Research Article

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
DOI: http://dx.doi.org/10.12681/mms.22508

Filling gaps: closing the life cycle of the endangered Mediterranean limpet *Patella ferruginea* Gmelin, 1791 (Gastropoda, Patellidae)

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Handling Editor: Serge GOFAS

Received: 1 March 2020; Accepted: 2 May 2020; Published on line: 30 June 2020

Abstract

Several reproductive issues and the larval development of the ferruginous limpet, *Patella ferruginea* Gmelin, 1791, an endangered species endemic from the western Mediterranean Sea, were studied to fill gaps in the knowledge of its life cycle.

Average diameter of mature oocytes was 141.83 µm and mean oocyte density in the ovary was 283,800 oocytes/gram. No significant correlations were found between both oocyte diameter or density and female shell length. Female fecundity (number of oocytes per gonad) was significantly correlated with shell length and varied between 189,200 oocytes in a 40.0 mm female and 5,019,200 oocytes in an 86.4 mm female. However, there was considerable variability, in particular for the largest females.

Spawning induction was not achieved using usual molluscan aquaculture methods. Thus, oocytes obtained after dissection of females were used for fertilisations trials. Alkalinisation treatments of seawater were used to test improvement in oocyte maturation and later fertilisation rates. Treatment at pH 9 during 2 h produced the highest increase in the percentage of mature oocytes and in the fertilisation rate; but these results showed high variability and were mainly significant when the initial degree of maturation was low. Sperm concentration experiments determined that best *in vitro* fertilisation was performed at 10^5 and 5×10^5 spermatozoids/ml.

The sequence and timing of the complete larval development of *Patella ferruginea* in laboratory conditions is described and illustrated here for the first time. At 20 °C, larvae became competent for metamorphosis 3 days after fertilisation, but some crawling pediveliger larvae with a still well-developed velum were found even 7 days after fertilisation. Recruits 1-2 mm in length were achieved in low numbers from two of the assays and were first detected between 131-141 days after fertilisation. The resulting juveniles were monitored for two years and sex determination of five survivors at the end of this period showed that two were mature males, two mature females and one indeterminate.

Our results show that the main reproductive traits or larval development of *P. ferruginea* hardly differ from those of other non-endangered Mediterranean or NE Atlantic limpet species. Therefore, its decline cannot be mainly attributed to constraints of these traits as was previously suggested, but to human impact.

On the other hand, it is feasible to complete the life cycle of this species in laboratory conditions, from fertilised eggs to mature individuals. However, an important part of the process like spawning induction was not achieved as gonads needed to be dissected fatally from females, although sperm could be obtained from males through non-lethal biopsies. At present, large-scale aquaculture production for reintroduction, restocking or stock enhancement purposes is neither possible nor an advisable conservation tool yet. Further study is required and meanwhile, the appropriate design of a network of effectively protected marine areas that ensures connectivity among extant populations is necessary.

Keywords: *Patella ferruginea*; Mollusca; Gastropoda; Patellidae; reproduction; fecundation; larval development; Mediterranean; endangered species; conservation.

Introduction

The ferruginous limpet (*Patella ferruginea* Gmelin, 1791) is a western Mediterranean endemic gastropod that is currently considered at risk of extinction. It was included in Annex IV of the "Habitats" Directive in 1992 and in 1996 in Annex II of Barcelona and Bern Conventions, and is under strict legal protection in the countries it still inhabits. The decline of this species has been attributed to human impact (harvesting, habitat degradation, development of coastal infrastructures and marine pollution) (Laborel-Deguen & Laborel, 1990a; 1991a; Templado, 2001; Paracuellos *et al*., 2003; Templado *et al.*, 2004; Moreno & Arroyo, 2008). Its distribution has been progressively reduced in recent times with most of its populations being much reduced or vanishing, mainly during the second half of the 20th century (Laborel-Deguen & Laborel, 1990a, 1991a; Porcheddu & Milella, 1991). The current distribution area of *Patella ferruginea* is very fragmented (see Luque *et al*., 2018 for a review), but the main populations are located in very few localities in North Africa: Chafarinas Islands, Melilla and Ceuta cities (Spain), Habibas, Plana (= Paloma) and Rachgoun Islands (Algeria) and Zembra Island (Tunis). There are relict populations or sparse specimens recorded in Corsica and Sardinia (Italy), the SE coast of Iberian peninsula (Andalusia and Murcia, Spain), Alboran Island, some north African localities like the Peñón de Vélez de la Gomera (Spain), several localities on the Mediterranean coast of Morocco (Al Hoceima National Park and other isolated coastal localities), and a few localities in France (Porquerolles, Port-Cros and Levant Island) and Italy (Tuscan Archipelago; Egadi and Pantelleria islands in Sicily). Recently it has been recorded in Liguria, on the mainland of the north Italian peninsula (Ferranti *et al*., 2019).

Reintroduction, restocking or stock enhancement are optional tools for the management of species whose populations are very depleted, but they need to be carefully evaluated before large-scale implementation (Bell *et al*., 2005). The Spanish National Strategy for the conservation of *Patella ferruginea* (MMAMRM, 2008) strictly limits these tools to actions based only on specimens obtained through aquaculture techniques. This limitation is envisaged to avoid the extraction of specimens from the few still well-preserved natural populations, since previous translocation attempts resulted in high mortalities, unacceptable for this strictly protected species (Laborel-Deguen & Laborel, 1991c; Espinosa *et al*., 2008; Zarrouk *et al*., 2018; see review in Luque *et al*., 2018). The Spanish Strategy thus encouraged the development of techniques for obtaining hatchery-reared juveniles to reintroduce or restock the species as one of the priority research lines, and the present study is an attempt in this direction.

Frenkiel (1975) and Laborel-Deguen & Laborel (1991b) noted that gamete fertilisation in artificial conditions of *P. ferruginea* was not difficult, but these authors did not provide additional data. Guallart *et al.* (2006) described a makeshift case of fertilisation during field work carried out in November 2005 in the Chafarinas Islands, resulting in pre-torsional veligers after 40 h. Later, Espinosa *et al*. (2010) tried to make controlled fertilisation experiments and described the early developmental stages, but they failed to continue beyond 48 h (at an anomalous early pre-torsional veliger stage).

The embryonic and larval development of several limpet species has been studied in detail in some classic works since the late 19th and during the first half of the 20th centuries by some authors who used limpets as models for the description of the larval development of gastropods (e.g., Patten, 1886; Wilson, 1904; Smith, 1935; Dodd, 1957). However, most of these works were based on fertilisation from gametes obtained through the dissection of adult specimens and in many cases, larvae did not reach metamorphosis. Spawning induction has been achieved only in a few limpet species by different approaches and often with unpredictable success (e.g., Kay & Emlet, 2002; Nhan & Ako, 2012, 2019; Ferranti *et al*., 2018). Although limpets are often harvested for human consumption (e.g., Pombo & Escofet, 1996; Hawkins *et al.*, 2000; Martins *et al.*, 2008; Riera *et al*., 2016; Henriques *et al*., 2017), they have not been the subject of intensive aquaculture studies (Mau & Jha, 2018). Nonetheless, several studies have shown techniques to improve maturation of artificially extracted oocytes from the ovary, among which the most common is their maintenance for a certain length of time in alkalinised seawater (e.g., Corpuz, 1981; Smaldon & Duffus, 1985; Hodgson *et al*., 2007; Aquino de Souza *et al*., 2009).

In the present study, we firstly address gaps in knowledge on the life cycle of adults and early post-settlement stages of *P. ferruginea* and secondly provide some techniques for fertilisation in laboratory, larval rearing and production of juveniles under controlled conditions. Ultimately, we aim to contribute to the advancement of our understanding of the early life history of this species. This has implications for its conservation management since developmental traits are related to its dispersal ability, the connectivity of populations, genetic structure, ecological resilience and evolutionary persistence, and must be taken into account in the design of any conservation strategies. Results presented here derive from various projects carried out from specimens collected from the Chafarinas Islands between 2006 and 2013, to study different aspects of the biology of *Patella ferruginea* with the aim of enabling its recovery and general conservation. The specific objectives of the study can be summarised as follows: 1) to develop methods to maintain broodstock in the laboratory, 2) to measure oocyte dimensions, 3) to estimate fecundity of females and its variation with size, 4) to test the effect of alkalinisation on oocyte maturation, 5) to determine optimum sperm concentration for fertilisation, and finally 6) to describe embryonic and larval development and metamorphosis and its implications for juvenile survival.

Material and Methods

Origin of specimens

Chafarinas Islands are a small Spanish archipelago located in SE of the Alboran Sea (35.1833326° N, -2.4333316° W), at 1.9 nm from the north coast of Morocco. It hosts one of the most important and healthy existing populations of *Patella ferruginea*, which has been studied and monitored since 1999. Guallart & Templado (2016) estimated a population stock of more than 42,000 adults for the entire archipelago, with annual recruitment being regular and sometimes very high (Guallart *et al*., 2011, 2012a; Guallart & Templado, 2016). This allowed not only the study of a natural population of this species in optimal conditions, but also the possibility to extract and sacrifice some specimens for research with a negligible impact on the local population. Notwithstanding, the conservation status of *P. ferruginea* necessitated minimising the sacrifice of specimens.

All specimens used for fertilisation assays were collected in November of several years, taking into account that spawning occurs in this month after the sudden decrease in the seawater surface temperature during October and with the onset of major autumn storms (Frenkiel, 1975; Guallart, 2006; Guallart *et al*., 2006). The short reproductive period of this species only allowed a few experiments each year.

From field work to aquaculture centres

After the first preliminary experiment in 2005 of *in vitro* fertilisation, several assays of spawning induction and fertilisation were performed between 2006 and 2012 in the laboratory facilities of the Chafarinas Islands Biological Station (CIBS). These studies also continued from 2011 to 2013 in two aquaculture centres away from Chafarinas Islands, by transferring sixty mature adults (39 females and 21 males) to the Instituto de Acuicultura de Torre de la Sal (IATS-CSIC, Castellón) and to the Institut de Recerca i Tecnología Alimentàries (IRTA, Tarragona). These specimens were sexed in Chafarinas following the technique of Wright & Lindberg (1979) successfully used for several limpet species (e.g. Lindberg & Wright, 1985; Le Quesne & Hawkins, 2006), but specifically adapted to *Patella ferruginea* by Guallart *et al*. (2013), and then maintained several days in aquaria at CIBS before being transported to IATS-CSIC and IRTA. For transport, each specimen was individually covered with a seawater-moistened cloth and carried in refrigerated isothermal boxes at temperatures between 8 and 15 °C. Transfer from CIBS to IATS-CSIC and IRTA lasted less than 24 h in all cases, with no mortality observed within the two-week period after collection and transportation.

During the 2006-2012 lab work, regular attempts to induce spawning in *Patella ferruginea* with methods usually employed for marine gastropods (thermal shocking, hydrogen peroxide or vigorous bubbling; see Kay & Emlet, 2002) were made in the three research centres, but they were unsuccessful, as usual in most patellid limpets (Aquino de Souza *et al*., 2009). Only once after a spawn induction trial at IRTA did one or more males release sperm several hours later during the night. Therefore, trials of *in vitro* fertilisation throughout these years were done obtaining gametes by direct extraction from mature specimens, a method formerly used by several authors (e.g., Dodd, 1957; Hodgson *et al*., 2007; Aquino-Souza *et al*., 2009).

In total, 26 experiments of *in vitro* fertilisation of *P. ferruginea* were carried out between 2006 and 2012. We describe here results of only 13 of them (A01 to A13, Table 1), performed between November 2009 and November 2012. In these experiments: 1) artificial matura-

Table 1. Summary of fertilisation experiments described in the present study. Abbreviations: CIBS, Chafarinas Islands Biological Station; IRTA, Institut de Recerca i Tecnología Alimentàries (Tarragona); IATS-CSIC, Instituto de Acuicultura de Torre de la Sal (Castellón). Changes of pH for oocyte maturation through seawater alkalinisation: 1, no alkalinisation (or control group); 2, 15 min at pH 9; 3, 2 h at pH 9. Sperm concentration control: use or non-use of different sperm concentrations during fertilisation. For each assay the number of females and estimated number of oocytes is indicated.

Code	Date	Location	pH	Sperm concentration control	Number of females	Estimated number of oocytes
A01	12/11/2009	CIBS	1, 2, 3	No	1	1,227,400
A02	13/11/2009	CIBS	1, 2, 3	No	1	3,352,600
A03	15/11/2009	CIBS	1, 2, 3	No	2	4,835,400
A04	21/11/2010	CIBS	1, 2, 3	No	1	2,116,300
A05	14/11/2011	IRTA	3	No	1	3,440,700
A06	28/11/2011	IATS-CSIC	3	No	1	1,069,400
A07	19/11/2012	IATS-CSIC	3	No	1	988,700
A08	20/11/2012	IATS-CSIC	3	Yes	1	2,000,300
A09	21/11/2012	IATS-CSIC	3	Yes	1	1,249,800
A10	26/11/2012	IRTA	1, 2, 3	Yes	1	2,531,300
A11	27/11/2012	IRTA	1, 2, 3	Yes	1	4,545,100
A12	28/11/2012	IRTA	1, 2, 3	Yes	1	2,754,000
A13	13/12/2012	IATS-CSIC	1, 2, 3	Yes	2	563,700

tion of oocytes by pH changes (seawater alkalinisation) was analyzed; 2) the influence of sperm concentration on fertilisation rate and percentage of non-anomalous larvae was studied; and 3) larval development to advanced stages was achieved.

Handling and maintenance of specimens

The maintenance of *Patella ferruginea* in culture tanks (from post-larvae through to juveniles and then to adults) entails some difficulties in comparison to other marine gastropods, most probably due to its midlittoral habitat. One of the major difficulties is related to a characteristic behaviour by which ferruginous limpets (and several other limpet species; Hawkins pers. obs.) tend to move upwards on the walls when placed in an aquarium or tank, and can remain out of the water until they die by desiccation (Peña *et al*., 2013). This behaviour also influences the design of strategies for settlement and metamorphosis of larvae (see below). A second key factor is the feeding of specimens, which graze on the biofilm of cyanobacteria and microalgae that covers the substrate in their natural habitat (Laborel-Deguen & Laborel, 1990b, 1991b; Burgos-Rubio *et al*., 2015), although it is a topic still little known in *P. ferruginea*.

To tackle these problems, similar techniques were followed at the three research centres, although with some differences. At CIBS, specimens were maintained in tanks for short periods (several days) on boulders extracted from the nearby infralittoral bottoms covered with a fine algal film. Wetting was ensured by keeping them under a permanent flow from jets of seawater. The water temperature in the tanks was maintained in the range 18-20 °C. At IRTA and IATS-CSIC, where reproductive adults (and later juveniles) remained for much longer periods, more elaborate techniques were employed (Pérez *et al*., 2012). At IRTA, automatic tides of 25-30 cm fluctuations in the water level were simulated with a periodicity of 2-4 h, the water temperature was maintained in the range 15-19 °C and the photoperiod produced with artificial light in a daily proportion 10:14 hours (light:dark) throughout the study. At IATS-CSIC, tides were simulated manually by varying the water level once a day to submerge or emerge the specimens. The water temperature oscillated within the range of 8-27 °C throughout the study period depending on the temperature of the water that reached the experimental tanks from the seawater reservoirs, and the photoperiod and light source were natural. Despite both of these tidal simulations, some specimens often tended to escape outside the tanks, which were placed back manually in a submerged position near the water level.

To promote a biofilm growth on the tank walls and other structures placed within (made of different materials: PVC, poliethylene, methachrylate, fiberglass-coated structures), these surfaces were previously maintained for several days in seawater enriched with nutrients and a high concentration of various species of planktonic microalgae commonly used in larval culture of molluscs (*Tetraselmis suecica*, *Isochrysis galbana* and *Phaeodactylum tricornutum*). When feeding by limpets reduced the biofilm covering the tanks, specimens were manually transferred to other tanks with a well-developed biofilm cover.

In most cases, juveniles and adults were maintained in "barely filtered seawater" (BFS) using 60-100 µm sand-filters that provided conditions relatively close to the natural environment and promoted the maintenance and growth of surface biofilms.

Obtaining gametes

Prior to gamete extraction, the maximum diameter (MD) of the shell (defined as the maximum shell length along its longitudinal axis, including in its profile prominences due to prolongation of the 'shell ribs' characteristic of the species) was measured with a Vernier caliper and this parameter was used as representative of size of specimens. Total weight, body weight (without the shell) and in several cases gonad weight were also recorded with an accuracy of ±0.01 g. Throughout the study, all samples of gametes were maintained in sterilised filtered sea water (SFSW), using UV lamps of different designs and powers and 1 µm filters.

Oocytes for fertilisation experiments were obtained by dissecting mature females. The bulky ovary surface observed after dissection under a stereomicroscope showed polygonal, compressed, mosaic-shaped oocytes indicating that they were in an advanced stage of maturation but spawning had not started (stage 3 of Frenkiel, 1975). Mature oocytes could only be obtained by dissection of one or two mature females per assay (Table 1). Ovaries were cut in several pieces and put into Petri dishes with SFSW, and oocytes carefully separated shaking these pieces using forceps and scissors, following Dodd (1957). The obtained material was then sieved through a 500 µm mesh to remove residues of the ovarian stroma and transferred to glass or plastic cylindrical containers of ca. 1 litre. Limpet oocytes are much denser than seawater and settle to the bottom after ca. 10 min: this allows removal of residues of broken oocytes and other material in suspension by decantation of ca. 80% of supernatant and refilling with SFSW. This procedure was repeated 4-6 times each time.

Unlike females, significant and enough amounts of sperm to perform fertilisation could be obtained from males through extraction by syringe using the non-lethal method of Guallart *et al*. (2013). Only sperm that showed at least some degree of mobility and no starry aggregates after extraction was used for fertilisation assays (see below). For the last six trials (A08-A13, see Table 1), in which the influence of sperm concentration on the fertilisation rate was studied, the amount of sperm obtained through biopsies was too low for experimental purposes. Therefore, two mature males were dissected for each of these assays, the gonad was removed and cut in several pieces in a Petri dish with SFSW, allowing the sperm to actively flow into the seawater, following Dodd (1957)

and Hodgson *et al.* (2007). Only males with bulky whitish gonads (stage 3 according to Frenkiel, 1975) were selected. Initial spermatozoid concentration was counted in a Neubauer chamber. Sperm motility was moderate or low in all cases.

For oocyte measurement, mature oocytes were selected from 67 females as follows: 1) females dissected for fertilisation experiments (n = 14, see below); 2) females dissected for the study of reproductive cycle (n = 13; Guallart *et al*., 2006); and 3) biopsies made during the sex change studies (n = 40; Guallart *et al*., 2013). Oocytes were kept at least 1 h in SFSW with agitation or gentle aeration after being extracted from the ovary. After this time, they were classified by their shape as "irregular", "oval" or "round" (i.e., spherical). The chorion condition was classified as "with chorion" (contour completely covered), "partially without chorion" (20-80% of covered contour) or "without chorion" (without chorion or with < 20% of covered contour). Although fertilisation could potentially occur in all kinds of oocytes (except the "irregular" and "completely covered with chorion" ones), throughout the study only "round and without chorion" (RN-NC) oocytes have been considered mature, following Dodd (1957) and Aquino de Souza *et al*. (2009). Oocyte samples were measured under a microscope using an ocular micrometre at 400× magnification. The two main perpendicular axes of 20 oocytes per female were measured, the oocyte diameter being considered the mean of the two values. The average oocyte diameter was then calculated for each female. The chorion, a jelly-like cover of the oocyte, was not considered in measurements since it dissolves within one to a few hours in seawater (Dodd, 1957) and consequently does not play any nutritive role for the embryo. The percentage of mature (RN-NC) oocytes was estimated in *Patella ferruginea* by counting at least 100 oocytes for each female.

For comparison, the same procedure was carried out to measure the diameter of oocyte samples from 3 mature females of *Patella caerulea* (length range 32.1-44.0 mm MD) and two of *P. rustica* (40.8, 42.8 mm MD), also collected in Chafarinas Islands.

Estimates of fecundity

Broadly speaking, the term "fecundity" refers to the number of offspring produced by a female in a certain time unit, normally in one breeding season (Ramirez Llodra, 2002). We refer here to fecundity as the estimated number of oocytes present in the mature ovary of a female, although the "actual fecundity" is possibly somewhat lower, since not all gametes are necessarily emitted during spawning (Ramirez Llodra, 2002).

To calculate the number of oocytes per female, the ovary was weighted in 35 mature females and a piece of ca. 0.30 g (weighted with an accuracy of ±0.01 g) was collected. All oocytes of this sample were extracted as previously described, then sieved through a 500 µm mesh and transferred into a measuring cylinder filled up to 1,000 ml with SFSW. After homogenising this suspension, a subsample of 1 ml was taken and put into a Sedgewick Rafter Counting Chamber (SRCC). Counts allowed to calculate the number of oocytes per gram of ovary ("oocytes density in ovary") and per ovary total weight ("fecundity" hereinafter). Values obtained for each specimen are probably underestimates, due to difficulties to collect all oocytes from the gonad sample or to oocyte damage during extraction.

Maturation of oocytes through water alkalinisation

After their extraction and before fertilisation, oocytes were experimentally matured in twelve trials by NaOH-alkalinisation of SFSW at 20 °C, seven of them under different conditions (A01-A04, A10, A12 and A13; see Table 1). In these seven trials, the obtained oocytes were divided into 3 similar portions, treating one sample at pH 9 for 15 min (pH 9-15m), another at pH 9 for 2 h (pH 9-2h) and comparing results with a control sample without any pH treatment (Table 1). The pH 9-2h treatment was the only one used in the other five fertilisation trials (A05-A09) given promising results of the first assays A01-A04 (see below). For alkalinisation treatments, oocyte samples were put into 1-4 litre containers with SFSW and concentrated NaOH solution was added until the solution reached pH 9. After each alkalinisation treatment, return to Chafarinas Islands seawater natural pH (pH 8.0) was reached by siphoning off the supernatant and replacing water with fresh SFSW 4-6 times. The whole process was carried out with gentle aeration. One hour after returning to natural pH, the oocyte maturation stage was characterised by shape and chorion condition counting the percentage of RN-NC oocytes in at least 100 oocytes taken from each of the treated and control samples using a SRCC.

A paired-Samples T-Test was used for comparing "pre *vs.* post" alkalinisation treatment effects on oocyte maturation and fertilisation rate for all treatments in experiments A01-A04 and A-10, A12 and A13. All calculations were made with the software IBM SPSS 22.0.0.0.

Sperm concentration

To determine the optimum concentration of spermatozoids for *in vitro* fertilisation, in six experiments (A08-A13) six groups of ca. 100,000 oocytes were separated and placed in 500 ml plastic beakers with SFSW at 20 °C. From the concentrated sperm mixture obtained by the dissection of mature males, progressive dilutions of a stock solution of sperm in SFSW were made to obtain concentrations of 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 spermatozoids/ml when added to each beaker. Spermatozoids were counted in a Neubauer Chamber. One hour later, to stop the fertilisation process, containers were refilled with SFSW, eggs allowed to fall to the bottom and then the supernatant was siphoned and refilled 2-6 times.

Fertilisation rate

To calculate the fertilisation rate, a sample of at least 100 eggs and embryos (considering embryos those with a clear polar body extruding or at least two blastomeres) obtained 2-3 h after fertilisation were counted under a microscope in a SRCC in trials A08-A13. After 20-24 h a homogenised 1 ml sample taken from each beaker was fixed with a drop of 4% formaldehyde, then counted in a SRCC the number of unfertilised oocytes, normal larvae (trochophores) and abnormal embryos and larvae. From these counts, the rate of fertilisation success was considered as the percentage of normal larvae (or at least not apparently abnormal) compared to total count (unfertilised eggs included) in each beaker.

The remaining eggs obtained in experiments A08-A13 were fertilised with a concentration of ca. 10^5 spermatozoids/ml and used for the study of embryonic and larval development.

Larval culture

To study embryonic and larval development, eggs were rinsed and placed in plastic or glass containers of different sizes (1-8 litres) wide enough to obtain a single layer of eggs on the bottom. Containers were only partially filled with SFSW and sperm added for fertilisation. Fertilisation was stopped by filling the containers, thus diluting considerably the amount of sperm, as previously described, and successive supernatant decantation and refilling with SFSW, repeated 4-6 times. No aeration was used during first 24 h post fertilisation, and after that, cultures were maintained under gentle aeration.

After ca. 16-18 h, two-thirds of the upper water layer with active swimming trochophore larvae was transferred by siphoning to glass or plastic beakers with SFSW. The original containers were refilled with SFSW and after 20-60 min, siphoning again and transferring the remaining active larvae to new beakers, reducing the density of larvae. Finally, the bottom layer of the containers with unfertilised eggs and abnormal larvae was discarded.

A similar procedure was used after 48 h with post-torsional veliger larvae to reduce larval concentration, renewing water and discarding abnormal larvae usually accumulated in the bottom. Fertilised eggs, embryos, and larvae were maintained at both IRTA and IATS-CSIC in SFSW at 20 °C in a phytoplankton temperature-controlled culture chamber, with a photoperiod of 24 h of light during first 72 h. No controls to calculate the concentration of larvae per volume of water were carried out in any case. No food was provided to the larvae during development (i.e., phytoplankton) in the assumption that development was most probably lecithotrophic.

Metamorphosis

Advanced pediveliger larvae were transferred 60-72 h after fertilisation to circular, glass fibre or methacrylate "metamorphosis tanks" of 200 to 1,000 l, partially filled with BFS. Water was not renewed during the subsequent days, but the level of water was progressively increased by slowly adding BFS to tanks. This procedure aimed to avoid loss of larvae and to ensure adequate humidity for post-larvae settled on tank walls, since it was assumed that larval settlement should take place close to the water-air interface. In most cases, different semi-submerged structures (a set of different plastic pieces made of materials similar to those of the adult tanks, of different shapes, composition and degree of surface rugosity) were placed into the tanks to increase the surface available for larval settlement. On all these structures, development of a biofilm coverage on the surfaces was previously promoted following the above described procedure for adult tanks. Additionally, some vertically semi-submerged plastic Petri dishes covered with biofilm were placed in the "metamorphosis tanks" and periodically examined under stereomicroscope and microscope (Guallart *et al*., 2017).

Ten days after larval transfer to settlement tanks, water was renewed and tides were simulated as described in the section regarding handling and maintenance of adult specimens.

Maintenance of juveniles

Recruits were obtained from larval culture trials in 2012 (from November 2011 experiment A06) and 2013 (from November 2012 experiments A07-A09), only at IATS-CSIC. They were maintained in the same tanks and conditions previously described for adults. Sex determination of specimens produced in fertilisation assays of November 2011 were made two years later, at the end of October, 2013 (month in which gonads are mature in the natural habitat), through biopsies following Guallart *et al*. (2013). Sex determination attempts were not carried out in 2012, when specimens from 2011 fertilisation experiments were only one year old, to avoid unnecessary risks for such small specimens, also considering that probably they were not yet mature at this age (Guallart & Acevedo, 2006).

Results

Oocyte size and density of oocytes in the ovaries

Diameter of mature oocytes of 67 females from 40.0 to 90.4 mm MD (average 71.4 ± 11.5 mm MD) of *P. ferruginea* ranged between 131.34 and 149.50 µm, with an average of 141.83 ± 3.67 µm (Fig. 1A). No significant correlation was found between oocytes diameter and shell length ($r = 0.136$; $p = 0.271$; $n = 67$). The mean diameter of mature oocytes found in *Patella caerulea* was 143.52 µm ± 2.75 ($n = 3$) and 143.97 µm ± 2.98 ($n = 2$) in *P. rustica.* Oocyte diameter of *P. ferruginea* was very similar or slightly smaller than the other two species, but small sample sizes of the latter preclude formal statistical analyses.

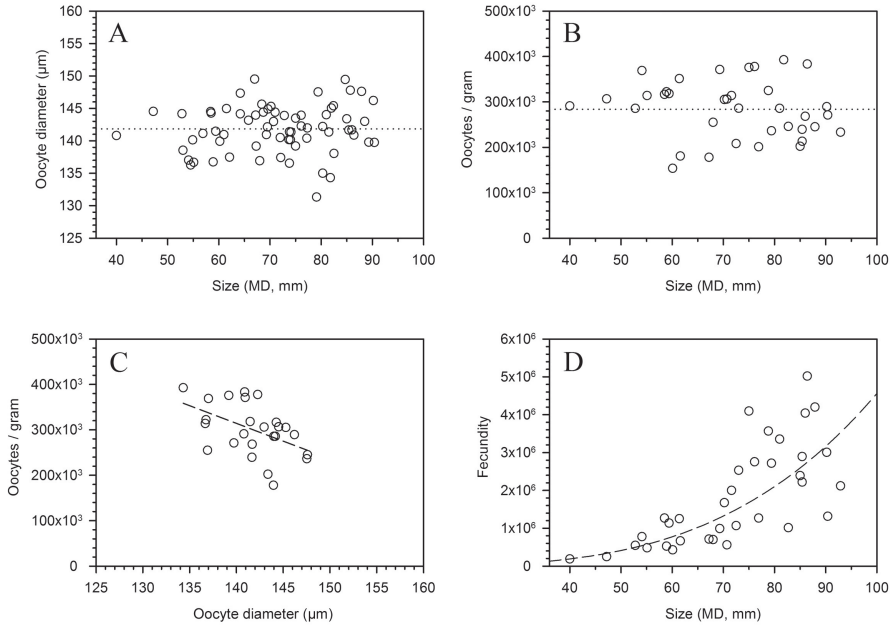


Fig. 1: Relationship between: A, size (shell maximum diameter, MD) and diameter of mature oocytes; B, size (MD) and density of oocytes (number of oocytes per gram of gonad); C, oocyte diameter and density of oocytes in the ovary; D, size (MD) and fecundity (number of oocytes in the gonad). The dotted lines in A and B indicate the mean of the values of the variable of the ordinate axis (where no correlation was detected between both variables). The dashed lines in C and D represent the fitted models obtained (see text).

From 36 females of *P. ferruginea* of 40.0 to 92.9 mm MD (average 72.02 ± 13.39 mm MD), oocyte density in the ovary ranged 153,600-392,600 oocytes/g, with an average of 283,807 ± 63,512 oocytes/g (Fig. 1B). No significant correlation was found between female size and oocyte density (r = -0,148; p = 0,390; n = 36).

However, there was a significant negative correlation between oocyte diameter and its density in the ovary (r = -0.484; p = 0.014; n = 25), suggesting an inverse correlation with females with somewhat smaller oocytes having slightly higher oocyte density (Fig. 1C).

Fecundity

Fecundity (as number of oocytes per gonad) was estimated from 35 females within the range 40.0-92.9 mm MD (average 71.7 ± 13.5 mm MD). The minimum value found was 189,200 oocytes in a female of 40.0 mm MD, and the maximum was 5,019,200 oocytes in an 86.4 mm MD female.

Fecundity showed a clear relationship with shell length (Fig. 1D). Best fit was obtained with the power function

$F = 0.514 * MD^{3.474}$ ($R^2 = 0.689$, $p < 0.001$, $n = 35$)

where F = fecundity and MD = maximum diameter of shell. The exponent was not significantly different than 3 ($p < 0.01$) which reflects an expected relation between length (size) and volume/weight (gonad content).

According to the equation obtained, a small female of 40 mm MD would have an average fecundity of ca. 190,000 oocytes and a quite large female of 90 mm MD ca. 3,150,000 oocytes, that is 16.7 times more. A "standard" mid-large female of 70 mm MD would have ca. 1,320,800 oocytes. However, there was a relevant variability, in particular for largest females. For the eight females within the range 85-95 mm MD, total fecundity varied between 1,316,800 and 5,019,200 oocytes with no significant correlation being found between size (MD) and number of oocytes (r = -0.476, p = 0.233, n = 8) in this large size specimens range.

Effects of water alkalinisation on oocyte maturation and fertilisation rates

The effect of water alkalinisation on the maturation of oocytes is shown in Fig. 2. The percentage of mature oocytes (those that had reached the category RN-NC) increased in all experiments with alkalinised water, both in pH 9-15m and pH 9-2h treatments. A maximum of 78.30% of mature oocytes was achieved in experiment A-11 at pH 9-2h treatment (Fig. 2A). Though there was a certain variability in the percentage of mature oocytes at the beginning of the experiments, treatment at pH 9-2 h improved maturation on average from 14.8% to 52.0%, considering all set of experiments together (Fig. 2B). Paired-Samples T-Test showed significant differences among initial and pH 9-15m treatment (t = -2.653, df = 7, p = 0.033), but differences were not significant when

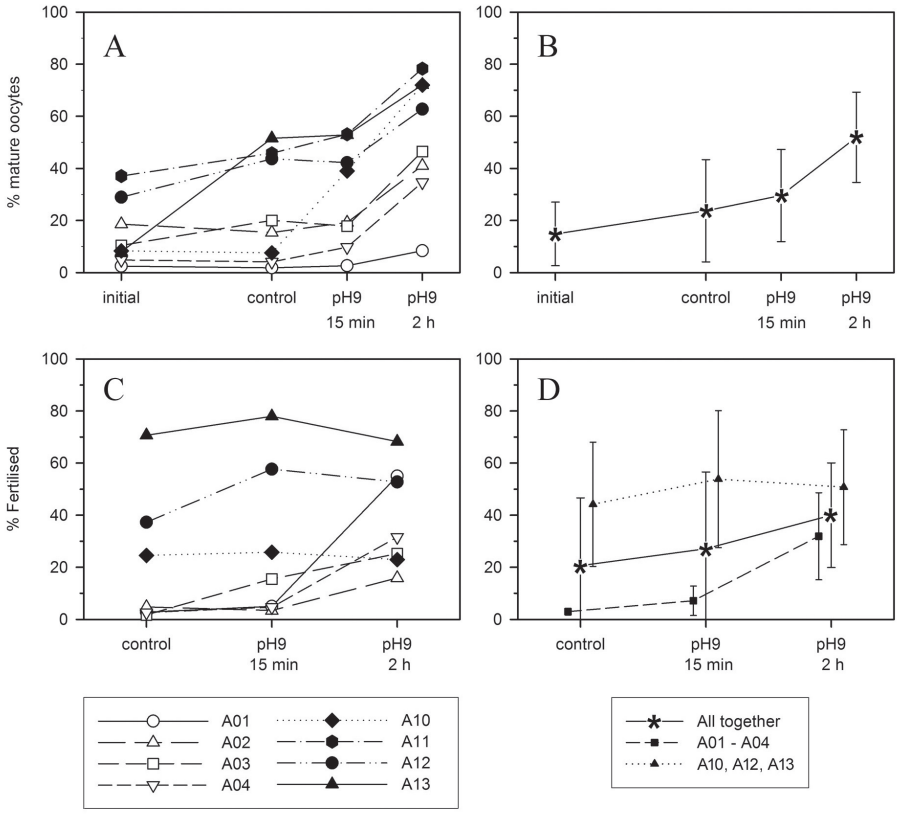


Fig. 2: Effects of alkalinisation on oocyte maturation and fertilisation rate. A, mature oocytes percentage before alkalinisation (initial), in control sample (no pH change) and in samples maintained at pH 9 for 15 minutes and 2 hours, respectively, for experiments A01-A04 and A10-A13. B, mean and standard deviation for all eight experiments. C, percentage of fertilised oocytes in control sample (no pH change) and in samples maintained at pH 9 for 15 minutes and 2 hours, respectively, for experiments A01-A04 and A10, A12, and A13. D, average and standard deviation values for all seven experiments and each of the two sets of experiments, A01-A04 and A10, A12, and A13 (see text).

compared with the control group ($t = -1.520$, $df = 6$, $p = 0.172$). In the pH 9–2h treatment, there were significant differences both when comparing with initial data ($t = -5.343$, $df = 7$, $p < 0.001$) and with the control group ($t = -4.775$, $df = 7$, $p < 0.001$).

Results were somewhat different when oocyte maturation was calculated taking into account the fertilisation rate (Fig. 2C-D). In the first set of experiments carried out in 2009 and 2010 (A01-A04), alkalinisation treatment apparently involved a clear increase in the fertilisation rate, which was low in the control group (< 5%), rose slightly in the pH 9-15m group, and sharply increased in the pH 9-2h group, reaching a maximum of 55.0% from 2.8% in experiment A-01. The average fertilisation rate for all the set of experiments was improved after treatment at pH 9-2h from 20.6% in control to 40.0%. Paired-Samples T-Test did not show significant average differences between control and pH 9-15m treatment ($t = -1,252$, $df = 3$, $p = 0.299$), but was just significant for pH 9-2h treatment ($t = -3.360$, $df = 3$, $p = 0.044$). However, in 2012 experiments (A10, A12 and A13) the fertilisation rate was higher (> 20%) in the control groups and alkalinisation treatment apparently had little influence. There were not significant differences between control data and pH 9-15m ($t = -1.705$, $df = 2$, $p = 0.230$) nor pH 9-15m treatments ($t = -0.918$, $df = 2$, $p = 0.455$). Analysing all seven experiments together (Fig. 2D), no significant differences were found among control group and pH 9-15m ($t = -2.341$, $df = 6$, $p = 0.058$) but they were found among control group and pH 9-2h treatment ($t = -2.767$, $df = 6$, $p = 0.033$).

Effects of sperm concentration on fertilisation rate and percentage of abnormal larvae

The fertilisation rate (measured 2 h post fertilisation) increased, initially, with sperm concentration in all six trials (A08-A013; Fig. 3A-B), with maximum values reached at concentrations between 10^5 and 10^6 spermatozoids/ml (10^6 spermatozoids/ml on average; Fig. 3B). The percentage of normal larvae at 19-22 h showed a

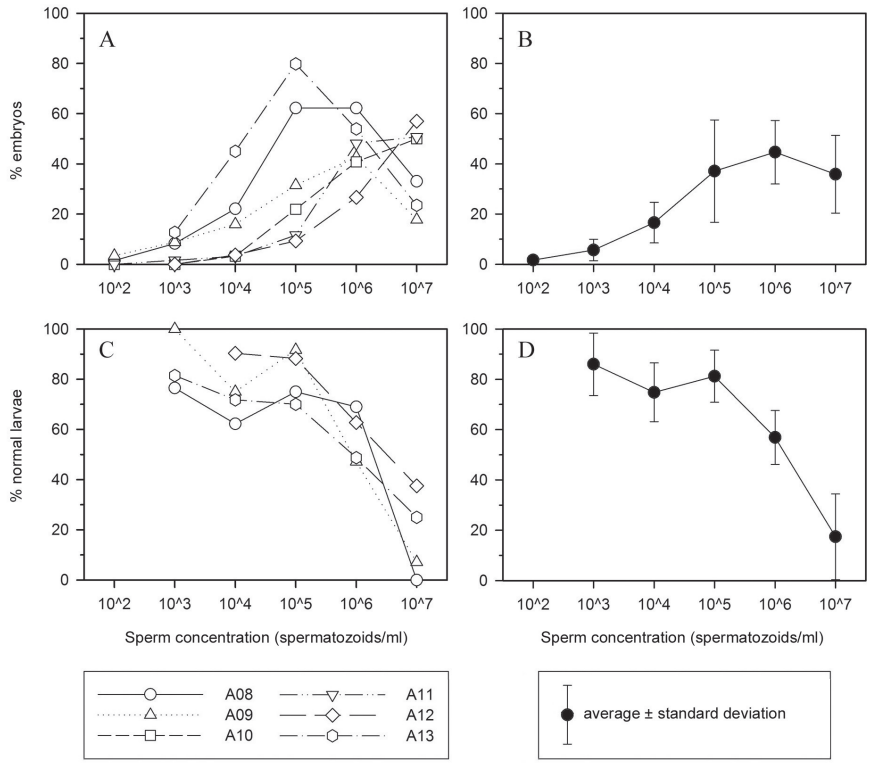


Fig. 3: Influence of sperm concentration on fertilisation rate (at 2 hours after fertilisation) and percentage of normal larvae (at 19-22 hours after fertilisation). A, percentage of embryos in each of experiments A08-A13. B, average and standard deviation values of embryos percentage for all six experiments. C, percentage of normal larvae (or not apparently anomalous) in each of experiments A08, A09, A12, and A13. D, average and standard deviation values of normal larvae percentage for the last four experiments.

Table 2. Summary of development in *Patella ferruginea* at 20 °C. Abbreviations: h, hours post-fertilisation; d, days hours post-fertilisation.

Developmental event or stage	Time after fertilisation	Figure
Frequent eggs with polar body, first cleavage, 2-cell embryos	0.3-3 h	4D, E
4-cell/8-cell embryos	2.3-4 h	4F
Morula	5 h	4G
Early swimming trochophores, with apical cilia	10-13 h	4H
Late swimming trochophores, telotroch (anal tuft) formed	16 h	4I
Pre-torsional veligers, protoconch initiated	23-30 h	4J
Late pre-torsional veligers	25-32 h	4K
Post-torsional veligers, foot rudiment, retractor muscles and protoconch well developed, operculum completely formed	39-45 h	4L
Ability to retract into the larval shell	41-45 h	4M
Appearance of eyespots, ciliation of metapodium, propodium formation, cilia on propodium	50-63 h	4N-O
Pediveliger larvae with developed foot and cephalic tentacles, alternating velar swimming with crawling	71.5 h	4P
Crawling pediveliger, still with ability to detach from substrata and velar swimming; operculum still present	6-7 d	4Q
Crawling post-larva, velum and operculum lost	8-10 d	4R
Crawling post-larva, beginning of teleoconch formation (212 μm)	15 d	4S
Crawling post-larva, teleoconch growth (279 μm)	28 d	4T
Crawling post-larva, teleoconch growth (290 μm)	42 d	4U

clear decreasing trend depending on sperm concentration (Fig. 3C-D). In general, it remained around 70-90% at 10^3 to 10^5 spermatozoids/ml, but decreased rapidly at higher concentrations. Using together both criteria, the optimum concentration found for *in vitro* fertilisation of *Patella ferruginea* was between 10^5 and 5×10^5 spermatozoids / ml.

Larval development

The sequence and timing of developmental events and stages of *P. ferruginea* at 20 °C is summarised in Table 2. Egg fertilisation is showed in Fig. 4C, D. Extrusion of polar body was observed between 20 min and more than 3 h after fertilisation (Fig. 4D). First cleavage took place within 2 h and two-cell embryos were common at this time (Fig. 4E). Early divisions were fast, with 4-8 cell stage observed within 2.3-4 h (Fig. 4F) and morula (Fig. 4G) within 5 h. Embryonic development was not synchronous, especially in these early stages. This may be due not only to possible differences between experiments along the study: for example, three hours after fertilisation in the same sample of trial A12 uncleaved eggs with polar body to embryos with more than 4 cells were simultaneously present. Early trochophores with apical cilia were observed at 10-16 h (Fig. 4H) and complete swimming trochophores with formed telotroch and refractive bodies at 24 h (Fig. 4I). At 23-30 h pre-torsional veliger larvae appeared and began secretion of the larval shell (Fig. 4J), that developed progressively (Fig. 4K). Torsion occurred at 30-40 h and at 39-45 h the veligers showed a well-developed protoconch, foot rudiment, both larval retractor muscles and a completely formed operculum (Fig. 4L). At this time, larvae also displayed the ability to retract into the shell (Fig. 4M). At 50 h, larvae showed developed eyespots, a ciliated metapodium (Fig. 4N), and a ciliated propodium at 63 h (Fig. 4O). Larvae reached the pediveliger stage at 71-72 h, showing well-developed cephalic tentacles and foot, and were able to alternate velar swimming with foot crawling on the substrate (Fig. 4P). These results indicate that larvae became metamorphically competent 3 days after fertilisation at 20 °C. However, some crawling pediveliger larvae still with a well-developed velum were found even ca. 7 days after fertilisation (Fig. 4Q). Crawling post-larvae with lost velum and operculum were found from 8-10 days (Fig. 4R). Beginning of teleoconch formation and subsequent teleoconch growth were observed from 15-42 days, in which the presence of radula and radular feeding movement were detected (Fig. 4S-U). It should be noted that this timing was produced without food supply (i.e., phytoplankton) to the developing larvae.

Recruitment

Recruitment was only achieved from fertilisation experiments A06 (2011) and A07-A09 (2012) carried out at IATS-CSIC. First recruits visible to the naked eye were detected on 17 April 2012, 141 days after fertilisation experiment A06, and on 2 April 2013, 131-133 days after A07-A09 experiments. At this time, recruits measured 1-2 mm in length and have been described in detail by Guallart *et al*. (2017). During the following weeks, 56 recruits were found in 2012 and 45 in 2013. Since juvenile detection was not simultaneous, these numbers are probably underestimates of recruitment due to the mortality of juveniles during the early stages. Most of the recruits were found on semi-submerged structures placed on culture tanks and it must be noted that only 4 of them (4.0%) were found on the tank walls.

Growth and sexual maturity of the juveniles

From fertilisation experiment A06 (28 November 2011), only 7 young specimens of lengths 12-28 mm survived for the next year (22 November 2012). To avoid risks due to their small size, biopsies for sex determination were not carried out; therefore, it was not possible to assess if they were sexually mature. One year later (23 October 2013) 5 specimens remained alive, with size ranging between 30.5-56.2 mm MD. Two of these specimens (30.5 mm and 36.1 mm MD) were sexed as males, two (45.1 mm and 56.2 mm MD) were females containing abundant oocytes, and the last specimen (33.2 mm MD) could not be sexed after 3 biopsy attempts. Recruits obtained from fertilisation experiments A07-A09 (19, 20 and 21 November 2012) were not sexed 1 year later due to their small size, and subsequent growth was not studied.

Discussion

Oocyte size

Egg size has been a central issue in the study of ecology and evolution of marine invertebrates and shows strong correlations with other life-history traits (Perron & Carrier, 1981). Thorson (1950) pointed out that egg size was correlated with fecundity, larval type, duration of the larval period and dispersal ability. In this sense, Laborel-Deguen & Laborel (1990b, 1991b) argued that large eggs of *P. ferruginea*, of "about 200 µm" were indicative of a short planktonic life and therefore indicated a low dispersal dispersion capacity of the species and this fact has been assumed as valid by various subsequent authors (e.g., Templado *et al*., 2004). The average diameter of mature oocytes found in our study (141.83 µm) is somewhat smaller than mean values (149.78 µm) obtained by Espinosa *et al*. (2006). Yet, this difference is not representative given that these authors used different methodologies, measuring the largest diameter of an oocyte including its chorion and after preservation in ethanol. However, both values are much lower than that of 200 µm size reported by Laborel-Deguen & Laborel (1990b, 1991b), which should be regarded as incorrect. It is remarkable that a 29.0% difference in diameter implies

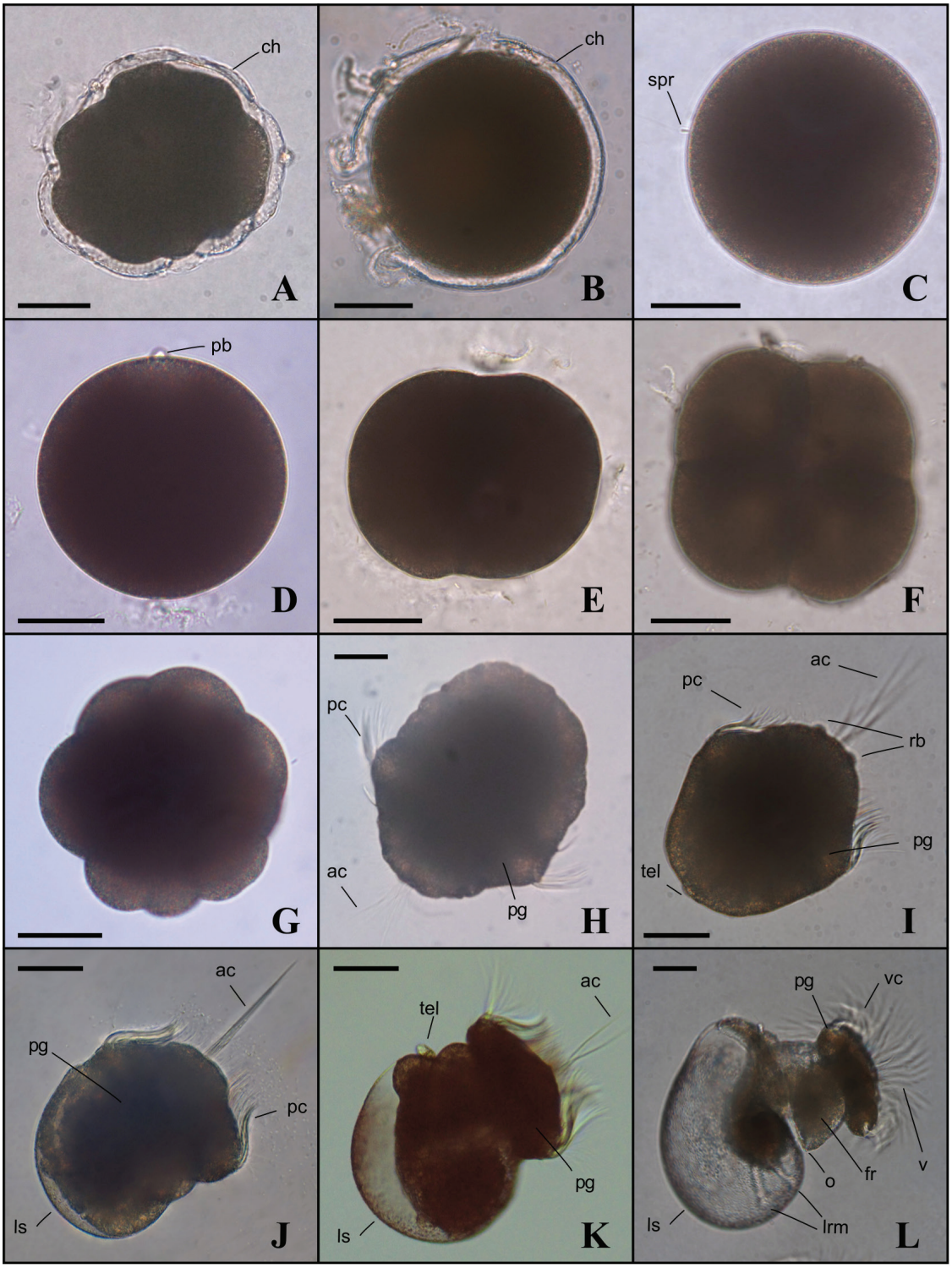
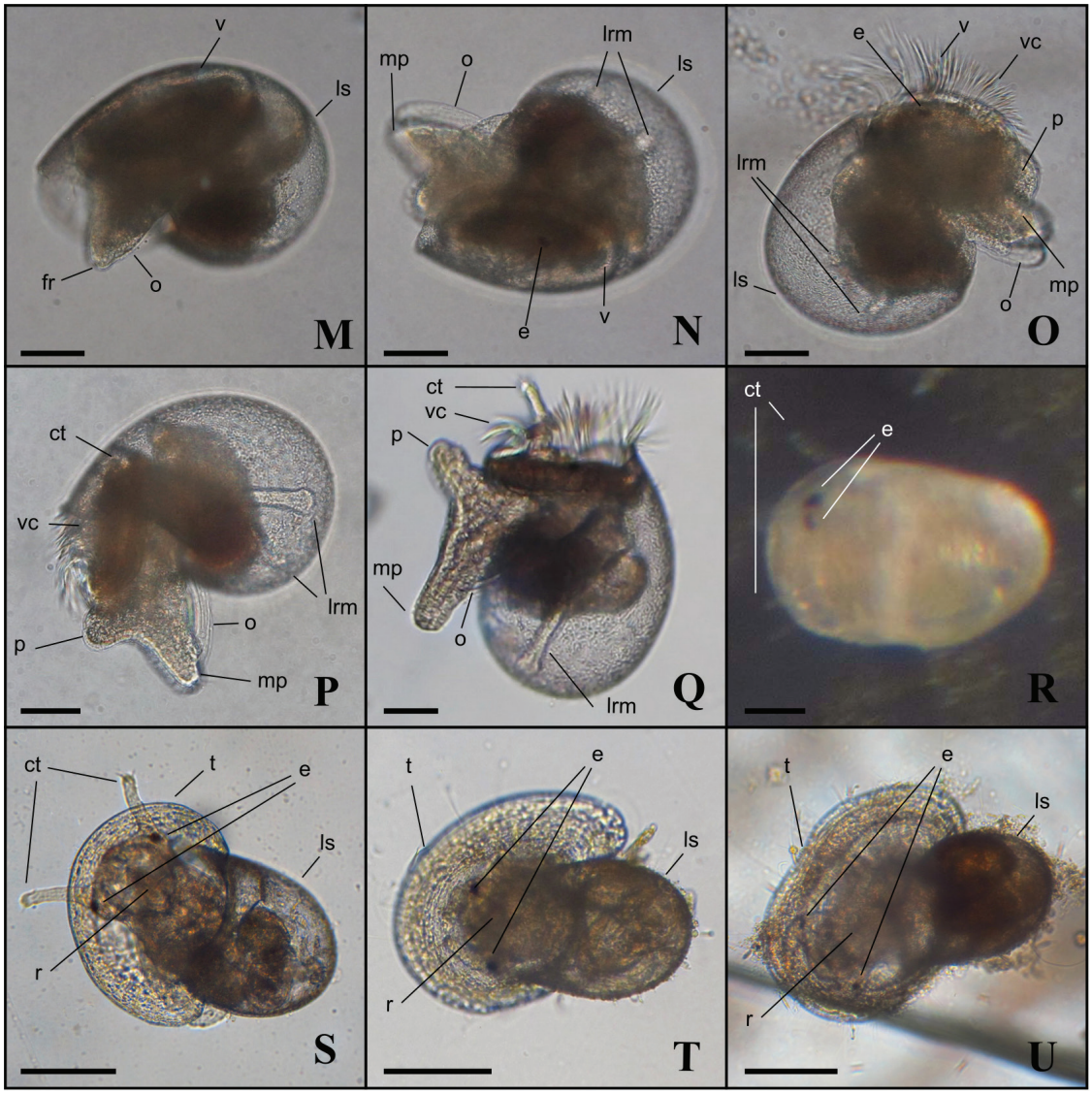


Fig. 4: Developmental stages and times post-fertilisation (min, minutes; h, hours; d, days) in *Patella ferruginea*, reared at 20 °C. A, irregular oocyte just extracted from ovary. B, round oocyte covered with chorion (not fully mature). C, Mature egg without chorion being fertilised by a spermatozoid (0 h). D, extrusion of polar body (20 min). E, two-cell stage showing rest of chorion (1 h, 22 min). F, 4-cell stage (2 h, 20 min). G, morula (4 h, 27 min). H, early swimming trochophore (13 h, 36 min). I, late swimming trochophore (15 h, 50 min). J, pre-torsional veliger (23 h, 15 min). K: late pre-torsional veliger (1 d, 3 h, 40 min). L, post-torsional



veliger (1 d, 15 h, 32 min). M, post-torsional veliger retracted into larval shell (1 d, 21 h, 43 min). N, post-torsional veliger with developed eyespots and ciliated metapodium (2 d, 2 h, 15 min). O, post-torsional veliger with ciliated propodium (2 d, 15 h, 31 min). P, pediveliger with well-developed foot and cephalic tentacles (2 d, 23 h, 30 min). Q, crawling pediveliger (6 d, 22 h, 58 min). R, crawling post-larva (8 d). S, crawling post-larva, beginning of teleoconch formation (212 µm, 15 d). T, crawling post-larva, teleoconch growth (279 µm, 28 d). U, crawling post-larva, teleoconch growth (290 µm, 42 d). Abbreviations: ac, apical cilia; ch, chorion; ct, cephalic tentacle; e, eye; fr, foot rudiment; lrm, larval retractor muscle; ls, larval shell (protoconch); mp, metapodium; o, operculum; p, propodium; pb, polar body; pc, prototrochal cilia; pg, prototrochal girdle; r, radula; rb, refractive body; spr, spermatozoid; tel, telotroch; v, velum; vc, velar cilia.

a considerably smaller volume (64.2%) and therefore vitellus/yolk reserves. Both Espinosa *et al.* (2006) and our results did not find a significant correlation between oocyte diameter and female size.

Indeed, mean diameter of mature oocytes of *Patella ferruginea* is quite similar to those obtained in the present study for two other limpets coexisting with *P. ferruginea*, 143.52 µm in *P. caerulea* and 143.97 µm in *P. rustica*. Dodd (1957) and Ferranti *et al.* (2018) reported a somewhat smaller diameter (135 µm) in *P. caerulea* and Prusina *et al.* (2014) provided a maximum diameter of 118.7 µm for *Patella rustica*, a lower value to that found in our study and also to that of 150 µm reported by Zegaoula *et al.* (2016). Within the Atlantic species, oocytes of *Patella vulgata* measure between 150 µm according to Dodd (1957) and 160 µm according to Lebour (1937), and those of *Patella pellucida* ca. 160 µm (Fretter & Graham, 1976). Data reported for mature oocytes of

other patellogastropod species are consistent with those of Mediterranean-Atlantic limpets, e.g., 130-156 µm in *Cellana exarata* (Corpuz, 1981), 120-150 µm in *Nacella* (*Patinigera*) *deaurata* (Morriconi, 1999), 134 µm in *Lottia asmi* and 155 µm in *L. digitalis* (Kay & Emlet, 2002), 110-170 µm in *Cellana grata* (Yang *et al*., 2017), 140 µm in *Cellana sandwicensis* (Mau *et al*., 2018) or ca. 190 µm in *Helcion pectunculus* (Gray & Hodgson, 2003). Therefore, there is no evidence that oocytes of *P. ferruginea* are particularly large compared to other *Patella* or patellogastropod species.

Fecundity (annually and throughout lifespan)

Fecundity in *P. ferruginea* (herein referred to the number of oocytes in the ovary per ripe female) is, as might be expected, significantly correlated with female size (shell length), which corresponds to a size (MD) - body volume (and consequently, ovary weight) relationship, which can be fitted to a power equation where the exponent is not significantly different from 3. This is also consistent with results of Espinosa *et al*. (2006), who reported 2.3 to 4.5 million oocytes in large females (68-90 mm MD) and agrees within the range estimated in the present study, between 1.3 and 5.0 million oocytes (in females 70-95 mm MD). Nevertheless, we found an important variability, in particular for largest females (85-95 mm MD). Among other factors, this could be related to the body volume that can vary considerably between specimens, even with a similar value of the parameter used to define their size, the maximum diameter of the shell (MD). In fact, two morphotypes have been described in *P. ferruginea*, *rouxii* (with a more conical and elevated shell, and therefore with greater body volume) and *lamarckii* (with a flatter shell, and consequently with a lower volume) (Porcheddu & Milella, 1991; Moreno & Arroyo, 2008; Coppa *et al.*, 2012). Although MD is the best parameter to define limpet size, shell height may influence body and gonad volume and thus fecundity estimations and be the origin of part of the aforementioned variability.

Low fecundity was indicated by Laborel-Deguen & Laborel (1990b) as being one of the aggravating factors of the decline of *P. ferruginea*, although these authors cited Frenkiel (1975) as origin of this information, who actually did not provide observations on this issue. However, according to our data and those of Espinosa *et al.* (2006), fecundity in *P. ferruginea* can be even higher than in other unendangered Mediterranean limpets, because females may reach a much larger size. It is noteworthy that according to our results the fertility of a 90 mm MD female can be up to 16.7 times larger than that of a 40 mm MD.

On the other hand, several studies indicate that selective harvesting pressure on larger specimens of *P. ferruginea* (with a higher probability of being females and with much greater oocyte production, i.e., fecundity) is one of the main threats that have led this species to be at risk of extinction (Laborel-Deguen & Laborel, 1990b, 1991b; Espinosa *et al.*, 2009b; Ceccherelli *et al.*, 2005; 2011; Rivera-Ingraham *et al.*, 2011a; Coppa *et al.*, 2016; Henriques *et al*., 2017) as with other protandrous limpet species (e.g., *P. aspera*, Hawkins *et al.*, 2000).

Moreover, the total fecundity referred to the total number of gametes produced throughout the entire lifespan will also depend on other hardly known population dynamics parameters. The may include at least: (1) age at which sexual maturity is reached, which is estimated to be 2 years for *P. ferruginea* (Guallart & Acevedo, 2006) and confirmed in our breeding of settled animals, but not for other coexisting limpets; (2) the extension of the annual reproductive period, which is much longer in *P. caerulea* than in *P. ferruginea* (Frenkiel, 1975), which could mean therefore a lower production of juveniles in the latter species; however, the reproductive cycle of *P. ferruginea* is very similar in duration and seasonality to *P. rustica* (Frenkiel, 1975; Prusina *et al.* 2014), an abundant and non-threatened coexisting limpet; (3) the growth rate and longevity in *P. ferruginea*.

Regarding this last point, Laborel-Deguen and Laborel (1990b, 1991b) attributed a very low growth rate to *P. ferruginea*. However, subsequent studies found a high variability in growth rate not only depending on specimen size (something common in many animal groups, in which the growth rate is faster in small specimens and slower in larger ones), but also depending on different environmental conditions (Espinosa *et al*., 2008) or even undetermined causes (Guallart *et al.*, 2012b). A low growth rate may imply that reaching the size range of maximum fecundity (e.g., > 90 mm MD), together with the aforementioned tendency to harvest large specimens could effectively limit its individual fecundity.

Laborel-Deguen and Laborel (1990b, 1991b) also suggested a high longevity in *P. ferruginea* which was supported by Espinosa *et al.* (2008), who proposed that depending on the environmental conditions and the calculation methodology used, it could exceed 30 years. However, Guallart *et al.* (2012b) could only confirm that the longevity of the species can certainly exceed 12 years. However, agreeing with the general high variability in growth rate indicated by Guallart *et al.* (2012b), Rivera-Ingraham *et al.* (2011d) provided direct evidence that, in certain cases, very high growth rates could allow some specimens of *P. ferruginea* to reach in < 5 years the higher fecundity size group (> 90 mm MD).

Therefore, more detailed knowledge of the population dynamics, apart from some little precise attempts (e.g., Rivera-Ingraham *et al.*, 2011b; Coppa *et al.*, 2016), is fundamental to understand if some biological parameters, as it has been suggested, make *P. ferruginea* vulnerable to decline or hamper its recovery once protection measures have been established.

Effects of water alkalinisation on oocyte maturation and fertilisation rates

Between the two alkalinisation treatments tested in this study, the pH 9-2 h produced the highest and significant increase in the percentage of mature oocytes and the

highest fertilisation rate, but these results were not consistent in the whole set of experiments. Oocyte maturation (evaluated as RN-NC oocytes) was improved on average from 14.8% to 52.0% and fertilisation rate from 20.6% to 40.0%, considering all set of experiments. However, the remarkable variability observed in the percentage of mature oocytes at the beginning of each experiment (carried out in different years) possibly reflects a so far unpredictable degree of natural gonadal maturation during trials. The usefulness of this treatment seemed to be much higher in not fully mature oocytes but apparently had a limited influence in oocytes in an advanced stage of maturation, in which alkalinisation hardly causes an increase in fertilisation rate.

Several authors improved oocyte maturation and fertilisation rates of different patellogastropods by pre-incubating oocytes in alkalinised seawater. The method (use of NH_4OH or NaOH in SFSW), the pH value (usually one or more values > pH 9) and the time spent in this alkaline medium (from ca. 10 minutes to several hours) are quite variable, as well as the optimal results obtained in each study for each species (e.g., Gould *et al*., 2001 for *Lottia gigantea;* Hodgson *et al*., 2007 for *Patella ulyssiponensis;* Aquino de Souza *et al.*, 2009 for *Patella depressa* and *P. vulgata;* Pérez *et al.*, 2016 for *Patella vulgata*, *P. depressa* and *P. ulyssiponensis*).

Present results on oocyte maturation and fertilisation rates after alkalinisation with NaOH in *Patella ferruginea* considerably improved the previous results presented by Espinosa *et al.* (2010). Yet, our results showed a high degree of unpredictable variability, possibly associated with the degree of maturation of gametes, *a priori* difficult to establish, apart from the external appearance (volume and examination under binocular microscope) of the gonad and the classifications that could be made based on these observations (e.g., Orton *et al.*, 1956; Frenkiel, 1975).

Nevertheless, for controlled reproduction of *P. ferruginea* for restocking purposes, further research is particularly needed not so much in the methodology of artificial maturation of extracted oocytes, but especially in spawning induction procedures.

Effects of sperm concentration on fertilisation rate and percentage of abnormal larvae

Fertilisation success depends, among other factors, on sperm concentration (Hodgson *et al*., 2007; Pérez *et al*., 2016). Usually, a too low concentration fails to fertilise most of eggs and results in a low fertilisation rate, while too high concentrations may cause polyspermy, lysis of the egg membrane or a high percentage of abnormal larvae (e.g., Helm & Bourne, 2004). The optimal sperm concentration value would be a compromise between both extremes, which can differ between species.

The best results obtained for *P. ferruginea* (concentrations between 10^5 and 5×10^5 spermatozoids/ml) are values quite similar to those provided by previous authors for other *Patella* species. Hodgson *et al*. (2007) indicated an optimum fertilisation success within the range of 10^5 and 10^7 spermatozoids/ml for *Patella ulyssiponensis* and *P. vulgata*; Aquino de Souza *et al*. (2009) indicated 6×10^5 spermatozoids/ml for *P. depressa* and *P. vulgata*, and Pérez *et al.* (2016), ca. 10^6 spermatozoids/ml for *Patella depressa* and *P. ulyssiponensis*.

Larval development

Larval rearing of *Patella ferruginea* up to crawling post-larval stages was completed for the first time on two occasions in the present study, later reaching sexual maturity after two years. Although previous authors had indicated the ease to achieve the fertilisation of gametes (Frenkiel, 1975; Laborel-Deguen & Laborel, 1991b) they did not provide further information. Previous attempts by Espinosa *et al*. (2010) failed to continue larval development beyond 48 h, only reaching an early pre-torsional veliger stage and suggesting that this difficulty may be related to "biological constraints" of the species that could contribute to its endangered status. The developmental sequence and timing described here up to the pre-torsional veliger stage agree well with results of those authors, despite the fact that they reared larvae at a lower temperature (18 °C).

The developmental sequence of *Patella ferruginea* described here does not differ substantially from that previously described for other *Patella* species (e.g., Patten, 1886; Smith, 1935; Dodd, 1957; Wanninger *et al*., 1999; Ferranti *et al*., 2018) and other patellogastropods (e.g. Corpuz, 1981; Kay & Emlet, 2002; Mau *et al*., 2018). Only the timing (i.e., duration of each phase) showed some differences between species that may be more related with rearing temperature rather than with specific developmental features. As in other molluscs, the timing and duration of the larval period may be greatly influenced by temperature (e.g., Kay & Emlett, 2002; Helm & Bourne, 2004; Ribeiro, 2008).

At 20 °C, larvae of *P. ferruginea* reached metamorphic competency ca. 3 days after fertilisation, with pediveligers showing a well-developed foot and an ability to crawl on the substrate. However, crawling pediveligers still able to swim were found even 7 days after fertilisation. Since the sea surface water temperature in Chafarinas Islands during the spawning period is ca. 18 °C (Guallart, 2008), somewhat lower than in our larval culture, the duration of larval development in the natural environment may be somewhat longer.

For larvae of other European limpets reared in laboratory conditions, Dodd (1957) reported a planktonic period of ca. 10 days at 12 °C in *Patella vulgata*; Ribeiro (2008) an average duration of precompetent periods of 3.7-14.0 days in *P. depressa*, 2.8-13.7 days in *P. ulyssiponensis*, and 5.7-14.6 in *P. vulgata*, varying inversely with temperature, and Ferranti *et al.* (2018) reported that the complete larval development of *P. caerulea* takes ca. 7 days at 14 °C.

Nevertheless, nothing is known about the duration of the planktonic phase or how often delayed metamorphosis occurs in patellid limpets in natural conditions. Ac-

cording to Ribeiro (2008), larvae of these species were capable of extending their planktonic life considerably after metamorphic competence in the absence of appropriate settlement substrata and might remain in the plankton longer than in laboratory cultures. Moreover, other factors such as food availability, larval mortality and swimming behaviour must be taken into account since they may significantly alter the duration of the planktonic phase (Cowen & Sponaugle, 2009). During the present study, *P. ferruginea* larvae have been considered as strictly lecithotrophic (i.e., they depend solely on their reserves of vitellus for their larval development and do not feed during their planktonic phase). Some authors have tried to provide phytoplankton feed during larval development of several *Patella* species, although without observing significant differences in development (Smith, 1935; Dodd, 1957; Ferranti *et al.*, 2018). No phytoplankton supply was provided to larvae of *P. ferruginea* in these experiments, since it was assumed that they probably had a lecithotrophic development. Although a late phase of phytoplankton feeding in *P. ferruginea* under certain environmental conditions cannot be ruled out, it is something that remains currently unproven and must be investigated.

All the above would indicate that the planktonic larval period of *P. ferruginea* could be extended enough to allow its dispersion over distances larger than initially thought (Templado *et al*., 2018; Ferranti *et al*., 2019). This would explain in part the genetic uniformity observed throughout most of its distribution range (Espinosa & Ozawa, 2006; Casu *et al*., 2012; Acevedo *et al.*, 2018), with only some exceptions for local populations in some MPAs of Sardinia described by Cossu *et al.* (2017). In this regard, current increasing sea temperatures due to climate change may affect the larval phase and thus the dispersal ability and population connectivity of *Patella ferruginea* and other invertebrate species.

Growth and sexual maturity of the juveniles

Five surviving recruits from fertilisation experiment A06 (28-11-2011) continued their growth until the age of two years, when they reached a size in which sexual maturity was expected (Guallart & Acevedo, 2006). Two of these specimens (30.5 and 36.1 mm MD) were sexed as males and two others (45.1 and 56.2 mm MD) as females, which is consistent with the minimum size described for each sex in *P. ferruginea* (Frenkiel, 1975; Espinosa *et al*., 2009b; Guallart *et al*., 2013; Rivera-Ingraham *et al.*, 2011a).

Until now, smaller sizes in sexually mature males was interpreted as the species first matures as male (protandric hermaphroditism) (Guallart *et al.*, 2006; Espinosa *et al*., 2009a; Guallart *et al*., 2010, 2013; Rivera-Ingraham *et al.*, 2011a) and that it can later (or not) change sex (and even doing it several times) (Guallart *et al.*, 2013). The results presented here, although isolated, suggest that the species could first mature both as male or female (apparently, as female only when size is > 40 mm MD), which would change the understanding of the model of sexual maturity and reproductive cycle of the species. Further study on this matter is required.

Implications for conservation

The pioneering works of Laborel-Deguen & Laborel (1990b, 1991b) suggested a limited dispersal ability for *P. ferruginea* based on the assumption that the large size of the oocytes (ca. 200 µm) would imply a short larval phase ("of several days duration"). These authors also added to this a supposed low fecundity, a short reproductive period, and a slow growth rate as biological factors that aggravate the decline of this species due to human impact. From these assumptions arose the argument that natural recovery of populations would be difficult due to low dispersal ability, even in localities where pressure on the species has apparently disappeared (e.g., marine protected areas). Therefore, reintroduction or restocking were considered as appropriate strategies for the recovery of these populations (Laborel-Deguen & Laborel, 1991a, 1991b, 1991c; MMAMRM, 2008). Yet, many of these assumptions have been proved to be wrong. As commented above, oocyte size in *P. ferruginea* are on average smaller than 150 µm and similar to oocyte size of other non-endangered Mediterranean limpets such as *P. rustica* or *P. caerulea*. In this study, larvae cultured in laboratory conditions showed metamorphic competence ca. 3 days at temperatures somewhat higher than in the natural environment, but we also found evidences that metamorphosis could be delayed for at least up to 7 days in the laboratory and could even delay more in the natural environment. This larval period may allow dispersal across at least tens of kilometers, taking into account that natural spawning and fertilisation occur in the autumn during major storms usually associated with strong surface currents (Laborel-Deguen & Laborel, 1990b; Frenkiel, 1975; Guallart, 2008). Better knowledge of the larval span of *P. ferruginea* achieved in this study provides relevant information to understand populations connectivity, and thus suggestions for the management of the species. Although the reproductive period is short with only one annual spawning event, *Patella rustica*, which coexists with *P. ferruginea*, has a similar reproductive cycle (Frenkiel, 1975; Prusina *et al*., 2014) and is nowadays abundant and not endangered. Likewise, the assumed low fecundity in *P. ferruginea* has been proven to be inaccurate, and it could be even higher than in other Mediterranean or eastern Atlantic limpets, because females can reach a much larger size. Nevertheless, this issue should be influenced by growth rate and population dynamics, which should be studied in more detail. Finally, as commented before, growth rates have been proven to be faster than previously assumed. In view of the above, it should be concluded that neither reproductive traits, larval development nor growth rates of *P. ferruginea* differ substantially from those of some other non-endangered Mediterranean limpets. Therefore, the decline of *P. ferruginea* can hardly be attributed to these biological traits but to human im-

pact, mainly harvesting, habitat degradation, and coastal development (Templado, 2001; Paracuellos *et al*., 2003; Moreno & Arroyo, 2008).

The most important impact of harvesting on marine species is to reduce their population sizes and densities, resulting in a particularly strong Allee (1931) effect, which leads populations to critical numbers and makes the species more vulnerable to extinction (Stephens & Sutherland, 1999). The major consequences of harvesting on populations of *Patella ferruginea* have been pointed out by many authors (see a review in Luque *et al*., 2018). As occurs in other limpet species, largest specimens of *P. ferruginea* are usually scarce because of their selective poaching (Laborel-Deguen & Laborel, 1990a, 1991b; Martins *et al.*, 2008; Espinosa *et al*., 2009b; Coppa *et al.*, 2011; Espinosa & Rivera-Ingraham, 2017; Henriques *et al*., 2017; Martins *et al.*, 2010, 2011, 2017) despite the fact that this limpet is strictly protected under European and some North African countries' laws. Since fecundity depends on size (Espinosa *et al*., 2006, and present study) this makes this species highly vulnerable to harvest. In this sense, the size and age at which sex change occurs under different conditions are key factors that could determine the reproductive success of the species, which in turn may result in low recruitments due to decreased reproductive output (Hawkins *et al*., 2000; Prusina *et al*., 2014; Henriques *et al*., 2017; Martins *et al*., 2017). A better knowledge of these aspects in *P. ferruginea* is needed to counteract its population decline and to improve its conservation.

As a result of all the experiments carried out in the present study, we report for the first time the complete life cycle of *Patella ferruginea* under laboratory conditions, with successful fertilisation, larval rearing to benthic post-metamorphic stages and subsequent growth of a few specimens until attainment of sexual maturity. This relative success strengthens the possibility to obtain numbers of recruits that could be used for reintroduction, restock or stock enhancement of populations with declining densities. However, the number of recruits obtained herein was very low and survival of first young stages up to reach sexual maturity was even lower.

Despite the progress made, actual closure of the biological cycle of *P. ferruginea* in culture conditions still appears to be rather difficult, since spawning induction has never been achieved, including in the present study. Although sperm could be obtained through non-lethal biopsies, it was not possible to obtain oocytes without sacrificing females. This is a common limitation in other patellogastropods, since most classic works on larval development study or experimentation in this group were carried out by sacrificing adults to obtain gametes (see above). However, this option must be discarded for such an endangered species as *P. ferruginea*. Even if it were possible to produce a high number of juveniles by this procedure, this would imply (1) to sacrifice quantities of females of this protected species and (2), whenever the number of the females was reduced would imply a low genetic diversity of the offspring, incompatible with its use for restocking.

Spawning induction has provided irregular and quite unpredictable results in other patellogastropods (Rao, 1973; Kay & Emlett, 2002). Even the most regular results described for *P. caerulea*, a limpet with an infralittoral habit (Ferranti *et al*., 2018) may be cautiously applied to limpets with midlittoral lifestyles like *P. ferruginea*, which, as described above, displays peculiarities not only for spawning induction but even for maintainance in aquaria. The results recently obtained using hormonal stimuli by Nhan & Ako (2012, 2019) also in a species with infralittoral habits (*Nacella sandwicensis*), are interesting and could be promising in further studies on *P. ferruginea*. Therefore, developing spawning induction techniques for *P. ferruginea* and improving its maintenance in aquaria to achieve higher post-larval and juvenile survival rates are still priority research issues that need resolution.

At the current state of knowledge, large-scale aquaculture production based on large numbers of reproductive adults of *Patella ferruginea* taken from some natural populations is not yet achievable. Therefore, we must stress the importance of ensuring connectivity of extant populations through an appropriate design of a network of effectively protected marine areas, improving surveillance to avoid illegal harvesting, properly monitoring the extant populations and increasing social awareness through an adequate policy of outreach and environmental education. Meanwhile, carefully designed research programmes are required to improve the applicability of aquaculture techniques to any *ex-situ* conservation strategies of *Patella ferruginea*.

Acknowledgements

This work was funded at initial stages (2006-2011) by contracts made by the Organismo Autónomo de Parques Nacionales (OAPN) and the Dirección General de Conservación de la Naturaleza of the former Spanish Ministerio de Medio Ambiente (1996-2008), Ministerio de Medio Ambiente, y Medio Rural y Marino (2008-2011) and Ministerio de Agricultura, Alimentación y Medio Ambiente (2011-2016). At its late stage (2011-2012), was funded by the project "Action plan for viability proposals of the endangered limpet, *Patella ferruginea*" (Project Cero of the Spanish Research Council-CSIC Foundation). We thank these institutions and especially to Javier Pantoja and Ainhoa Pérez Puyol and the staff of División para la Protección del Mar (Dirección General de Sostenibilidad de la Costa y del Mar) for permission to handle specimens of a species protected by Spanish law. We are also indebted to Javier Zapata and the staff of the Chafarinas Islands Biological Station (OAPN) and the military personnel of the Spanish Ministerio de Defensa on the archipelago for facilities provided during the fieldwork. We also thank to Iván Acevedo, Marta Calvo, Annie Machordom, Patricia Cabezas (Museo Natural de Ciencias Naturales, CSIC) and Juanjo Villalón (Melilla) their valuable contribution to logistics, field or laboratory work. We are also indebted to Dr. Stephen J. Hawkins and an anonymous review-

er for their detailed reviews and suggestions, which have helped to considerably improve this manuscript.

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