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Implementation of controlled reproduction techniques on marine invertebrates: gastropod molluscs of *Patella* genus

Sperimentazione di tecniche di riproduzione controllata di invertebrati marini: Molluschi gasteropodi, Genere *Patella*

Dottorando:

Maria Paola Ferranti

Tutor:

Mariachiara Chiantore

Javier Guallart

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Summary

The PhD project was mainly focused on the implementation of controlled reproduction techniques in the *Patella* genus. The species considered were *P. caerulea* and more particularly *P. ferruginea*, a protected and endangered species, whose repopulation is the subject of the European ReLife project (LIFE15 NAT/IT/000771).

Being *P. ferruginea* a protected species, we initially preferred to test the non-lethal induction treatments on a cogeneric species, *P. caerulea*, common and abundant along the Italian coasts. The various treatments tested have provided excellent results, and the "bubbling" treatment was the best, both in terms of time of response to stimulation of the specimens and of replicability and ease of use. The results obtained made it possible to draw up a non-lethal spawning protocol, and to carry out artificial fertilization with subsequent larval development and settlement of the juveniles (Ferranti et al., 2018).

In addition, monitoring was carried out along the Ligurian coast, which allowed reporting the presence of *P. ferruginea*, considered extinct along these coasts (Espinosa et al., 2014). *P. ferruginea* distribution, along the Ligurian coasts, is extremely scarce and fragmented; in fact the species has been found as isolated specimens or small groups, such as in the Cinque Terre MPA. However, these findings permitted to expand the mapping of the presence of *P. ferruginea* along the Mediterranean coasts (Ferranti et al., 2019).

Furthermore, to better understand the reproductive cycle and the timing of *P. ferruginea* natural spawning at our latitudes, we placed specimens at sea for maturation in natural conditions, up to the beginning of the expected reproductive period. Specimens were later subjected to a biopsy of the gonad, both before being placed in the sea to determine sex (Guallart et al., 2013a), and at the beginning of the expected reproductive period to understand the stage of maturation. The observation of the material obtained through biopsy made it possible to determine that the specimens had reached gonadal maturation and that one of them had performed sex change, an event known in the literature, but observed here for the first time at the north-western Mediterranean latitude (Ferranti et al., 2021). This implies that the species can complete its reproductive cycle along the Ligurian coasts.

Always in view of a better understanding of the timing of gonad maturation, we applied the Magnetic Resonance Imaging (MRI) techniques on limpets. MRI, through a non-invasive approach, allowed to observe the thickness of the gonad and also to understand whether or not a specimen had released gametes, in order to eventually apply stimulation techniques to spawning only at the right time, reducing stress on specimens. The results indicated that MRI can be very useful as an additional tool, both in evaluating the sexual maturity of *P. ferruginea* and in minimizing the number of induction trials for each specimen, and consequently the stress (Guallart et al., 2020).

Even more relevant is having provided evidence, for the first time, that spawning can be induced in *P. ferruginea* and demonstrated the feasibility of obtaining juveniles, under controlled conditions through low-invasive methods. These results allowed drawing up a preliminary protocol on the induction of spawning of *P. ferruginea*, which allows for the spontaneous release of gametes, without sacrificing the broodstock of a protected species. Furthermore, this allowed us to carry out an artificial fertilization that led to larval development, settlement, metamorphosis and growth of a pretty large number of *P. ferruginea* juveniles (Ferranti et al., in prep.).

Consequently, knowing that the species is present along the Ligurian coast, that it manages to reach maturity even at these latitudes, and that it is possible to reproduce *P. ferruginea* under controlled conditions until it reaches the juvenile stage, we provide evidence of the feasibility of *P. ferruginea* restocking and

reintroduction in the natural environment, recovering this species in this area, as expected from the ReLife project.

Moreover, the reintroduction made from specimens obtained through aquaculture techniques, without substantially affecting donor populations, is also in accordance with the Spanish strategy for the conservation of the species (MMAMRM, 2008), and generally, repopulation actions through controlled reproduction, could allow in the future to repopulate other coastal areas, where populations of *P. ferruginea* are disappeared and/or in regression, for the recovery of the species at a global level.

Riassunto

Il progetto di dottorato è stato principalmente focalizzato sull'implementazione delle tecniche di riproduzione controllata nel genere *Patella*. Le specie oggetto di studio sono state *P. caerulea* e più in particolare *P. ferruginea*, specie protetta e in via di estinzione, il cui ripopolamento è oggetto del progetto europeo ReLife (LIFE15 NAT/IT/000771).

Essendo *P. ferruginea* una specie protetta, inizialmente abbiamo preferito testare i trattamenti di induzione non letale su una specie cogenerica, *P. caerulea*, comune e abbondante lungo le coste italiane. I vari trattamenti testati hanno dato ottimi risultati, e il trattamento del "bubbling" è risultato il migliore, sia in termini di tempo di risposta alla stimolazione degli esemplari, sia perché si tratta di un metodo poco costoso e facilmente ripetibile. È stato possibile stilare un protocollo di spawning non letale, ed effettuare una fecondazione artificiale con successivo sviluppo larvale e insediamento dei giovanili (Ferranti et al., 2018).

Inoltre, sono stati condotti dei monitoraggi lungo la costa ligure, che hanno permesso di riportare la presenza di esemplari di *P. ferruginea*, considerata estinta lungo queste coste (Espinosa et al., 2014). La distribuzione di *P. ferruginea*, lungo le coste liguri, è estremamente scarsa e frammentata, infatti la specie è stata rinvenuta come esemplari isolati o piccoli gruppi, come nell'AMP delle Cinque Terre. Questi ritrovamenti hanno però permesso di ampliare la mappatura della presenza di *P. ferruginea* lungo le coste del Mediterraneo (Ferranti et al., 2019).

Inoltre, per comprendere meglio il ciclo riproduttivo e i tempi di rilascio dei gameti in *P. ferruginea* alle nostre latitudini, abbiamo posizionato degli esemplari in mare per la maturazione in condizioni naturali, fino all'inizio del periodo riproduttivo. Gli esemplari successivamente sono stati sottoposti a biopsia della gonade sia prima di essere messi in mare per determinare il sesso (Guallart et al., 2013a), che all'inizio del periodo riproduttivo previsto per comprendere lo stadio di maturazione. L'osservazione del materiale ottenuto tramite biopsia ha permesso di determinare che gli esemplari avevano raggiunto la maturazione gonadica e che uno di loro aveva effettuato il cambio di sesso, evento noto in letteratura, ma osservato qui per la prima volta alla latitudine del Mediterraneo nord-occidentale (Ferranti et al., 2021). Tali risultati implicano che la specie può completare il suo ciclo riproduttivo lungo le coste liguri.

Sempre al fine di comprendere meglio i tempi di maturazione delle gonadi, abbiamo applicato le tecniche della Risonanza Magnetica su esemplari di patelle. La Risonanza Magnetica, attraverso un approccio non invasivo, ha permesso di osservare lo spessore della gonade e anche di capire se un esemplare avesse rilasciato o meno gameti, al fine di applicare eventualmente tecniche di stimolazione al rilascio dei gameti solo al momento giusto. I risultati indicano che la risonanza magnetica può essere molto utile come strumento aggiuntivo, sia per valutare la maturità sessuale di *P. ferruginea*, che per ridurre al minimo il numero di prove di induzione per ogni esemplare, e di conseguenza lo stress (Guallart et al., 2020).

Ancora più rilevante è l'aver fornito l'evidenza, per la prima volta, che l'emissione delle uova può essere indotta in *P. ferruginea* e dimostrare che è possibile ottenere giovanili, in condizioni controllate, attraverso

metodi non invasivi. Questi risultati hanno permesso di elaborare un protocollo preliminare sull'induzione all'emissione spontanea dei gameti da parte di *P. ferruginea*, senza sacrificare i riproduttori di una specie protetta. Inoltre, questo ci ha permesso di effettuare una fecondazione artificiale che ha portato allo sviluppo, insediamento, metamorfosi e crescita larvale di un numero piuttosto elevato di individui giovanili di *P. ferruginea* (Ferranti et al., in prep.).

Di conseguenza, sapendo che la specie è presente lungo la costa ligure, che riesce a raggiungere la maturità anche a queste latitudini e che è possibile riprodurre *P. ferruginea* in condizioni controllate fino allo stadio giovanile, possiamo affermare che esiste la possibilità di ripopolamento e reintroduzione in ambiente naturale di esemplari di *P. ferruginea*, recuperando questa specie nell'area in oggetto, come previsto dal progetto ReLife.

Inoltre, la reintroduzione realizzata da esemplari ottenuti mediante tecniche di acquacoltura, senza influenzare sostanzialmente le popolazioni donatrici, è anche in accordo con la strategia spagnola per la conservazione della specie (MMAMRM, 2008), e in generale, le azioni di ripopolamento attraverso la riproduzione controllata, potrebbero consentire in futuro di ripopolare altre aree costiere, dove le popolazioni di *P. ferruginea* sono scomparse e/o in regressione, per un recupero della specie a livello globale.

1. AIM OF THE PhD THESIS

My PhD project aimed at the implementation of controlled reproduction techniques on *Patella* genus. Two were the species mainly examined: *Patella caerulea* and *Patella ferruginea*.

The *Patella* genus, but more generally some species of limpets (Patellograstropoda), are overharvested in different regions of the world, and this has led some species to the risk of extinction. Consequently, the development of reproduction techniques could contribute to increase knowledge on limpet aquaculture techniques, which unfortunately are not as well developed as for other marine invertebrates, despite the overfishing of natural populations. Such implementation could reduce pressure on natural populations and move forward establishment of new job opportunities in the light of the Blue Growth.

Furthermore, the studies carried out for the implementation of limpet reproduction techniques may contribute to the production of juveniles of endangered species, under international and national directives, such as *P. ferruginea,* to be restocked or re-established in light of e.g. the EU Biodiversity 2030 Directive, which foresees ecological restoration as a necessary approach to the biodiversity maintenance.

2. INTRODUCTION

2.1 General conditions state of the exploitation of limpets in the world

Limpets (Patellogastropoda) are conspicuous gastropod species of the midlittoral and shallow sublittoral zone, from the tropics to the poles (Branch, 1981; Mau & Jha, 2018a), largely influenced by abiotic (e.g. desiccation) and biotic factors (competition and predation, Connell, 1972; Paine, 1994; Raffaelli & Hawkins, 1996; Mau & Jha, 2018a). In rocky coastal ecosystems, they play a key role through their grazing activity, controlling composition and structure of the benthic community (Southward, 1964; Underwood, 1980; Moreno & Jaramillo, 1983; Farrell., 1988; Anderson & Underwood, 1997; Fernández & Castilla, 2005). For example, they control barnacle numbers by removing the juveniles with their grazing, and at the same time they limit the growth of macroalgae (Southward, 1964; Hawkins & Hartnoll, 1983), which could impede barnacle recruitment (Branch, 1981; Underwood et al., 1983). In this way, through the grazing activity, the limpets maintain some open spaces in the intertidal zone enabling many species to coexist, thus contributing to increased marine biodiversity on rocky shores (Espinosa & Rivera-Ingraham, 2017).

Although the limpets are resilient to environmental and biological pressure from other invertebrates, they have experienced negative anthropogenic influences since a long time (Mau & Jha, 2018a), inasmuch they are considered culinary delicacies and harvested worldwide, as food and as bite for fishery, since at least the Neolithic Age, also for their ease of harvesting (Laborel-Deguen & Laborel, 1990a; Laborel-Deguen & Laborel, 1991a; Ghisotti, 1997; Espinosa et al., 2009).

Several species of limpets are harvested for human consumption in many regions of the world (Boudouresque & Laborel-Deguen, 1986; Porcheddu & Milella, 1991; Martins *et al*., 2008; Nhan & Ako, 2012). In some regions, this has led to the population decline of many species (Laborel-Deguen & Laborel, 1991a; Erlandson et al., 2011; Martins et al., 2011; Coppa et al., 2015; Sousa et al., 2019), with some species being threatened of extinction.

Among the overexploited species we can quote for example the owl limpet *Lottia gigantea* along the Californian coast, whose selective harvesting of the larger individuals by humans, caused a reduction in mean size and population abundance, unlike natural predators, such as seabirds, which tend to prey primarily on smaller owl limpets (Lindberg et al., 1998; Erlandson et al., 2011). In fact, *L. gigantea* is a protandric species: therefore, harvesting focused on larger specimens (normally females) causes males to change into females at smaller sizes, further reducing reproductive output and recruitment in future generations (Erlandson et al., 2011).

As another example, in the Hawaii three are the main endemic limpet species, locally known as "opihi", including black foot or makaiauli (*Cellana exarata*), yellow foot or ālinalina (*Cellana sandwicensis*), and the largest species, giant opihi or Kōʻele (*Cellana talcosa*) (Kay & Magruder, 1977). These species were overharvested for a long time, for private and commercial consumption, causing a decrease of population during the XX century (Kay & Magruder, 1977; Tom, 2011; Nhan & Ako, 2012; Nhan, 2014). They are considered as high-value market food and high-potential candidate species for commercial aquaculture.

Both genera, *Lottia* and *Cellana,* are considered not just as a good source of protein, vitamin A, vitamin D, phosphorus and iron (Miller & Robbins 1940), but also they were used as scraping and scaling tools, drinking bowls and medicine, and in the Hawaiian society the opihi were considered as a cultural symbol (Tom, 2011; Nhan & Ako, 2019).

Also, in the Azores archipelago, the limpets and other littoral organisms (e.g., barnacles, seaweeds) have been exploited since the 15th century (Martins et al., 2008). This exploitation of limpets, gathered as food at both commercial and recreational levels (Fernandes et al., 2019; Sousa et al., 2019), was one of the largest anthropogenic impacts on these shores (Hawkins et al., 2000; Núñez et al., 2003; González-Lorenzo et al., 2015). Two species of the *Patella* genus, *P. aspera* and *P. candei*, were overharvested with consequent drastic population reductions. For this reason, a specific Regional Regulatory Decree (RRD 14/93/A) was issued to create Limpet Protected Zones (LPZ), where harvesting is prohibited (Martins et al., 2008; Martins et al., 2011). Moreover, legal regulations and management measures were imposed to reduce the anthropogenic impact and restore the native stocks also in Madeira (Sousa et al., 2019a, b) and in the Canary archipelagos (DECRETO 182/2004; Núñez et al., 2003).

Another species of *Patella* genus is *Patella ferruginea,* which is an endemic species of the Western Mediterranean Sea where it is considered one of the most threatened invertebrates and is emblematic of conservation in the region (Ramos, 1998; Luque et al., 2018). Its decline has been attributed to human impact, mainly harvesting but also habitat degradation, development of coastal infrastructures and marine pollution (Laborel-Deguen & Laborel, 1990a; Laborel-Deguen & Laborel, 1991a; Templado, 2001; Paracuellos et al., 2003; Templado et al., 2004; Moreno & Arroyo, 2008). Such pressures have drastically reduced its distribution since at least the middle of the 20th century (Laborel-Deguen & Laborel, 1990a; Laborel-Deguen & Laborel, 1991a). For this reason, it is currently protected by various international country binding conventions (Annex II of Barcelona and Bern Conventions) and specific laws in various Mediterranean countries (Spain, France and Algeria).

Continual decline in wild populations of limpets has implications not only ecologically, but also socioeconomically and culturally (Mau & Jha, 2018a). Despite this, aquaculture techniques at commercial level are not yet as developed, as occurs for other molluscs (e.g., oyster, clams and mussels). However, the aquaculture of limpets, although extremely limited, is currently being developed with hopes to supply the market with sustainably sourced seafood and improve wild stocks (Gibson, 1978; Corpuz, 1981; Nhan, 2014), not only of marketable species, but also of endangered species.

For example, in the Macaronesian Region, the limpets can be representing an opportunity to diversify the local production in aquaculture, a strategy that could promote the consolidation of the industry through a sustainable development (Schmidt et al., 2011), and an alternative to the exploitation of the natural resources, besides being able to be applied for restocking purposes (Castejón et al., 2020).

2.2 Aquaculture and controlled reproduction in limpets

Controlled reproduction is a requirement for developing effective mollusc culture for commercial or restoration purposes.

While the spawning of abalones has become routinary and breeding and rearing protocols are well established (Morse et al., 1977; Setyono, 2004; Hannon et al., 2013; Mau & Jha, 2018a), the sustainable aquaculture of limpets still requires successful spawning and larval rearing techniques.

The aquaculture of limpets is a recent research field, so few bibliographical and methodological resources are available (Corpuz, 1981; Guallart, 2010; Ferranti et al., 2018; Mau & Jha, 2018a; Guallart et al., 2020). Most efforts on limpet aquaculture research have been focused on the acclimation of adult stocks (Nhan & Ako, 2019), design of artificial diets (Nhan & Ako, 2016; Mau & Jha, 2018b), artificial reproduction in controlled conditions (Kay & Emlet, 2002; Hodgson et al., 2007; Aquino De Souza et al., 2009; Ferranti et al., 2018; Mau et al., 2018; Nhan & Ako, 2019; Guallart et., al 2020; Castejón et al., 2020; Ferranti et al., in prep). Larval production is a mandatory step for further studies focused on the larval cycle and aquaculture production of limpets (Guallart et al., 2013a).

Actually, artificial reproduction had been often carried out in different limpet species by way of gametes obtained through gonad dissection since more than one century: *Cellana* spp. (*C. exarata*: Corpuz, 1981; *C. tramoserica:* Anderson, 1962), and *Patella* spp. (*P. caerulea*: Lo Bianco, 1899; Wilson, 1904; *P. vulgata*: Smith, 1935; Orton et al., 1956; *P. vulgata & P. caerulea:* Dodd, 1957; Wanninger et al., 1999; *P. vulgata* and *P. ulyssiponensis*: Hodgson et al., 2007; *P. depressa* and *P. vulgata:* Aquino de Souza *et al*., 2009; *P. ferruginea*: Espinosa et al, 2010; Guallart et al., 2013a; Guallart et al., 2020; *P. aspera*: Castejón et al., 2020). In some cases, the male gametes were obtained through non-lethal biopsy (Guallart et al., 2013a, b; Guallart et al., 2020; Ferranti et al, in prep). Yet, gametes from dissection cannot be considered as an option in case of endangered and protected species.

Another issue related to gamete extraction by dissection relies on the state of maturation and fertilization success. Several studies have shown that the fertilization success can be enhanced, improving maturation of artificially extracted oocytes from the ovary, maintaining the same oocytes for a certain length of time in alkalinised seawater at pH 9 (e.g., Corpuz, 1981; Wanninger et al., 1999; Gould et al., 2001; Hodgson *et al*., 2007; Aquino de Souza *et al*., 2009, Guallart, 2010; Guallart et al., 2013a; Guallart et al., 2020; Castejón et al., 2020). NaOH and NH_4OH are the most common alkalinizing agents used for the activation in several limpet species. However, the best method for obtaining ripe gametes for fertilization would be to stimulate adult specimens to induce them to spontaneously release their gametes, avoiding sacrificing the broodstocks.

Spawning induction has been achieved only in few limpet species with different methods: (1) chemical: hydrogen peroxide (H_2O_2), hormonal stimulation, and (2) physical: thermal shock, vigorous aeration (Corpuz, 1981; Kay & Emlet, 2002; Nhan & Ako, 2012, 2019; Mau et al., 2018a; Ferranti *et al*., 2018).

Hydrogen peroxide is a common method for spawning invertebrates (e.g. abalone, Morse et al., 1977). The release of hydroperoxy free radicals and peroxy radicals from H_2O_2 interacting with seawater is believed to activate prostaglandin endoperoxide synthetase and induce spawning in the mature specimens (Morse 1984). This treatment was adopted to successfully spawn *L. digitalis* and *L. asmi* by Kay and Emlet (2002), *P. caerulea* by Ferranti et al. (2018) and *C. sandwichensis* by Nhan (2014), which has observed lethal effects on broodstock opihi, making this treatment unsuitable. Also, this is not a sustainable practice for the limpet aquaculture industry because it requires capture of mature animals (according to Mau et al., 2018; Ferranti et al., 2018; in prep.).

Another method successfully implemented for spawning limpets is hormonal stimulation, trough injecting gonadotropin-releasing hormone (GnRH) directly into the specimen gonad (*C. sandwichensis,* Mau et al., 2018; Nhan & Ako, 2012, 2019). Nhan & Ako (2012, 2019) tested salmon GnRH analogue (sGnRHa) on *Cellana sandwichensis,* injecting the hormone for both gonad maturation (250ng.g^{-1} BW/week) and spawning (single dose of 1000ng.g^{-1} BW). This method resulted to be the most practical for *C. sandwichensis* and did not implicate high mortality of broodstock specimens compared to experiments using hydrogen peroxide.

Recently hormonal stimulation has also been applied to some specimens of *P. ferruginea*. Instead of using salmon gonadotropin-releasing hormone analog (sGnRHa), a similar hormone, luteinizing hormone-releasing hormone (LHRH ethylamide acetate salt hydrate) was used, and the dosage used was the one reported for spawning induction (1 µg/g BW, Nhan and Ako, 2019). However, it did not produce any results (Ferranti et al., in prep).

The physical methods, vigorous aeration ("bubbling") and thermal shock, simulate natural triggers for inducing spawning (temperature changes and/or rough sea conditions), and for this reason they were considered possible good candidates since the spawning of genus *Patella* is apparently positively affected by wave mechanical shock, during rough sea events (Orton et al., 1956; Dodd, 1957; Frenkiel, 1975; Branch, 1981). These two methods were applied successfully on *C. exarata* (vigorous aeration, Corpuz, 1981), *L. digitalis* and *L. asmi* (thermal shock, vigorous aeration, Kay & Emlet, 2002) and *P. caerulea* (thermal shock, vigorous aeration, Ferranti et al., 2018). Furthermore, vigorous aeration is an economic and simple method to replicate, and in *P. caerulea* it induced an immediate release of gametes, resulting in the best of the treatments tested (Ferranti et al., 2018).

The choice of totally non-invasive and non-lethal approaches is particularly useful in the case of reproduction of endangered species for repopulation and restocking purposes. Such as for example for *Patella ferruginea* on which different non-lethal stimuli have been tested (Ferranti et al., in prep).

2.3 Focus on *Patella ferruginea*

The ferruginous limpet, *Patella ferruginea* Gmelin, 1791, is an endemic species of the Western Mediterranean Sea, presently considered the most threatened and at risk of extinction marine macroinvertebrate in the region (Ramos, 1998). For this reason, it is included in the list of the strictly protected species in Annex IV of the Directive 92/43 (Directive "of Habitats"), in Annex II of Barcelona and Bern Conventions, and protected by specific laws in various Mediterranean countries (e.g., Spain, France, and Algeria). The decline of this species has been attributed to human impact mainly harvesting, but also habitat degradation, development of coastal infrastructures and marine pollution (Laborel-Deguen & Laborel, 1990a; Laborel-Deguen & Laborel, 1991a; Templado, 2001; Templado et al., 2004; Moreno & Arroyo, 2008; Luque et al., 2018). The species is easily accessible to humans since it inhabits in the high mid-littoral zone (Laborel-Deguen & Laborel, 1990a, b; Templado, 2001; Templado et al., 2004), but can also be found in the supra-littoral (Paracuellos et al., 2003; Guerra-García et al., 2004). Moreover, it is very visible, being a large limpet, in fact it may normally reach 80 mm in maximum diameter of the shell, but may even reach more than 100 mm (Guallart & Templado, 2012; Luque et al., 2018).

Reproductive biology

Knowledge about the biology of the species has increased in the last two decades. The reproductive cycle of the species has only been studied in the Alboran Sea along the Algerian coasts (Frenkiel, 1975) and in the Chafarinas islands (Guallart et al., 2006). Like other species of *Patella*, *P. ferruginea* is a broadcast spawner, the gametes are emitted in the sea, where fertilization occurs (Branch, 1981). The species displays a short reproductive cycle, which begins in late summer and culminates in late autumn, where the emission of gametes is generally synchronized between November and December, when the first strong storms of late autumn occur. The rest of the year, the species appears to be in complete sexual rest (Frenkiel, 1975; Laborel-Deguen & Laborel, 1990b; 1991a, b; Guallart, 2006; Guallart et al., 2006; Luque et al., 2018).

Patella ferruginea was initially described as a protandrous hermaphrodite (Frenkiel 1975; Laborel-Deguen & Laborel 1991b; Espinosa et al. 2006). Laborel-Deguen and Laborel (1991b), on basis of the results reported by Frenkiel (1975), described that individuals of *P. ferruginea* reach sexual maturity as males at a size of about 24 mm, and then, change sex when they are about 40 mm long. However, subsequent studies have shown the inaccuracy of this information (Guallart et al., 2006; Espinosa et al., 2009). In fact, these authors have clarified that in the populations studied in the Chafarinas Islands (Guallart et al., 2006) and along the coast of Ceuta (Espinosa et al., 2009), male specimens were found also within the largest size groups, although it is true that the proportions of males compared to females tended to decrease with size. Moreover, Rivera-Ingraham et al. (2011) highlighted the high variability in the size or age at which sex change occurs in this species, suggesting that it is driven by environmental factors, such as density or population structure. However, it was later shown that *P. ferruginea* is a sequential protandrous hermaphrodite showing alternating sex reversal (Guallart, 2010; Guallart et al., 2010; Guallart *et al*., 2013b). In fact, these authors, through the continuous monitoring of various specimens in the Chafarinas Islands, observed both the sex change from males to females in some specimens during the study period, but also reported that several specimens initially sexed as females had transformed into males in the following years. Recently, Ferranti et al. (2021) reported the first sex change observation of *P. ferruginea* in the Ligurian Sea, previously reported only in the Alboran Sea (Espinosa et al., 2009; 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, 1990b; 1991b). There are few studies about the fertilization and larval phase: some authors described that the fertilization of *P. ferruginea* was not difficult, although they did not provide other relevant information (Laborel-Deguen & Laborel, 1990b; 1991b; Frenkiel, 1975). Guallart et al. (2006) obtained some preliminary controlled fertilization assays, following larval development up to the pretorsional veliger stage 40 hours after fertilization. Subsequently, some attempts were conducted for reproduction under controlled conditions, reaching the larval stage of early pretorsional veliger (Espinosa et al., 2010). Conversely, Guallart et al. (2020), managed to "close the cycle" of the species, describing larval development, metamorphosis and the subsequent achievement of sexual maturity. All these results were achieved starting from oocytes obtained exclusively from the dissection of mature females. In the present thesis, I describe for the first time the successful spawning induction in *P. ferruginea* (through a not lethal approach), the following gamete fertilizations, the whole larval cycle until settlement and metamorphosis (Ferranti et al., in prep). These results contribute to the advancement of our understanding of this species biology and ecology.

Mediterranean distribution

P. ferruginea populations were widespread throughout the Western Mediterranean in the late Pleistocene (Tyrrhenian period) and primarily maintained this range until the 19th century (Laborel-Deguen & Laborel, 1990a; 1991a). During the second half of the 20th century its distribution has been progressively reduced to a few restricted areas (Laborel-Deguen & Laborel, 1990a, 1991a; Porcheddu & Milella, 1991; Doneddu & Manunza, 1992; Guerra-García et al., 2004). In fact, the current distribution area is very fragmented, and Espinosa et al. (2014) and Luque et al. (2018) provided an exhaustive review of its recent overall Mediterranean distribution (Fig. 1).

The conspicuous populations are located along the North African coast: Chafarinas Islands (Guallart et al., 2012a; Guallart & Templado, 2016), Melilla (Guallart et al., 2013c), Ceuta (Rivera-Ingraham et al., 2011), Habibas Islands (Espinosa, 2009; Boumaza & Semroud, 2001), Rachgoun Island (Taibi et al., 2013), Plane Island (Algeria, Kallouche et al. 2020), and Zembra Island (Espinosa & Bazaïri, 2009; Zarrouk et al., 2016).

There are relict populations or sparse specimens recorded along the coasts of Morocco, Cala Iris Islet and Al Hoceima National Park (Bazairi et al., 2004; Guallart et al., 2012b); in Spain: along Alboran Island (Paracuellos et al., 2003; CMA-JA, 2014), Peñón de Vélez de la Gomera (Orozco et al., 2013), along the south of Spain

(Espinosa et al., 2005; Moreno & Arroyo, 2008; Guallart & Templado, 2012; CMA-JA, 2014); in France: Porquerolles, Port-Cros and Levant Island (Laborel-Deguen & Laborel, 1991c; Astruch et al., 2012) and in Corsica, within and surrounding the Marine Park of Scandola (Galéria region) at Cap Corse, and in the Strait of Bonifacio (Laborel-Deguen & Laborel, 1991a; Laborel-Deguen *et al*., 1993; Blacher *et al*., 1998; Pascal, 2002; Vela *et al*., 2007).

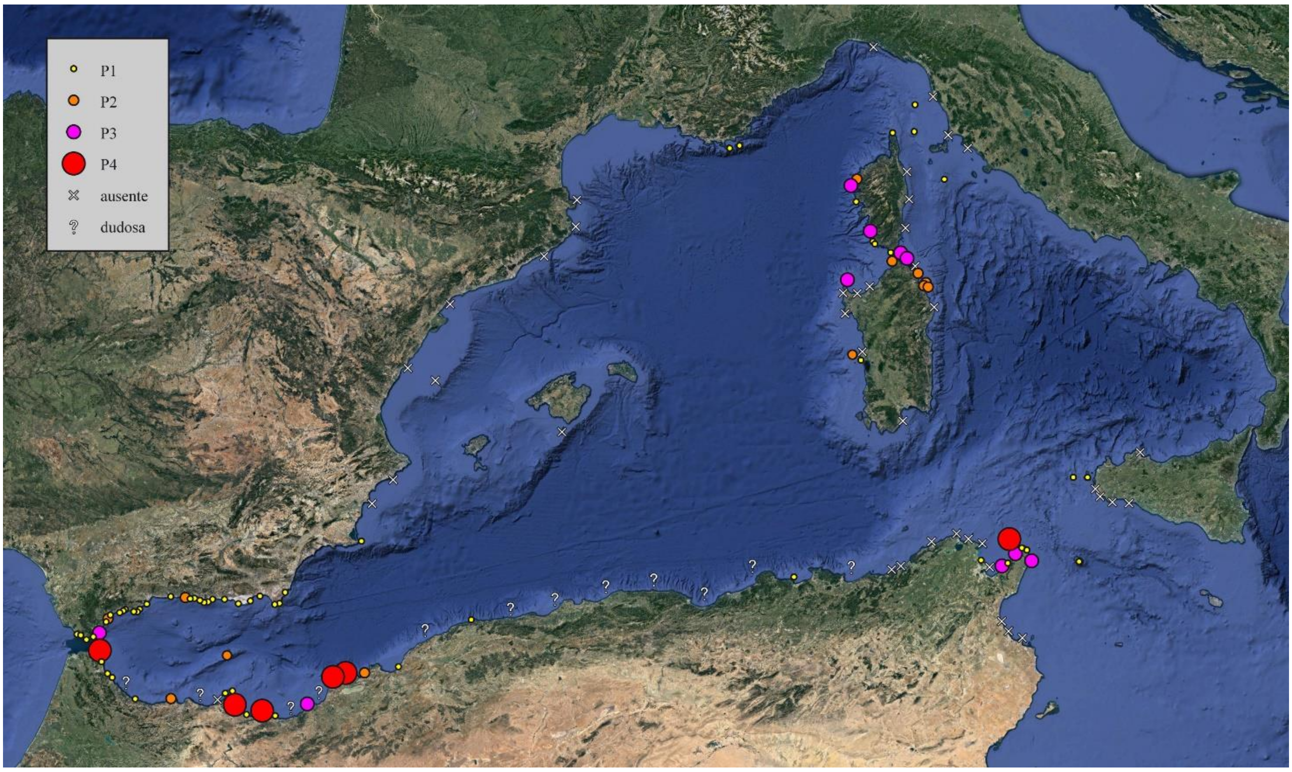

Fig. 1: Mediterranean distribution of *Patella ferruginea* (from Luque et al., 2018).

Also along the Italian coasts the distribution of *Patella ferruginea* is fragmented (Fig. 2). Most of the localities where it is found correspond to isolated specimens or groups in order of tens of limpets, which therefore represent populations that cannot self-sustain. The most healthy populations are in the Northern Sardinia, Asinara and Tavolara MPAs (Casu et al., 2004; 2006; 2010; 2011; Cossu & De Luca, 2014; Cossu et al., 2017), the Maddalena Archipelago (Cristo, 2005; Cossu *et al*., 2006), the Ceraso Cape (Cristo & Caronni, 2008), the Gulf of Olbia (Cristo *et al*., 2007) and smaller populations are found in the MPA of "Penisola del Sinis - Isola di Mal di Ventre" (Casu et al., 2006; Coppa *et al*., 2011; 2012; 2015). *P. ferruginea* has been reported also in Sicily: in the Egadi Archipelago, in Pantelleria islands, the Sicily Channel, and in the Island of Lampedusa (Scotti & Chemello, 2000). Finally, in the Tuscan Archipelago, where *P. ferruginea* is scantly distributed and records are episodic, is present in the Montecristo, Gorgona and Capraia Islands (Appelius, 1869; Terreni, 1981; Porcheddu & Milella, 1991) and in Piombino (Biagi & Poli, 1986). Recently, to this distribution, the reporting of some specimens has been added along the Ligurian coast (Ferranti et al., 2019), where the species was reported as missing by Espinosa et al. (2014).

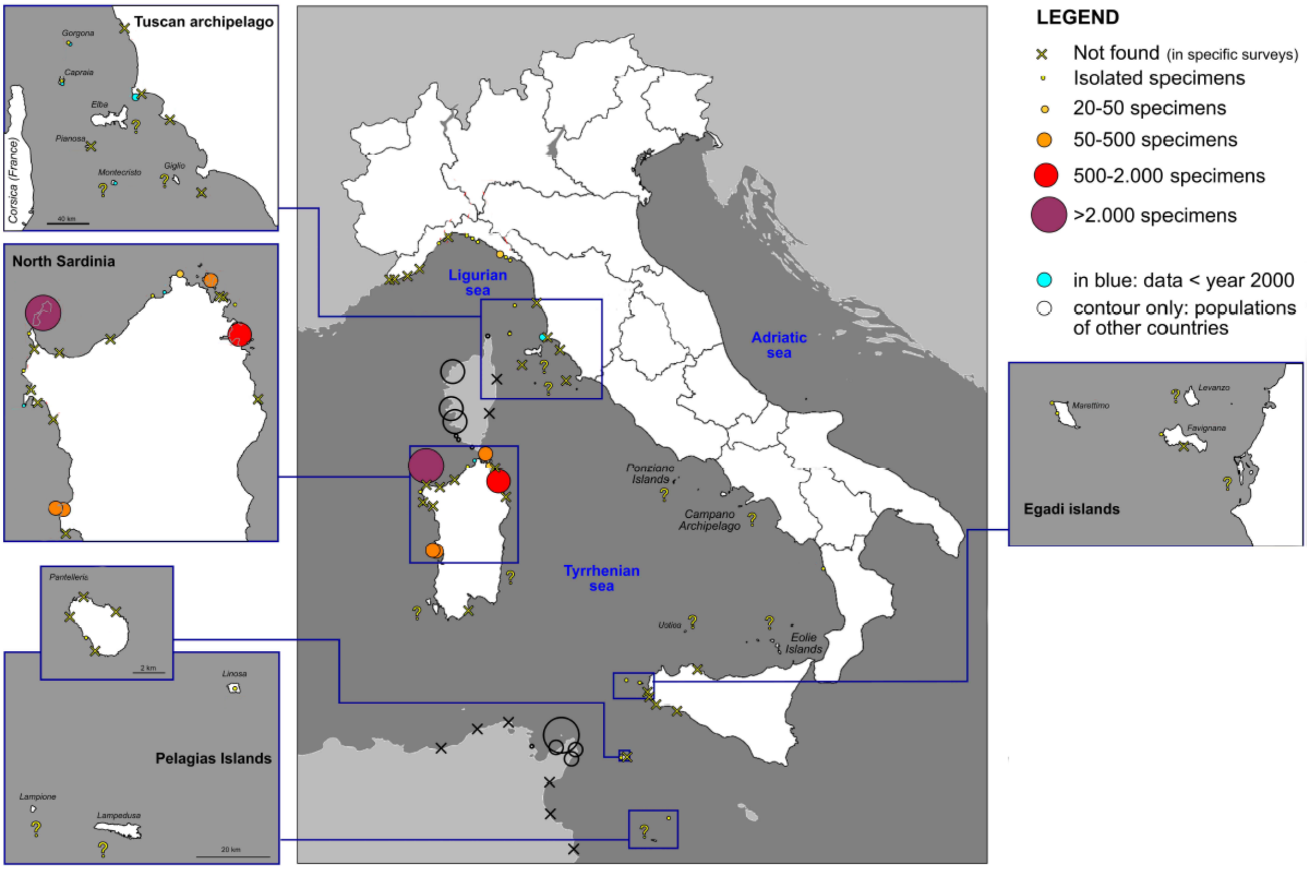

Fig. 2: Present distribution of *P. ferruginea* along Italian coasts.

2.4 References

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3. THESIS OUTLINE

The PhD thesis was carried out in the framework of the ReLife project, Re-establishment of the Ribbed Limpet (*Patella ferruginea*) in Ligurian MPAs by Restocking and Controlled Reproduction (LIFE15 NAT/IT/000771), co-funded by the EU LIFE programme. The project partners are Portofino, Bergeggi and Cinque Terre AMPs (receiving sites), Tavolara Punta-Coda Cavallo AMP (donor site), University of Genoa, Aquarium of Genoa, Algowatt S.p.A.

The project aims at reintroducing *P. ferruginea* within the Portofino MPA and in the other Ligurian MPAs (Bergeggi and Cinque Terre). Two main actions are planned: (i) the re-introduction of *P. ferruginea*, by transferring adult specimens from the Tavolara MPA (Northern Sardinia), where a rich population in good health is still present; (ii) the production of new specimens in hatchery, adopting specifically culturing protocols, to extend the repopulation process and replicate it in other MPAs. Moreover, the original population in Tavolara MPA will be restored by transferring reared specimens and an optimized rearing process will be setup to replicate active reintroduction and restocking in other MPAs.

Before testing the spawning induction techniques on *Patella ferruginea* specimens, we carried out some trials on individuals of *P. caerulea*, a common and very abundant species along the Italian coasts. The spawning induction treatments tested were all non-lethal methods for the specimens, and produced good results, allowing us to draw up an artificial reproduction protocol to be applied to other limpet species as well, in particular protected species, such as *P. ferruginea* (Ferranti et al., 2018; see Chapter 1).

Furthermore, to better understand the reproductive cycle and more particularly the timing of *P. ferruginea* natural spawning at our latitudes, we placed specimens at sea for maturation in natural conditions. Specimens were subjected to a biopsy of the gonad, a method tested by Guallart et al. (2013a) to determine sex. Subsequently, at the beginning of the expected reproductive period, the specimens were subjected to a second biopsy of the gonad to understand the stage of maturation. From the observation of the gametes obtained through biopsy, we were able to determine that the specimens had reached gonadal maturation and, moreover, that one of them had performed sex change, an event known in the literature, but observed for the first time at the North-western Mediterranean latitude (Ferranti et al., 2021; see Chapter 2). Such results were extremely encouraging, providing evidence that the species is reproctively viable in the area where the re-establishment of the species is foreseen by the project.

Always with a view to better understand the timing of gonad maturation, in order to apply stimulation techniques to spawning only at the right time, we have also developed an alternative technique to biopsy, the Magnetic Resonance Imaging (MRI). MRI allowed to observe the thickness of the gonad and thus understand whether a specimen had released or not gametes, through a totally non-invasive approach (Guallart et al., 2020; see Chapter 3).

An unexpected but extremely positive finding during the PhD project implementation was the recording of a few dozen of specimens of *P. ferruginea* along the Ligurian coast. Herein the species was considered extinct, although present in the past: this had actually lead to the conceptualization of the ReLife Project. Such records are extremely relevant, not only to update the known distribution of the species (Ferranti et al., 2019; see Chapter 4), but to support the present suitability of the area for the species and the potential of the project to re-stock the species in the area. Ferranti et al. (2019) reported 32 specimens, but over the following years, monitoring along the Ligurian coast continued and additional 33 specimens were recorded, for a total of 65 specimens of *P. ferruginea* to date known along the Ligurian coast.

Finally, we were able for the first time to draw up a non-lethal spawning induction protocol for *P. ferruginea*. This result was achieved thanks to the combination of different approaches: maturation in the natural environment, stimulation techniques already tested on *P. caerulea* added with new triggers specifically implemented for *P. ferruginea*, a species that mostly lives in the low supra-littoral habitat. All this has also allowed to increase basic knowledge on reproduction and larval stage duration of *P. ferruginea*, and, most relevant for the project, to produce juveniles to be introduced into the natural environment for repopulation (Ferranti et al., in prep.; see Chapter 5).

4. PUBLISHED PAPERS

4.1 CHAPTER 1

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

Maria Paola Ferranti[1*], Davide Monteggia[1], Valentina Asnaghi[1], Mariachiara Chiantore[1]

[1]Department of Earth, Environment, Life Sciences, DISTAV, University of Genoa, Genoa, Italy

*Corresponding author: Maria Paola Ferranti, mariapaola.ferranti@edu.unige.it

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ABSTRACT

Controlled reproduction is a requirement for developing effective mollusc cultivation for commercial or restoration purposes. In this study, a protocol for spawning induction using noninvasive methods in limpets was developed, using the common Mediterranean species, *Patella caerulea* Linnaeus, 1758. Six nonlethal spawning induction treatments were tested: three chemical (two concentrations of H2O2 and KCl) and three physical (bubbling, warm and cold thermal shock). All treatments, except thermal shocks, induced the spawning of fertile gametes. Bubbling resulted the best treatment in providing spawning response, being the easiest and least invasive method tested. After eggs fertilization, larval development was followed until metamorphosis, testing fed and unfed conditions. Settlement took place after 7 days. The developed protocol represents a benchmark for further application to other limpets, for aquaculture or repopulation.

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

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4.2 CHAPTER 2

Are there life-history constraints on restoration of the endangered limpet *Patella ferruginea* (Mollusca, Gastropoda) in the Northern Mediterranean Sea?

Maria Paola Ferranti[1*‡], Javier Guallart[2‡], Virginia Cortella[1], Giacomo Terenziani[1], Mariachiara Chiantore[1]

[1] DiSTAV, Department for Earth, Environment and Life Sciences, University of Genoa, Italy
[2] Marine Biology Laboratory, Department of Zoology, University of Valencia, Spain

‡ Equal contribution

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ABSTRACT

1. Effectiveness of restoration/repopulation measures may be strongly hindered by the ability of the target species to cope with the environmental conditions of the receiving area.
2. Although a species has been reported previously for a given area, the environmental conditions and human pressures may have changed, potentially making any restoration attempt ineffective. Evidence is provided showing that the endangered ferruginous limpet *Patella ferruginea* can complete its reproductive cycle in the northernmost sector of the western Mediterranean.
3. This supports the idea that the reintroduction and the strict protection of *P. ferruginea* in the extreme north of its historical geographic range could be effective for the recovery of the species in this area and ultimately the establishment of reproductively viable and stable populations.
4. Verification of gonadal maturation of specimens experimentally established on the Gulf of Genoa (Ligurian Sea) coastline, as well as evidence of sex change in one individual (the first outside the Alboran Sea), provides further support of the potential for individuals to complete their reproductive cycle in this area.
5. The results support the potential of actions for the restoration of viable populations of the species along the Ligurian coasts through the introduction of juveniles obtained exclusively by aquaculture techniques, in order to mitigate any impact on donor sites.

KEYWORDS: benthos, coastal, endangered species, ferruginous limpet, hermaphroditism, intertidal, invertebrate, reproduction, restoration, sex change.

4.3 CHAPTER 3

In vivo magnetic resonance imaging to assess the sexual maturity of the endangered limpet *Patella ferruginea*

Javier Guallart[1,‡], Maria Paola Ferranti[2,‡,*], Lorenzo Bacigalupo[3] and Mariachiara Chiantore[2]

[1] Marine Biology Laboratory, Department of Zoology, University of Valencia, 46100 Burjassot, Valencia, Spain;
[2] DiSTAV, Department for Earth, Environment and Life Sciences, University of Genoa, Corso Europa 26, 16132 Genoa, Italy
[3] S.C. Radiodiagnostica, E.O. Ospedali Galliera, Mura delle Cappuccine, 14, 16128 Genoa, Italy

*Corresponding author: Maria Paola Ferranti, mariapaola.ferranti@edu.unige.it

‡ Equal contribution

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Knowledge on the reproductive biology of a species is vital for understanding its life cycle. This is particularly important in the case of threatened species because it may affect decision making for their management or support juveniles rearing in captivity and reintroduction strategies. Studies of the reproductive cycle of marine limpets, key species in rocky intertidal ecosystems, are numerous. Patellid limpets are not sexually dimorphic and their reproductive stage cannot be evaluated externally. Consequently, most published studies have been based on collecting and dissecting a significant number of specimens, sometimes in the order of thousands of individuals (e.g. Orton, 1928; Frenkiel, 1975; Sousa et al., 2017).

4.4 CHAPTER 4

Distribution of the Mediterranean ribbed limpet *Patella ferruginea* Gmelin, 1791 along the Ligurian coast and implications for conservation actions

Maria Paola Ferranti[1*], Davide Monteggia[1], Valentina Asnaghi[1], Alessandro Dagnino[2], Federico Gaino[2], Paolo Moretto[2], Veronica Parodi[2], Luca Tixi[3], Valentina Cappanera[4], Claudio Valerani[5], Simone Bava[6] and Mariachiara Chiantore[1]

[1] DiSTAV, Department for Earth, Environment and Life Sciences, University of Genoa, Corso Europa 26, 16132 Genoa, Italy
[2] ARPAL, Regional Agency for the Environmental Protection Liguria, Via Bombrini 8, 16149 Genoa, Italy
[3] Outdoor Portofino, Via Duca degli Abruzzi 62, 16034 Portofino (Ge), Italy
[4] Portofino Marine Protected Area, Viale Rainusso 1, 16038 S. Margherita Ligure (Ge), Italy
[5] Cinque Terre Marine Protected Area, Via Discovolo snc, 19017 Riomaggiore (Sp), Italy
[6] Bergeggi Marine Protected Area, Via A. De Mari 28d, 17028 Bergeggi (Sv), Italy

*Corresponding author: Maria Paola Ferranti, mariapaola.ferranti@edu.unige.it

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ABSTRACT

Patella ferruginea is a limpet endemic to the Western Mediterranean Sea. It is presently considered the most threatened marine macroinvertebrate in the region and has been included in several international conservation directives. Its populations were widespread throughout the Western Mediterranean in the late Pleistocene period, and remained broadly distributed until the 19th century. Presently this species is confined into small populations in a few restricted areas due to human harvesting for food and baits, construction of coastal infrastructures and the effects of seawater pollution. In particular, the species is reported as presently disappeared from the whole of the Italian continental coast and measures are in progress to reintroduce the species through translocation and reproduction in controlled conditions along the Ligurian coasts of the Northwestern Mediterranean.

Recent surveys implemented in the framework of the present work along the Ligurian coasts, to assess the most suitable sites for reintroduction, resulted in the discovery of 32 specimens of this endemic limpet, which previously was thought to have vanished from the area. These findings shed new light on the ability of species to naturally disperse, the relevance of the measures set in place to restore presently rarefied populations and may provide information to aid in the selection and management of sites within the Natura 2000 Ecological network.

KEYWORDS: *Patella ferruginea*, distribution, Ligurian Sea, conservation

4.5 CHAPTER 5

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

Running head: *Patella ferruginea* controlled reproduction

Maria Paola Ferranti[1*], Javier Guallart[2*], Giorgio Fanciulli[3], Pier Augusto Panzalis[4], Mariachiara Chiantore[1]

[1] DiSTAV, Department for Earth, Environment and Life Sciences, University of Genoa, Italy
[2] Marine Biology Laboratory, Department of Zoology, University of Valencia, Spain
[3] Portofino Marine Protected Area – Viale Rainusso 1 –16038 S. Margherita Ligure (Ge), Italy
[4] Tavolara Punta Coda Cavallo Marine Protected Area - Via Dante 1, 07026 Olbia, Italy

[*]*Both authors have contributed equally to the preparation of the paper*

Corresponding author: Maria Paola Ferranti, mariapaola.ferranti@edu.unige.it

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ABSTRACT

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

Keywords: artificial fertilization, ferruginous limpet, Mollusca, Mediterranean Sea, Patellogastropoda, reproduction.

5. DISCUSSION AND CONCLUSIONS

The results obtained from the studies carried out during the PhD allowed a further understanding of the biology and ecology of *Patella* genus and more particularly of*Patella ferruginea*.

Being *P. ferruginea* a protected species, we initially preferred to test the induction treatments on *P. caerulea*, common and abundant species along the Italian coasts. The trials performed on *P. caerulea* and reported in Ferranti et al. (2018), reprensented the first successful spawning induction in the *Patella* genus using non-invasive techniques. Six treatments were tested: 3 physical (Vigorous aeration ("bubbling"), Warm and Cold thermal shock) and 3 chemicals (Oxygen peroxide 6%, Oxygen peroxide 10% and Potassium chloride 0.2%). The vigorous bubbling treatment resulted the best, both in terms of response speed to stimulation by the specimens, and because it is an inexpensive and easily repeatable method. Moreover, compared to what is reported in the literature, during artificial fertilization process, using a sperm concentration 10^6 cell/ml for 30 minute exposure, we did not experience polyspermy, nor found any need to increase fertilization medium pH (e.g. by way of Tris or NH_4OH), as reported by other authors (Dodd, 1957; Espinosa et al., 2010; Guallart et al., 2020). This happens if spawning is induced when the gametes are actually ready for spontaneous emission. These non-lethal treatments can be applied and/or adapted to other species of limpets, as for example *P. ferruginea.*

In the case of *P. ferruginea*, the bubbling was found to be one of the induction treatments to spawning useful for this species, although only in combination with other stimuli (Ferranti et al., in prep). In fact, the stimuli for spawning induction in *P. ferruginea* can be assumed to be different from those of other species: since *P. ferruginea* thrives in the upper mesolittoral (Laborel-Deguen & Laborel, 1990b; Templado, 2001; Templado et al., 2004), subaerial factors (e.g. winter cooling and strong waves, Frenkiel, 1975; Laborel-Deguen & Laborel, 1990b; 1991a) are possibly stronger drivers compared to species living in the infralittoral habitat (Baker et al., 2001; Ferranti et al., 2018; Mau & Jha, 2018b). For this reason, we have implemented bubbling wich other stimulation techniques, some already used on other limpet species, as Thermal Shock (Kay & Emlet, 2002; Ferranti et al., 2018) and others totally new, developed in order to catch the subaerial habitat triggers: Dry and Cold maintenance.

Furthermore, the maturation of the specimens directly at sea, in natural conditions, resulted very important, before subjecting them to various spawning induction treatments. To evaluate the state of gonad maturation of *P. ferruginea* specimens, we used the biopsy, following the methods of Wright & Lindberg (1979) and Guallart et al. (2013a). The biopsy was carried out both before and after the maturation period in the sea, and through this method we could observe some gamete samples to valuate the maturity state. In this way, it was possible to determine the achievement of maturation and the sex change of specimens along the Ligurian coast (Ferranti et al., 2021). Although the sex change has already been described in *P. ferruginea* (Espinosa et al., 2009; Guallart et al., 2013a), these studies were carried out only in the southernmost areas of its distribution range, in the Alboran Sea (Ceuta and Chafarinas Islands respectively). Consequently, the present observations imply that the species can complete its reproductive cycle along the Ligurian coasts, at its northernmost distribution limit, where the species, previously considered extinct, was recently recorded (Ferranti et al., 2019). These findings provide support for the idea that the reintroduction of *P. ferruginea* along the Ligurian coasts has the potential to be effective for the recovery of the species in this area. As expected by with one of the objectives of the ReLife project, these reintroductions should only be made from specimens obtained through aquaculture techniques, without substantially affecting donor populations.

Always in order to evaluate the moment of maximum gonad development for spawning stimulation, we have also developed an innovative technique, Magnetic Resonance Imaging on limpets (Guallart et al., 2020b).

This technique has resulted in an additional tool to assess gonad size, to minimize the number of induction trials for each specimen, and consequently the stress. The method cannot be applied to estimate gonad maturity, which can still be assessed only by observing gamete samples collected by gonad biopsy. However, this approach can be applied to a limited number of specimens used for the artificial reproduction, and it is easily replicable although has logistic limitations due to necessity to have the equeipment for magnetic resonancewhich it is not possible in many field study conditions.

Consequently, the combination of maturation at sea just before the reproductive period with the various treatments tested for spawning induction on *P. ferruginea* specimens, allowed us to draw up, for the first time, a preliminary spawning induction protocol (Ferranti et al., in prep). This protocol has allowed for the controlled stimulation release of gametes, without sacrificing the broodstock of a protected species, demonstrating also the feasibility of obtaining juveniles, under controlled laboratory conditions through low-invasive methods. The application of this protocol has allowed us not only to obtain the gametes, but also to proceed with artificial fecundations, which showed higher fertilization rates, between 97.40% and 98.90%, than those obtained from dissections of the gonad (almost 3%, Espinosa et al., 2010; range: 20.6-40 %, Guallart et al., 2020a). This allowed us to obtain a good larval development, with subsequent larval settlement and metamorphosis and growth of *P. ferruginea* juveniles.

The results obtained in Ferranti et al. (in prep), provided an even more detailed information on the larval cycle of this species compared to previously provided information in Guallart et al. (2020a), thanks to a broader graphic documentation. Larval development and timing from our study fundamentally fit with the results from Guallart et al. (2020a) on *P. ferruginea* (gamete obtained from dissection) and general larval development phases are very similar to those described for other *Patella* species (e.g. Patten, 1886; Smith, 1935, Dodd, 1957; Wanninger et al., 1999; Ferranti et al., 2018).

In particular, it was possible to provide evidence that the time interval in which *P. ferruginea* larvae carry out settlement and metamorphosis is longer than previously known. In fact, the first post-larvae completing metamorphosis were observed 7 dpf, but last pediveligers with swimming capacity were detected up to 32 dpf. Moreover, we have observed a large variability in growth rate from post-larvae, probably due the variability at the time of metamorphosis, which suggests that it can delay up to 40 dpf. The interval, 25 days (or even 32) apart, when individual larvae decide to settle and undergo metamorphosis may explain the variability in the size of the post-larvae. Such long larval phase considerably broadens the survival range and dispersal capacity of *P. ferruginea* larvae, conversely to that reported by some authors Laborel-Deguen & Laborel (1990b; 1991b) and assumed by later authors (e.g. Templado, 2001; among others). On the basis of these observations, of the current regime of the Ligurian Sea (Pinardi et al., 2015) and of the distribution of populations of *P. ferruginea* in the NW Mediterranean (Luque et al., 2018; Ferranti et al., 2019) we can hypothesize that the adult specimens recorded along the Ligurian coast represent a stochastic recruitment of larvae from populations located at least several hundred kilometers away (coasts of NW or S Corsica or NE Sardinia). Moreover, this finding could be in agreement with the genetic uniformity observed in the distribution of this species by Casu et al., 2011.

Although, the specimens recently recorded along the ligurian coasts show an extremely scarce and fragmented distribution and unfortunately do not constitute a self-sustainable population, their presence was useful both for expanding the distribution of *P. ferruginea* in the Mediterranean Sea, and to better understand, for the purposes of the ReLife project, in which areas to reintroduce juveniles produced under controlled conditions.

In the future, it will be necessary to be able to use a larger number of broodstock to avoid excessive reduction of the genetic variability of the populations in which restocking will take place. Currently, as there is a small population along the Ligurian coast, the reintroduction of these juveniles should not decrease the genetic variability along these coasts. Moreover, if the introduced specimens will manage to reach reproductive size, together with those already present naturally they could become a source for a local spread of the larvae.

In conclusion, on the basis of all the results obtained, we can affirm the repopulation of *P. ferruginea* through controlled reproduction and reintroduction in the natural environment is feasible where populations of *P. ferruginea* are disappeared and/or in regression in order to enhance the recovery of the species at a global level.