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Artificial reproduction of *Holothuria polii*: A new candidate for aquaculture

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ABSTRACT

Holothuria polii (Delle Chiaje, 1823) is a common Mediterranean sea cucumber and has recently become a highly exploited target species destined mainly to the Asian markets. Unregulated harvesting is putting the natural stocks at risk, with potential consequences for the benthic communities to which these organisms belong. In response aquaculture, sea ranching and restocking could become solutions to this issue in the Mediterranean region. In this study, we present the first results on artificial breeding of *H. polii,* describing the spawning induction, larval development and early juvenile growth in hatchery cultures, our goal is to develop a rearing protocol for this species. The trials were conducted between July and September 2016. *Holothuria polii* was induced to spawn by testing two different methods. The thermal shock proved a simple and effective method for obtaining healthy gametes from this species. Larval development in *H. polii* progressed through five stages, reaching the pentactula stage in 10 days. Three different microalgal feeding regimens were tested for larval breeding; the higher feeding concentration 20,000–40,000 cells mL^{-1} proved to be the best, with 14% of the larvae metamorphosed into settled juveniles by adhering to the artificial substrates at 15 days. Our results indicate that *H. polii* performs very well in the hatchery rearing during the larval and juvenile phases; this indicates that this species could be a valuable new candidate for aquaculture in the Mediterranean region both for production and for restocking purposes.

1. Introduction

With the surge in demand and fishing pressure in recent decades, the risk of stock collapse for many species of sea cucumbers has increased (Anderson et al., 2011; Purcell et al., 2013). These marine invertebrates are harvested worldwide, however the main part of the catch is consumed in Asia, mainly China and Japan where they are considered as luxury seafood (Kinch et al., 2008; Purcell et al., 2012; Toral-Granda et al., 2008). In addition, sea cucumbers have also a long history as a traditional medicine with many beneficial effects and a wide-ranging pharmacological potential in modern medicine (Bordbar et al., 2011; Dakrory et al., 2015; Janakiram et al., 2015).

A large part of the commercially important species are deposit feeders which ingest sediment and organic material, such as vegetal and animal detritus, including bacteria, protozoa and diatoms (MacTavish et al., 2012; Purcell et al., 2016; Uthicke, 1999). They play an important role in processing organic matter, this involving both material cycling and energy flow in benthic ecosystems (Purcell et al., 2016). Given their trophic behaviour, some sea cucumbers have been successfully used in integrated multitrophic aquaculture (IMTA) (Ahlgren, 1998; Chopin et al., 2012; Hannah et al., 2013; Paltzat et al., 2008; Slater and Carton, 2009; Slater and Carton, 2007; Yokoyama, 2013).

Sedentary nature, easy harvesting and high market demand, all make sea cucumbers vulnerable to overexploitation (Purcell et al., 2013, 2010). Thus, in many countries overfishing poses wild stocks at risk, threatening biodiversity of benthic communities and the health of the sediment they inhabit (Purcell et al., 2016). Aquaculture appears to be a viable solution to overcome the wild exploitation for human consumption as food, as well as supplying the biomedical, pharmaceuticals and nutraceuticals sectors (Anderson et al., 2011; Benkendorff, 2009). Moreover, hatchery-produced juveniles could be used to reconstitute wild breeding populations (Bell and Nash, 2004; Eriksson et al., 2012).

In the face of an increasingly interconnected and globalized market, pioneering research into sea cucumber aquaculture has also been developed in regions where they are not consumed directly, as in the case of the Atlantic and Mediterranean regions (Domínguez-Godino et al., 2015; Domínguez-Godino and González-Wangüemert, 2018; Rakaj et al., 2018). In the Mediterranean Sea, species such as *Holothuria polii*, *Holothuria tubulosa* and *Holothuria mammata* have become a novel fishing target (González-Wangüemert et al., 2014, 2015, 2018). In these areas, the market interest is expanding rapidly due to both the high

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value of the product and the lack of fishery management measures (González-Wangüemert et al., 2018; González-Wangüemert and Domínguez-Godino, 2016). Illegal, unreported and unregulated (IUU) fishing is therefore one of the main problems affecting the natural stocks in many Mediterranean countries. Recently, *H. tubulosa* and *H. polii* fishing has been increasingly reported along the Italian coasts with massive seizures of the product by law enforcement agencies (Ansa, 2016; L.S., 2016; R.B., 2016; I.F.Q., 2017). This has led the Italian Ministry of Agriculture, Food and Forestry (MIPAAF) to put a moratorium on sea cucumbers fishing by means of Order 156/2018.

Against this background, the present study sets out to explore the potential of *Holothuria polii* for aquaculture as an opportunity to diversify seafood production while limiting damage due to overexploitation.

Holothuria polii (Delle Chiaje, 1823) is a common Mediterranean sea cucumber which lives in the sublittoral zone (Tortonese, 1965), where it inhabits soft sediment and seagrass meadows. According to ecological observations, *Holothuria polii* emerges as an important deposit feeder, thus confirming its fundamental role in benthic dynamics (Boncagni et al., 2018; Massin and Jangoux, 1976; Tortonese, 1965). It is a gonochoric species without sexual dimorphism; spawning and fertilization are external during the summer, with a distinct annual reproductive cycle (Aydin and Erkan, 2015). *H. polii* is one of the most exploited sea cucumber species in the Mediterranean basin. From about 720,000 to 1.080.000 sea cucumbers collected daily in Turkish waters (González-Wangüemert et al., 2014), this species alone accounting for 80% of the catches. With the exception of some studies concerning the Turkish coast (González-Wangüemert et al., 2014, 2015), little is known about the conservation status of this species in other countries since stock assessment is still lacking.

From the nutritional viewpoint, *H. polii* has a content high in proteins, micronutrients, polyunsaturated acids but is very low in lipids. It is therefore a “functional” and nutraceutical food for human consumption (Aydin et al., 2011; Sicuro et al., 2012). *Holothuria polii* has been highlighted as a potential source of bioactive compounds with significant antifungal, anti-inflammatory and antibacterial activity (Canicatti and Roch, 1989; Ismail et al., 2008; Omran and Allam, 2013). This suggests that research on *H. polii* aquaculture could have high applicative aspects on several fronts.

The present study extends the knowledge of the life history of *Holothuria polii*, describing for the first time this species' embryonic, larval and juvenile development. Our research provides new information on spawning behaviour, larval feeding, larval growth, settlement rates and juveniles' post-settling growth, our aim being to draw up a rearing protocol under hatchery-based conditions.

Finally, these outputs pave the way for new aquaculture developments for *H. polii*, which could provide solutions for production or restocking actions in the future.

2. Materials and methods

2.1. Broodstock collection and maintenance

From July to September in 2016 adult specimens of *H. polii* (240 individuals; mean weight: 106.24 ± 9.47 g; mean ± SE) were collected by snorkeling (1–5 m depth) at Santa Marinella in the central Tyrrhenian Sea, Italy (42°3′0″Nord, 11°49′9″Est). The broodstock were then transported to the Laboratory of Experimental Ecology and Aquaculture (University of Rome Tor Vergata) inside 30 L tanks equipped with aerators and dry ice (to maintain the temperature below 28 °C). Once in the lab, the breeders were acclimatized in 600 L indoor tanks in a recirculating aquaculture system (RAS) at a density of around 40 specimens per tank. Sediment collected from the sampling site was placed in each tank and the water temperature was maintained at 24 °C.

2.2. Spawning induction and fertilization

Spawning in *H. polii* was induced in concomitance with the species gonosomatic peak from July to September (Authors, personal communication). The spawning induction protocol used was based on that developed for *H. tubulosa* (Rakaj et al., 2018). Prior to stimulation, breeders were moved in substrate-free aquaria where they were kept for 48 h in order to void their gut contents; then they were washed with filtered seawater to remove sediment and other organisms. Finally, they were transferred to 600 L spawning tanks containing 5 μm filtered and UV-sterilized seawater.

To induce spawning, mechanical shock and thermal shock methods were tested in parallel on broodstock groups of 20 individuals for eight replicates.

- Mechanical shock (dry shock): broodstock were kept dry for approximately 30 min before being subjected to a strong water jet; they were then returned to the spawning tanks (Rakaj et al., 2018; Renbo and Cheng, 2004; Yanagisawa, 1998).
- Thermal shock: water temperature was rapidly increased by 3–5 °C (from 24 °C to 27–29 °C), maintained over 1.5 h, and then returned to the starting temperature (Battaglene et al., 2002; Dabbagh et al., 2011; Domínguez-Godino et al., 2015; Rakaj et al., 2018).

When spawning induction was successful, the broodstock showed signs of pre-spawning movements. Males were immediately identified since they began spawning first, and were transferred to separate tanks. The remaining specimens manifesting pre-spawning behaviour were assumed to be female and isolated in 20 L spawning buckets until they released eggs. Water in the buckets was stirred to uniformly distribute the eggs. Five samples of 1 mL were collected to estimate the number of released eggs/female; eggs were counted using a Sedgewick-Rafter cell chamber under a light stereomicroscope (for 34 females). At this stage, the spermatozoa solution (obtained from isolated males) was added at low concentration (circa $1–5 \times 10^4$ spermatozoa mL^{-1}), in order to minimize polyspermy (Hamel and Mercier, 1996), in the female spawning buckets. After fertilization, the eggs were continuously monitored and the timing of embryo development tracked. Thirty minutes after fertilization, the eggs were collected by a siphon and washed with sterilized seawater in order to remove the residual sperm.

2.3. Larval and juvenile culture

The fertilized eggs were transferred and maintained in 10 L round-bottom flasks at a density of 1 egg mL^{-1} (Battaglene and Bell, 1999; Domínguez-Godino et al., 2015; Domínguez-Godino and González-Wangüemert, 2018; Pitt, 2001; Rakaj et al., 2018; Ramofafia et al., 2003). Into each flask, air driven pipettes were placed to maintain sufficient aeration and ensure gentle water circulation.

Embryonic and larval development was tracked by collecting ten mL water samples (5 replicates) in order to record measurements and development stages. During the first 24 h, development was followed constantly by taking samples every hour; for the following two days, samples were taken every 6 h and, from the third day, they were collected daily. The larval rearing was conducted indoors with a natural circadian photoperiod and the temperature maintained at 24 °C by conditioning the hatchery room. Circa 80% of the culture water was changed each day using a 60 μm mesh screened siphon; dead larval deposits were removed by siphoning the bottom of the flasks with a pipette. For the daily change of rearing medium, natural seawater was used (5 μm filtered and UV sterilized) with a salinity maintained between 36 and 37 PSU. When a fully developed gut was observed (third day following the fertilization), larvae were fed daily using a mixture of microalgae, under exponential growth, of *Isochrysis galbana* and *Chaetoceros calcitrans*, in equal proportions. The microalgae were cultured in 2 L plastic bag photo-bioreactors using Guillard's f/2-β growth medium (Guillard and Ryther, 1962) at a 12:12 h light-dark photoperiod. Three different feeding regimes were tested for larval rearing of *H. polii* with three replicates for each condition:

- High feeding regime of 20,000–40,000 cells mL^{-1} from early to late auricularia, as recommended in the literature for several sea cucumber species (Agudo, 2006).
- Intermediate feeding regime of 5000–10,000 cells mL^{-1} from early to late auricularia, as recommended in the literature for *H. tubulosa* (Rakaj et al., 2018).
- Low feeding regime of 1000–2000 cells mL^{-1} similar to that recommended in the literature for *A. mollis* (Morgan, 2008).

Water samples (20 mL, five replicates) were taken daily in order to follow larval development, record mean length and width of the larvae (n = 30) and estimate survival on the different feeding treatments. The percentage of late auricularia larvae with hyaline spheres was determined for each feeding condition. At the doliolaria stage, the larvae were transferred from the round-bottom flask to 30 L aquaria with 6 Polymethyl methacrylate (PMMA) settlement panels (0.20 m × 0.40 m stacked with a 30 mm gap). The settlement panels had previously been cultured with diatom-biofilm (*Navicula* sp., *Nitzschia* sp., and *Phaeodactylum tricornutum*) raised in naturally illuminated tanks with f/2-β growth medium (Rakaj et al., 2018). Two air stones were placed on the bottom of each aquarium to provide aeration and water circulation. The water was partially changed (circa 50%) every day using 60 μm mesh siphons. Starting from the fifteenth day, ALGAMAC 3050 and dry *Spirulina* was added as supplementary feed into the juveniles' aquaria (0.01 g in each aquarium daily). After settlement, the number of competent larvae and juveniles was determined for each treatment (at 15 and 30 days) by counting them in five subareas of a sampling plate.

After 30 days, the juveniles were gathered and transferred to 600 L tanks, where it was possible to administer increasing rates of ALGAMAC and *Spirulina* (0.1 g in each tank daily); then, at day 60 their survival was estimated through partial counts. Finally, juvenile survival was determined through a complete count at day 90 from fertilization.

Eggs, embryos, larvae and juveniles were relaxed and anaesthetized in 7.5% $MgCl_2$ solution isotonic to seawater before being fixed in 4% Phosphate Buffered Formalin solution. The specimens were observed, photographed and measured using a Leica MZ12 stereomicroscope and a Leica DMLB 2000 light microscope (Leica, Wetzlar, Germany).

3. Results

3.1. Spawning induction

Among the eight spawning induction treatments performed in parallel on *H. polii* broodstock, no response was recorded after the mechanical shock treatment. Instead, the thermal shock treatment induced spawning successfully in 7 out of 8 trials. Thermal shock emerged as the best approach for inducing spawning in this species. When pre-spawning behaviour occurred in broodstock, individuals adopt a standing position, many of them aligning on the tank walls close to the water surface. Breeders cling the substrate with the posterior part of the body, while extending the anterior end to help gamete dispersion (Fig. 1). Males spawned before females, releasing a thin and steady stream of sperm from the apical gonopore with short interruptions. Females' started spawning approximately 0.5–1 h after males, rising the anterior body and twisting upward to release a vigorous squirt of eggs. Each female completed spawning with 2–4 distinct jets (of 2–4 s) with an intermittence of 10–15 min between each squirt. This powerful ejection dispersed the eggs much more widely relative to the streams of sperm. Mature eggs of *H. polii* were spherical, visible to the naked eye, with a mean diameter of 203.7 ± 10.21 μm (Fig. 2a). The quantity of eggs varied widely among individuals, ranging from 48.000 to 655.000 eggs per spawning female (176.194 ± 15.900; n = 34). Since the eggs were heavier than seawater, they sank to the bottom of the bucket.

3.2. Embryonic and larval development

The eggs fertilization membrane swelled within 5 min after the sperm addition, increasing their size to a mean diameter of 247.46 ± 15.48 μm (n = 30 larvae) (Fig. 2b). The first cleavage was holoblastic and completed 90 min after fertilization (Fig. 2c). The second and third cleavages were observed at 120 and 150 min, respectively, after fertilization (Fig. 2d, e). The morula stage (Fig. 2f, g) appeared six h after fertilization and the blastula stage was reached 12 h after fertilization, with a typical central blastocoele surrounded by a single layer of cells (Fig. 2h). The embryo started moving and at 20 h the blastopore start forming and the first gastrula was observed (mean length 246.25 ± 9.19 μm) (Fig. 2i).

During the following day, the gastrula continued to elongate (Fig. 3a) until they reached the early auricularia stage (Fig. 3b). At this stage, the third day after fertilization was subjected the larval cultures to the three feeding conditions. Here we present the developmental time and size of larvae registered under the high feeding regime (Table 1).

As has been noted in other Aspidochirotida, *Holothuria polii* progressed through the five larval development stages: early auricularia, mid auricularia, late auricularia, doliolaria and pentactula.

The early auricularia appeared completely developed the third day post fertilization and mean length and width were 513.8 ± 30.03 μm and 295.36 ± 17.98 μm, respectively. The larvae in this stage are translucent with a functional digestive tract, including a buccal cavity, stomach and a wild developed axohydrocoel (Fig. 3b). The first feeding instance was observed at this stage. Four days after fertilization, the larvae increased in size, reaching the mid auricularia stage (Fig. 3c), with a mean length and width of 623.42 ± 49.04 μm and 368.46 ± 24.18 μm and the left somatocoel made its appearance during this stage. Larvae reached the late auricularia stage on day 6 post fertilization (Fig. 3d), and had a mean length of 704.8 ± 42.42 μm and width of 442.4 ± 26.19 μm, respectively, these being the maximum dimensions registered during any of the planktonic stages. Larval intestines increased in size throughout the development of late auricularia while hyaline spheres also appeared (Fig. 3d). Eight days post fertilization a rapid metamorphosis from the late auricularia to the doliolaria stage took place. Doliolaria larvae of *H. polii* displayed the characteristic barrel shape with five ciliary bands and well developed hyaline spheres (Fig. 3e). At this stage, the larvae became smaller than in the previous stages, with a mean length and width of 375.07 ± 28.37 μm and 217.55 ± 13.98 μm, respectively and the digestive tract disappeared. In the late doliolaria tentacles became visible, the larvae stated swimming close to the substrate. The first pentactula appeared nine d post fertilization when the barrel shaped doliolaria pull out their five anterior tentacles and podia (Fig. 3f). The mean length and width of pentactula larvae were 408.1 ± 36.21 μm and 234.3 ± 15.1 μm respectively, at which point they started feeding on the predisposed panels. At 15 d post fertilization the juveniles became visible to the naked eye, measuring a mean size of 511.7 ± 24.7 μm, their bodies being covered with ossicles of varying shapes (Fig. 3g). At 30 d juveniles reached the size of 1044.3 ± 62.9 μm (Fig. 3h) with an average growth rate from 15 to 30 days of 0.062 mm day^{-1}.

After 60 d of culture, juveniles reached the mean size of 7.24 ± 1.6 mm (Fig. 4a) with an average growth rate from 30 to 60 days of 0.21 mm day^{-1}. From 60 to 90 days the average growth rate was 0.46 mm day^{-1}, reaching the mean size of 21 mm ± 8 mm (Fig. 4b). At this stage, the body color of the juveniles began to darken, their body size being variable, with some specimens considerably larger than others.

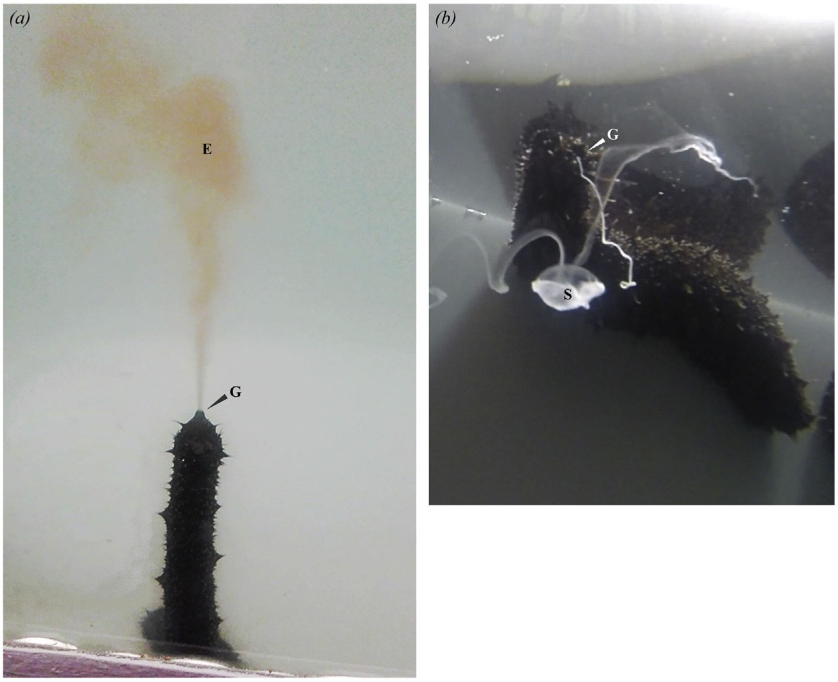

Fig. 1. Spawning of *Holothuria polii*. a) Female: gonopore (G), eggs (E). b) Male: gonopore (G), sperm (S).

3.3. Larval survival and feeding protocols

In the present study, 99% of deposited eggs of *H. polii* were mature. We observed a high proportion (about 95%) of successfully fertilized embryos that reached first cleavage, while 52% developed into the early auricularia stage. All three feeding regimes enabled the larvae to reach the doliolaria stage but with differences in larval growth, development and survival (Fig. 5).

Food administered at high concentration (20,000–40,000 cells mL^{-1}) resulted in the highest metamorphosis of larvae into juveniles. Under this feeding regime, 43% of larvae reached the late auricularia stage (82% of then displaying hyaline spheres), while 32% metamorphosed into doliolaria. We estimated that 14% of fertilized eggs metamorphosed into juveniles at day 15 and day 30, the survival rate was estimated to be 9%.

Food administered at an intermediate concentration (5000–10,000 cells mL^{-1}) resulted also in a good yield of metamorphosed larvae reaching the juvenile stages, although under this condition the development was circa two days slower than in high feeding regime. Under this feeding regime, 54% of larvae reached the late auricularia stage (76% displaying hyaline spheres), while 26% metamorphosed into doliolaria. We estimate that 12% of fertilized eggs metamorphosed into juveniles at day 15 and day 30; the survival rate was estimated to be 9%.

Food administered at low concentration (1.000–2.000 cells mL^{-1}) resulted in a moderate yield of metamorphosed larvae. The development was slower and a large portion of the larvae does not complete metamorphosis. Under this feeding regime, 48% of larvae reached the late auricularia stage (7% displaying hyaline spheres), while 11% metamorphosed into doliolaria. We estimate that 4% of fertilized eggs metamorphosed into juveniles at day 15 and day 30, the survival rate was estimated to be 2%.

Considering that some juveniles may have escaped the count by adhering to the panels' support, the survival rate of juveniles reported here might be a slight underestimate.

At day 30, all juveniles from the different conditions were transferred into 600 L tanks. Their survival rate was estimated to be 5.7% at day 60 and 3.2% at day 90 (Fig. 6).

4. Discussion

The commercially valuable sea cucumber *H. polii*, a high potential Mediterranean species for aquaculture, was successfully reared for the first time in this study, using a hatchery-based protocol. The development of *H. polii* was consistent with that of previously reared aspidochirotes (Asha and Muthiah, 2007; Battaglene, 1999; Domínguez-Godino et al., 2015; Rakaj et al., 2018; Ramofafia et al., 2003), nevertheless variations emerged regarding spawning response, female fecundity, egg size, larval development and larval feeding behaviour, when compared with the other congeneric species.

4.1. Spawning response

The ability to control spawning is a key step for large-scale seed production in a hatchery-based system in the process of adopting/introducing a novel species for aquaculture.

A variety of physical, chemical and biological methods have been used to induce spawning in sea cucumbers with different and often species-specific responses (Abdel Razek et al., 2012; Agudo, 2006; Battaglene et al., 2002; Domínguez-Godino et al., 2015; Hu et al., 2010, 2013; Kumara et al., 2013; Zacarías-Soto et al., 2013). Two common methods for spawning induction were tested on *H. polii*: mechanical shock and thermal shock. The mechanical shock treatment, despite having been effective for *Apostichopus japonicas*, *Bohadschia marmorata*, *Holothuria scabra* and *Stichopus horrens* (Al Rashdi et al., 2012; Hu et al., 2013; Laxminarayana, 2005; Renbo and Cheng, 2004) did not induce *H. polii* to spawn. Similarly, this treatment also proved to be unsuccessful for another Mediterranean species, *H. tubulosa* (Rakaj et al., 2018). Thermal shock, a widely used treatment to induce spawning in species such as *Actinopyga mauritiana*, *Australostichopus mollis*, *Holothuria arguinensis*, *Holothuria atra*, *Holothuria fuscogilva*, *Stychopus horrens* and *Holothuria scabra* (Battaglene et al., 2002; Domínguez-Godino et al., 2015; Hu et al., 2013; Laxminarayana, 2005; Morgan, 2009a; Ramofafia et al., 2003) proved to be highly effective for *H. polii*. By manipulating water temperature, we successfully induced spawning through a method that is both simple and convenient, so proving unnecessary the recourse to more complex methods employed for other

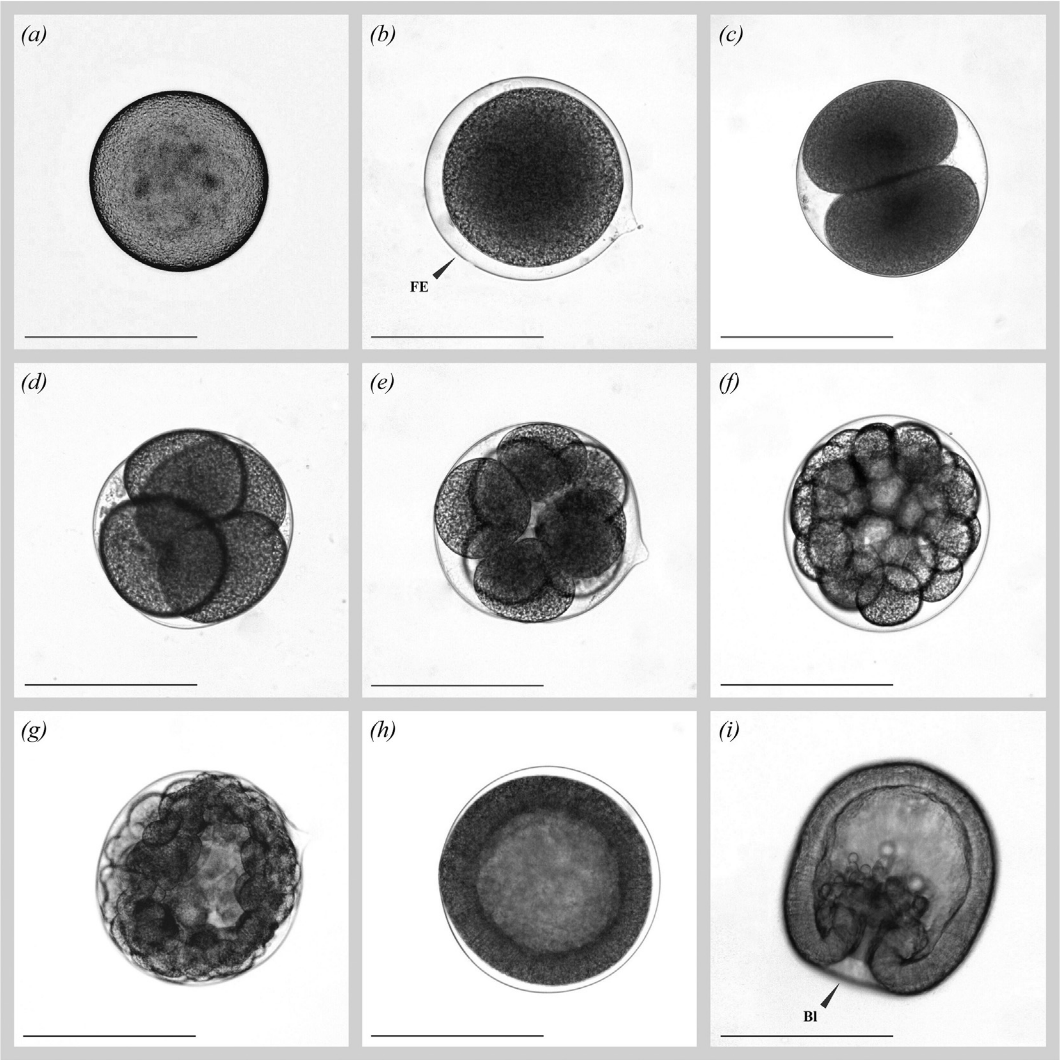

Fig. 2. Embryonic development of *Holothuria polii*. a) Unfertilized egg. b) Fertilized egg with a fertilization envelope (FE). c) First cleavage. d) Second cleavage. e) Third cleavage. f) Morula. g) Blastula h) Rotary blastula. i) Early gastrula with blastopore (Bl). Scale bars = 200 μm.

species. Spawning was successfully induced during the summer months in concomitance with the species gonad index peak.

As observed in many holothuroid species during spawning, *H. polii* seek the highest positions in their surroundings, rising the anterior end of the body from the substrate in order to maximize the gametes dispersion (Battaglene et al., 2002; McEuen, 1988). It is possible to distinguish sexes only once they start spawning; the males start first, releasing a thin flow of sperm that potentially stimulates egg maturation and subsequent spawning in females.

4.2. Reproductive strategy

The allocation of reproductive energy into a large versus small number of offspring and a large versus the small size of offspring, is an important life-history strategy for any species (Stearns, 1976). Harriot (1985) found heterogeneous reproductive strategies when comparing tree tropical sea cucumbers of the same genus: *H. atra* with a large number of small eggs, *H. impatiens* with a small number of large eggs and *H. edulis* with an intermediate number of medium-sized eggs.

In this study, *H. polii* egg size (203.7 ± 10.21 μm) turned out to be considerably larger than that reported for other congeneric species (Table 2) as *H. spinifera* 143.59 ± 22.83 μm (Asha and Muthiah, 2002), *H. scabra* 156.4 ± 2.9 μm (Ramofafia et al., 2003), *H. arguinensis* 170.33 ± 10.51 μm (Domínguez-Godino et al., 2015), *H. mammata* 142.54 ± 7.35 μm (Domínguez-Godino and González-Wangüemert, 2018), *H. tubulosa* 151.2 ± 1.7 μm (Rakaj et al., 2018), *H. fuscogilva* 151.7 ± 5.2 μm (Ramofafia et al., 2003) and *H. leucospilota* 140.80 ± 0.84 μm (Huang et al., 2018).

The average number of eggs produced per *H. polii* female (or species fecundity) of 0.176 million ± 0.015 (*n* = 34; ranging from 48.000 to 655.000) instead proved to be significantly lower than that observed in the case of *H. arguinensis* (1.5–9.6 million per female) (Domínguez-Godino et al., 2015), *H. mammata* (0.011–6.95 million per female) (Domínguez-Godino and González-Wangüemert, 2018), *H. tubulosa* (3.48 ± 1.41 million; *n* = 12) (Rakaj et al., 2018), *H. scabra* (1.9 ± 0.6 million; *n* = 17) (Battaglene et al., 2002), *H. fuscogilva* (2.6 ± 0.2 million; *n* = 5) (Ramofafia et al., 2003) and *H. leucospilota* (1.30 ± 0.47 million; n = 5) (Huang et al., 2018). These features support the hypothesized inverse relationship between the number of eggs and egg size, so indicating an allocation strategy of the reproductive effort for *H. polii* toward reducing egg number and increasing egg size.

4.3. Larval development

For most benthic marine species with complex life cycles, the larval stage is the dominant dispersal stage, this being directly correlated to the temporal extension of the larval phase (Cowen and Sponaugle,

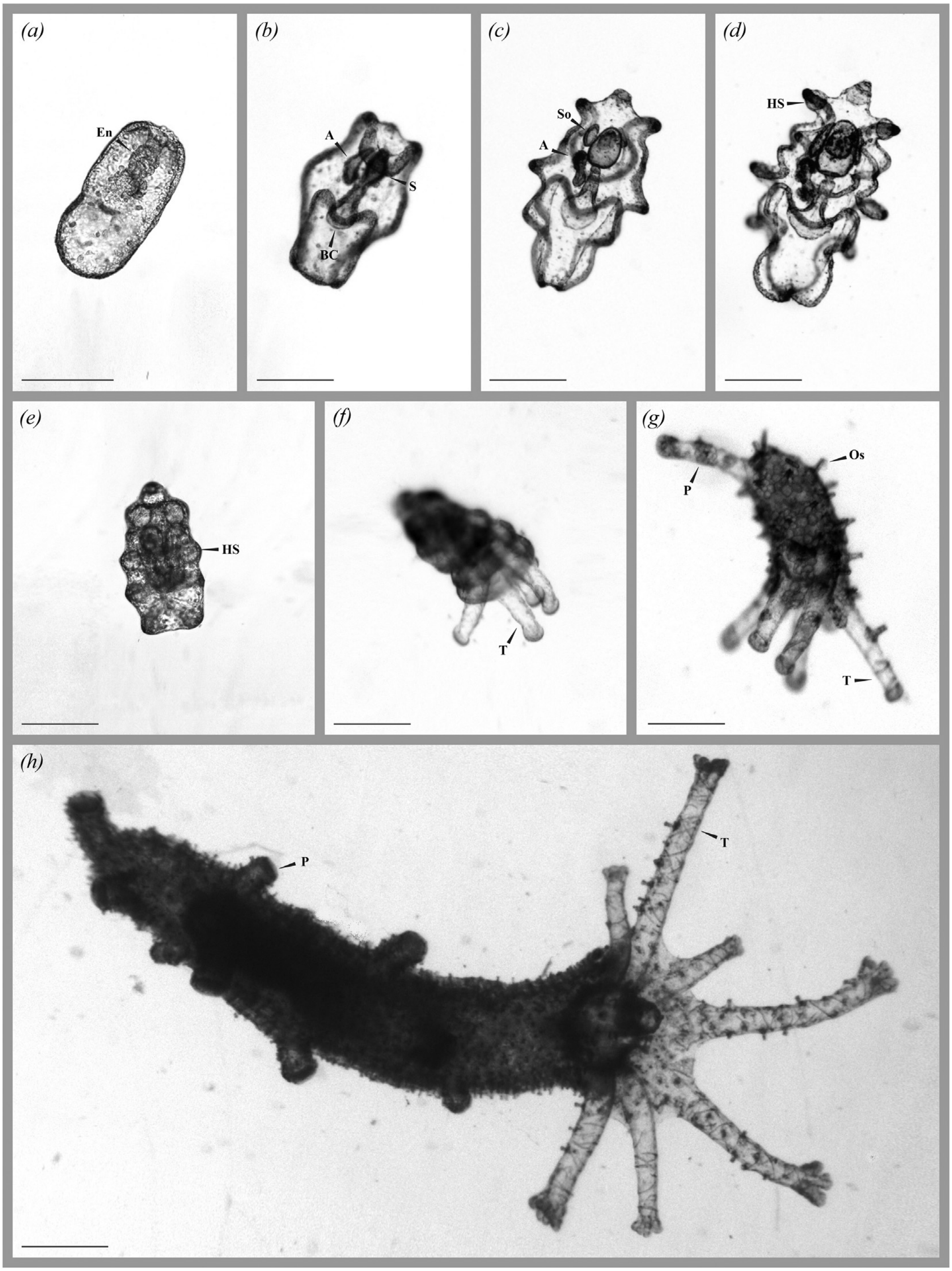

Fig. 3. Larval development of *Holothuria polii.* a) Late gastrula: enterocoeles are forming (En). b) Early auricularia with widely developed axohydrocoel (A), buccal cavity (BC), oesophagus (O), stomach (S). c) Mid auricularia: axohydrocoel (A) and somatocoele (SO). d) Late auricularia and hyaline spheres (HS). e) Doliolaria with hyaline spheres (HS). f) Pentactula with tentacles (T). g) 15 d juvenile: podia (P), ossicle (Os) and tentacles (T). h) 30 d juvenile: Scale bars = 200 μm.

2009).

In *H. polii* the duration of the larval stages differed from other species (Agudo, 2006; Asha and Muthiah, 2002; Battaglene et al., 2002; Domínguez-Godino et al., 2015; Ramofafia et al., 2003; Ramofafia et al., 2000). The development period to reach the doliolaria stage (eight d) in this species is shorter than that observed in many other reared holothurians such as *Holothuria atra* (20 d) (Laxminarayana, 2005), *Holothuria scabra* (14 d) (Agudo, 2006) and *Holothuria arguinensis* (12 d) (Domínguez-Godino et al., 2015) while it is still shorter than that observed in *H. tubulosa* (24 d) (Rakaj et al., 2018). A similar development period (10 d) has been reported only for *H. spinifera* (Asha and Muthiah, 2002), although this species was reared at a significantly higher temperature (29–31 °C) than *H. polii* (24 °C), higher temperatures being known to accelerate larval development (Asha, 2004; Hamel and Mercier, 1996).

Vance (1973) points out that development time is inversely proportional to egg size in marine benthic invertebrates. This reproductive strategy matches what we observed in *H. polii,* were the development time appear to be the shortest, among the species artificially reared belonging to the genus *Holothuria* and, at the same time, the egg size the

Table 1
Embryonic and larval development of *H. polii* with size (mean ± SE; n = 30) at 24 °C and high feeding condition.

Stage	Time (min, h, d)	Size (μm)
Unfertilized egg	–	203.7 ± 10.21
Fertilization envelope	0–5 min	247.46 ± 15.48
2-cell division	90 min	–
4-cell division	120 min	–
8-cell division	150 min	–
16-cell division	240 min	–
Morula	6 h	–
Blastula	12 h	250.03 ± 12.20
Early gastrula	20 h	246.25 ± 9.19
Late gastrula	40 h	330.73 ± 28.34
Early auricularia	3 d	513.8 ± 30.03
Mid auricularia	4 d	623.42 ± 49.04
Late auricularia	6–7 d	704.8 ± 42.42
Doliolaria	8–9 d	375.07 ± 28.37
Pentactula	9–10 d	408.1 ± 36.2
Juvenile	15 d	511.7 ± 24.7
Juvenile	30 d	1044 ± 62.9
Juvenile	60 d	7240 ± 1600
Juvenile	90 d	21,012 ± 8173

h: hours; min: minutes; d: days.

largest among them.

In *H. polii*, during the early auricularia stage, the axohydrocoel developed prematurely. This structure appearance was observed taking place only prior to larval metamorphosis in species such as *H. tubulosa*, *H. arguinensis* and *H. mammata* (Domínguez-Godino et al., 2015; Domínguez-Godino and González-Wangüemert, 2018; Rakaj et al., 2018). The precocious appearance of this trait in *H. polii,* commonly related to the development of the buccal podia in the metamorphosed juveniles (Balser et al., 1993), might also reflect its rapid larval development as a life history strategy.

4.4. Larval feeding and Hyaline Spheres

Food availability plays a key role in larval development and survival (Thorson, 1950). Growth and survival data are of paramount importance in understanding the effects of artificial diet when proposing for aquaculture of novel species with still unknown foraging behaviour. Hence, experiments under the diverse feeding regimes were conducted with the larvae of *H. polii,* the aim being to understand the larval development and feeding behaviour in this species.

Algal concentrations of 2–4 × 10^4 cells mL^{-1} were indicated as optimal in larval rearing of *S. japonicus*, *H. spinifera*, *H. scabra,* and *H. atra* (Asha and Muthiah, 2002; Asha, 2004; James et al., 1994; Laxminarayana, 2005). Nevertheless, Archer (1996) stated that under high feeding concentration the larval ingestion rate of *A. mollis* declines. Morgan (2001) observed that *H. scabra* larvae at high algal concentration inhibited larval development, while Rakaj et al. (2018) observed larval degeneration under high algal concentrations in *H. tubulosa*. Duy et al. (2016) recently, demonstrated a relationship between algal diet, presence and size of hyaline spheres and final settlement yields in terms of the early juvenile in *H. scabra*. In line with this, several authors have suggested that hyaline spheres (HS) could play a role in energy storage during larval development (Chen et al., 1991; Dautov and Kashenko, 1995; Mortensen, 1938; Ramofafia et al., 2003).

In the present study, the settlement rate into juveniles for *H. polii* was more or less similar for algal concentrations of 2–4 × 10^4 and 0.5–1 × 10^4 in cells mL^{-1}. However, under 0.5–1 × 10^4 cells mL^{-1} the metamorphosis lasted two days more (10 d), the occurrence of hyaline spheres showing similar average percentages. The low feeding concentration of 0.1–0.2 × 10^4 cells mL^{-1} resulted in a low number of larvae metamorphosing into juveniles. A substantial proportion (98%) of larvae did not develop HS during late auricularia stage, while the remaining part, which developed HS in this condition, were smaller than those observed under the higher feeding regimes and developed only in the anterior part of the larvae. In *H. polii* HS development proved to be also influenced by the feeding concentration on the larval cultures, while there was a positive correlation between HS formation and feeding concentration among the different conditions as suggested for other sea cucumbers (Duy et al., 2016; Morgan, 2009b; Morgan, 2008; Ramofafia et al., 2003). The above results lead us to conclude that high and intermediate algal concentration are both good feeding regimes; however the high regime of 2–4 × 10^4 cells mL^{-1} should be considered the optimal feeding concentration for larval rearing of *H. polii*.

4.5. Juvenile culture

Our results highlight that Algamac and dry *Spirulina,* under the administered rate, is an appropriate food for the first juvenile rearing until day 90. The attained size of 21 mm in only three months is comparable to that recorded for *A. japonicus* and *H. spinifera* (Asha and Muthiah, 2007; Ito and Kitamura, 1998). This confirms that these feeding integrators can be used successfully to replace live micro-algae during hatchery culture of *H. polii* juveniles.

To date, rearing protocols have only been developed for three European sea cucumbers species (Domínguez-Godino et al., 2015; Domínguez-Godino and González-Wangüemert, 2018; Rakaj et al.,

Fig. 4. a) 60 d juvenile. b) 90 d juvenile. Scale in cm.

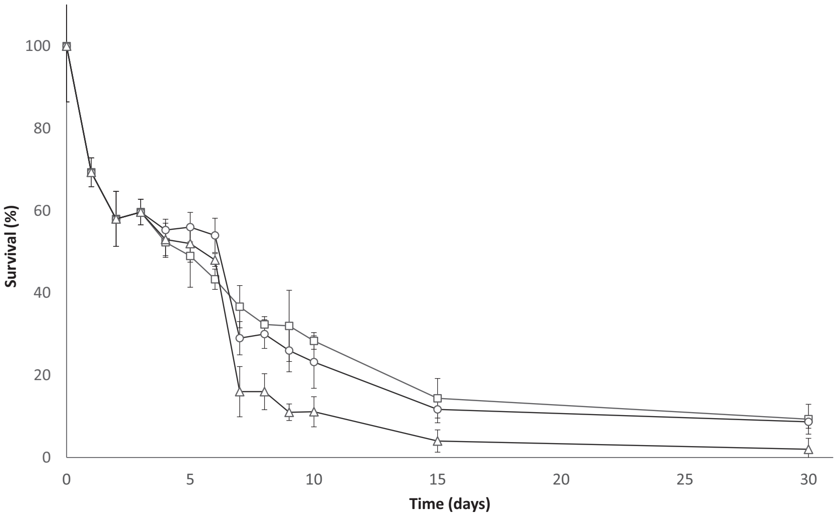

Fig. 5. Survival of larval and juvenile stages of *Holothuria polii* among the three feeding conditions. High algal concentration (squares) 20,000–40,000 cells mL^{-1}; intermediate (circles) 5000–10,000 cells mL^{-1} and low algal concentration (triangles) 1000–2000 cells mL^{-1}; (mean ± SE; n = 3).

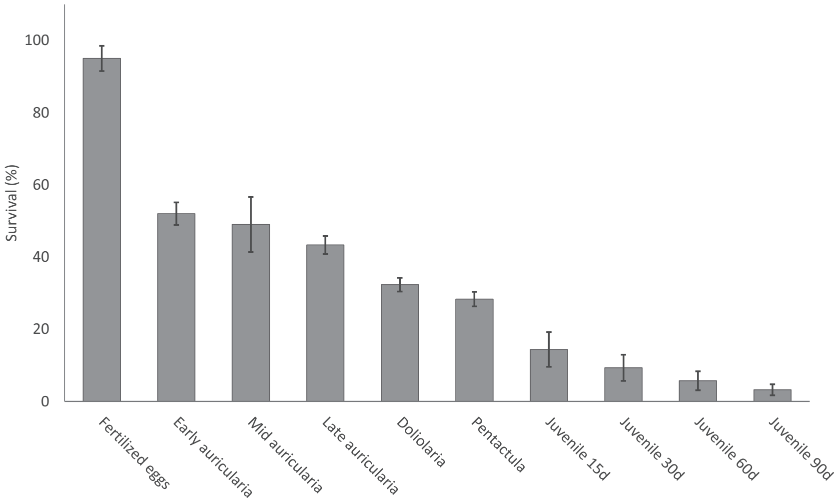

Fig. 6. Survival rates of *Holothuria polii* larvae and juveniles during 90 days of rearing under the best feeding condition.

Table 2
Comparison of female fecundity, egg size and development time between *H. polii* and the other artificially reared species belonging to the genus *Holothuria*.

Species	Female fecundity (eggs × female)	Egg size (μm)	Metamorphosis time (days)	References
H. polii	176,000 ± 15,000 (n = 34) 48,000–655,000	203.7 ± 10.21 μm	8–9	
H. tubulosa	3,480,000 ± 1,410,000 (n = 12) 400,000 - 12,000,000	151.2 ± 1.7 μm	24	Rakaj et al., 2018
H. arguinensis	– 1,500,000 - 9,600,000	170.33 ± 10.51 μm	12–13	Domínguez-Godino et al., 2015
H. mammata	– 11,000 - 6,950,000	142.54 ± 7.35 μm	17–18	Domínguez-Godino and González-Wangüemert, 2018
H. scabra	1,900,000 ± 600,000 (n = 17) 9,207,000–17,303,000	156.4 ± 2.9 μm	12–13	Ramofafia et al., 2003 Conand, 1993
H. atra	800,000 (n = 1) –	88 μm	15	Laxminarayana, 2005 Harriot, 1985
H. spinifera	– –	143.59 ± 22.83 μm	10	Asha and Muthiah, 2002
H. fuscogilva	2,600,000 ± 100,000 (n = 6) 6,387,000–14,210,000	151.7 ± 5.2 μm	–	Battaglene et al., 2002 Conand, 1993
H. leucospilota	1,300,000 ± 470,000 (n = 5) –	140.80 ± 0.84 μm	17–20	Huang et al., 2018

2018). Furthermore, to our knowledge, this is the first study to describe the development of *H. polii* as a novel candidate in aquaculture.

5. Conclusions

The production of marine invertebrates for direct human consumption is playing a growing role globally. In this scenario, the implementation of a reliable protocol for the aquaculture of Mediterranean Sea cucumber is a desirable goal. The availability of farmed holothurians could become a solution for reducing the exploitation and IUU impact on wild populations. Furthermore, the use of cultured juveniles could also be of crucial relevance for restocking programs.

The present study shows that *H. polii* can be reared successfully up to the juvenile stage, producing healthy gametes in captivity with high settlement yields and very rapid larval development if compared to congeneric species. Feeding investigations conducted in this study have helped to identify some key traits, thus providing a basis to support future development of hatchery production. All these features make this species an excellent candidate for aquaculture.

Further research is needed to evaluate the best settlement substrate for large-scale juvenile production, the optimal stocking density and this species' ability in IMTA.

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