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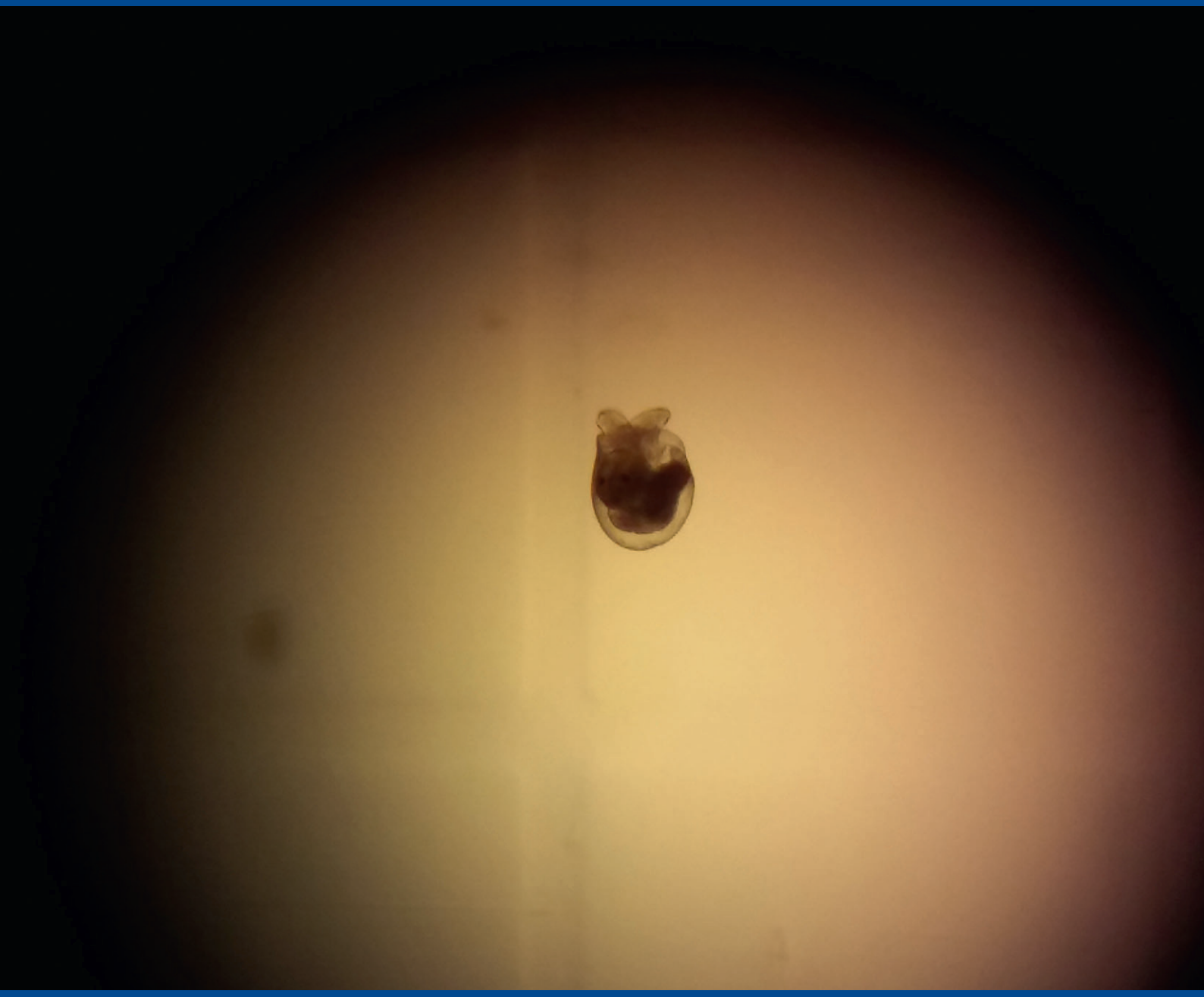

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ORIGINAL ARTICLE

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Artificial reproduction protocol, from spawning to metamorphosis, through noninvasive methods in *Patella caerulea* Linnaeus, 1758

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Abstract

Controlled reproduction is a requirement for developing effective mollusc cultivation for commercial or restoration purposes. In this study, a protocol for spawning induction using noninvasive methods in limpets was developed, using the common Mediterranean species, *Patella caerulea* Linnaeus, 1758. Six nonlethal spawning induction treatments were tested: three chemical (two concentrations of H_2O_2 and KCl) and three physical (bubbling, warm and cold thermal shock). All treatments, except thermal shocks, induced the spawning of fertile gametes. Bubbling resulted the best treatment in providing spawning response, being the easiest and least invasive method tested. After eggs fertilization, larval development was followed until metamorphosis, testing fed and unfed conditions. Settlement took place after 7 days. The developed protocol represents a benchmark for further application to other limpets, for aquaculture or repopulation.

KEYWORDS
artificial reproduction, limpet aquaculture, *Patella caerulea*, spawning induction

1 | INTRODUCTION

Limpets (Patellogastropoda) and abalones (Vetigastropoda) are conspicuous gastropod species of the midlittoral and shallow sublittoral zone, where play a key role through their grazing activity. These species are also appreciated as culinary delicacy and harvested worldwide, as food and as bite for fishery.

Harvesting has lead to the overexploitation of several populations. As an example, in the Hawaii, three endemic limpets belonging to the *Cellana* genus (*C. exarata*, *C. sandwicensis* and *C. talcosa*), locally known as "opihi", were overharvested for centuries, causing a decline in harvested biomass of about 90% during the XX century, fostering the interest to develop culture techniques for these species (Corpuz, 1981; Kay & Magruder, 1977). Also in coastal regions of North America (Californian coast), harvesting of the owl limpet, *Lottia gigantea*, caused a reduction in population abundance and shell size (maximum and mean size, Erlandson et al., 2011).

As far as the *Patella* genus, in the Azores archipelago, two species (*P. aspera* and *P. candei*) were overharvested and experienced drastic reductions. For this reason, a specific Regional Regulatory Decree (RRD 14/93/A) was issued to create *Limpet protected zones* (LPZ), where harvesting is prohibited (Martins et al., 2011; Martins, Jenkins, Hawkins, Neto, & Thompson, 2008). Also along the Mediterranean coasts, *Patella* spp. harvesting occurs for food supply, as a bite for fishery or shell collection. *Patella ferruginea*, the largest Mediterranean species, collected as the Pleistocene (Espinosa, Rivera-Ingraham, & García-Gómez, 2009), is considered the most threatened marine macro-invertebrate in the western Mediterranean (Ramos, 1998) and is included in several directives at European and country levels (MMAMRM, 2008; Directive 92/43 CEE "Habitat," Annex IV; Berna Convention Annex 2; Barcelona Convention - Annex 2) and has been proposed by the Italian Marine strategy (d.lgs. n. 190, 13th October

2010) as a target species to define a Good Environmental Status of a specific location).

Introduction of mollusc species on a commercial production level is largely determined by its seed production. While the spawning of abalone has become routine and breeding and rearing protocols are well established (Mau & Jha, 2018), allowing an overall production from aquaculture of 140,614 tons (FAO, 2018a) compared to only 7,227 tons from harvesting (FAO, 2018b) in 2015, the sustainable aquaculture of limpets still requires successful spawning and larval rearing techniques.

In this context, we approached controlled reproduction of the common Mediterranean species, *P. caerulea*, to develop a benchmark protocol for spawning induction using noninvasive methods in limpets. The choice for totally noninvasive approaches was made to make the protocol suitable also for endangered species for repopulation and restocking purposes.

Patella caerulea is present in the lower part of the intertidal and in the upper infralittoral zones (Della Santina, Sonni, Sartoni, & Chelazzi, 1993; Mauro, Arculeo, & Parrinello, 2003), is protandrous hermaphrodite (Bacci, 1947; Belkhodja, Jaafoura, Missaoui, & Romdhane, 2011) and is considered a predominantly winter breeder. The period of sexual maturity along Naples coast (Southern Italy) extends from the beginning of November to May (Bacci, 1947). In a different manner, in Algeria, spawning occurs from March to June (Frenkiel, 1975). In the Northern Adriatic Sea, breeding season of *P. caerulea* extends from mid-December to September (Wanninger et al., 1999). These evidences support the potential for this species to reproduce along the whole year in controlled conditions.

Little information is reported in literature regarding reproduction in controlled conditions of *Patella* spp. Artificial reproduction has been carried out for *Patella* spp. only by way of gametes obtained after gonad dissection, or at least there is no clear indication of spawning inducement (*Patella* spp.: Patten, 1885; Patten, 1886; *P. caerulea* and *P. vulgata*: Dodd, 1955; Wanninger et al., 1999; *P. vulgata* and *P. ulyssiponensis*: Hodgson, Le Quesne, Hawkins, & Bishop, 2007; *P. ferruginea*: Espinosa, Rivera-Ingraham, & García-Gómez, 2010; Guallart, Peña, Pérez-Larruscaín, Calvo, & Acevedo, 2013) or through nonlethal biopsy (*P. ferruginea*: Guallart, Calvo, Acevedo, & Templado, 2013; Guallart, Peña, et al., 2013).

Guallart, Peña, et al. (2013) performed artificial spawning trials using noninvasive methods on *Patella* spp. but without any evidence of success. These methods can be schematically divided into chemical (e.g., hydrogen peroxide and potassium chloride) and physical (e.g., thermal shocking and bubbling) treatments. Physical treatments that simulate natural triggers for inducing spawning (temperature changes and/or rough sea conditions) were considered possible good candidates as the spawning of genus *Patella* is apparently positively affected by wave mechanical shock, during rough sea events (Dodd, 1955; Orton, Southward, & Dodd, 1956).

Second step in the design of an efficient culturing protocol is represented by egg fertilization. For *Patella* spp., fertilization of eggs in literature has been carried out in different ways to get the higher fertilization rate; different authors pointed out that alkalinization of eggs before contact with sperm (Espinosa et al., 2010; Hodgson et al., 2007; Wanninger et al., 1999) and sperm concentration (Dodd, 1955; Espinosa et al., 2010; Hodgson et al., 2007; Wanninger et al., 1999) were important factors controlling fertilization rate and larval abnormalities occurrence.

Following fertilization, larvae need to be reared until metamorphosis. Only two studies followed larval development until metamorphosis in *P. caerulea* and *P. vulgate* (Dodd, 1955; Wanninger et al., 1999). Early stages were described and metamorphosis was reported to be completed in 170 hr postfertilization (hpf; Wanninger et al., 1999). As far as larval feeding, very scarce and discordant information is available in literature: feeding with diatoms after 4 days (Dodd, 1955) or no feed until metamorphosis (Wanninger et al., 1999).

Final step for an artificial reproduction protocol is represented by settlement and metamorphosis into the juvenile stage, where a key role is played by eventual substrate preferences and/or adult cue. In fact, the addition of substrate from the aquarium where adults were cultured or of adult specimens in the settlement tanks (Wanninger et al., 1999) or the use of a film of macroalgae germlings (*Ulva linza*, *Ulva lactuca* and *Cylosiphon* sp.; Dodd, 1955) have been reported to increase the metamorphic rate.

In this study, we developed a complete artificial reproduction protocol for *P. caerulea*. In particular, we tested different noninvasive methods applied to commercial species for inducing gamete release: (a) vigorous bubbling (*Cellana exarata*: Corpuz, 1981; *Lottia* spp.: Kay & Emlet, 2002); (b) hydrogen peroxide (*Haliotis* spp.: Morse, Duncan, Hooker, & Morse, 1977; *Lottia* spp.: Kay & Emlet, 2002; *Mytilus galloprovincialis*: Turolla, Castaldelli, Barbin, & Rossi, 2002); (c) thermal shocking (*Lottia* spp.: Kay & Emlet, 2002); (d) potassium chloride (*M. galloprovincialis*: Turolla et al., 2002). We decided to test all these four different treatments in order to assess their effectiveness and potentially to select the least invasive, both for the adults and the offspring.

Embryos were then followed until metamorphosis testing fed and unfed conditions, larvae were induced to metamorphose and juvenile growth was followed for around 40 days after fertilization.

2 | MATERIAL AND METHODS

Two successive spawning trials were set up, each with 30 specimens of *Patella caerulea* collected along the Ligurian coast, near Genoa (length: 24.6 ± 5.1 mm, width: 19.6 ± 4.4 mm) and transferred to the tanks of the marine laboratory of Camogli (Genoa, Italy). Herein, they were maintained for acclimation purposes for at least 2 weeks, in a recirculation system, where water was changed twice a week. In both trials, for all the experimental steps (maintenance, spawning, larval rearing), ambient water temperature was 14°C (±1) and natural photoperiod was used (sun light).

2.1 | Spawning induction

Spawning trials were performed, respectively, on 6th (trial 1) and 20th March (trial 2) 2017. Six treatments (5 limpets/treatment) were

tested: physical (Bubbling, Warm and Cold thermal shock), chemical (Oxygen peroxide 6%, Oxygen peroxide 10% and Potassium chloride 0.2%).

After been gently detached from the aquarium, the limpets were packed for 1 hr with a seawater wet cotton gauze at room temperature (about 18–20°C), as suggested by Morse et al. (1977) and each group of limpets was exposed to one of the following treatments:

1. Bubbling treatment: The limpets were placed in plastic baskets, in filtered seawater (FSW) at ambient temperature. Seawater was vigorously bubbled for 2 hr, using standard aquarium air stones positioned below the baskets (Kay & Emlet, 2002).
2. Thermal Shock: The limpets were located in bowls in FSW. Water temperature was increased (placing the bowls into a warm bath) or decreased (using ice coolers) of about 3–5°C compared to the acclimation temperature. The treatment lasted 2 hr (Kay & Emlet, 2002).
3. Hydrogen peroxide (H_2O_2) treatment: Following Morse et al. (1977), specimens were placed in bowls with FSW with aeration and enough water to cover the specimens. In each spawning tank, 6.6 ml of 2 M Tris for each litre of filtered seawater were added. After 15 min, 4 ml of freshly prepared 6% or 10% hydrogen peroxide solution for each litre of water were additionally added and the water was mixed. The treatment lasted 2.5 hr.
4. Potassium chloride (KCl) treatment: As in the Hydrogen peroxide treatment, the limpets were placed in bowls with FSW with aeration and enough water to cover the specimens and 6.6 ml of 2 M Tris for each litre of filtered sea water were added. After 15 min, 2 g/L of KCl 0.2% were added. The treatment lasted 4 hr.

After being exposed to the induction treatments, specimens were rinsed and placed in bowls filled with clean FSW and observed for several hours to detect spawning. In case of spawning, the number of gametes released was estimated and fertilization rate was assessed. In the next days, adult survival rate was recorded.

2.2 | Larval rearing (fertilization, larval development, metamorphosis and settlement)

Eggs and sperm from the different spawning trials were mixed, because it was not always possible to have both male and female gametes from the same spawning induction treatment.

Egg fertilization is related to the disappearance of the chorion around the egg (Frenkiel, 1975). As egg fertilization may be affected by the alkalinization of the eggs and by sperm concentration, we tried different fertilization conditions.

As far as alkalinization, we tested the addition of Tris or NH_4OH for removing the chorion, but no need was observed, probably because when we performed the experiments the specimens were ripe (late in the natural spawning season) and consequently the oocytes were fully mature.

To test sperm concentration effects, we tested sperm concentration reported in literature (between 10^6–10^8 cells/ml; Hodgson et al., 2007—*P. vulgata* and *P. ulyssiponensis*) with an eggs exposure time to sperm of about 30 min. However, using these concentrations, we observed polyspermy affecting larval development. As a consequence, lower concentrations were tested (10^6–10^7 cells/ml) and fertilization solution was continuously mechanically stirred. Fertilization volumes and eggs concentrations were 750 ml, 111 eggs/ml in trial 1 (sperm/eggs ratio around 10^3) and 500 ml, 35 eggs/ml in trial 2 (sperm/eggs ratio around 10^2). Following fertilization, to remove the sperm in excess, the solution was filtered using a 120 μm mesh sieve (considering average ripe eggs diameter of about 135 μm, according to Dodd (1955) and Authors personal measurement) and eggs were re-suspended in FSW and fertilization rate was assessed.

Larvae were maintained in 600 ml FSW beakers, gently paddled. For only the second trial, every day the number and stage of larval development were monitored (counting larvae in 1 ml/beaker, 3 replicates per beaker: Table 1). Water in the larval beakers was changed three times a week by filtering larvae through a 120 μm mesh sieve and re-suspending them in filtered seawater.

In order to test the need for feeding the larvae, we applied fed and unfed regimes to two different subsets of beakers. The fed set of trochophore larvae was supplied with a mix of *Dunaliella tertiolecta* and *Tetraselmis suecica* (500 algal cells per larva) from 2 days postfertilization (dpf).

At 4 dpf, a subset of veliger larvae was moved in four small bowls containing an adult, as settlement cue. In addition, in two of these bowls, *Amphora* sp., a diatom species well known as a potential settlement inducer in invertebrate larvae (Daume, Krsinich, Farrell, & Gervis, 2000; Hannon, Officer, & Chamberlain, 2017) was provided, as an additional settlement cue. Length and width of spats were measured between 25 and 34 days postfertilization, to assess the growth rate of juveniles.

3 | RESULTS

Survival rate of adults, subjected to the different spawning induction treatments, was monitored for 4 days post-treatment for the first trial and for 14 days in the second one; in both case 100% survival was obtained.

3.1 | Spawning induction

During the first test, gametes release occurred in three treatments: Bubbling, H_2O_2 10% and KCl 0.2%. In the second one, spawning induction occurred in the Bubbling and in the H_2O_2 6% treatments (Figure 1).

The treatment that performed better was the Bubbling one: 40% of the treated specimens released gametes, followed by KCl and H_2O_2 10% (20% of the specimens) and H_2O_2 6% (10% of the specimens).

TABLE 1 Density of the different larval stages (Average/ml ± *SE*) of *Patella caerulea* over the 168 hr postfertilization: comparison between fed and unfed trials

hpf	Eggs	Trochophore	Veliger	Pediveliger	Total
Fed					
18	3.0 ± 0.6	7.5 ± 1.8			10.5 ± 2.4
42		1.3 ± 0.3	9 ± 1.5		10.3 ± 1.9
96			9.0 ± 0.6		9.0 ± 0.6
168				10.0 ± 2.1	10.0 ± 2.1
Unfed					
18	5.0 ± 1.0	8.0 ± 1.6			13 ± 2.6
42		1.3 ± 0.9	9.3 ± 2.4		10.7 ± 3.3
96			9.0 ± 0.6		9.0 ± 0.6
168				6.7 ± 2.0	6.7 ± 2.0

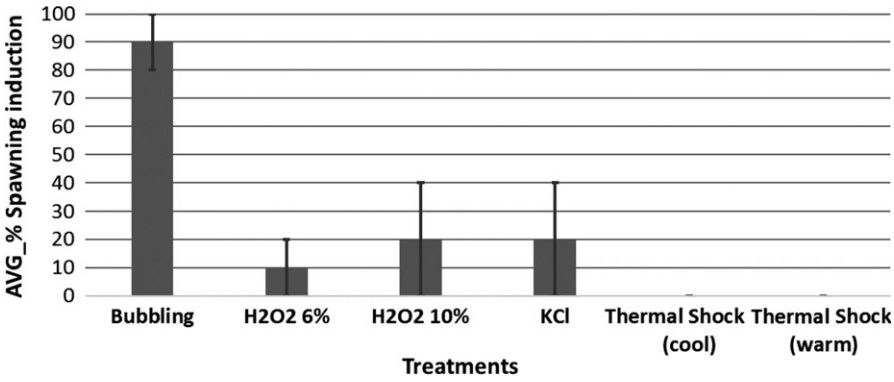

FIGURE 1 Spawning induction treatments: percentage of success per each treatments across the two trials (avg ± *SE*)

3.2 | Larval rearing (larval development, metamorphosis and settlement)

First cleavage displaying polar body (evidence of fertilization), was observed in both trials from 2–3 hpf (hours postfertilization).

In the first trial, a slower development was observed: the trochophore stage was observed from 22–24 hpf and lasted until 72 hpf, when the test was stopped because of ciliate contamination.

In the second trial, instead, we observed larval development consisting of a trochophore stage (18 hpf), veliger (42 hpf) and pediveliger (168 hpf), until the settlement of juveniles (Table 1; Figure 2). Seven days postfertilization, larvae looked ready to metamorphose and a subset of larvae was moved from beakers to bowls with adults and small epiphytes substrates. Preliminary settlement has been observed after 7 days.

No differences in larval development and in survival rate were observed, suggesting that there is no need to feed larvae (i.e., larvae are lecithotrophic). However, we observed a difference in survival rate at 168 hpf between fed (55%) and unfed (37%) pediveligers (Figure 3).

Spats have been measured over 9 days, recording length and width. Growth rate in terms of length was estimated to be on average 63 μm/day and in terms of width 41 μm/day. After 34 days postfertilization, spats were on average 608 μm in Length and 448 μm in Width (Figure 4).

Both Length and Width growth followed a linear trend, with length growing faster than width, causing the change in shape of the tiny juvenile, which shifts from a round to an oval shape during growth.

4 | DISCUSSION

4.1 | Spawning induction

The main result of the present study is represented by the first successful induction of spawning in *Patella* genus using noninvasive techniques. The method that provided the larger success was the vigorous bubbling, as applied in other limpets, belonging to the genus *Cellana* (Corpuz, 1981) and *Lottia* (Kay & Emlet, 2002). So far, the few successful artificial fertilization experiments were performed through adult dissection (*Patella* spp.: Patten, 1885; Patten, 1886; *P. caerulea* and *P. vulgata*: Dodd, 1955; Wanninger et al., 1999; *P. vulgata* and *P. ulyssiponensis*: Hodgson et al., 2007; *P. ferruginea*: Espinosa et al., 2010; Guallart, Peña, et al., 2013; *Cellana exarata*: Corpuz, 1981) or biopsy (*P. ferruginea*: Guallart, Calvo, et al., 2013, Guallart, Peña, et al., 2013).

To have a successful induced spawning, it is necessary that the specimens are acclimated and ripe. An appropriate acclimation period (at least 2 weeks) to the artificial environment is recommended before performing the trials (Authors' personal observation).

Previous trials performed in autumn and winter 2016–2017, using exactly the same approach were nonsuccessful, until March. This observation contrasts with the larger reproduction period for this species in the Mediterranean Sea that is reported to span from

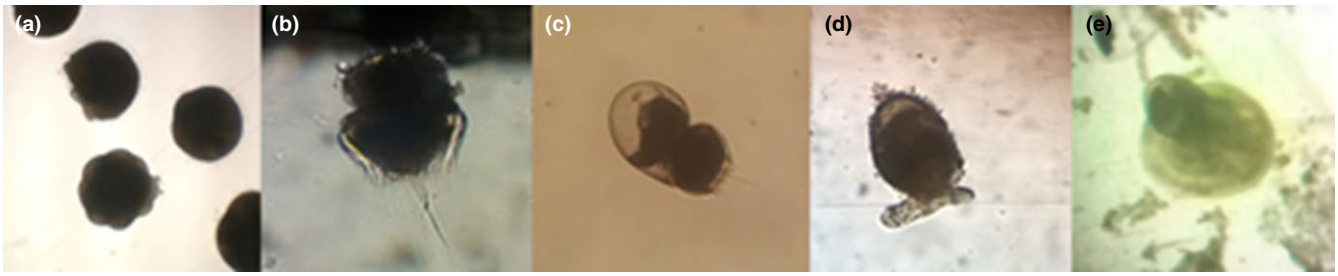

FIGURE 2 *Patella caerulea* larval development: (a) fertilized eggs and morula; (b) Trochophore, 18 hpf; (c) Veliger, 42 hpf; (d): Pediveliger, 168 hpf; (e) settled spat, over 168 hpf [Colour figure can be viewed at wileyonlinelibrary.com]

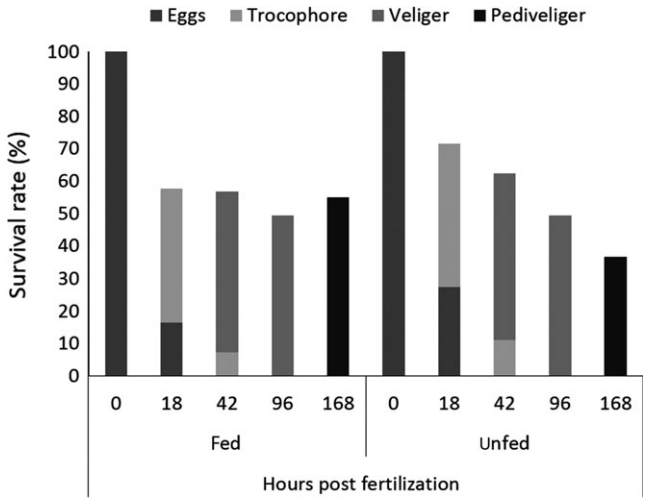

FIGURE 3 Survival rate and larval development in *Patella caerulea* over 168 hr postfertilization, comparison between fed and unfed trials

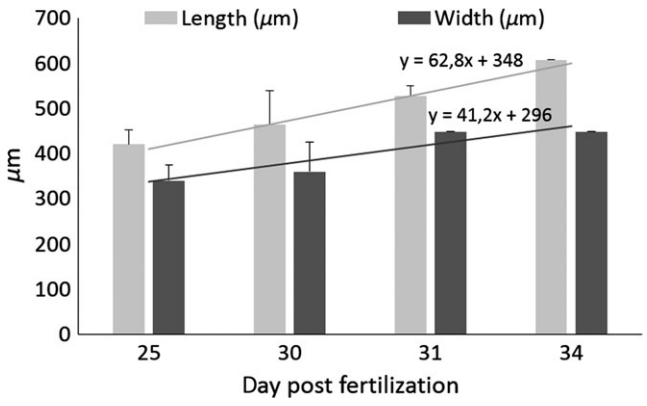

FIGURE 4 Growth of *Patella caerulea* spats in terms of Length and Width at around 30 days postfertilization

autumn to spring (Bacci, 1947; Frenkiel, 1975; Wanninger et al., 1999). To increase our knowledge on the effective reproductive potential of the species, it would be necessary to perform spawning induction trials during spring and summer seasons and to formally test the relevance of the acclimation period. All this information could strongly enhance the feasibility of *Patella caerulea* breeding along the year, with relevant fallouts for other species, for commercial or repopulation purposes.

4.2 | Larval rearing (fertilization, larval development, metamorphosis and settlement)

Different factors may affect fertilization success, mainly polyspermy and fertilization medium pH. From the trials performed on *P. caerulea* we did not experience polyspermy (at sperm concentration 10^6 cell/ml for 30 min exposure) nor found any need to increase fertilization medium pH (e.g., by way of Tris or NH_4OH), as reported by other authors (Dodd, 1955; Espinosa et al., 2010), if spawning is induced when the gametes are actually ready for spontaneous emission.

The complete larval development takes around 170 hr at the tested temperature (14°C). This finding is consistent with Wanninger et al. (1999) results. Larval feeding was not necessary during early larval development and this result is in agreement with Wanninger et al. (1999). However, a difference in mortality at 168 hpf has been observed between fed and unfed trials. These evidences support the conclusion that larvae need to be fed when the pediveliger stage is reached (at about 5 or 6 dpf), providing an algal biofilm, partially supporting previous results that suggest to start feeding the larvae at day 4 postfertilization (Dodd, 1955).

The relatively short larval development duration and the reduced feeding requirements increase feasibility of *P. caerulea* rearing and easy replicability of the applied techniques to other species for commercial or repopulation purposes.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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