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Advancements towards restoration of the endangered limpet *Patella ferruginea* Gmelin, 1791 through controlled reproduction

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Abstract

The ferruginous limpet is one of the most threatened invertebrate species in the Mediterranean Sea. Its controlled reproduction has been considered one of the potentially most effective strategies for the production of juveniles for population restocking or for recolonization of areas where the species was brought to extinction by human pressure but is now under protection (e.g., MPAs). This approach has been pursued for at least two decades but with partial results: at most, some juveniles have been obtained and have reached adult stage, yet not starting from spawning induction but through sacrifice of female specimens (lethal approach), additionally resulting in low genetic diversity of the juveniles produced. Herein, we describe, for the first time, positive results of the spawning induction in *P. ferruginea*. The fertilizations made after these experiences allow describing the larval development of the species from its early stages to its metamorphosis. The fertilization rate in these spawning events was much higher (>97%) than in previous studies that were based on the extraction of oocytes by dissection. The rate of non-anomalous larvae after 15 hours was not negligible but variable, depending perhaps on the quality of the sperm available at each fertilization. The timing of larval development of the species and its variability, particularly regarding metamorphosis, are described. Settlement ability is reached at 3–4 days after fertilization, depending on water temperature, while a large variability is observed in metamorphosis, occurring between 7 and 32 days (probably up to 40 days), although inducing factors are still unknown.

KEYWORDS
artificial fertilization, ferruginous limpet, Mediterranean Sea, Mollusca, Patellogastropoda, reproduction

Maria Paola Ferranti and Javier Guallart contributed equally to the preparation of the paper.

1 | INTRODUCTION

The ferruginous limpet (*Patella ferruginea*) is an endemic species of the western Mediterranean, where it is considered one of the invertebrate species at higher known risk of extinction. Abundant since the Neolithic in the whole basin, it seems to have suffered a very important regression especially during the 20th century. The causes of this regression are considered to be of human origin, including its harvesting (for consumption, use as bait or others), degradation of the littoral habitat, pollution and coastal works (Laborel-Deguen & Laborel, 1990a, 1991a; Luque et al., 2018; Moreno & Arroyo, 2008; Templado, 2001; Templado et al., 2004).

Therefore, restocking of this species, as well as of other endangered limpets has been approached, in order to restore population, maintain biodiversity and related ecosystem functions and services. A first approach to restocking was through translocation of adult specimens. A first trial was carried out by Laborel-Deguen and Laborel (1991c) on specimens from Corsica and the Port-Cros Reserve, although the result was poor specimen survival. Since then, several studies have shown high mortality in the translocation of *P. ferruginea* specimens from areas with still healthy populations ("donors") to others ("recipients") in which it was intended to reintroduce or reinforce *P. ferruginea* populations (see a summary in Luque et al., 2018). Other actions related to harbour maintenance have generally resulted in high mortalities of *P. ferruginea* specimens (e.g., Espinosa et al., 2008) except for the recent paper by Fa et al. (2018) in the Gibraltar harbour. For this reason, the Strategy of the Spanish Administration for *P. ferruginea* advised against the translocation of specimens from natural populations, promoting less invasive methods, among which is the production of juveniles through aquaculture techniques (MMAMRM, 2008). Reproduction of the species in controlled conditions, using non- lethal techniques, represents, therefore, the most sustainable approach, with the least impact on donor populations, but knowledge of the fundamental biology of the species is required.

Understanding about the biology of the species has increased in the last two decades, undoubtedly due to its protected status under several national and international regulations. The reproductive cycle of the species has been studied in the Alboran Sea by Frenkiel (1975) for the Algerian coasts and by Guallart et al. (2006) in the Chafarinas Islands. The species displays a short reproductive cycle, which begins in late summer and culminates in late autumn, where spawning is generally synchronized, between November and December, when the first strong storm of late autumn occurs. During the rest of the year, the species appears to be in complete sexual repose. Laborel-Deguen and Laborel (1990b, 1991b) suggested that the species might have a quite short larval life. This would hamper species dispersal and especially recolonization of suitable areas where it had previously suffered significant human pressure, although at present regression causes have been reduced or eliminated, e.g., Marine Protected Areas (MPAs).

Some authors described that the fertilization of *P. ferruginea* was relatively simple, mixing gametes obtained from the dissection of males and females, although they did not provide other relevant information (Frenkiel, 1975; Laborel-Deguen & Laborel, 1990b, 1991b). Espinosa et al. (2010) made some attempts for reproduction in laboratory conditions, reaching an early stage, which the authors define as early pretorsional veliger, but which probably corresponds to an anomalous larva. Conversely, Guallart, Peña, et al. (2020) managed to "close the cycle" of the species, describing larval development, metamorphosis and the subsequent achievement of sexual maturity, but without being able to manage spawning induction of the specimens and starting from oocytes obtained exclusively from the sacrifice of mature females.

In the present paper, we describe for the first time the successful spawning induction in *P. ferruginea* (through a non-lethal approach), the following gamete fertilizations and the whole larval cycle until settlement and metamorphosis. These results contribute to the advancement of our understanding of this species biology and ecology, and represent a first step towards the definition of an artificial reproduction protocol for this protected species, for the production of juveniles that can be used for the reinforcement of scarce and resilient populations or for the recolonization of areas where the species was present but is nowadays extinct. Such approach can substantially be tested on other endangered limpet species, such as *Patella candei* and *P. aspera*. These are fundamental steps forward species and habitat conservation and restoration, envisaged by several EU Directives, most recently the EU Biodiversity Strategy for 2030.

2 | MATERIAL AND METHODS

2.1 | Ethics statement

Our manuscript does not need an ethical approval.

2.2 | Sampling adult reproductive specimens

Within the framework of the ReLife project (LIFE15 NAT/IT/000771) in October 2018, 2019 and 2020 a total of 88 specimens of *P. ferruginea* were collected along the coasts of the Tavolara-Punta Coda Cavallo MPA and transported to the facilities of the CNR-IBF laboratory in Camogli. The specimens were detached from the rocks using a blunt knife when they were outside of their home scar; they were covered by a cloth soaked with seawater and placed in a portable refrigerator along with coolers (at around 12–16°C). Transport was performed by van and ferry in less than 24 hours.

All specimens were adults, according to their size range (38.3–78.0 mm, average 58.42 ± 8.03 mm, maximum shell diameter – MSD) (Frenkiel, 1975; Luque et al., 2018). In the laboratory, specimens were placed in the maintenance tanks, where they were kept in a recirculation system. Tides were generated to ensure water movement similar to natural conditions and to avoid the particular mortality issues in captivity, due to the attitude of the specimens to move to the uppermost and not immersed portion of the tank walls where they

often remain until death due to desiccation (Guallart, Peña, et al., 2020; Peña et al., 2013; Figure S1). The seawater was renewed at least two or three times per week from a seawater intake near the Camogli Dragonara castle (at about 1.5 m depth), with limitations due to weather conditions or unexpected logistical situations. In the tanks, ambient water temperature was not controlled and artificial photoperiod was adjusted to time of the year conditions using sun light neon (Sylvania Growlux® and Aquastar®).

The specimens were biopsied for sex determination (Guallart et al., 2013; Wright & Lindberg, 1979) and additional evaluation of sexual maturation conditions. Specimens were then labelled with tags (plastic pieces with identification numbers and colour identifying the sex; Figure S1). Gonadal biopsies in limpets were initially designed for specimen's sex identification (Guallart et al., 2013; Wright & Lindberg, 1979), but they can also provide information about their reproductive stage. For example, the difficulty in obtaining gonad samples in biopsies may indicate that the specimens are not yet mature or that have already spawned, at least partially. Additionally, microscopic examination of material obtained from biopsies can also provide information on reproductive status.

The strategies for obtaining mature adults changed over the 3 study years (2018–2020). The collection of specimens in 2018 was carried out at the end of October under the idea, based on the scarce existing literature (Frenkiel, 1975; Guallart, 2006; Guallart et al., 2006), that (a) they had almost reached the condition of sexual maturity and that (b) spawning in *P. ferruginea* has been observed on a few occasions but so far always in the month of November.

For the above considerations, in 2018 the specimens were collected on October 25, but the difficulties encountered for obtaining material in the biopsies in several specimens indicated that they were either not mature or that they had already spawned. The autumn of 2018 was meteorologically unusual and at the end of October an unprecedented strong storm occurred in the Ligurian Sea, causing serious damage to various coastal areas and ports. This apparently affected the maturation of *P. ferruginea* specimens. For this reason, in the following years, specimens were collected earlier, on October 4 (2019) and October 9 (2020) with the aim of ensuring that they were collected during the maturation phase and that they had not yet spawned.

2.3 | Maturation of specimens

Two strategies have been used to achieve the final maturation of the specimens in 2019 and 2020. The first consisted in simulating in the laboratory the natural conditions, as close as possible, by (a) changing the sea water in the tanks as often as possible to maintain the physical-chemical parameters, in particular temperature, more closely mirroring the sea conditions, (b) controlling the photoperiod. The second strategy, after an acclimation period from their transport from Tavolara MPA (to check for survival), consisted in the transfer of several specimens (males and females) on the natural rocky shore near Camogli, in order to facilitate sexual maturation in the natural environment.

In 2019, in some specimens, the state of maturation was evaluated using MRI techniques (Guallart, Ferranti, et al., 2020). In addition, when biopsies were performed, the material obtained was used to evaluate the degree of maturation of the specimens. In all cases the material from biopsy was placed in 1–10 ml containers with sea water and examined under a microscope after 1 hour. For males, maturation was evaluated by the degree of sperm motility and the presence of "star-shaped structures" (considered aggregates of spermatids, therefore not fully mature sperm). For this purpose, a subjective scale of "motility" (m) was established between 0 (no sperm movement detected) and 5 (generalized and intense mobility) and of "presence of star-shaped structures" (s) between 0 (absent) and 3 (abundant). For females, the percentage of eggs considered macroscopically "mature" was estimated. When directly extracted from the ovary, the eggs display an irregular shape and are covered by a gelatinous cover (often referred to as chorion), but after some time in seawater they are considered to be mature when they become completely round and lose (completely or almost completely) their chorion (Dodd, 1957; Guallart, Peña, et al., 2020). The percentage of round eggs without chorion was calculated by counting the morphology of at least 100 eggs at the microscope in a Sedgwick Rafter Counting Chamber (SRCC) and this value was considered as reference for the degree of maturation (Dodd, 1957; Guallart, Peña, et al., 2020).

November 2020 was unusually warm and characterized by uncommon calm weather conditions. Given these optimal conditions, it was decided to move several specimens at sea, experiencing, therefore, natural conditions which could speed up the achievement of the mature stage, trying to minimize (a) the possibility of loss of specimens in the wild by predators, (b) the natural spawning at sea due to occurrence of storms. Therefore, specimens were kept at sea 11 days for one stock and 19 days for another. Then, assuming that they could have reached sexual maturity, specimens were collected and moved in the laboratory and immediately subjected to spawning induction trials. The specimens were biopsied after being collected from the coast and the material obtained was evaluated as described above.

2.4 | Spawning induction trials

Various methods of spawning induction commonly used in limpets (Patellogastropoda) have been tried (Corpuz, 1981; Ferranti et al., 2018; Kay & Emlet, 2002; Mau, Bingham, et al., 2018; Nhan & Ako, 2019). These include: (a) Thermal shock (TS); (b) Vigorous aeration (VA, "Bubbling treatment") and (c) Hormonal stimulation (HS). Noteworthy, only female specimens require necessarily to be subjected to spawning induction stimuli, since sperm from different males may be collected through biopsies that, although initially designed to determine the sex of the specimens (Guallart et al., 2013;

Wright & Lindberg, 1979), also allow to obtain sufficient amounts of active sperm for fertilization (Guallart, Peña, et al., 2020).

Thermal shock is usually performed by moving molluscs from normal water tanks to warmer ones, with a difference of 4–6°C, although this is done with species that normally spawn in spring (Kay & Emlet, 2002). In the case of *P. ferruginea*, the stimulus applied was water cooling since the species spawn in autumn. Therefore, TS consisted in temporarily placing the specimens in tanks filled with water several degrees colder than normal conditions. TS was performed by placing freezer blocks in seawater, lowering the water temperature by approximately 5°C (from 19 to 14°C; from 16 to 12°C) over a period of 1 hour, generally concurrently with VA.

In VA, specimens were placed in small baskets with the ventral side down and were bubbled through aeration produced by strong air flow (102 L/minute) through large aquarium air stones (according to Ferranti et al., 2018) from the bottom and directed to the specimens foot. The VA method was used also by other authors (Corpuz, 1981; Kay & Emlet, 2002) and aims to simulate the weather conditions that trigger emission of gametes in many limpet species (Branch, 1981; Dodd, 1957; Frenkiel, 1975; Guallart, 2006; Kay & Emlet, 2002; Orton et al., 1956).

Hormonal stimulation started in 2019, using the injection of human chorionic gonadotropin (Gonasi® HP, IBSA) in a dose of 0.05 ml/10 g body weight (BW) in six specimens (one female and five males). The BW (body mass without shell) in living specimens was calculated from the correlation between size (MSD) and BW (according to Luque et al., 2018) and from data between total weight with shell (TW) and without shell (BW) for Chafarinas Islands specimens (Guallart, unpublished data). In 2020, the spawning stimulation strategy was planned following papers by Mau, Bingham, et al. (2018) and Nhan and Ako (2019) who applied such technique to the limpet *Cellana sandwicensis*. Instead of using salmon gonadotropin-releasing hormone analogue (sGnRHa), a similar hormone, luteinizing hormone-releasing hormone (LHRH ethylamide acetate salt hydrate; Sigma-Aldrich®) was used, because it was proven to be more stable and effective in other species (Fornie et al., 2003). The HS tests were carried out assuming that the specimens had mature or almost mature gonads. Therefore, the dosage used (1 μg/g BW) was the one reported for spawning induction and not the lower dose used for gonad maturation, according to Nhan and Ako (2019). The injection was carried out in the gonad, following a similar procedure, although inverse, to that described to perform the biopsies (Guallart et al., 2013). In some specimens of *P. ferruginea*, several injections (up to 4) were made with this dose, at 2–3 days intervals.

Additionally, other techniques have been used trying to mimic environmental triggers for this species living in the upper mid-littoral. The first, Osmolarity shock (OS) was immersion for a short time (<15 minutes) in fresh water or in reduced salinity conditions (about 10‰), assuming that the storms during which spawning occurs are usually accompanied by heavy rains.

A second one consisted of keeping the specimens dry (D) for a while upside down. Although this is clearly not a natural situation, the technique was intended to subject these specimens to a strong stress. Since this species may spend several days out of the water (in low tide and extreme calm sea conditions), when specimens get submersed, they could perceive such condition as a trigger to spawning. Two different procedures were used: Room temperature and Dry (RD) conditions; Cold Dry (CD), maintained in a refrigerator at 4–6°C. Both treatments were implemented for half an hour.

In some cases, at some point during spawning induction tests, a puncture was performed, either in order to perform a biopsy (B, to evaluate the state of the gametes) or for the injection of hormones. Although it was not done for this purpose, puncture has also been considered as a stimulus for the spawning of Patellogastropoda species (Rao, 1973).

Finally, in some cases, we added a few drops of sperm (S) obtained by biopsy to the water of the VA tank to provide an additional trigger to female spawning.

The technique of immersing the specimens in a hydrogen peroxide solution, usually used in gastropods particularly *Haliothis* spp. (Courtois De Viçose et al., 2007; Hahn, 1989; Morse et al., 1977), but also in some limpets (*Lottia* spp.; Kay & Emlet, 2002) was not used because only little positive results were reported in *Patella* species and, sometimes, produced larger mortality (Ferranti et al., 2018; Guallart pers. obs.).

During autumn 2019 and 2020, different spawning induction trials were carried out, 13 (2019) and 11 (2020) respectively (Table 1). After being submitted to the induction stimuli, specimens were placed in trays with seawater and without aeration and observed to detect spawning, for up to few hours.

Throughout the study, the following different conditions of seawater have been used in the various culture phases: (a) barely filtered seawater (BFSW), i.e., water collected from the sea and filtered with only a sand filter at approximately 60 μm; (b) filtered and sterilized seawater (FSSW), which was further filtered through cartridge filters down to 1 μm and sterilized through UV filters (Odyssea UV PRO-36W®); (c) AFSSW: FSSW added with antibiotics (Penicillin G sodium salt 10 mg/L and Streptomycin sulphate salt 20 mg/L, according to Huggett et al., 2005) to prevent bacterial proliferation in larval cultures.

2.5 | Fertilization

During autumn 2019 and 2020, out of the total induction trials, a total of four successful fertilizations were performed in eggs from females that had spawned: one in 2019 and three in 2020. These fertilizations will be indicated as F1 in 2019, F2-F3-F4 in 2020 (Table 1, Figure 1).

The eggs released were collected and placed in glass beakers. Eggs were counted by homogenizing the sample in a known seawater volume, taking a 1 ml subsample, and counting in a Sedgwick Rafter Counting Chamber (SRCC). Egg state of maturity was established on the basis of observation of the eggs obtained by biopsy. The eggs spontaneously released were completely round and without chorion, therefore mature eggs.

TABLE 1 Spawning induction trials in *P. ferruginea* specimens between 2019 and 2020

Year	Date	N° F, N° M	Treatment	Observed spawning	Fertilization event
2019	24/10/19	2 M	RD (0.5 hour) + OS (3 minutes) + VA (1 hour) + RD (0.5 hour) + VA (1 hour)	-	
	11/11/19	1 F, 3 M	RD (0.5 hour) + B + [VA − TS − S] (1.5 hours)	1F	F1
	12/11/19	1 F, 4 M	RD (0.5 hour) + B + [VA − TS − S] (2.5 hours)	-	
	14/11/19	1 F, 4 M	RD (0.5 hour) + B + [VA − TS − S] (2.5 hours)	-	
	18/11/19	1 F, 4 M	RD (0.5 hour) + B + [VA − TS − S] (2.5 hours)	-	
	18/11/19	1 F, 4 M	RD (0.5 hour) + OS (1 minute) + VA (10 hours)	-	
	20/11/19	1 F, 3 M	[RD (0.5 hour) + VA (0.5 hour)] (3 times) + B + VA (2 hours) + [VA − TS] (0.5 hour)	-	
	22/11/19	1 F, 3 M	RD (0.5 hour) + VA (2.5 hours) + TS + VA (1.5 hours)	-	
	26/11/19	1 F, 3 M	RD (2 hours) + B + [VA − S] (2 hours)	-	
	02/12/19	1 F, 6 M	RD (0.5 hour) + B + [VA + S] (3 hours) + RD (0.5 hour)	-	
	03/12/19	1 F, 6 M	RD (0.5 hour) + VA (2.5 hours)	1M	
	06/12/19	1 F, 3 M	B + HS	-	
	11/12/19	1 F, 5 M	RD (1 hour) + B + HS + VA (2 hours)	1F, 2M	-
	11/12/19	1 M	Spontaneous spawning in tank	1M	
2020	31/10/20	3 F	HS		
	02/11/20	2 F	HS + RD (0.5 hour) + [VA − TS] (2 hours)	-	
	03/11/20	5 F, 2 M	HS + RD (0.5 hour) + [VA − TS − S] (2 hours)	1M	
	06/11/20	5 F	HS	-	
	09/11/20	2 F, 1 M	HS + RD (0.5 hour) + VA (2 hours)	-	
	10/11/20	3 F	HS + RD (0.5 hour) + [VA − TS] (2 hours)	-	
	15/11/20	1 F, 1 M	CD (0.5 hour) + B + [VA − TS] (1 hour) + RD (0.5 hour) + HS	1M, 1F	F2
	16/11/20	6 F	CD (0.5 hour) + [VA − TS] (1 hour) + RD (0.5 hour) + B	-	
	19/11/20	1 F	CD (0.5 hour) + B + [VA − TS] (1 hour) + RD (0.5 hour)	1F	F3
	24/11/20	3 F	CD (0.5 hour) + B + [VA − TS] (1 hour) + RD (0.5 hour)	-	
	30/11/20	2 F	CD (0.5 hour) + B + [VA − TS] (1 hour) + RD (0.5 hour)	2F	F4

Note: The order of the treatments indicates the temporal sequence in which the triggers were applied.

Abbreviations: B, biopsy; CD, cold dry; HS, hormonal stimulation; OS, osmolarity shock; RD, room temperature-dry conditions; S, addition of a few drops of sperm; TS, thermal shock; VA, vigorous aeration.

The eggs are much denser than seawater (Corpuz, 1981; Dodd, 1957; Guallart, Peña, et al., 2020; Kay & Emlet, 2002) and settle to the bottom after ca. 10 minutes. This allows removal of residues of broken oocytes and other material in suspension by decantation of ca. 80% of supernatant and refilling with FSSW. This removal through decantation process was repeated successively 2–3 times. Total amount of eggs was distributed in several glass beakers (volume 1–2 L, each). The number of beakers depended on the number of eggs available, in order to obtain a single layer of eggs on the bottom.

Fertilization was performed at a concentration between 5×10^5 spermatozoa/ml, indicated as adequate by Guallart, Peña, et al. (2020), and 10^6 spermatozoa/ml. In general, a higher concentration was used subjectively within this range when the available sperm had a lower mobility. These values match those described by other authors for limpets (Baker & Tyler, 2001; Ferranti et al., 2018; Hodgson et al., 2007; Kay & Emlet, 2002). The initial concentration of the available solution of sperm was calculated by counting in a Neubauer Chamber and this was used to calculate the amount to add in each container. The eggs, immediately before their fertilization, were placed in containers with AFSSW that represented approximately one-fifth of the total volume of the container.

One hour after fertilization and in order to stop it, containers were refilled with AFSSW, eggs were allowed to sink to the bottom and then the supernatant was siphoned and refilled three times to remove the sperm in excess (Guallart, Peña, et al., 2020). Fertilization rate was assessed 1–2 hours after sperm addition by resuspension and homogenization in the water of the eggs and embryos present on 1 ml samples; counting was performed in a SRCC. Eggs were considered fertilized when a clear polar body appeared or when they already turned into embryos formed by the division of several cells.

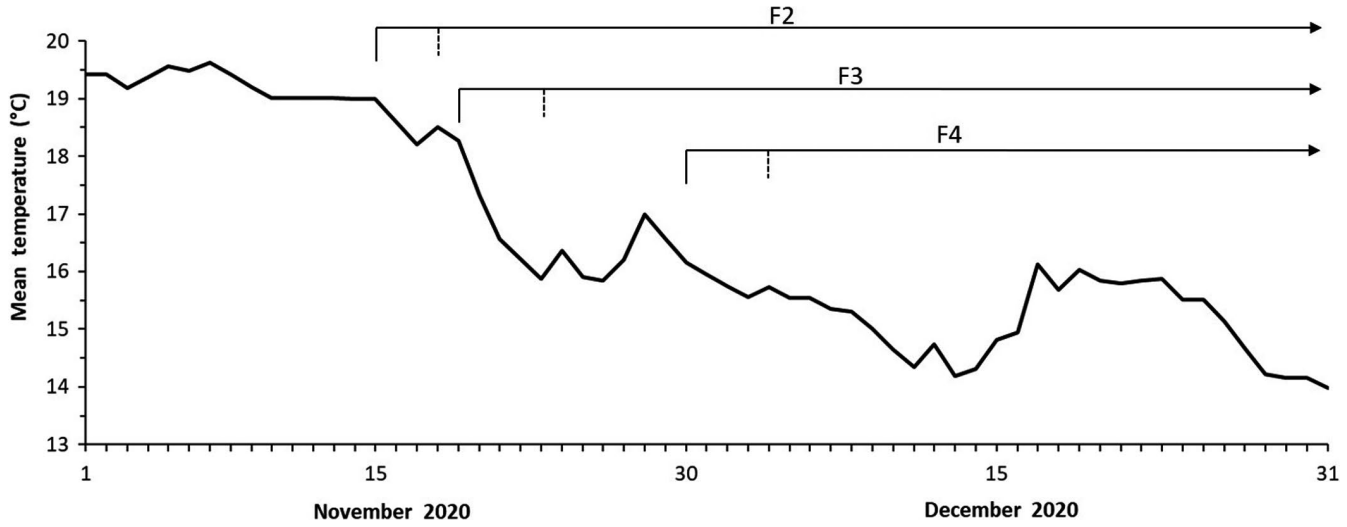

FIGURE 1 Evolution of the average seawater temperature in the maintenance tanks of the Camogli laboratory from the beginning of November to the end of December 2020. The start date of the three fertilization trials (F2; F3; F4) is reported. The dotted vertical line indicates the first appearance of crawling pediveliger larvae: note the different larval duration (ca. 3 days vs. ca. 4 days) related to temperature in F2 versus F3 and F4

2.6 | Larval rearing

After ca. 16–18 hours post fertilization (hpf) cultures with swimming trochophore larvae occupying the entire body of water in the containers were processed with two objectives: (a) to eliminate the unfertilized eggs accumulated on the bottom of the containers and the anomalous larvae, herein more common; and (b) to reduce the density of the larvae. To this purpose, the upper half of each beaker was transferred by siphoning to other beakers of similar volume, partially filled with AFSSW. We assumed that the most active healthy larvae were distributed in the upper part of the water volume. The original beakers were then filled with AFSSW: 1 hour was waited for the trochophores to naturally distribute and again about 70% of the beaker content was siphoned into new containers. All of these containers were numbered and monitored.

The remaining content was used to calculate the number of unfertilized eggs. The count of these eggs, abnormal larvae, and normal larvae was used to calculate the "non-fertilization" rate and to estimate the percentage of abnormal larvae. The larvae remaining in the unutilized water were delivered at sea, near the Camogli laboratory, instead of being discarded, in order to potentially favour in situ larval settlement.

The density of larvae in a beaker, originating from a monolayer of eggs after fertilization, resulted to be very high, between 100 and 150 larvae (trochophores)/ml. This first water change involved a reduction in density by about half, but still much higher than that suggested by other authors (e.g., Corpuz, 1981; Courtois de Viçose et al., 2007; Kay & Emlet, 2002).

At approximately 40 hpf, a new distribution of the available larvae (now veligers) was made. The veligers were transferred by siphoning ca. 50%–70% of surface water into new containers with AFSSW, again diluting the concentration of the larvae. In this case, the dilution was 1:10, reaching an approximate density of 10–15 larvae/ml. The remaining material on the bottom was again discarded at sea.

At about 72 hpf, the larvae (already pediveligers) were moved by siphoning to the settlement tanks, where biofilmed substrates had been placed.

The larval cultures were carried out at room temperature (between 15 and 19°C, in autumn 2020; Figure 1). The temperatures at which the first phases of the larval cycle took place, up to the crawling pediveliger phase, depended on the date on which each fertilization was carried out. Furthermore, no aeration was used during the first 24 hours post fertilization, and after that, cultures were maintained under gentle aeration.

The larval stage identification was performed from fertilization to metamorphosis using an optical microscope.

2.7 | Settlement and metamorphosis

A larva was considered to have completed metamorphosis when (a) it had lost the cilia of the velum; (b) had modified the "disc shape" of the velum of the anterior region; (c) had acquired a morphology in the cephalic region without a disc shape and, whenever possible, presented movement of the buccal area for feeding purposes on the substrate.

Other features were also considered to evaluate the processes of "settlement" and "post-larval metamorphosis." The development of a conical structure (Figure S2) on the edge of the protoconch was considered as the prove that the larva had completed the settlement and metamorphosis, in an early stage before elaborating the definitive teleoconch from the initial protoconch. Some of these morphologies were observed not in living specimens, but in protoconchs found on the bottom of the culture tanks. Thus, in the last

fertilizations (F3, F4), observations were made to determine the timing when: (a) velum and at least some cilia were still displayed in the larvae, although these structures did not seem functional and the specimens showed a clear benthic behaviour (Video S1); (b) the edge of the proto-shell morphology started to change, developing a conical structure (Video S2).

Different structures were used for the larval settlement but with a basic common strategy: to provide a high surface area of substrate covered with biofilm and to keep the larvae at a much lower density than in the previous stages. The larvae were transferred to settlement tanks of between 40 and 900 L where, in order to increase the available settlement surface, several plastic (polycarbonate) plates were placed vertically in a fixed position. Furthermore, in order to monitor the settlement process (under a stereomicroscope), several plastic Petri dishes were placed, vertically, floating but partially submerged. Such positioning was implemented assuming that, due to the habitat preferences of *P. ferruginea*, the settlement would take place near the air-water interface. In all these structures, from the tanks to the polycarbonate pieces and the floating plastics, the growth of a biofilm was promoted to serve as a stimulus to the settlement of the larvae and as feed for the post-larvae. To this purpose, in the previous weeks these structures were maintained in tanks under conditions of continuous artificial light, addition of nutrients and of random amounts of macro- and microalgae normally used in aquaculture systems (*Ulvella lens*, *Dunaliella tertiolecta*, *Tetraselmis suecica* and *Amphora* spp.).

The monitoring of settlement and metamorphosis was performed almost exclusively on the floating Petri dishes, through their periodic observation under a microscope. Post-larvae size measurements were made using a micrometer eyepiece and/or from photographs elaborated through ImageJ software. The maximum teleoconch diameter (MTD; equivalent to MSD) was considered as the size of the post-larvae (regardless of whether the protoconch could initially present a profile external to it; Figure S3). An exhaustive monitoring or count was not carried out in order to minimize the mortality that the manipulation of the substrates could cause.

In the settlement tanks the bottom was periodically siphoned, and the extracted seawater was sieved through a 69 μm mesh. The observation of the collected material allowed to record both the presence of empty proto-shells in different stages of development (some with the beginning of the development of the teleoconch, as reported above), of some apparently normal larvae that had not started the settlement/metamorphosis and finally to have clues on the overall mortality of the post-larvae.

In the days immediately following the positioning of the larvae in the settlement tanks, water level was increased from day to day, preventing the larvae (crawling pediveligers) to remain dry for too long, as they tended to crawl on the substrates at the air-water interface (Figure S4). When all the larvae were supposed to have settled, the water circulation was re-established. When only post-larvae and no crawling pediveligers were present in the settlement tanks, the tidal system was activated to ensure water movement similar to natural conditions, as for the adults in the maintenance tank.

3 | RESULTS

3.1 | Spawning induction

Out of the 24 spawning induction trials in *P. ferruginea* specimens between 2019 and 2020 at least partial emission of gametes occurred seven times, six in males and six in females; an additional spontaneous spawning of one male occurred in the maintenance tank (Table 1).

In 2019, 13 spawning induction trials were performed, but only three (11 November, 3 and 11 December) achieved gamete release from, overall, two females and three males. In at least one case, spontaneous emission by a male was observed (11/12/19), in a specimen that was stimulated in the previous days/weeks.

In 2020, 11 spawning induction trials were performed, but only in four events (3-15-19-30 November) gametes were released, overall, from two males and four females. A large part of the trials was based on the use of hormonal stimulation. However, in almost all cases where it was used ($n = 7$), spawning was not achieved. Only in two cases, in the trays where the females were placed after the injection of hormones, a small number (several hundreds) of oocytes was observed: yet, these eggs were assumed to have been released by the mechanical syringe puncture performed to inject the hormone (Figure S5).

A proper oocyte emission in a significant amount was achieved in four events (Table 2). In the last three events, in 2020, the emission occurred after an almost identical stimulation procedure (Table 1), so it can be considered as a basis for a best protocol for spawning stimulation in *P. ferruginea* females.

The successfully implemented protocol for spawning induction can be summarized as follows:

1. The specimens were left to dry upside down at a temperature of 5°C inside a plastic box in a refrigerator for half an hour;
2. Biopsy was performed to determine the state of gamete maturity;
3. Specimens were maintained in a cold bubbling tank for at least 1 hour; temperature was around 5°C lower than ambient seawater temperature;
4. The specimens were left to dry upside down at ambient temperature (18°C) for half an hour;
5. Specimens were positioned in trays in FSSW at room temperature and without aeration and regularly inspected for spawning for a few hours.

3.2 | Spawning process description

The onset of spawning in almost all cases occurred after 0.5–2 hours after being subjected to the set of stimuli. In males, spawning occurred by the emission of a whitish fluid, from the anterior part of the specimen, and was observed to last more than 1 hour (Figure S6 and Video S3). In females, usually the largest number of eggs (between

TABLE 2 Summary of fertilization events described in the present study (2019 and 2020): code of the fertilization event, date, number and size of spawning females, total number of eggs released, number of males and sperm origin (B = biopsy; S = induced spawning), fertilization rate (% embryos vs. number of eggs released) and trochophore rate (% normal trochophores vs. number of eggs released)

Code	Date	N° females	MSD (mm) females	Total N° eggs	Sperm	Fertilization rate	Trochophore rate
F1	11/11/2019	1	52.0	129.600	4 (B)	14.90%	3.08%
F2	15/11/2020	1	67.4	778.200	1 (S)	97.40%	na
F3	19/11/2020	1	57.3	247.700	3 (B)	99.05%	47.60%
F4	30/11/2020	2	51.2–72.4	846.000	3 (B)	98.90%	32.60%

Abbreviation: na, not available.

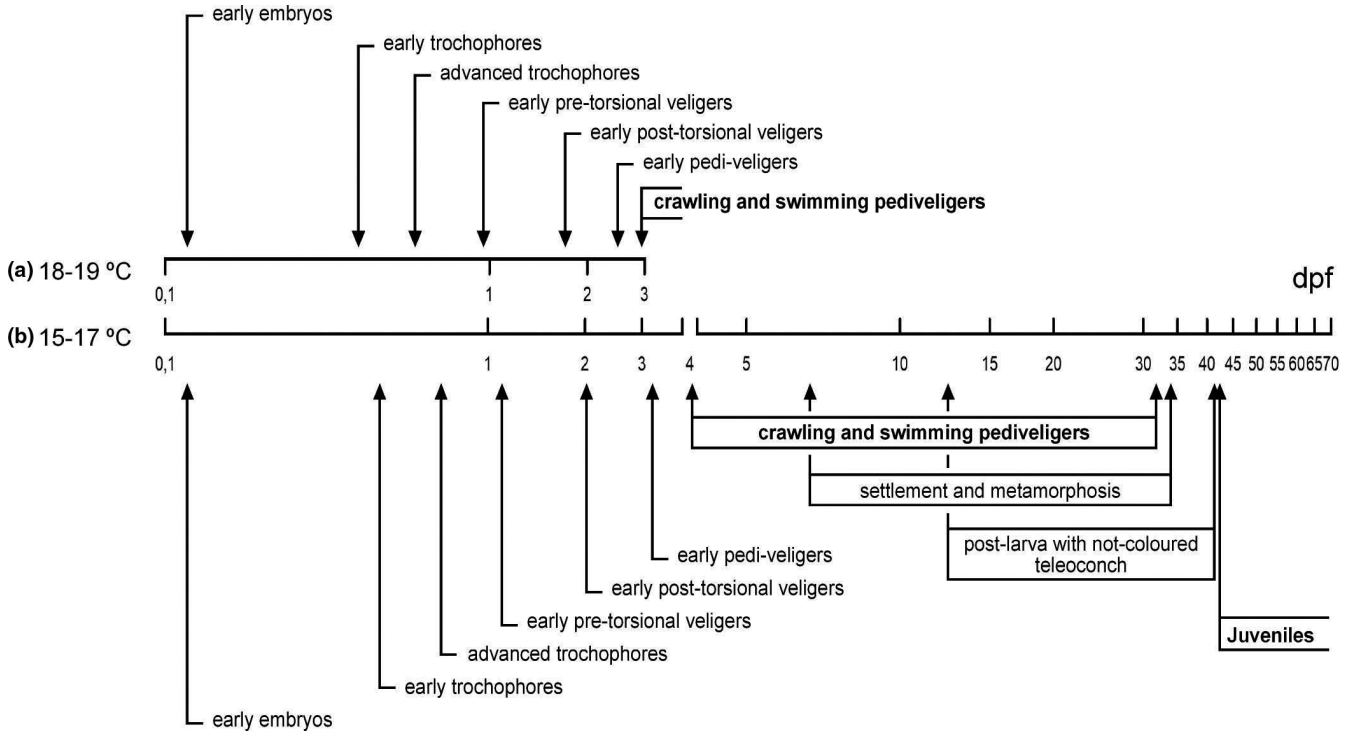

FIGURE 2 Timing of larval development in *Patella ferruginea* (left: a: fertilization F2, b: fertilizations F3 and F4; right: b: fertilizations F2, F3 and F4)

60% and 80% of the total), reddish in colour, was released approximately in less than 1 hour (Figure S7 and Video S4). After this first release, the eggs were collected and placed into the beakers and the females were placed again in trays with renewed FSSW. In all these occasions, subsequent emissions were observed: the eggs of each release event were separated on each occasion. In total, the females laid eggs over several hours, in decreasing numbers. In one case a small number of eggs were observed in the tray up to 8 hours from the start. Eggs after spawning were already mostly round in shape and had no chorion.

After male emission, sperm motility was often difficult to observe due to dilution in seawater. However, in some cases it was surprisingly observed that motility was much lower compared (m = 1: see Video S5) with that observed in some cases from samples obtained by biopsy (m = 3: see Video S6; m = 5: see Video S7).

3.3 | Fertilization rate

Table 2 shows the main data and the fertilization rate (initial; and reaching the trochophore stage) in the four fertilization events described. In 2019, F1 showed a low fertilization rate, 14.90% of the 129.600 spawned eggs. Conversely, the fertilizations carried out in 2020 (F2, F3 and F4) showed very high fertilization rates, above 97%.

3.4 | Larval development

In the F1 fertilization event (2019), characterized by a very low fertilization and trochophore rate (Table 2), larval development was observed, although after 48 hours, most of the veliger larvae showed an

abnormal shape. Although they were placed in metamorphosis tanks, no post-larvae were observed.

Therefore, the description and timing of larval development is based on the observations of the F2, F3 and F4 fertilizations, all performed in autumn 2020: F2 fertilization occurred at higher temperature (room condition: 18–19°C), while F3 and F4 were performed at lower temperature (15–17°C), according to the decrease of environmental air temperature during the month of November. Healthy trochophore rate in F3 and F4 was 47.60% and 32.60% respectively, while for F2 it was not calculated (Table 2). Information about larval stage onset, duration and timing is summarized in Figure 2 and in Table 3 (and in Supporting information, Figures S1 to S9, Videos S1 to S11).

The first cell divisions were observed starting from 30 minutes after fertilization. During the first 2–3 hours, embryos were observed in relatively different stages of development, from oocytes with the polar body (Figure 3a) to embryos with more than 8 cells (Table 3; Figure 3c), a variability that can be attributed to the exact fertilization time. However, between 5 and 10 hpf, the embryos displayed a marked homogeneity in their degree of development: morulae with ciliated cells on the surface (Figure 3e, similar to those described by Smith, 1935, Figure 1 for *Patella vulgata*). Remarkably, until the onset of this "ciliated morula" stage, the embryos did not display mobility: they remained at the bottom of the containers, allowing water changes (if considered necessary) by siphoning most of the superficial seawater. Such habit was displayed even when most embryos were "ciliated morulae," since they moved horizontally over the bottom.

At 10–18 hpf the larvae already displayed an early swimming trochophore stage (Figure 3f, Table 3) and were distributed throughout the volume of the beakers (Figure S8). From this moment, although the larvae showed a fairly marked synchrony in their development, differences were observed regarding the onset of each stage according to temperature conditions (different speed between F2 and F3–F4).

The larval development shown in Figure 3 for *P. ferruginea* is very similar to that described by Guallart, Peña, et al. (2020). One of the most relevant steps is the achievement of the "crawling pediveliger" stage, when the larvae display a well-developed foot with a ciliated surface and the ability to crawl on the substrate, but still present a ciliated velum that they use to swim freely (Videos S1 and S8). When the larvae reach this stage, they may be considered already able to settle on the substrate and start metamorphosis. The main differences between F2 and F3-F4 refer to the evidence that at a temperature of 18–19°C the crawling pediveliger stage is reached at ca. 67 hpf (i.e., about 3 days post fertilization, dpf), while at a temperature of 15–17°C the stage is reached at ca. 94 hpf (i.e., about 4 dpf) (Figure 3o, Table 3).

3.5 | Settlement and metamorphosis

When the larvae reached the state of crawling pediveliger (at 3–4 dpf depending on fecundation) they were transferred into the settlement tanks. For the fertilizations carried out in autumn 2020, the water temperature when settlement and metamorphosis were performed was, in all cases, in the range of 15–17°C (Figure 1).

Settlement time was largely variable. In fact, although most of the larvae carried out the settlement and metamorphosis between 7 and 32 dpf, at 32 dpf some crawling pediveligers were still observed in the settlement tanks (Table 3).

When crawling post-larvae performed the settlement and metamorphosis (between 7 and 32 dpf), they lost the veil and the operculum, but the teleoconch was not yet visible (Figure 3p,q, Table 3). The start of the teleoconch formation occurred starting from 12 dpf (Figure 3s, Table 3).

It has often been difficult to determine the exact timing of the transformation process from settlement to metamorphosis. Settling could be considered to occur, in principle, when a crawling pediveliger was attached to the substrate by the foot, retracted or not. In some cases, it was still possible to detect the velum disc thanks to the transparency of the protoconch (Figure 3n,o), suggesting that the metamorphosis had not yet started. In some cases, this was confirmed when, often after crawling over the substrate, the larva pulled out the veil and swam (Video S9-S10). In other cases, although without a substantial modification of the protoconch shape, metamorphosis could be considered occurred, because a clearly cephalic structure was seen through transparency of the proto-shell (Video S11). Some cases were considered uncertain because, although the structure of the velum disc was apparently maintained, the cilia of the velum were not observed or were scarce and did not display any movement. In these cases, the specimen was considered in the process of metamorphosis.

The first growth of the shell in the post-larvae after metamorphosis is carried out with a funnel or fan morphology (Figure 3p,q; Video S2). This shell had a conical appearance with a much smaller angle (more closed) than that of the teleoconch of growing juveniles later. Although strictly this portion can be considered as "teleoconch," due to its different morphology, clearly recognizable in the later juveniles (Figure 3u; Figure S2), it has been named here as "intermediate shell." When the intermediate shell reaches a maximum diameter of between 195 and 265 μm, the growth pattern changes and a more open structure is formed that corresponds to the teleoconch feature of the juvenile limpet.

3.6 | Post-larval growth

After observing the first post-larvae (7 dpf), teleoconch growth monitoring was implemented from 10 dpf up to 100 dpf (Figure 4). After day 55 the measurements were made using photographs: therefore, the smaller post-larvae (e.g., <750 μm) could have not been recorded. In the time frame in which the teleoconch measurements were taken, temperature showed a decreasing trend, from an average value of 17.04 ± 1.11°C in November to an average value in January and February of 13.36 ± 0.83°C.

Minimum teleoconch size is about 150 μm, which represents the average opening in the shell of the crawling pediveligers, from which the growth of the "intermediate conch" begins.

A large variability was observed in the evolution of the size of post-larvae, mostly in relation to the variability of the onset of metamorphosis. The first post-larvae with complete metamorphosis were observed 7 dpf (Figure 3p). On the other hand, pediveligers with swimming capacity were found up to 32 dpf in the same tanks, although probably

TABLE 3 Summary of developmental events or stages in *Patella ferruginea* at two different temperature ranges (hpf: hours post-fertilization; dpf: days post-fertilization)

Developmental event or stage	Time after fertilization		Figure/SM
Temperature range (Fertilization event)	**18–19°C (F2)**	**15–17°C (F3, F4)**	
Eggs with polar body	0.5–2 hpf	0.5–2 hpf	3A
2-cell embryos	0.5–2 hpf	0.5–2 hpf	3B
8-cell embryos	1–2.5 hpf	1–2.5 hpf	3C
Morula	4–6 hpf	4.5–6.5	3D
Ciliated morula (swimming)	6– 8 hpf	7–9 hpf	3E
Early trochophores (swimming)	<15–18 hpf	<15–20 hpf	3F
Early pre-torsional veliger	19–21 hpf	21–23 hpf	3G
Pre-torsional veligers, protoconch initiated	21–25 hpf	24–28 hpf	3H
Post-torsional veligers, foot rudiment, protoconch well developed, operculum visible. Larval retractor muscles present and ability to retract into the larval shell	42–47 hpf	46–52 hpf	3J-K-L
Pediveliger larvae with well-developed foot (propodium and metapodium differentiation) covered by fine ciliation. Velum disc with eyespots and primordium of cephalic tentacles.	<63– 68 hpf	<70–87 hpf	3N/SM 18
Pediveligers alternating crawling and velar swimming. Eyespots well visible, cephalic tentacles elongated and with prominences. Noticeable ability to move the foot. Operculum present.	From 68 hpf (ca. 3 dpf)	From 94 hpf (ca. 4 dpf)	3O/SM 3, 16, 17
Temperature range (Fertilization event)	**15–17°C (F2, F3, F4)**		
Post-larva, velum and operculum lost, head visible. No visible start of teleoconch formation (FIRST DAY observed)	From 7 dpf	3P	
Post-larva, beginning of teleoconch ("intermediate conch") formation (FIRST DAY observed)	From 12 dpf	3P-Q/ SM 19	
Pediveliger alternating crawling and swimming; velum disc present and velum cilia functional (LAST DAY observed)	Until 32 dpf	3O/SM 3, 16	
Post-larva, with well-developed teleoconch, but transparent, only protoconch coloured	12–42 dpf	3S	
Small juveniles with teleoconch well coloured with a characteristic pattern of radial bands (FIRST DAY observed)	From 43 dpf	3U/SM 2, 20	
Global settlement and metamorphosis ability interval (observed)	4–32 dpf		
Global metamorphosis interval (observed)	7–32 dpf		

the metamorphosis can extend even up to about 40 dpf (Figure 4). This interval, 25 days apart (or even 33 days), when individual larvae decide to settle and undergo metamorphosis may explain the variability in the size of the post-larvae during these first 3 months of life.

From day 43 dpf, largest specimens started to show the typical coloured teleoconch of small juveniles (according to Guallart et al., 2017; Figure 3u; Figure S9). Starting from 57 dpf, small juveniles of about 1500 μm could be observed with the naked eye both on the polycarbonate plates and on the floating petri dishes. It is noteworthy that the acquisition of colour seems to be more related to age than to size: at 40–45 days in post-larvae larger than 1 mm the teleoconch was still transparent, while after 55 days even post-larvae <750 μm were observed coloured.

We took measurements periodically to avoid stress or damage on the post-larvae or small juveniles. For the same reason, we avoided checking all the available substrates: therefore, at present, an estimate of the total number of juveniles is not feasible.

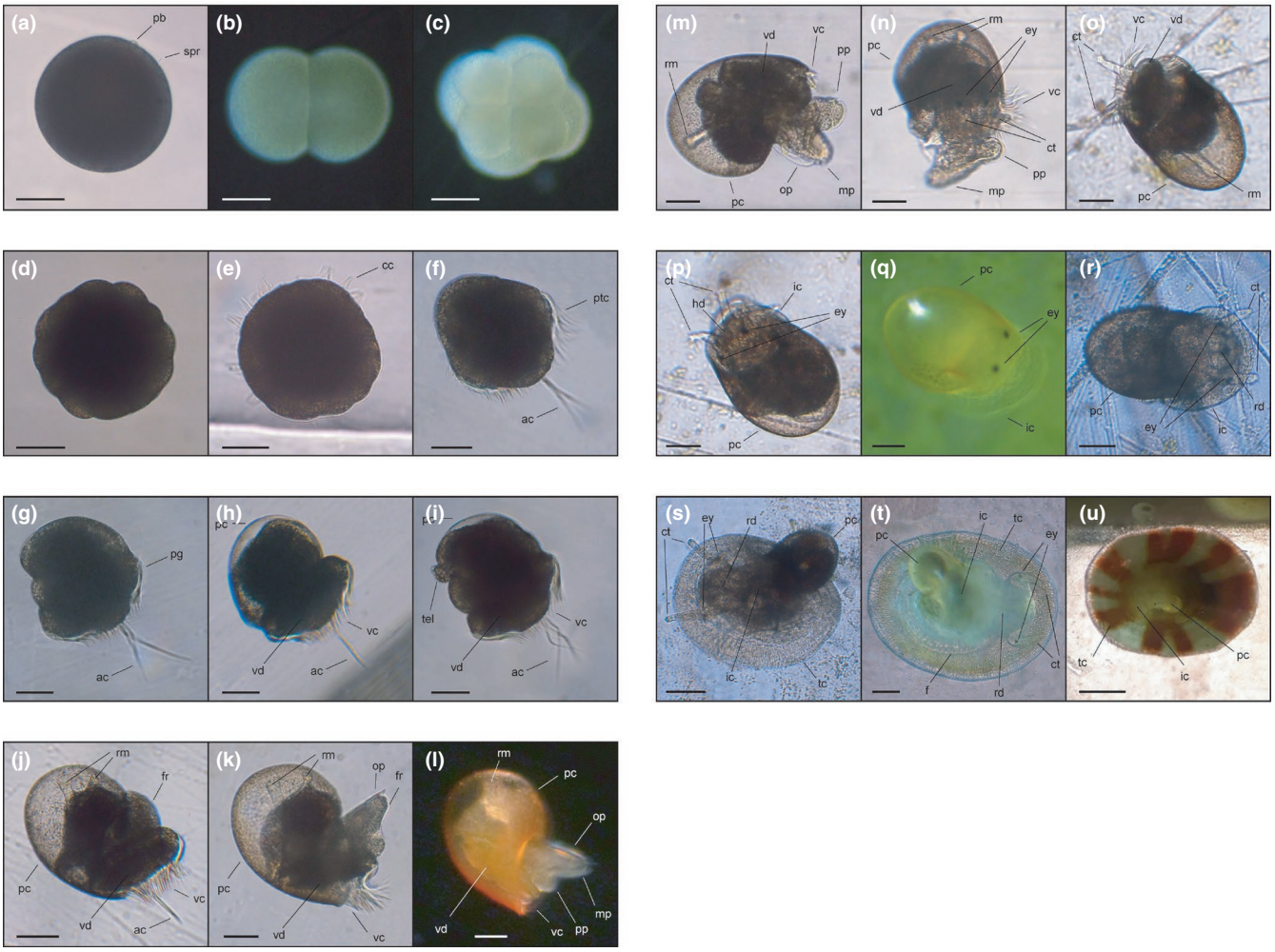

FIGURE 3 Developmental stages in *P. ferruginea*, reared at temperature range between 15 and 19°C. a: Mature egg fertilized, with polar body, and with one spermatozoid visible on its surface; b: 2-cell embryo; c: 8-cell embryo; d: morula; e: morula with ciliated cells; f: early swimming trochophore; g: early pre-torsional veliger; h: pre-torsional veliger, protoconch initiated; i: pre-torsional veliger with telotroch; j: post-torsional veliger with foot rudiment; k, l: post-torsional veliger partially retracted in the protoconch; m: post-torsional veliger with well-developed foot, covered with small cilia; n: pediveliger with well-developed foot and cephalic tentacles, front view; o: crawling pediveliger; p: post-larva, start of intermediate conch; q: post-larva with intermediate conch; r: post-larva with visible radula; s: post-larva 430 μm MSD, with well definite teleoconch, but still prominent protoconch; t: post-larva 686 μm with transparent teleoconch, that surpasses and includes the protoconch; u: small juvenile 1.88 mm, with well-coloured teleoconch. Abbreviations: ac, apical cilia; cc, ciliated cell; ct, cephalic tentacle; ey, eye; fr, foot rudiment; ft, foot; hd, head; ic, "intermediate conch"; mp, metapodium; op, operculum; pb, polar body; pc, protoconch; pg, prototrochal girdle; pp, propodium; ptc, prototrochal cilia; rd, radula; rm, larval retractor muscle; spr, spermatozoid; tc, teleoconch; tel, telotroch; vc, velar cilia; vd, velar disc. Scale bar: a–r = 50 μm, s and t = 100 μm, u = 500 μm

4 | DISCUSSION

4.1 | Spawning stimulation and laboratory fertilization techniques

Until now, spontaneous release of gametes by stimulation had never been achieved in *P. ferruginea*. Guallart, Peña, et al. (2020) managed to complete the larval cycle in *P. ferruginea*, but did not achieve stimulation techniques for the spawning: they obtained eggs by dissecting mature adult females and sperm by biopsies (Guallart et al., 2013; Wright & Lindberg, 1979). Despite the relevant information they provided, this technique could not be considered as a strategy to produce *P. ferruginea* specimens for the purpose of reintroduction or reinforcement of populations for two reasons: (a) as an endangered and strictly protected species, the number of specimens that need to be sacrificed for this purpose must be minimized; (b) genetic diversity from a culture based on a small number of adults would be too low for reintroduction purposes.

The results herein reported, providing, for the first time, gamete release by spawning stimulation using exclusively non-lethal techniques, represent an additional step towards the same objective, paving the way for a best practice technique for juvenile production with the aim of repopulation of *P. ferruginea*.

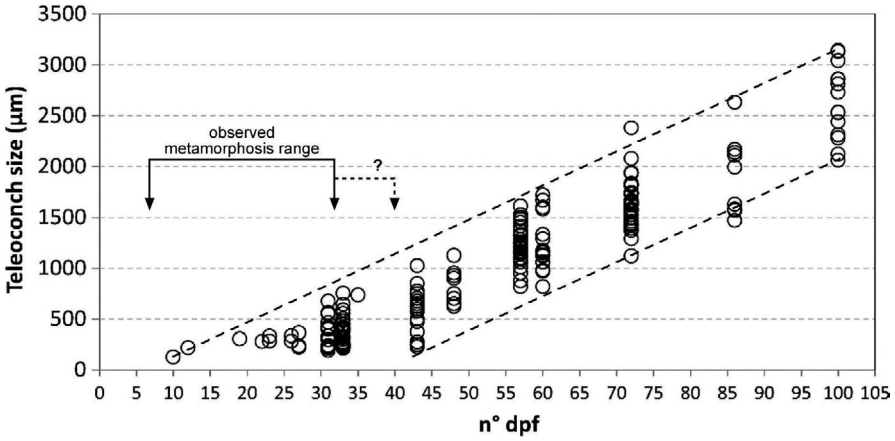

FIGURE 4 Size of the post-larva maximum diameter ("intermediate conch" or teleoconch) in relation to dpf in *P. ferruginea*. Dashed parallel lines show growth trend including 95% of data point. The observed metamorphosis range is displayed by the solid line with arrows; dotted line with arrows represents probable prolonged range, inferred from occurrence of smallest sized post-larvae at day 43

Spawning induction techniques are often based on: (a) simulating natural conditions in which laying occurs; (b) harshening above conditions, creating a stressful situation that may induce mature adults to release gametes (Corpuz, 1981; Dodd, 1957; Ferranti et al., 2018; Kay & Emlet, 2002; Orton et al., 1956).

The stimuli for spawning induction in *P. ferruginea* can be assumed to be different from those of other commonly laboratory-grown species due to its habitat. As it is a species of the upper mesolittoral (Laborel-Deguen & Laborel, 1990b; Templado, 2001; Templado et al., 2004), the influences of subaerial factors (e.g., desiccation, winter cooling and strong waves, Frenkiel, 1975; Laborel-Deguen & Laborel, 1990b, 1991a) are possibly stronger drivers compared with species thriving in the infralittoral habitat (Baker & Tyler, 2001; Ferranti et al., 2018; Mau & Jha, 2018).

The applied techniques were all already somehow applied by other authors on different species: Vigorous Aeration (VA) and Thermal Shock (TS) by Ferranti et al. (2018) on *P. caerulea*, Kay and Emlet (2002) on *Lottia* spp. and Corpuz (1981) on *Cellana exarata*; Hormonal Stimulation (HS) by Mau, Bingham, et al. (2018) and Nhan and Ako (2019) on *Cellana sandwicensis*. Instead, another approach was totally new, developed in order to catch the subaerial habitat triggers: the Dry and Cold maintenance (CD) approach.

Vigorous Aeration and Thermal Shock provided very limited results either in 2019 and 2020 (in five spawning induction trials). Hormonal Stimulation was not effective, conversely to other limpets of the family Nacellidae (Mau, Bingham, et al., 2018; Nhan & Ako, 2019). On the other hand, the novel Dry and Cold maintenance, carried out in sequence with other stimulation techniques (VA, TS and others; see Table 1) provided positive and quite promising results.

During the autumn 2019, in 13 trials using a combination of classical techniques, only one proper emission was achieved from a female, which released a small number of eggs (129.600), with a low fertilization rate (14.90%).

During the autumn 2020, four out of 11 spawning induction trials using combined classical and new techniques were successful, inducing gamete release from two males and four females. The number of eggs released was very high for each female (on average 624.000), with a fertilization rate between 97.40% and 98.90%. Although the described protocol undoubtedly needs to be revised and improved, the fact that 4 successful female spawning events were obtained in three successive trials in 2020, using the same sequence of triggers, in a species such as *P. ferruginea*, that seems quite resistant to spawning stimulation, suggests that it is not a stochastic event and that an appropriate set of triggers has been identified.

Concurrently, the results from our study provide evidence that spawning stimulation needs to be performed on specimens with fully mature gonads. It is noteworthy that 3 of the 4 females that spawned in 2020 had been kept in the natural environment for their complete maturation and that spawning was achieved by using all the stimuli immediately (less than 1 hour) after their recapture and transport to the laboratory. When eggs are released from fully mature females the fertilization rate turns out to be largely higher than in eggs from dissection (almost 3%, Espinosa et al., 2010; range: 20.6%–40%, Guallart, Peña, et al., 2020). Low fertilization rate recorded in the present study from the 2019 event was most probably due to low sperm motility and prolonged addition of sperm in the solution.

Biopsy, as described by Guallart, Peña, et al. (2020), represents a necessary step to assess the maturity stage (oocyte shape; motility for the sperm) and a potential tool for sperm collection (instead of gamete release stimulation in males). However, the quality of the sperm obtained by biopsy (e.g., the degree of maturity, its mobility) is a factor that must be taken into account and can highly affect the fertilization rate. For example, the relatively low fertilization rate registered in F4 of the present study could be due to the fact that the sperm came from males that, already at the end of November, had started the regression of the gonad. The quality of sperm during fertilization may also explain the relative variability in the calculation of the optimal sperm concentration for fertilization, as observed in the results of Guallart, Peña, et al. (2020).

4.2 | Larval development, settlement and timing

Larval development and timing from the present study fundamentally fit with the results from Guallart, Peña, et al. (2020) on *P. ferruginea* (larvae obtained from dissection) and are very similar to those described for other *Patella* species (e.g., Dodd, 1957; Ferranti et al.,

2018; Patten, 1886; Smith, 1935; Wanninger et al., 1999). Compared with Guallart, Peña, et al. (2020), in the present work a broader graphic documentation is presented (see especially Supplementary material, Figures S1 to S9, Videos S1 to S11) and, most of all, the settlement and metamorphosis process is analysed in more detail.

Laborel-Deguen and Laborel (1990b, 1991b, 1991c), on the basis of the size of the eggs (diameter around 200 μm), suggested that the species might have a quite short larval life (although they actually suggested "from a few days up to a few weeks"). This would hamper the species dispersal and especially the recolonization of areas where the species is presently missing because previously suffered significant human pressure, also in case such human pressure has been reduced or removed.

Marine gastropods display a wide spectrum of development strategies in their larval phase. At one extreme, planktotrophic larvae have the ability to feed on phyto- and/or zooplankton after a certain stage of development (Thorson, 1950). This strategy provides a long persistence of the larvae in the plankton (often many months) and implies a high dispersal capacity for the species (Scheltema, 1971). At the other extreme, lecithotrophic larvae depend exclusively on the reserves present in the egg until they manage settling, undergoing metamorphosis and feed in their post-benthic larval form (Kempf & Hadfield, 1985; Thorson, 1950). Such larval style largely limits the time they can remain in the plankton and therefore their dispersal capacity (Sponaugle et al., 2002). Lecithotrophy would also act as a limiting factor in the ability of a larva to reach the optimal place to settle: the "desperate larva hypothesis" postulates that lecithotrophic larvae become less discriminating in their settlement requirements over time, due to depletion of energy reserves (Botello & Krug, 2006). Patellid limpets are generally considered species with lecithotrophic larvae (Guallart, Peña, et al., 2020; Wanninger et al., 1999). In fact, some previous studies compared the effect of larval culture performance (with and without planktonic microalgae addition), but the microalgae addition, a few drops or, most generally, a microalgal biofilm on substrates, was relevant only as a settlement cue (Dodd, 1957; Ferranti et al., 2018; Smith, 1935).

The results of the present study provide evidence that the time frame in which *P. ferruginea* larvae carry out settlement is longer than previously known. Guallart, Peña, et al. (2020) indicated that at 20°C larvae reach the crawling pediveliger stage at about 70 hpf (ca. 3 dpf), so they can start the substrate selection process. They also described the first post-larvae at 10 dpf and advanced post-larvae with a well initiated developing teleoconch at 15 dpf. However, they indicated that, under natural conditions in the Chafarinas Islands, the natural spawning of the population usually takes place when the sea surface temperature is somehow lower (18°C) than the one experienced in the laboratory conditions (20°C): such difference could imply a slower larval development in the natural environment.

Our results, with cultures reared at even lower temperatures (between 15 and 19°C), also showed that the stage of crawling pediveliger larvae is reached between 3 and 4 dpf and the first post-larvae have been observed at 7 dpf. However, a large variability has been observed in the timing of settlement: in fact pediveligers were still found in the tanks up to 32 dpf (possibly even up to 40 days, following the smallest post-larvae measured at 43 dpf, Figure 4). This finding considerably broadens the survival range and dispersal capacity of *P. ferruginea* larvae. The causes of this variability have not been determined, since advanced post-larvae and pediveligers have been found simultaneously for many days in the same tanks and therefore under identical environmental conditions.

Settlement and metamorphosis are sometimes considered equivalent events, but are clearly different. The first involves fixation on the substrate although the larva can still move by swimming to another spot on the substrate. Metamorphosis implies the irreversible loss of swimming ability and therefore of the potential to change the selected substrate, as well as a whole series of body changes, including the development of cephalic structures that allow it to feed on the benthic biofilm (Botello & Krug, 2006; Rodríguez et al., 1993).

Different criteria can be considered to assess that a larva has performed metamorphosis. For example, Scheltema (1961) for the gastropod *Nassarius obsoletus* (=*Ilyanassa obsolete*) indicates the loss of the veil as indicative, while Alfaro Jeffs and King (2014) for *Haliotis iris* considered individuals to be metamorphosed when distinct fan-shaped growth of the peristomal shell (named here "intermediate conch") was detectable.

From the observations made, the following sequence of events is proposed in the metamorphosis of *Patella ferruginea*. Starting from a crawling pediveliger, with a ciliated veil that allows it to swim but with a well-developed, elongated foot (with distinction between propodia and metapodium), with the surface covered with small cilia, with operculum, with eyes, with cephalic tentacles:

1. The cilia of the velum become non-functional (it still has a disc-shaped anterior part of the body)
2. The velum cilia are lost (it still has an anterior disc-shaped part)
3. The operculum is lost
4. The velum disc is transformed into a defined cephalic area
5. The mouth develops in the cephalic region
6. The transition zone between the protoconch and the teleoconch, the "intermediate shell," starts to grow
7. Teleoconch starts developing as the beginning of the definitive shell morphology.

4.3 | Post-larval growth

The growth rate of post-larvae was also found to be largely variable. It is noteworthy that the growth rate of *P. ferruginea* post-larvae in the present study is considerably higher than that previously described by Guallart, Peña, et al. (2020). In the present study, juveniles achieved 1–2.5 mm MSD, with the typical colouration of the teleoconch which characterizes the small juveniles (Guallart et al., 2017), after only 1.5–2.5 months: same size was achieved in more than 4–5 months according to Guallart, Peña, et al. (2020). Such faster growth rate may be ascribed to food availability (quality and quantity of biofilm) or to temperature, although not specific information is available for both studies.

4.4 | Restocking and conservation management implications

As reported by several studies the translocation of adult *P. ferruginea* specimens for restocking purposes is not recommended as it causes high mortality (90% after 2 years, Laborel-Deguen & Laborel, 1991c; 60%–80% after 2 years, Espinosa et al., 2008; 20%–80% after about 2 years, Zarrouk et al., 2018; see Luque et al., 2018 for a review). In fact, for this reason, the Strategy of the Spanish Administration for *P. ferruginea* advised against the translocation of specimens from natural populations and highlighted the importance of doing it, if necessary, exclusively from specimens obtained through aquaculture techniques (MMAMRM, 2008). Yet, an established protocol for controlled reproduction of the species was not available and the only specimens obtained in captivity were from dissection of adult females (Guallart et al., 2017; Guallart, Peña, et al., 2020).

Our study, for the first time, provides evidence that spawning can be induced in *P. ferruginea* and demonstrates the feasibility of obtaining juveniles under controlled conditions through low-invasive methods. Furthermore, fertilization of eggs released through spawning induction turned out to be much higher than from dissection (almost 3%, Espinosa et al., 2010; range: 20.6%–40%, Guallart, Peña, et al., 2020), further supporting the potential of the approach to produce a large number of larvae. These results allow to establish a preliminary protocol for spawning induction of the species and to step forward the possibility of *P. ferruginea* restocking and reintroduction in the natural environment. In addition, this preliminary protocol may represent a useful reference for other protected or endangered limpet species, such as *Patella candei* and *P. aspera* in the Azores archipelago, species subject to overfishing and whose harvesting is now prohibited (Martins et al., 2008, 2011; Santos et al., 2010). For these species, work is underway to achieve aquaculture production (*P. aspera*, Castejón et al., 2020).

Larval growth from the present study fundamentally mirrors that from eggs obtained from dissection (Guallart, Peña, et al., 2020), although some temperature effects may be detected and larvae cultured at 18–19°C may reach the crawling and swimming pediveliger stage at ca. 3 dpf, compared with ca. 4 dpf at 15–17°C. Therefore, these results can provide insights for best practice maintenance of larvae for aquaculture purposes. Only data are available regarding survival rate of the trochophore stage (F3: 47.60%; F4: 32.60%; F2: not calculated; Table 2). Survival of subsequent larval stages is not available, since we preferred to avoid excessive manipulation of the substrates in order to minimize stress on post-larvae and juveniles.

Post-larval growth observed so far is remarkably faster than in the specimens monitored by Guallart, Peña, et al. (2020) who reported juveniles 1 mm MSD at 4 months. At present, in fact, some of our juveniles exceed 3 mm at 100 dpf. If the difference in growth rate is maintained, we expect in a year to reach a size that we consider suitable for introduction in the natural environment (2–3 cm).

Even more remarkably, our results also show that the dispersal capacity of *P. ferruginea* is not as low as previously assumed (Laborel-Deguen & Laborel, 1990b, 1991b, 1991c; Templado, 2001). This finding could be in agreement with the genetic uniformity observed in the distribution of this species between Corsica, NE Sardinia and Ligurian coastline (Casu et al., 2011), and further reinforces the observations by Guallart, Peña, et al. (2020) that larval duration could allow dispersal across at least tens of kilometres, taking into account that natural spawning and fertilization occur in the autumn during major storms usually associated with strong surface currents (Frenkiel, 1975; Guallart, 2008; Laborel-Deguen & Laborel, 1990b). Therefore, the longer larval duration than expected further reinforces the conclusions by Guallart, Peña, et al. (2020) that the cause of the observed regression in the current distribution of the species should not be ascribed to biological limitations to recolonize areas where it used to be distributed but, above all, to the persistence of strong human pressures (mostly harvesting) that had caused their decline/disappearance.

The duration of the swimming larval phase (between 7 and 32 dpf, maybe even 40), observed in the present study, could make possible exchanges between the populations of NE Sardinia and Corsica and the Ligurian coasts. In fact, taking into consideration the direction and maximum speed of the surface layer of the Northern Current (10 cm/s, Esposito & Manzella, 1982; Millot & Taupier-Letage, 2005; Pinardi et al., 2015), a larva could have a dispersal capacity of 8.64 km/day. This velocity turns into a minimum of about 60.5 km dispersal in 7 days to a maximum of about 276.5 km dispersal in 32 days or even 345.6 in 40 days. On the basis of these observations, of the current regime of the Ligurian Sea (Pinardi et al., 2015) and of the distribution of larger populations of *P. ferruginea* in the NW Mediterranean (Ferranti et al., 2021; Luque et al., 2018) we can hypothesize that the adult specimens recorded along the Ligurian coast, and more abundant in the Cinque Terre MPA area (Ferranti et al., 2019), certainly represent stochastic recruitment of larvae from populations located at least several hundred kilometres away. Most reasonably, these larvae could originate from the coasts of NW Corsica (about 210 Km between Marine Park of Scandola and Cinque Terre MPA and 117 Km Cap Corse- Cinque Terre MPA; Figure 5), or from NE Sardinia (about 350 km distance between Tavolara and Cinque Terre MPAs). Moreover, the reintroduction of *P. ferruginea* juveniles in the Ligurian MPAs, as foreseen in the ReLife project, could make them work as a source of individuals for stretches of coast in the range of 60–300 km, creating hotspots in the distribution of the species along the coast, which could be considered "reproductively viable populations." The reproductive potential of the species in the area has, additionally, been proven by Ferranti et al. (2021), who provided evidence of achievement of sexual maturation and even sex-change at the northern limit of the species distribution.

The results of the present study reinforce the feasibility of restocking actions implementation, highly encouraged by several EU strategies, most of all the Biodiversity Strategy 2030, also following the recommendations made by the IUCN (e.g., McGowan et al., 2017). Therefore, the approach of carrying out reintroduction or reinforcement actions by implantation of aquaculture-produced specimens of *P. ferruginea* in areas where the species is currently severely

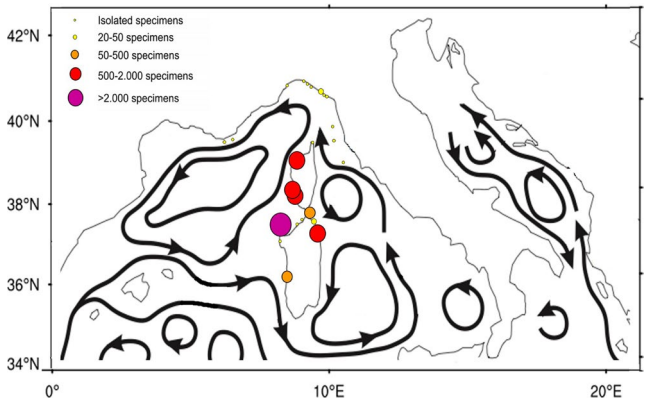

FIGURE 5 Mean surface circulation structures from Pinardi et al. (2015) superimposed to the present known distribution of the species in the North Mediterranean. Different size of the circles refers to adult population size of *P. ferruginea*. Data regarding species distribution and abundance are from Luque et al. (2018), updated by Ferranti et al. (2019)

depleted or has disappeared seems quite reasonable, under the assumption that local human pressures have been removed, although further studies are required to convert these results into a routine protocol for aquaculture production.

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AUTHOR CONTRIBUTION

Maria Paola Ferranti: Writing - Original Draft, Writing - Review & Editing, Investigation, Conceptualization, Visualization. Javier Guallart: Writing - Original Draft, Writing - Review & Editing, Investigation, Conceptualization, Visualization. Giorgio Fanciulli: Resources, Supervision, Project administration, Funding acquisition. Pier Augusto Panzalis: Investigation, Resources. Mariachiara Chiantore: Resources, Writing - Original Draft, Writing - Review & Editing, Conceptualization, Visualization, Supervision, Project administration, Funding acquisition.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

DATA AVAILABILITY STATEMENT

Not applicable.

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