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Where have all the youngest gone? The post-larval and young stages of the Mediterranean endangered limpet *Patella ferruginea* Gmelin, 1791

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Abstract

Recruits of *Patella ferruginea* are hardly seen in their habitat; hence, the precise level where they settle and the morphology of the earliest growth stages remain unknown. The earliest post-larval stages of this species are described here for the first time, based on specimens obtained both through controlled reproduction and in its natural environment in Chafarinas Islands (North Africa). Young post-larval specimens of 290 μm in length settled slightly below the water level on floating Petri dishes in the aquaculture tank. Specimens of approximately 4 mm were found in the natural habitat at the level of the vermetid gastropod *Dendropoma lebeche*, whereas those larger than 8 mm were also found at this level, but also in the upper midlittoral, at the *Chthamalus* spp. fringe.

The characteristic star-like shell and colour pattern of young specimens, with alternating concentric dark and light bands, appeared at approximately 8 mm in length. Smaller specimens have a more or less oval shell and dark radial bands. This information is useful for future non-invasive studies on the recruitment of this endangered species, but further information about the post-larval stages of the other three Mediterranean patellid species is needed to distinguish specimens smaller than 3 mm.

Keywords: *Patella ferruginea*, Mollusca, Gastropoda, Patellidae, post-larval stages, Mediterranean, endangered species, conservation.

Introduction

The ferruginous limpet (*Patella ferruginea* Gmelin, 1791) is an endemic western Mediterranean species that is considered one of the most endangered marine invertebrates (Laborel-Deguen & Laborel, 1991a; Templado, 2001).

At the end of the 19th century, *Patella ferruginea* was distributed throughout most of the western Mediterranean coasts, suffering an important decline during the 20th century (Laborel-Deguen & Laborel, 1991a; Porcheddu & Milella, 1991; Templado *et al.*, 2004; Espinosa *et al.*, 2013). Currently, it is considered extinct on the European continental coasts except for some localities of southern Spain (Templado *et al.*, 2004; Espinosa, 2009; see Espinosa *et al.*, 2013 for an updated review of global distribution). The main populations are currently found along the North African coasts, from Tunisia to Morocco (Rivera-Ingraham *et al.*, 2011; Guallart *et al.*, 2013c; Guallart & Templado, 2016; Zarrouk *et al.*, 2016). The decline of this species is attributed to human impact, such as overexploitation, habitat degradation, development of coastal infrastructures, and marine pollution (Templado, 2001; Paracuellos *et al.*, 2003; Moreno & Arroyo, 2008).

For all these reasons this limpet has been included in Annex II of the Berna Convention, Annex II of Barcelona Convention and Annex IV of the "Habitats" Directive, and also in the Spanish Catalogue of Endangered Species as "in risk of extinction" since 1999 (BOE, 1999). A Spanish national strategy to protect this species was approved in 2008 (MMAMRM, 2008), being the first strategy to protect a marine invertebrate in this country.

Patella ferruginea is a large limpet that may reach more than 100 mm of maximum shell diameter (MD) and lives in the upper midlittoral zone (Templado, 2001). Like other limpets, it is a broadcast spawner and is a sequential protandrous hermaphrodite showing alternating sex reversal (Guallart *et al*., 2013b). It is assumed that this species has a low fecundity, a short planktonic larval phase with limited dispersal ability (Laborel-Deguen & Laborel, 1991b; Guallart *et al.*, 2010), and a slow growth rate (Espinosa *et al.*, 2008; Guallart *et al.,* 2012b). However, nothing is known about the larval phase or on the recruitment processes.

Smaller specimens of *Patella ferruginea* are hardly seen in their habitat (Frenkiel, 1975; Laborel-Deguen & Laborel, 1991b). The latter authors suggested that this may be due to the fact that the first benthic stages probably refuge in the upper infralittoral zone, at the level of the *Cystoseira* belt, where they would be very difficult to find.

Shell morphology of young specimens (MD 20-30 mm) is well known and quite characteristic, with a star-like outline due to prolongation of axial ribs and a colour

pattern of concentric dark and light bands. They are common in the midlittoral zone of localities with dense populations of adults (Frenkiel, 1975; Espinosa, 2009; Espinosa *et al.*, 2011; Rivera-Ingraham *et al.*, 2011; Guallart *et al.*, 2013b; Guallart & Templado, 2016). Nevertheless, several authors considered unfeasible the identification of young specimens less than 20 mm (Mari *et al.*, 1998; Giudicelli *et al.*, 1999; Meinesz *et al.*, 2001; Pascal, 2002) or 10 mm (Coppa *et al.*, 2012) on the basis of the external shell characters, and thus did not include them in censuses.

As far as we know, the smallest specimen of *Patella ferruginea* illustrated is that of 7 mm by Scaperrotta *et al.* (2011, p. 20). Laborel-Deguen & Laborel (1991a) briefly described specimens less than 5 mm shell diameter from Corsica as having an elevated conical shell, no visible radial ribs, and ten white radial bands standing out against the dark background of shell. According to these authors, radial ribs appear gradually in specimens larger than 5 mm, which have the characteristic colour pattern of concentric beige bands forming a star-like outline when crossing the ribs.

Casu *et al.* (2010) used genetic (and presumably lethal) methods to identify two juveniles of *Patella* of approximately 2.5 mm found on the shell of an adult specimen of *Patella ferruginea* since "species' diagnostic features may not be apparent" in such small specimens.

Therefore, almost nothing is known about the precise habitat and the morphology of the earliest growth stages of *P. ferruginea*. The early post-larval stages still remain undescribed despite that detailed information about recruitment success and young survival rates are essential for conservation purposes. Morphologic and non-invasive identification criteria for the young stages of this endangered species are therefore necessary.

Accordingly, our aim is to describe here for the first time the sequence of evolution of shell morphology and colour pattern of *Patella ferruginea* from early post-settlement stages until 20 mm MD.

Materials and Methods

Data presented here come from two sources: specimens studied in its natural environment (Chafarinas Islands, North Africa) and those obtained through controlled reproduction.

Chafarinas Islands house one of the most important and healthy existing populations of this species, estimated at more than 42,300 adults (Guallart & Templado, 2016). Recruitment, although variable, has been regular in this archipelago during the last years, sometimes exceptionally successful with up to 50 recruits/meter (Guallart *et al.*, 2012a). This facilitated the observations of recruits of different sizes. As a result of thorough censuses carried out in these islands between 1999 and 2013, a total of 7,922 specimens smaller than 20 mm MD were found; among them, 74 specimens had a size of less than 5 mm MD. Nevertheless, we were unable to find specimens smaller than 3.8 mm, due to the difficulty of locating them in a heterogeneous substrate with dense biological coverage.

On the other hand, controlled reproduction under laboratory conditions at the "Instituto de Acuicultura de Torre de la Sal", Castellón (IATS-CSIC), Spain, allowed obtaining newly settled post-larval specimens in 2012 and 2013. The description of the first stages of growth up to 2.5 mm MD is based upon 42 specimens obtained from *in vitro* fertilisation using eggs and sperm from reproductive individuals coming from the Chafarinas Islands (Guallart *et al.*, 2012c, 2013d). Growth monitoring in the laboratory of these specimens provided data on sizes up to 25 mm MD and larger, consistent with those observed in the natural habitat. Most observations come from specimens kept alive in aquaria but some post-larval shells were obtained dead from the filters at the bottom of the aquaculture tanks. Descriptions of the inner surface of the shell are based on specimens which died during laboratory studies; a representative series of 14 voucher shells in the range 5.6-28.7 mm have been deposited at Museo Nacional de Ciencias Naturales, Madrid (MNCN 15.05/60855).

Scanning Electron Micrographs were taken at MNCN under a low vacuum mode on a FEI QUANTA 200 SEM and at high vacuum mode on a JSM-840 SEM.

Results

The smallest studied post-larval specimens were found on the algal biofilm covering some Petri dishes placed at the air-water interface, floating in the culture tanks, and were observed nine days after *in vitro* fertilization of eggs. In its natural habitat, specimens longer than 3.8 mm MD were found at the level of the vermetid gastropod *Dendropoma lebeche* Templado, Richter & Calvo, 2016 and the crustose coralline red algae *Neogoniolithon brassica-florida* Harvey (Setchell & Mason, 1943) (Fig. 1A), whereas those larger than 8-10 mm were mainly found in the upper midlittoral, at the *Chthamalus* spp. fringe, together or even on adult specimens (Fig. 1B, C).

The smallest settled specimen measures 267 μm in total length (Fig. 2A), showing a translucent teleoconch (212 μm in maximum diameter). The protoconch (embryonic shell or protoconch I) measured in 12 post-larvae ranged between 210 and 245 μm in length (mean 232.7 ± 12.1 μm). It is smooth, symmetrical, but somewhat left-tilted with regard to the longitudinal teleoconch axis (Fig. 3A, B). The protoconch can remain in specimens up to 3 mm or even more in juveniles hatched and maintained in aquaria, although probably disappear much earlier in natural conditions due to erosion. At this time, the teleoconch shows a dense sculpture of fine radial lines, and remains translucid up to nearly 400 μm in length. Post-larval specimens of 267-290 μm and juveniles around 2 mm long were repeatedly observed scraping the algal film on the surface where they were settled with the radula (Supplementary material, S1, S2).

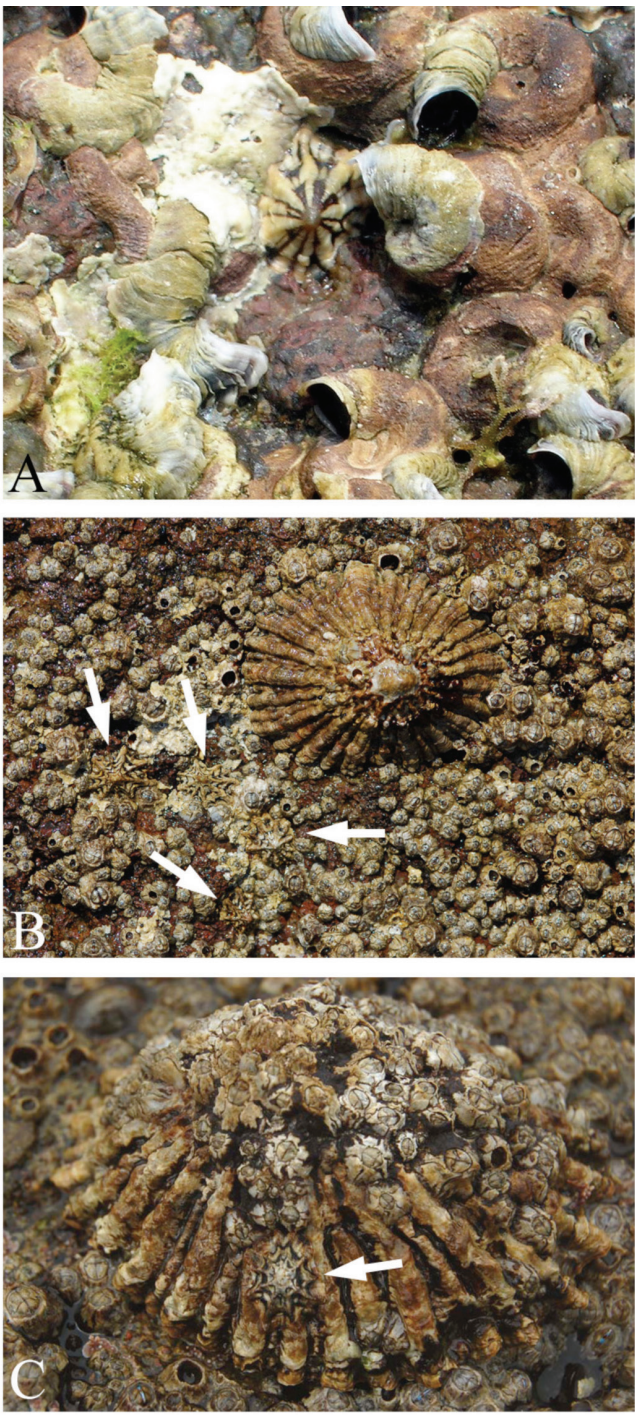

Fig. 1: *Patella ferruginea*. Young specimens in their habitat. A, 3.8 mm limpet on the *Dendropoma lebeche* / *Neogoniolithon brassica-florida* level. B, four young specimens of approx. 10-18 mm (arrows) and one adult of approx. 55 mm, at the *Chthamalus* level. C, one specimen of approx. 18 mm (arrow) on an adult of nearly 75 mm, at the *Chthamalus* level.

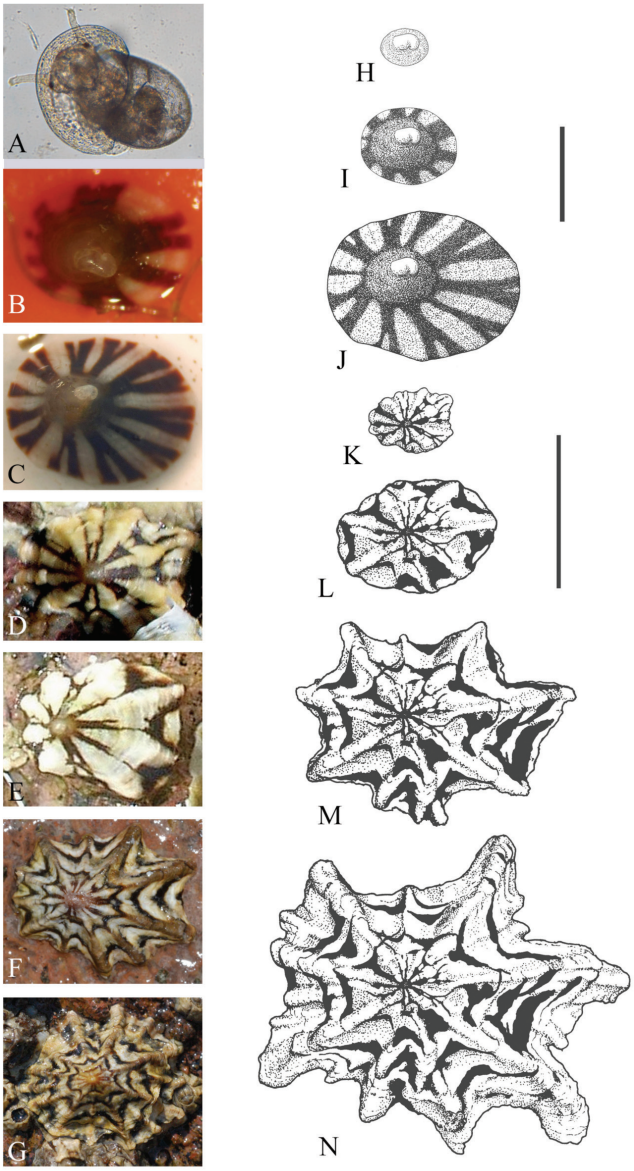

Fig. 2: *Patella ferruginea.* A-G. Living young post-larval specimens. A 0.29 mm total length (0.21 mm teleoconch MD). B 1.1 mm. C 2.4 mm. D 3.8 mm. E 8.0 mm. F 11.5 mm. G 25.0 mm. H-N. Schematic reconstruction of the evolution of shell shape and colour pattern. H 0.5 mm. I 1 mm. J 2 mm. K 4 mm. L 8 mm. M 12 mm. N 17 mm. Scale bars: H-J 1 mm; K-N 10 mm.

Young specimens of 1-2 mm MD have an ovate shell and a distinct colour pattern of radial bands alternating light (whitish) with 8-12 dark (brown to blackish), most of them forking near the margin (Fig. 2B, C). Some of these forked bands appear later to be grouped in pairs in larger juveniles (Fig. 2 D, E). In 1-2 mm specimens, the initial teleoconch, at approximately 500 µm in diameter, shows an almost uniformly dark brown colour. The inner shell shows the same radial banded pattern by transparency (Fig. 5A). This pattern remains in specimens up to 3-4 mm length, both observed in the natural environment and reared in laboratory (Fig. 2D).

Specimens between 5 and 8 mm show dark spots near the shell edge (Fig. 2E). These spots begin the development of the first dark ring of a series of concentric bands, which are characteristics of specimens larger than 10 mm MD (Fig. 2F, G). The banded radial pattern of the smaller specimens often disappears in larger specimens

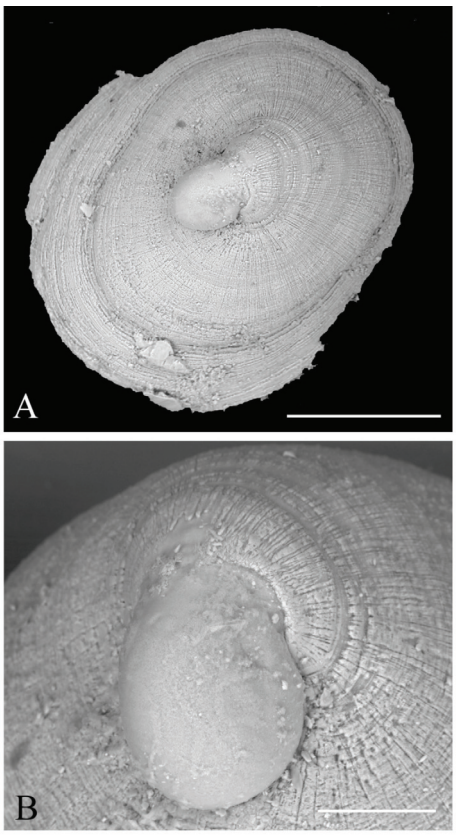

Fig. 3: *Patella ferruginea*. A SEM micrograph of the shell of a recently settled post-larval specimen 0.99 mm in length. B Detail of the protoconch of the same specimen. Scale bars: A, 400 µm; B, 100 µm.

(25-30 mm) due to shell erosion in its natural habitat, but remains in laboratory specimens up to 40 mm MD. Figure 2H-N represents the schematic evolution of shell morphology and colour pattern from new settled juveniles 0.5 mm in length until 17 mm.

The shell inner surface in individuals more than 5 mm up to at least 20 mm is darker, predominantly reddish brown and dark blue (Fig. 4A-J). The pattern is ill-defined, with blue under the radial ribs and the concentric dark bands of the external surface. Sometimes, the growth edge is more evident due to its whitish colour. The inner surface of the shell becomes gradually whitish in specimens from 20-25 mm (Fig. 4K-N).

The colour pattern of the soft parts differs between young and adult specimens. Adults (>30 mm MD) show the foot sole a yellowish cream colour tarnished with grey; dark grey or blackish foot sides, head and cephalic tentacles; and translucent pallial tentacles (Fig. 5C). Smallest specimens are paler, showing a translucent whitish foot sole, head, and cephalic tentacles at a length of 2 mm (Fig. 5A). Cephalic tentacles are the first to become darker, being grey at 10-12 mm MD (Fig. 5B); head, foot sole, and sides have a light cream, almost yellowish colour. Specimens more than 25 mm have dark grey or blackish cephalic tentacles and the head becoming greyish, while the foot sole and sides remain light cream to sizes over 30 mm. Foot sides darken to grey or blackish colour at approximately 40-50 mm.

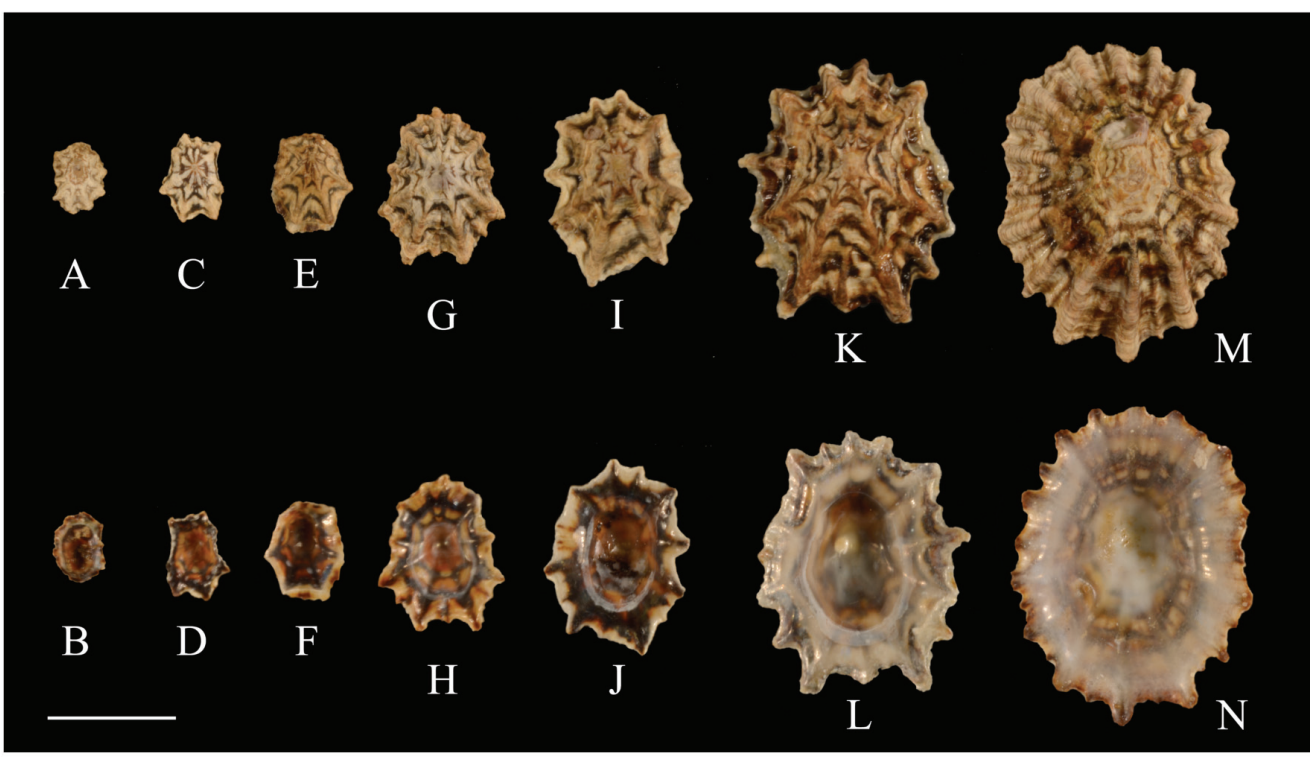

Fig. 4: *Patella ferruginea.* External and internal views of the shells of some young specimens stored at MNCN as voucher material. A-B 5.6 mm. C-D 7.0 mm. E-F 7.9 mm. G-H 12.5 mm. I-J 15.2 mm. K-L 20.6 mm. M-N 25.0 mm. Scale bar: 10 mm.

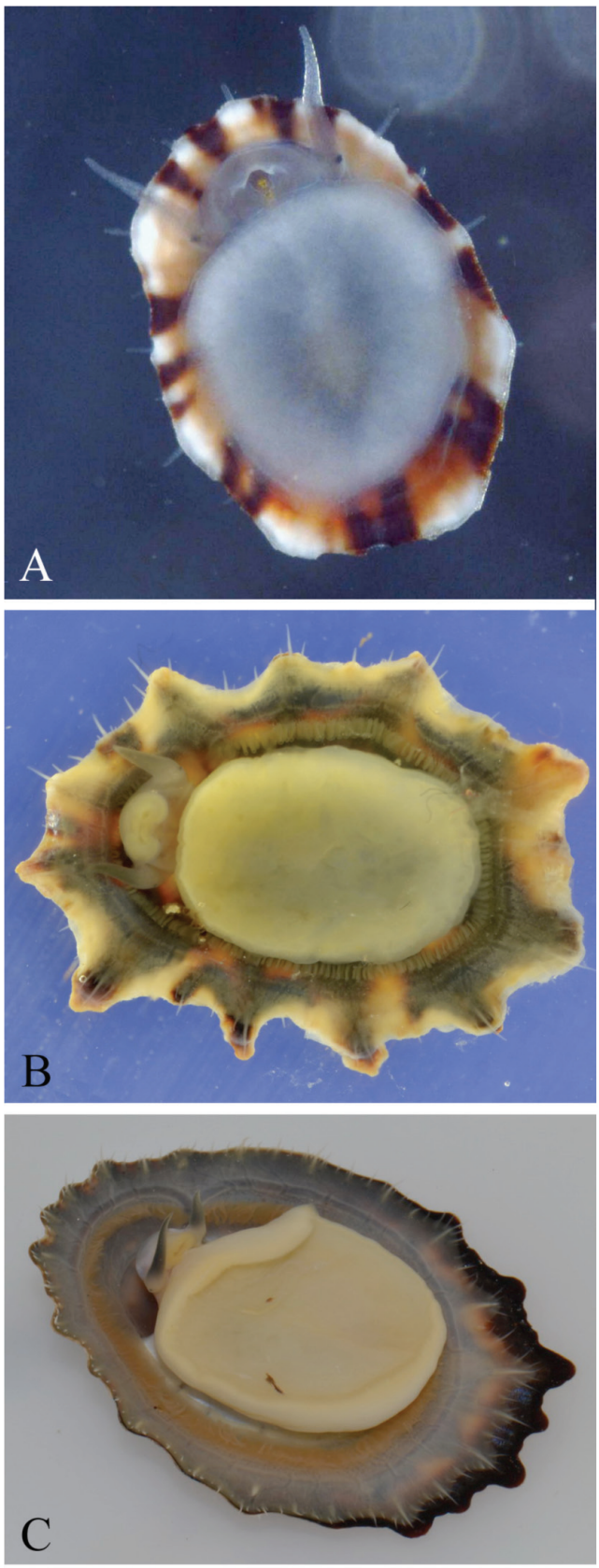

Fig. 5: *Patella ferruginea*. Ventral views of living animals. A 2.56 mm. B 12 mm. C 30 mm.

Discussion

According to our laboratory observations, the youngest post-larvae settled just at the air-water interface, and the subsequent early stages live slightly above this level, probably to avoid desiccation. The youngest specimens were found in the natural habitat at the *Dendropoma lebeche* belt (which coincides with the mean sea level), and only specimens more than 8-10 mm long climb to the upper *Chthamalus* spp. level (upper midlittoral), sharing this habitat with adults. Our observations on the location of the recruits partially agrees with the suggestion of Laborel-Deguen & Laborel (1991b) that the very first benthic stages live at lower levels than adults, but we consider it unlikely that they settle at the *Cystoseira* spp. belt (infralittoral). Instead, our observations suggest that they could settle at the lower midlittoral, on the irregular surface covered by the *Dendropoma lebeche-Neogoniolithon brassica-florida* belt. Indeed, Guallart & Templado (2016) found a significant association between maximum values of density of *Patella ferruginea* and the presence of a more or less continuous belt of *D. lebeche-N. brassica-florida* crusts with moderate vertical development in Chafarinas Islands, and suggested that a more detailed study of this association is needed.

The protoconch of *P. ferruginea* is illustrated and described here for the first time. Little information is available on the protoconchs of other Mediterranean-Atlantic limpet species. The protoconch of *Patella caerulea* Linné, 1758 is approximately 200 mm in length according to measurements made on Fig. 4 from Wanninger *et al.* (1999). It shows also a symmetrical but not sculptured embryonic shell (protoconch I); the right part of the juvenile teleoconch apparently grows faster than the left part, also giving a left-tilted protoconch with respect to the longitudinal axis of teleoconch (Wanninger *et al.*, 1999). Nevertheless, the early teleoconch of *P. caerulea* lacks the radial sculpture of *P. ferruginea*. Thompson (1912) described a similar asymmetry with the posterior apex of the protoconch being to the left of the teleoconch axis in *Acmaea* sp. (Acmaeidae). Similarly, the teleoconch of *Lottia digitalis* (Rathke, 1833) and *L. asmi* (Middendorf, 1847) (Lottiidae) was initially symmetrical, but in slightly older post-larvae the protoconch appeared to be displaced slightly clockwise relative to the long axis of the teleoconch (Kay & Emlet, 2002).

Smith (1935) observed that post-larvae of *Patella vulgata* Linné, 1758 loss the protoconch at approximately 0.5 mm length, while in specimens studied here, the protoconch remained in specimens up to 3 mm in length, and even longer in laboratory-cultured juveniles.

Post-larvae and young specimens of *Patella ferruginea* under 5 mm MD are described and illustrated in this work for the first time. They show a remarkably different shell morphology and colour pattern when compared to larger specimens, and can be recognised from a young age. The 7 mm specimen of *Patella ferruginea* illustrated by Scaperrotta *et al.* (2011, p. 20) from Corsica shows an identical shell shape and colour pattern to those we have studied of similar sizes.

Patella ferruginea shares its upper midlittoral habitat with *Patella rustica* Linné, 1758. This species lives in a slightly higher level, but no studies about its youngest stages have been published. We have not studied specimens of *P. rustica* under 5 mm MD, but at this size, *P. rustica* has a more regular profile of the shell margin and a greater number (12) of more or less discontinuous radial dark bands than *P. ferruginea*, which are not grouped in pairs: thus both species can be easily differentiated. Scaperrotta *et al.* (2011, p. 21) illustrated a 5.5 mm specimen of *P. rustica* also showing 12 radial black bands. According to Lima *et al.* (2006), small (*ca*. 5 mm) specimens of *Patella rustica* show the characteristic black spots decorating the shell of this species.

Casu *et al.* (2010) compared the sequences of the cytochrome c oxidase subunit I (COI) of two young limpets of approximately 2.5 mm in length found attached to an adult shell of *P. ferruginea* from Isole Le Camere (NE Sardinia, Italy), that they considered morphologically unidentifiable (although they were not described). These authors concluded that COI may be useful as DNA barcoding to differentiate Mediterranean species of *Patella*, and identified one of these two specimens as *P. rustica* and the other as *P. ferruginea*. They also stated the unfeasibility of a confident identification of young individuals, "even sacrificing the specimen, a practice not to be encouraged when dealing with a species threatened with extinction". Although they used a tissue sampling protocol by excising samples of foot muscle and gill (Casu *et al.*, 2006), there is no comment on the survival of the two young specimens after this treatment. According to our own experience (unpublished observations) it is unlikely that such small specimens could have survived that procedure, in contrast to the high survival rates observed in adults by Casu *et al.* (2006) and by our sampling protocol (Guallart *et al.*, 2013a) that did not include such very small specimens. Therefore, this invasive, expensive, and slow genetic method seems to be inapplicable in practice to report the abundance of very young individuals in ecological surveys or in censuses of this species facing risk of extinction, and thus must be discarded.

Although further information is needed about specimens smaller than 5 mm of *P. rustica* (and also of the other two Mediterranean patellid limpets, *P. caerulea* and *P. ulyssyponensis* Gmelin, 1791), the present paper provides information with which to identify with confidence young specimens of *Patella ferruginea* at least 3 mm MD exclusively on the basis of the external shape and colour pattern, a non-invasive and far preferable method than genetic ones.

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Supplementary Material

Video footages of two young stages of *Patella ferruginea*, scraping the transparent substrate where they settled.

S1. Post-larva of 267 μm in total length, 15 days after fertilisation, seen in dorsal view.
S2. Young specimen 2.1 mm MD, approx. 5 months old, seen in ventral view.