spawning period. Gonadal  
134 tubules in mature female was dark orange to red and white to pale pink in mature male (fig. 2 A-B). In April and  
135 during the spawning period, mature gonads were observed, corresponding to stage IV: mature and pre-spawning  
136 gonads (Santos et al., 2015; Tuwo & Conand, 1992). Gonads occupied a large place in the body cavity and tubules  
137 were more than 10 centimeters long and 1 to 2 mm in diameter, corresponding to class T4 fecund tubules (Tuwo &  
138 Conand, 1992). Male mature tubules were filled with agglomerated spermatozoa, composed by a tail and a head (fig.  
139 2C). Microscopic examination of female tubules shown mature and densely packed ovaries (fig. 2D-E), composed by  
140 mature oocytes (165 $\mu\text{m}$  in diameter) with their germinal vesicle (50-55 $\mu\text{m}$  in diameter) and enclosed with their  
141 follicle. The newly released oocytes had lost their follicle coat and were surrounded by a 20  $\mu\text{m}$ -thick translucent jelly  
142 layer. The nucleus (5 $\mu\text{m}$  in diameter) was well visible in the germinative vesicle (fig. 2F).

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150 **Fig. 2.** Macro- and microscopic morphology of pre-spawning gonadal tubules in female (A, D, E, F) and in male (B, C) of *H.*  
151 *forskali*. A: Macroscopic aspect of mature gonadal tubule in female (T4 fecund tubules, stage IV pre-spawning). B: Macroscopic  
152 aspect of mature gonadal tubule in male (T4 fecund tubules, stage IV pre-spawning). C: active spermatozoa (spz) with a focus on  
153 spermatozoa morphology, tail (tl) and head (hd) in the black box. D-E: Microscopic morphology of ovarian tubules and oocyte:  
154 mature vitellogenic oocytes (vo) with their follicle (f), and germinal vesicle (vg). F: view on mature oocyte with a well-visible  
155 nucleus (nc) in the germinative vesicle (vg), and the translucent jelly layer (tjl) around the oocyte.

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### 157 3.2 Spawning behavior

158 Spawning started in the night of the 8<sup>th</sup> of April 2018, after a 2 day- thermal stimulation. Prior to spawning, male and  
159 female climbed on tank walls, to the upper water level, and then extended and erected the anterior region of their body,  
160 ventral face in front of the surface (fig. 3A). More than half of their body sought the standing position during  
161 spawning. Male and female ejected their gametes by the gonopore (fig. 3B), localized dorsally above the mouth

162 (arrow, fig. 3C). Male ejected continuous streams of sperms and female streams of orange mature oocytes (fig. 3C, fig.  
163 4A). Male and female performed oscillations to help the dispersal of the gametes.

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**Fig. 3.** Posture and spawning behavior of *H. forskali*. Males and females erected their body (A) and ejected a stream of sperm (sp) or oocytes (oo) (arrows, B, C) by the gonopore. Scale bars = 3 cm.

175 Animals were spawning successively during 3 days, mainly during the night, starting at the end of the day,  
176 corresponding to the decrease of photoperiod. Eggs were recovered, washed and placed in incubation.

### 177 3.3 Embryonic and larval development

178 The average diameter of the mature eggs released by females was  $165.46 \pm 4.29 \mu\text{m}$  (n=15). Because fertilization  
179 was not controlled by adding sperm, duration of fertilization can be only estimated at 30 minutes. Embryonic  
180 development of *H. forskali* started with the elevation of the fertilization envelop (fig. 4, table 1) and the expulsion of  
181 the polar bodies (fig. 4B). The embryonic development of *H. forskali* was radial holoblastic. The first cleavage  
182 appeared 2 hours after fertilization and generated 2 equal and hemispheric blastomeres (fig. 4C, table 1). The two  
183 blastomeres were symmetrically divided in the following cleavages (fig. 4D-F), egg diameters evolved from 180 to  
184  $195 \mu\text{m}$  until the morula stage (table 1). The blastula (mean  $193.33 \pm 7.99 \mu\text{m}$ ) stage was observed 24 hours after  
185 fertilization, exhibiting a central blastocoele and a peripheral ciliary cover able to ensure continuous rotary movements  
186 (fig. 4G). The blastula stage was the first mobile stage in *H. forskali* embryonic development. The early gastrula stage  
187 (mean  $242.50 \pm 8.2 \mu\text{m}$ ) was reached between 36 hours and 2 days after fertilization by invagination and elongation of  
188 the blastula wall and evolved to the late gastrula (mean size  $296.67 \pm 9.43 \mu\text{m}$ ) 3 to 4 days after fertilization (fig. 4H-I,  
189 table 1).

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**Fig. 4.** Embryonic development of *H. forskali* at  $17^\circ\text{C}$ . A: mature spawned oocyte / unfertilized egg, and a spermatozoon (spz). B: fertilized egg with a fertilization envelop (fe) and polar bodies (pb). C: First cleavage, 2 cells egg formed with 2 blastomeres (b). D: second cleavage, 4 cells egg. E: 8 cells eggs. F: Morula. G: Rotary blastula, blastocoele (blc), ciliated cove (ci), and ectoderme (ect). H: early gastrula with blastopore (blp). I: mid gastrula. Scale bars =  $100 \mu\text{m}$ .

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**Table 1.** Embryonic and larval development and size (mean  $\pm$  SD; N > 15; \*N = 5; size range and mean in  $\mu\text{m}$ ) of *H. forskali* at  $17^\circ\text{C}$ . Time is represented in weeks (w), days (d), hours (hr) and minutes (min), time after fertilization (AF).

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211 The common larval stages in *Holothuroidea* species are auricularia, doliolaria and pentactula, before metamorphosis  
212 and settlement. The auricularia stage hatched 4 days after fertilization (table 1). The early auricularia was characterized  
213 by a functional, well visible mouth, esophagus, cloaca (fig. 5A-B) and a ciliated band used for feeding and locomotion.  
214 At this stage, the larvae was able to feed. The mean length and width were respectively  $639.87 \pm 40.93 \mu\text{m}$  and  $394.08$   
215  $\pm 89.30 \mu\text{m}$  (table 1). During the auricularia stages, the larvae continuously elongated and their size evolved from 300  
216 in early stages to  $1450 \mu\text{m}$  in late auricularia (table 1). The mid auricularia was obtained 7 days after fertilization (fig.  
217 5C), axohydrocoel was forming, the intestine increased in size and larvae began to accumulate hyaline spheres, to  
218 obtained the late auricularia stage (fig. D-E), with a mean length and width of  $1060 \pm 138.83 \mu\text{m}$  and  $790.29 \pm$   
219  $111.25 \mu\text{m}$  respectively, the maximum length registered was  $1417 \mu\text{m}$ . The late auricularia was reached after 25 to 35  
220 days post-fertilization, accumulated 10 well-formed hyaline spheres and the edges of the larvae were invaginating.  
221 After 4 weeks, the late auricularia progressively began to compact, round and shrink down to obtain a mean length of  
222  $772.33 \pm 48.61 \mu\text{m}$  at the non-feeding doliolaria stage reached 36 days after fertilization. Doliolaria larvae performed

223 rotary movements with their 5 transverse ciliary bands, hyaline spheres were aligned (fig. 5G-H). Quickly, the larvae  
224 evolved towards the pentactula stage less than a week later (fig. 5I, table 1), the ultimate stage before metamorphosis,  
225 and changed in behavior from a planktonic larvae to a benthic larvae. Ciliated bands disappeared the 5 tentacles were  
226 well-visible and the larvae moved to the bottom of the tanks to enhance metamorphosis (fig. 5I). During  
227 metamorphosis, aerating was decreased, feeding was stopped and larvae were transferred in settlement tanks.  
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**Fig. 5.** Larval development of *H. forskali* at 17°C. A: newly hatched auricularia; intestine (int), buccal cavity (bc), esophagus (eso) and enterocoel (ent). B: early auricularia; cloaca (clo). C: Mid auricularia; ossicle (oss), axohydrocoel (axo), ciliary band (cb). D-F: Late auricularia; hyaline sphere (hs), mouth (mou), cardiac sphincter (cs). G: Early doliolaria. H: Doliolaria. I: Pentactula stage; tentacles (ten). Scale bars = 200 µm.

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### 239 3.4 Metamorphosis and juvenile development

240 Metamorphosis and settlement of *H. forskali* began 42 days after fertilization. Duration of settlement still remained  
241 undetermined. First juveniles visible to the naked eye were observed 2 months after metamorphosis. More than 200  
242 juveniles were found in the settlement tank. The body of 120-day juvenile was elongated, yellowish and translucent  
243 colored (fig. 6A-B). The dermis was covered with ossicles of varying shapes and with papillae along the dorsal body  
244 wall; ambulacral podia appeared in the ventral face and helped for movement on substrate. In the oral pole, 10 well-  
245 ramified tentacles were present. The digestive tract was visible through the dermis and exhibit peristaltic movements.  
246 Most of the juveniles had a length between 0.5 and 1.5 mm (mean size 8 mm) and presented a large heterogeneity  
247 between specimens. The 150 day-juveniles began to darken and were less translucent (fig. 6C-D); ossicles, papillae  
248 and podia were well-developed. The average length was comprised between 5 and 30 mm (mean size 12 mm). The  
249 250-day juveniles were small replicas of adults (fig. E-H), the body color were darkening, a part of the juveniles were  
250 dark brown (fig. E-F-H); the length was between 0.5 and 4 cm. The 300-day juveniles sized had a large range size  
251 comprised between 1 cm and 6 cm (fig. 6I) and presented an important heterogeneity. The growth of juveniles was  
252 approximately 0.1mm per day from the settlement to 300 days post-fertilization (fig. 7). This heterogeneity of juvenile  
253 growth may result from the feeding system and otherwise due to the difficulty of length measurements because of the  
254 retraction capacity of the body and the fragility of the juveniles during manipulations.  
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**Fig. 6.** Juveniles of *H. forskali*. A-B: 120-day juvenile; A: dorsal view; B: lateral view; tentacle (ten), podia (pod), calcareous ring (cr), ossicle (oss), papillae (pap), anus (an). C-D: 160-day juvenile; digestive tract (dt), ramified tentacle (rten). E-H: 250-day juveniles. G: zoom on juvenile dermis. I: 300-day juveniles.

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**Fig. 7.** Average growth of the juveniles *H. forskali*. Grey bars represented minimum and maximum sizes, the black square the mean size and the blue line the regression line with the equation and R<sup>2</sup>.

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278 **4. Discussion**

279 The purpose of this study on *H. forskali* was to describe spawning, embryonic and larval development,  
280 metamorphosis, juvenile growth, and associated breeding times in hatchery, prior to aquaculture development.

281 *4.1 Reproductive behavior and spawning*

282 Broodstocks of *H. forskali* were collected in West Brittany in April, during the breeding season as previously  
283 described in the Atlantic Region and more precisely in Glenan Archipelago (Santos et al., 2015; Tuwo & Conand,  
284 1992) and confirmed by gonadal examination of the collected broodstock. The maturation cycle of *H. forskali* differed  
285 according to the site, as described in the Adriatic Sea (Despalatovic et al., 2003) or in the Atlantic Ocean (Tuwo &  
286 Conand, 1992; Santos et al., 2015); and these variations were also recorded between different populations of *H.*  
287 *arguinensis* and *H. mammata* in the Iberian Peninsula (Marquet et al., 2017).

288 Induction of spawning is an important challenge to optimize spawning and fertilization rates in invertebrate  
289 hatchery. In hatchery culture of bivalves, different techniques for spawning induction are used: thermal shocks,  
290 thermal induction, mechanical shocks, biochemical induction, and lethal methods such as gonad laceration (Bourne et  
291 al., 1989; Helm & Millican, 1977; Helm et al., 2004; Loosanoff & Davis, 1963; Utting & Pencer, 1991). In  
292 Holothuroidea, these techniques have been used and the response to the type of stimulation could be different  
293 according to the species (Rakaj et al., 2018). Thermal stimulation demonstrated the best results during the spawning  
294 season, both for temperate species from North-Eastern and Mediterranean regions such as *H. polii*, *H. tubulosa*, *H.*  
295 *mammata* and *H. arguinensis*, (Dominguez-Godino et al., 2015; Dominguez-Godino & Gonzalez-Wangüemert, 2018;  
296 Rakaj et al., 2018; Rakaj et al., 2019) and for tropical species of the Indo-Pacific Region such as *H. scabra* (Kumara et  
297 al., 2013; Mazlan & Hashim, 2015). Others techniques have shown very contrasting results such as mechanical shocks  
298 by dry stimulation for the tropical species (Hu et al., 2013; Huang et al., 2018), thermal shocks (Battaglione et al.,  
299 2002; Morgan, 2009; Rakaj et al., 2018) or phytoplankton induction (Dominguez-Godino & Gonzalez-Wangüemert,  
300 2018; Hu et al., 2013; Mazlan & Hashim, 2015). Otherwise, artificial induction of oocyte maturation by chemical  
301 inducers or by maturation inducing biochemical fractions from echinoderms shown high efficiency on Holothuroidea  
302 (Léonet et al., 2009; Mercier & Hamel, 2002) and on *H. forskali* (Léonet et al., 2009), but this technique requires the  
303 sacrifice of a large number of mature animals.

304 Spontaneous and synchronous spawning of *H. forskali* was observed for both males and females in a delay of 2 days  
305 after the thermal stimulation. Therefore, the release of the gametes occurred mainly during the night. The amount of  
306 produced and spawned gametes was high, which is a common reproductive strategy for marine animals characterized  
307 by an external fecundation and a planktonic embryonic development. Reproductive behavior was similar to those  
308 observed in other species, thereby broodstock erected their body, below to the surface of the tank, and ejected sperms  
309 or eggs by the gonopore.

310 This preliminary study demonstrates the necessity to acquire data on the maturation cycle and spawning season of *H.*  
311 *forskali* in its natural habitat. Additional works on spawning induction and fertilization control have to be processed, to  
312 optimized hatchery procedures. Minimally invasive methods and non-lethal methods would be preferred to limit the  
313 sacrifice of broodstock. Moreover, to enhance a new and sustainable aquaculture, broodstock selection and genetic  
314 diversity will be an important point.

315 *4.2 Embryonic and larval development*

316 For this first complete description of embryonic and larval development of *H. forskali*, the larval rearing duration  
317 was 40 days (table 2, fig. 8). The embryonic development was holoblastic and the first larval stage, auricularia,  
318 hatched after 4 days and lasted more than 30 days (table 2). Pentactula and metamorphosis were obtained after 40  
319 days, this stage was longer than the other European species and Indo-pacific species (12-15 days for *H. scabra*,  
320 Ramofafia et al., 2000; 15-17 days for *Apostichopus japonica*, Ito & Kitamura, 1998; Soliman et al., 2013; Yang et al.,  
321 2015) (table 2). However, in other cultivated Echinoderms, larval life duration can be variable, as in the edible sea-  
322 urchin *Strongylocentrotus intermedius* whose duration was 20 days (Lawrence et al., 2019), in comparison with  
323 *Tripneustes depressus*, whose duration can reached 54 days (Sonnenholzner-Varas et al., 2018).

324 The breeding temperature may explain these results in part. In invertebrate hatchery, the temperature plays an  
325 important role and can decreased breeding time, for the bivalve *Crassostrea gigas*, the larval life duration decrease from  
326 27-32 days at 18°C, to 22 days at 21°C, and 18 days at 28°C (Ben Kheder et al., 2010). For comparison with temperate  
327 holothurians, breeding temperature is 24°C for *H. polii* (Rakaj et al. 2019), *H. arguinensis* (Dominguez-Godino et al.,  
328 2015) and *H. tubulosa* (Rakaj et al. 2018) (table 2). Generally, an increase of temperature reduced breeding time  
329 (Asha, 2004; Asha & Muthiah, 2005; Hamel & Mercier, 1996), therefore, larval development of temperate species is  
330 longer than tropical species (Chen & Chian, 1990; Ramofafia et al., 2003).

331 Another way to reduce larval life duration is to reduce larval density. In this present study, a high larval density has  
332 been chosen (5 to 10 larvae/mL). However, in other species, an average density of 1-2 larvae per mL is commonly

333 used (Dominguez-Godino et al., 2015; Dominguez-Godino & Gonzalez-Wangüemert, 2018; Rakaj et al. 2019). In this  
334 experiment, the larvae density was high during all the auricularia stage and a high larval density can increase the larval  
335 duration, the risk of pathogen development and diseases, and finally mortality (Kennedy et al., 2016). Moreover, the  
336 high density decrease the food availability (Battaglione, 1999; Ramofafia et al., 2003).

337 The food sequencing and the food availability, from the first feeding larval stage, auricularia, to the non-feeding  
338 stage, doliolaria, are crucial points which have consequences on duration, shape, and development of larvae (Morgan,  
339 2009; Morgan, 2001; Knauer, 2011; Militz et al., 2018) and metamorphosis (Ito & Kitamura, 1997; Yang et al., 2015).  
340 The phytoplankton species *Chaetoceros muelleri* and *Isochrysis galbana* are commonly used in hatchery and for  
341 holothurians breeding (Hu et al., 2010; Hu et al., 2013; Mazlan & Hashim, 2015; Morgan, 2001; Rakaj et al., 2018).  
342 Feeding larvae with *C. muelleri* can increase growth, survival and metamorphosis (Knauer, 2011). The use of  
343 microalgae concentrates can be explored to diversify food (Duy et al., 2016; Hair et al., 2011).

344 Additional experiments will be performed to monitor survival, in order to measure zootechnical efficiency and to  
345 optimize protocols.

346  
347  
348 **Fig. 8.** Rearing time from embryonic to the benthic larval stage pentactula in different species of sea-cucumbers. In blue: embryonic  
349 stages; in light grey: auricularia stage; in green: doliolaria stage; in dark grey: pentactula (sources in table 2).

350  
351  
352 **Table 2.** Comparison of embryonic and larval development: rearing time, size and temperature of cultivated sea-  
353 cucumbers in aquaculture.

#### 354 355 4.3 Metamorphosis and juveniles growth

356 Metamorphosis represents a critical point in hatchery, with mortality associated. Succeed the metamorphosis stage  
357 depends on parameters such as water quality, substrate, settlement material, food, hydrology, or light intensity  
358 (Altaramino et al., 2017; Battaglione et al., 1999; Ramofafia et al., 2000; Gunay et al., 2015; Indriana et al., 2017; Ito &  
359 Kitamura, 1997; Mercier et al., 2000) Growth and feeding methods of sea cucumbers are well-known for cultivated  
360 species, but remains elusive for this species (Navarro et al., 2013; Robert et al., 2000; Uthicke, 1999). Metamorphosis  
361 and settlement were effective, which represent a technical challenge. For *H. forskali*, the use of Plexiglas plates  
362 covered with a microalgae fouling was successful. This type of support is common in aquaculture and is used for  
363 holothurians settlement (Ito & Kitamura 1997; Morgan, 2001). An additional work on fouling will have to be done, on  
364 periphitic diatoms, to optimize settlement rate and to homogenize juvenile growth (Ito & Kitamura 1997; Morgan,  
365 2001), on substrate, on density and on the moment to transfer the pentactula larvae in the settlement tanks (Battaglione  
366 et al., 1999). Because of it efficiency in shellfish hatchery, settlement on fine crushed shell chips could to be explored.  
367 Juveniles have been observed only after 60 days for this first rearing of *H. forskali*. The mean juvenile size of *H.*  
368 *forskali* was 0.8 cm after 120 days and more than 4 cm after 300 days (fig.7, table 2). In the first 200 days, the juvenile  
369 growth followed a polynomial equation ( $y = 0,0004x^2 - 0,0022x + 1,3443$  with  $R^2 = 0.9672$ ), described in other species  
370 such as *Isostichopus fuscus* (Mercier et al., 2005). The growth rate of approximately 0.1 mm per day (fig. 7), lower  
371 than the tropical cultivated species *Stichopus horrens* (0,6mm per day, Hu et al., 2013) and 0.2-0.3 mm per day after  
372 60 days of culture for *H. leucospilota* (Huang et al., 2018). These growth values were lower than commercialized  
373 species but similar to temperate species (table 2).

## 374 5. Conclusion

375  
376 Taking into account over fishing of sea cucumbers in many places such as Indo-Pacific, Mediterranean regions and  
377 in the Overseas Collectivity of Saint-Pierre-et-Miquelon, European species are a new target for the Asian Market.  
378 Moreover, sea cucumber play an important ecological role in marine benthic ecosystems by taking part in bioturbation  
379 process and nutrient recycling. *H. forskali* presents an interesting candidate as deposit feeder in integrated multi-  
380 trophic aquaculture, and thus offering many opportunities to develop a sustainable aquaculture. In this context, the  
381 development of aquaculture is in fact a guarantor for the sustainable management of a new fisheries resource.

382 This first description of *H. forskali* embryonic, larval development and juvenile growth aimed to enhance a new  
383 aquaculture to promote diversification of production and potential export market.

384 Even if this work was carried out in a lab-scale, these preliminary results are very encouraging for the domestication  
385 of this species, and 5 cm long juveniles have been produced after 250 days of breeding. This experiment is promising  
386 and further research is needed to optimized zootechnical procedures and then to create a new innovative and profitable  
387 aquaculture.



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393

394

## 395 7. Authors' contributions

396 HL designed the aquaculture experiments and analysed data. HL wrote the first draft of this manuscript and PLC  
397 reviewed it. All authors read and approved the final manuscript.

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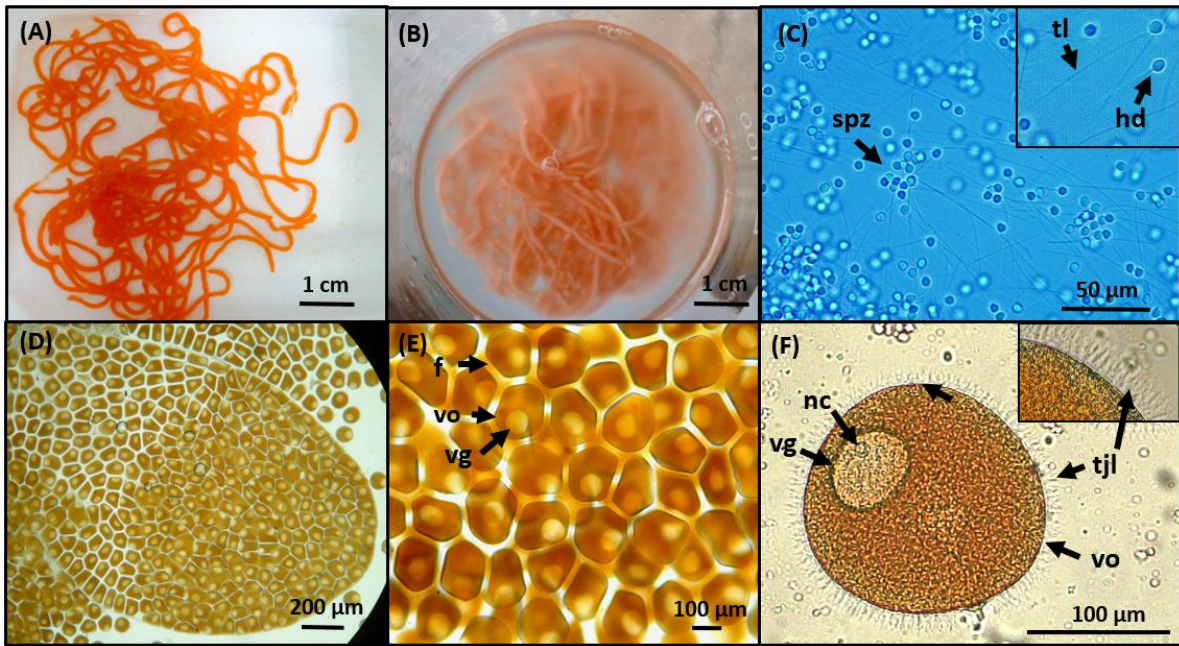
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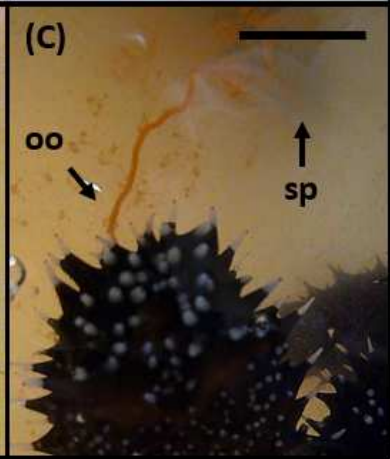
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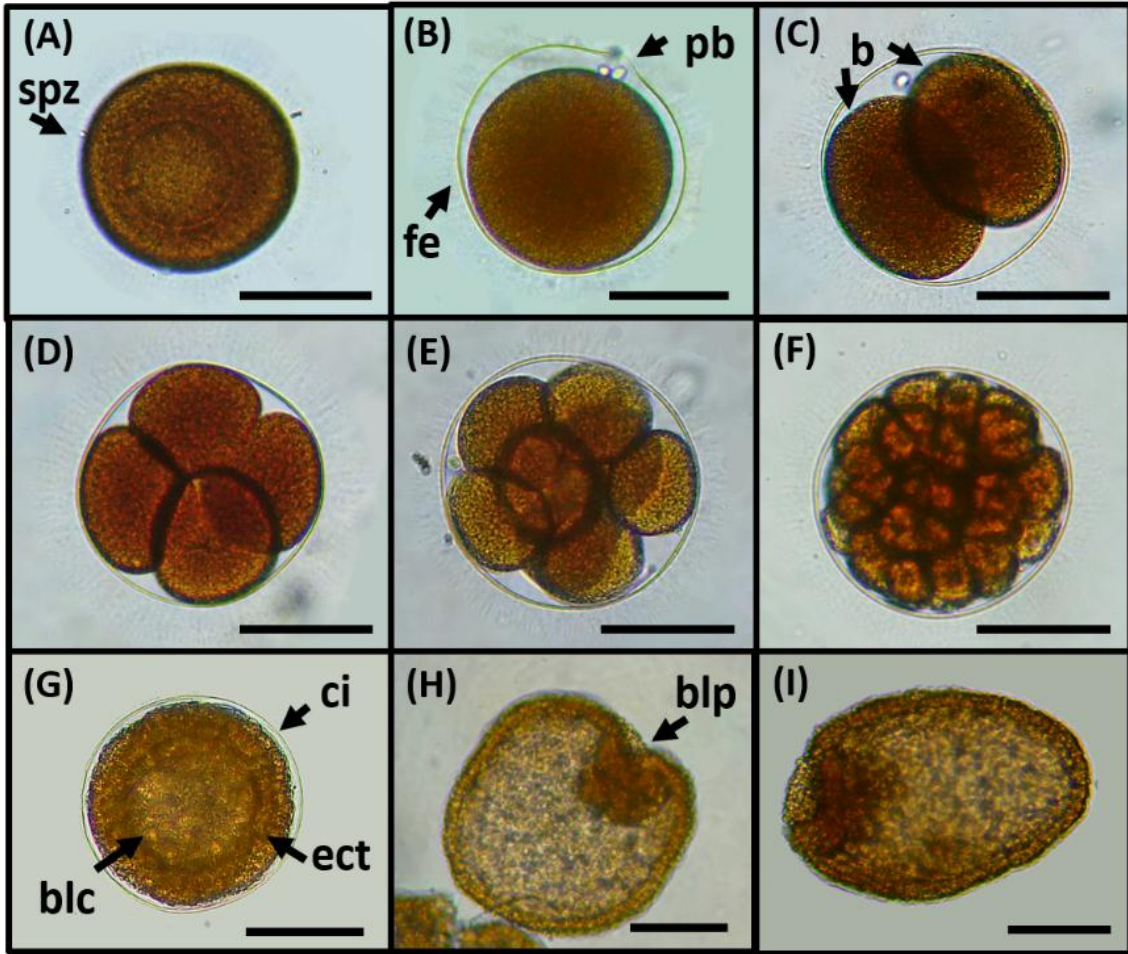


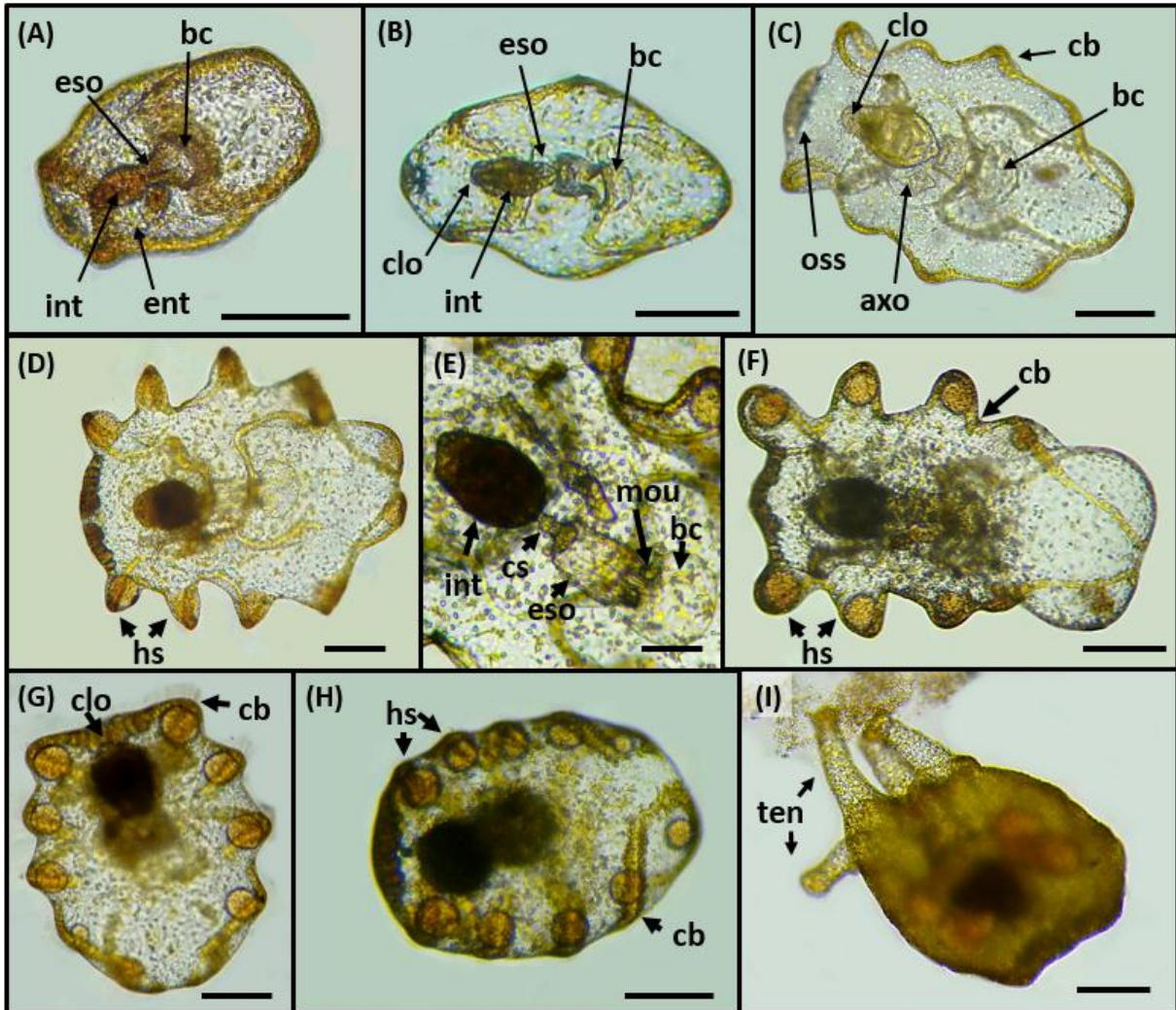








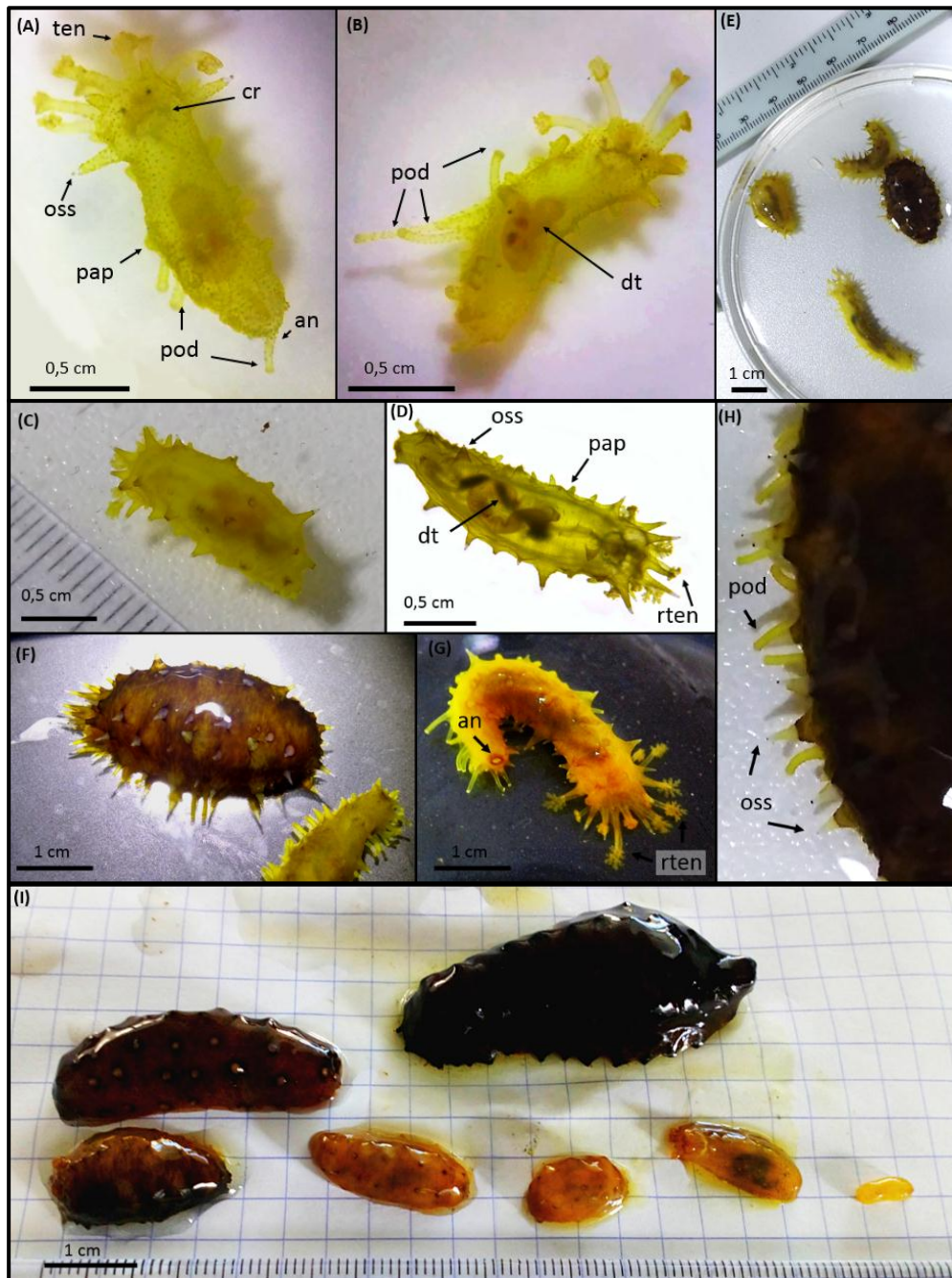




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**Fig. 5.** Larval development of *H. forskali* at 17°C. A: newly hatched auricularia; intestine (int), buccal cavity (bc), esophagus (eso) and enterocoel (ent). B: early auricularia; cloaca (clo). C: Mid auricularia; ossicle (oss), axohydrocoel (axo), ciliary band (cb). D-F: Late auricularia; hyaline sphere (hs), mouth (mou), cardiac sphincter (cs). G: Early doliolaria. H: Doliolaria. I: Pentactula stage; tentacles (ten). Scale bars = 200 μm.

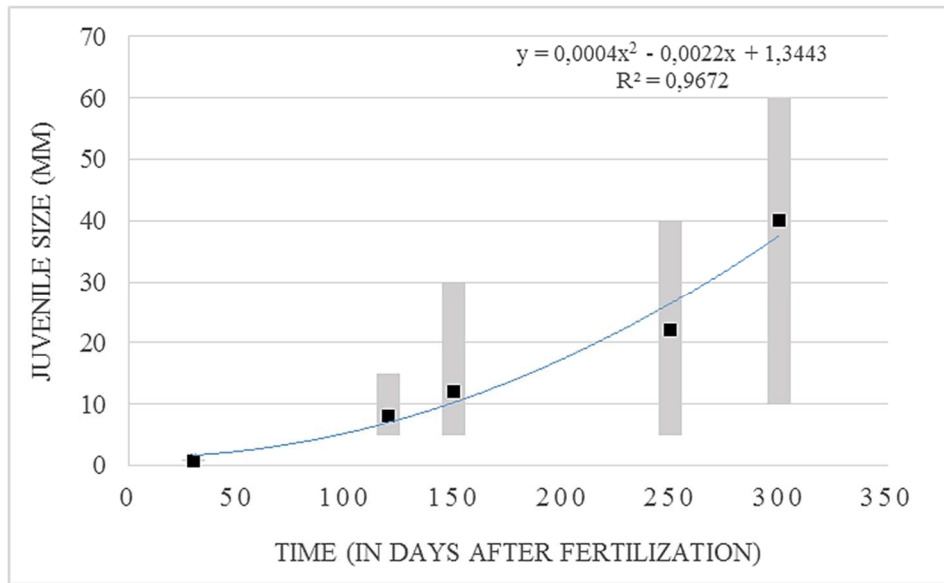


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**Fig. 6.** Juveniles of *H. forskali*. A-B: 120-day juvenile; A: dorsal view; B: lateral view; tentacle (ten), podia (pod), calcareous ring (cr), ossicle (oss), papillae (pap), anus (an). C-D: 160-day juvenile; digestive tract (dt), ramified tentacle (rten). E-H: 250-day juveniles. G: zoom on juvenile dermis. I: 300-day juveniles.

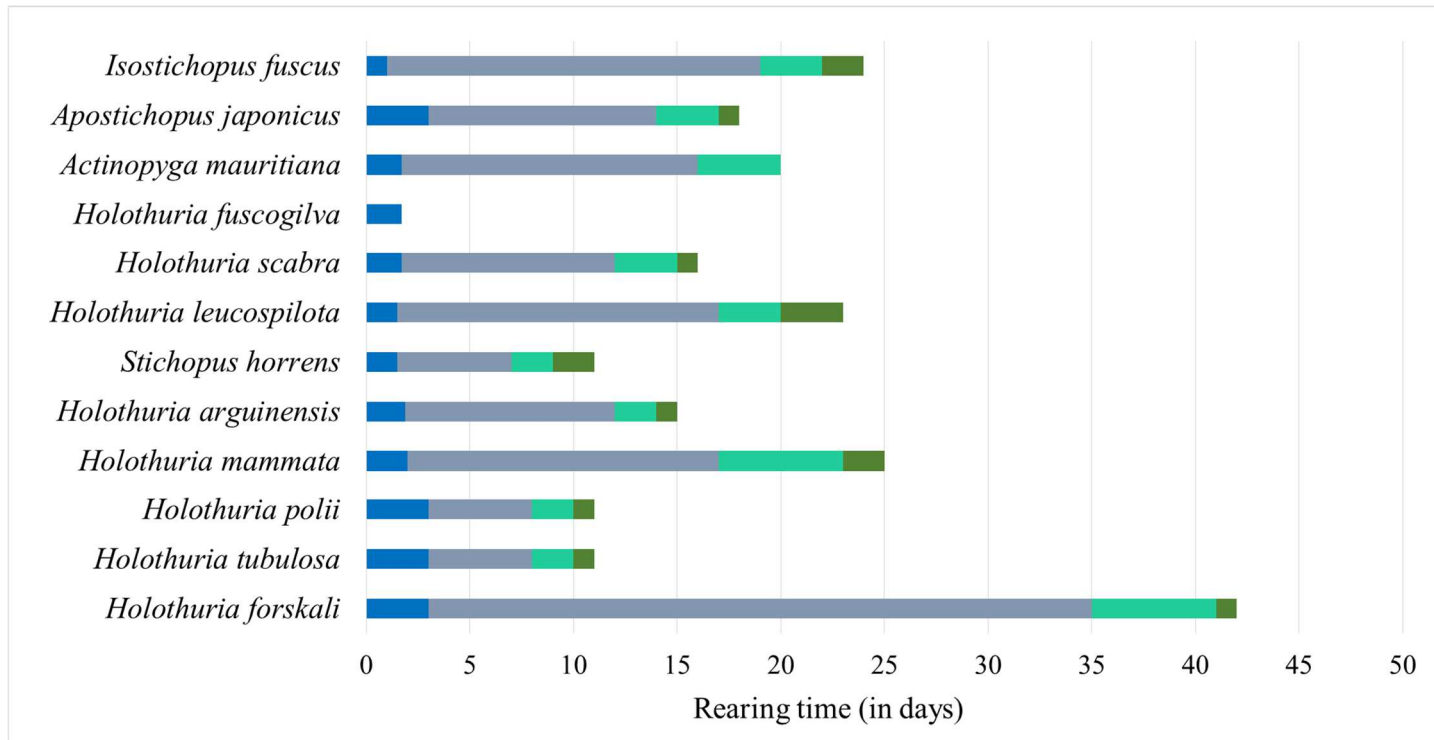
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**Fig. 7.** Average growth of the juveniles *H. forskali*. Grey bars represented minimum and maximum sizes, the black square the mean size and the blue line the regression line with equation and  $R^2$ .

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**Fig. 8.** Rearing time from embryonic to the benthic larval stage pentactula in different species of sea-cucumbers. In blue: embryonic stages; in light grey: auricularia stage; in green: doliolaria stage; in dark grey: pentactula (sources in

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**Table 1.** Embryonic and larval development and size (mean  $\pm$  SD; N > 15; \*N = 5; size range and mean in  $\mu\text{m}$ ) of *H. forskali* at 17°C. Time is represented in weeks (w), days (d), hours (hr) and minutes (min), time after fertilization (AF).

Embryonic and larval stage	Size range	Mean $\pm$ SD	Time AF
Unfertilized oocyte	[160-175]	165.46 $\pm$ 4.29	-
Fertilized egg	[180-190]	189.07 $\pm$ 3.63	-
2 cells egg	[185-195]	189.00 $\pm$ 2.79	2 hr
4 cells egg	[185-200]	190.37 $\pm$ 3.12	4 hr
8 cells egg	[185-200]	195.00 $\pm$ 4.77	5 hr
16 - 32 cells egg*	[190-205]	195.00 $\pm$ 4.08	7 hr
Rotary blastula	[190-210]	193.33 $\pm$ 7.99	24 hr
Early gastrula	[210-270]	242.50 $\pm$ 8.29	36 hr
Late gastrula	[270-310]	296.67 $\pm$ 9.43	48 hr
Early auricularia	[300-700]	639.87 $\pm$ 40.93	4 -6 d
Mid auricularia	[700-900]	822.74 $\pm$ 58.30	7-25 d
Late auricularia	[900-1450]	1060 $\pm$ 138.83	25-35 d
Doliolaria*	[700-850]	772.33 $\pm$ 48.61	36 d
Pentactula*	[700-850]	-	42 d

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**Table 2.** Comparison of embryonic and larval development for cultivated sea-cucumbers in aquaculture: rearing time, size and breeding temperature and distribution area.

Species	Fertilized egg size ( $\mu\text{m}$ )	Newly-hatched auricularia - in time AF (days or hours)	Auricularia size range ( $\mu\text{m}$ )	Pentactula stage in time AF (days)	Breeding temperature ( $^{\circ}\text{C}$ )	Juvenile size after 90-120 days AF (mm)	Origine	References
<i>Holothuria forskali</i>	189,07 $\pm$ 3,63	4 d	300-1450	42	17-18	8	NE Atlantic, Mediterranean - temperate waters	This study
<i>Holothuria tubulosa</i>	172,1 $\pm$ 1,7	3 d	430-800	26	24	8,7	NE Atlantic, Mediterranean - temperate waters	Rakaj et al., 2018
<i>Holothuria mammata</i>	167,17 $\pm$ 8,29	2 d	350-630	19-20	25-38	mortality	NE & E Atlantic, E mediterranean - temperate waters	Dominguez-Godino & Gonzalez-Wanguemert, 2018
<i>Holothuria polii</i>	247,46 $\pm$ 15,48	3 d	300-750	9-10	24	21,0 $\pm$ 8,2	Mediterranean - temperate waters	Rakaj et al., 2019
<i>Holothuria arguinensis</i>	171,66 $\pm$ 5,21	45 hr	500-920	13-14	27-28	-	NE & E Atlantic, E mediterranean - temperate waters	Dominguez-Godino et al., 2015
<i>Stichopus horrens</i>	120,60 $\pm$ 11,53	45 hr	350-1200	19-27	25-27	18-40	Indo-Pacific, Red Sea, Australia - warm waters	Hu et al., 2010; Hu et al., 2013
<i>Holothuria leucospilota</i>	140-145	36 hr	300-1500	20-23	29-33	40-70	Indo-Pacific - warm waters	Huang et al., 2018
<i>Holothuria scabra</i>	154,4 $\pm$ 2,9	40-70 hr	300-890	12-15	25-27	30	Indo-Pacific - warm waters	Ramofafia et al., 2000; Ramofafia et al., 2003; Yan et al. 2012; Knauer 2011; Battaglione et al., 1999; Altamirano et al., 2017; Kumara et al., 2013; Mazlan & Hashim, 2015
<i>Holothuria fuscogilva</i>	151,7 $\pm$ 5,2	40-70 hr	420-730	mortality	25-27	mortality	Indo-Pacific - warm waters	Ramofafia et al., 2000; Ramofafia et al., 2003
<i>Actinopyga mauritiana</i>	109,9 $\pm$ 0,99	40-70 hr	300-740	mortality	25-27	mortality	Indian Ocean, Red Sea - warm waters	Ramofafia et al., 2000; Ramofafia et al., 2003
<i>Apostichopus japonicus</i>	150-200	36 hr	500-1100	15-17	17-21	> 10-20	SE Asia - temperate waters	Yang et al., 2015; Soliman et al., 2013; Ito and Kitamura, 1997
<i>Isostichopus fuscus</i>	120	1-2 d	540-1300	21-26	22-29	80	E Pacific - temperate waters	Mercier et al., 2005
<i>Athyonidium chilensis</i>	360 $\pm$ 16	-	-	7	13	1,4	Chile, Peru - temperate waters	Guisado et al., 2012

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