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PERSPECTIVE NOTE OPEN ACCESS

Pilot studies for stock enhancement of purple sea urchins (*Paracentrotus lividus,* Lamarck, 1816): usefulness of refuges and calcein marking for the monitoring of juveniles released into the natural environment

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Abstract – In the framework of stock enhancement, field experiments were conducted between March and July 2021 in rocky shores of Central Portugal to test the usefulness of refuges and calcein marking for juvenile sea urchins releases. Individuals with 10–20 mm in test diameter were captured in nature and tagged through immersion in a calcein bath with a concentration of 150 mg L^{-1}, during 48 hr. Artificial shelters were used to provide refuge and an acclimatization structure for the released sea urchins, and *in situ* monitoring was carried out by counting the marked specimens over three months. Results point out to the importance of using shelters to provide protection to sea urchins, and validated the efficiency of the calcein tagging protocol for *in situ* monitoring. Sea urchins' test diameter growth during the experiment was estimated to be 0.470 mm $month^{-1}$ (SD = 0.181).

Keywords: reseeding / sea urchin release / tagging / shelters / Portugal west coast

1 Introduction

The demand for the high prized gonads of sea urchins has produced over the last decades an alarming decline in the wild populations of *Paracentrotus lividus* due to intense harvesting (Boudouresque and Verlaque, 2020). Stock enhancement programs are considered a potential management tool to increase the abundance of sea urchin populations, and have been widely used for the improvement of fisheries, particularly in Asian countries (Lorenzen et al., 2012; Agatsuma, 2020). In recent years, there has been an increased interest in the exploitation of *P. lividus* in Portugal, and in the north of the country intense harvesting reduced density and biomass of the species and changed the length structure of the populations (Bertocci et al., 2014, 2018). The important role that sea urchins play in ecological and economic terms (Boudouresque and Verlaque, 2020) might be endangered by overfishing, being the aquaculture production and the stock enhancement a potential solution that meets both the demand of the market and the species conservation. Therefore, it is important to increase the knowledge on the efficiency of stock enhancement to counterbalance scenarios of threatened populations, either in a conservation or a fisheries perspective. Particularly relevant is the development of methodologies for sea urchins reseeding and/or translocations procedures, to optimize the success of the operations.

Several studies on the effects of sea urchin reseeding (grazing pressure, predation survival, growing rates, genetic impact on wild populations) have been conducted (Tegner, 1989; Juinio-Meñez et al., 2008; Couvray et al., 2015; de la Uz et al., 2018). However, the effectiveness of releasing sea urchins into the natural environment has been rarely evaluated

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from a fisheries management perspective and the economic benefit of reseeding remains uncertain (Agatsuma, 2020). It is fundamental to estimate the survival and growth rates of released sea urchins, in order to assess the cost/benefit of stock enhancement for sustaining the fisheries. One of the main causes for the mortality of released juveniles into the natural environment is predation (de la Uz et al., 2018), and a key aspect for the success of sea urchin reseeding is to reduce their vulnerability to predators (e.g. octopus and starfish). Therefore, the main goal of this pilot study was to test release strategies of juvenile sea urchins, as well as validate the use of calcein marking for monitoring reseeding operations. The sea urchins' growth rates were also analysed, to assess the potential influence of the manipulation in their metabolism and feeding behaviour.

2 Methods

P. lividus specimens (10–25 mm on horizontal test diameter without the spines) were manually collected during the low tide from intertidal habitats around the region of Peniche (central Portugal; 39°19′08.5N, 9°21′24.1W). Collection sections occurred in March 2021 in less than one week, in locations where sea urchins' densities were higher than 50 individuals m^{-2} and achieve 300 individuals m^{-2} (average densities of 160 ± 75 individuals.m^{-2}). Sea urchins were handpicked from intertidal pools using tweezers or other tools (e.g., a fork or a small knife) to detach sea urchins from the substrate, their burrows, or shelters, and only specimens that could be easily removed without damaging their spines were targeted. Low density batches of sea urchins were transported in boxes, for maximum 15 min, to the nearby MARE – Marine and Environmental Sciences Centre (Polytechnic of Leiria) facilities. They were kept for 15 days in fiberglass tanks in a recirculating aquaculture system (RAS) and fed every two days *ad libitum* with dry *Ulva rigida* and *Porphyra dioica*,12:12 h light:dark photoperiod, and 18 °C temperature. During the 15 days of acclimation, in the marine facilities, ≈ 70% of the sea urchins survived and were used for field experiments.

To distinguish the released animals from the wild ones and monitor the success of the operation, after the 15 days of acclimation sea urchins were marked by exposure to calcein, a method already tested to mark and detect large numbers of small sea urchins without sacrificing the animals (Johnson et al., 2013; Santos et al., 2022). This method offers high effectiveness for the development of field studies, since calcein tagging shows high success marking rate (Santos et al., 2022) and the produced fluorescent mark can be viewed under blue light portable flashlights (e.g., GoBe NIGHTSEA Fluorescence Exploration Light; 440–460 nm range) with appropriate filter glasses (Lü et al., 2020). To ensure that the fluorescent mark was recognisable either on sea urchins oral and aboral surface, and at any field conditions (sunny and cloudy days), animals were submersed in a well-aerated calcein sea water solution of 150 mg L^{-1} for a period of 48 hr, during which they were not fed.

The marked juveniles were transported to the release sites in a seawater tank with an air pump. Two intertidal sites (Cavalinho and São Sebastião rocky shores at Ericeira, Portugal; 38°59'47.20N, 9°25'33.45W and 38°58'12.05N, 9°25'17.20W, respectively) were chosen for the release, both characterized by abundant macroalgae and with high sea urchin densities (19 and 46 individuals.m^{-2}, respectively), to secure good environmental conditions (food and refuge) for the released specimens.

Preliminary experiments were performed using 60 sea urchins marked with calcein and released in two rocky pools at Cavalinho site. Monitoring was conducted in the following days using the blue light portable flashlights and after 7 days, no marked sea urchins were observed anymore in both pools. Following the disappearance of all the juveniles implanted during the preliminary study, it was decided to use only artificial shelters for this experiment.

Artificial shelters were constructed using red clay bricks (20 cm × 20 cm × 10 cm) with four big holes, protected on the top and on the bottom with plastic nets, and used as acclimatization and protection structures. The artificial shelters were fixed to the seafloor of Cavalinho and São Sebastião rocky shores (14 in each site), distributed in an area of approximately 200 m^2. To fix each artificial shelter, two holes were drilled in the seafloor to install the eye bolts that anchored the structure, by means of cable ties. The distance between the shelters was subject to the pool area available, being at least 1 m separated whenever possible. In each site, a total of about 800 juvenile sea urchins (807 and 847 respectively in Cavalinho and São Sebastião) averaging 16 ± 1.4 mm in test diameter ($n = 1654$) were equally distributed in each artificial shelter (average number of 58 and 60 individuals per shelter, respectively in Cavalinho and São Sebastião). The plastic nets were closed with cable ties ensuring that individuals were confined to the enclosures during the first 10 days. Afterwards, the plastic nets were removed, and shelters were monitored during low tide, using the blue light portable flashlight and filter glasses. The release experiment was carried out over three months, between April and July 2021. Monitoring of the marked sea urchins started ten days after the release of animals, and two days after opening the shelters, with the following periodicity: in the first week every 2 days, then after one week, nearly fortnightly in the following month, and finally with an interval of one month. To assess the releasing strategy, the total number of individuals observed with the fluorescent mark in every shelter and in the immediate vicinity (up to 2 m of distance from structures) was registered over the studied period, and dead sea urchins (tests) counted and removed from the structures. The sea urchin presence in the study area (presence rate) was computed as the percentage of sea urchins marked with calcein and alive at each monitoring date. The global presence rate is expressed as a fraction of the total released sea urchins, and the shelter presence rate is expressed as a fraction of the sea urchins present in each shelter. After three months the sea urchins remaining in the shelters were transported to the laboratory to estimate the respective growth rates.

In the laboratory, the sea urchins recovered with calcein mark were measured (test diameter without the spines – *TD*, in mm) and dissected to remove the jaw structures, which were examined with a stereomicroscope under ultraviolet illumination and equipped with a filter. Growth data were obtained from a total of 18 individuals (11 and 7 from SS and CAV, respectively) averaging 17.0 ± 2.7 mm in test diameter and that exhibited a clear line of visible fluorescence in the jaws

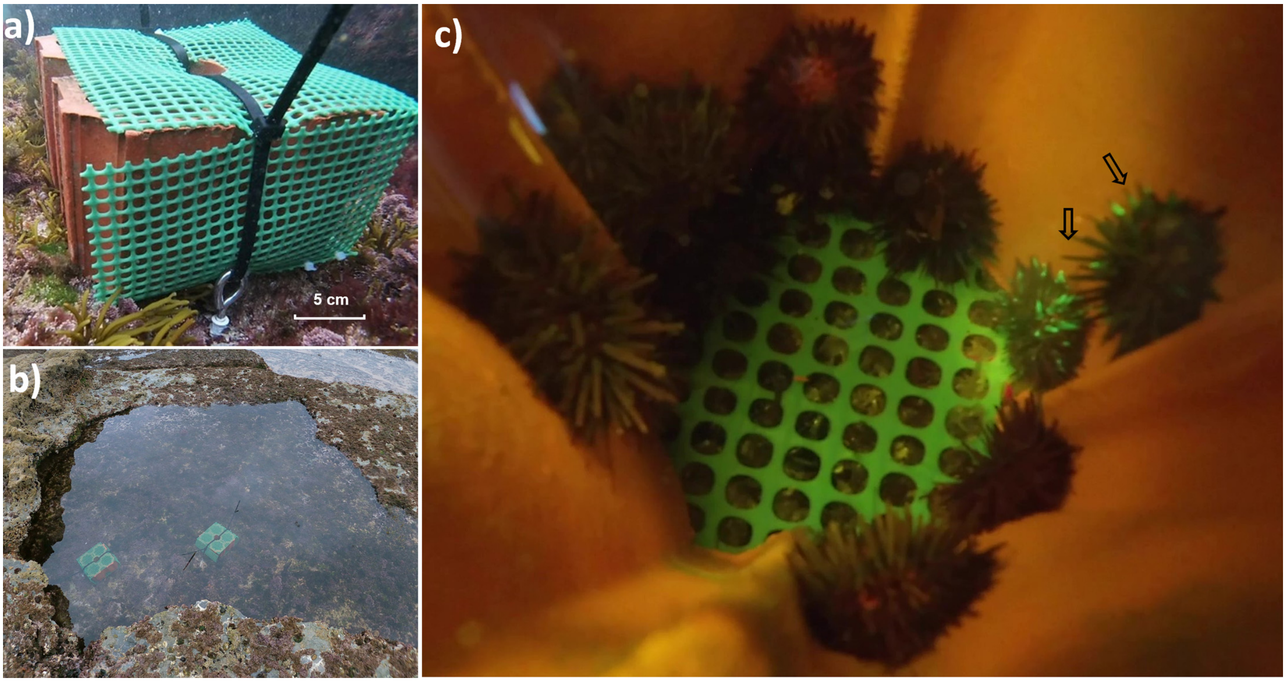

Fig. 1. Artificial shelters used as acclimatization and protection structures for the release of sea urchins *Paracentrotus lividus*. (a) detail of the fixation mechanism; (b) overview of the structures in a tide pool; (c) sea urchins inside structures, fluorescent marks indicated by the black arrows.

allowing measurement of the increment from the time of calcein marking to the time of recapture after three months. Digitalized images were captured using a video camera (Leica DFC280, Germany) coupled to the stereomicroscope, and a Leica LAS (4.1.0, Germany) system was used to measure the total jaw length and the jaw length at the fluorescent line. The increment of the jaw length was computed as the difference between the jaw length at the fluorescent line and the total jaw length.

To estimate the test diameter from the jaw length, the allometric relationship between the jaw length (*J*, mm) and test diameter without the spines (mm) (Ebert, 2020) was established from the marked sea urchins sampled and recorded in the laboratory:

$$\mathrm{TD} = aJ^b,$$

where a and b are regression coefficients. Log-log plots of J and TD length values ($\log \mathrm{TD} = \log a + b^* \log J$) were created for the identification of outliers before the linear regression fitting procedure. To analyse the growth differences between the sea urchins from Cavalinho and São Sebastião, the mean growth was compared using the Student's *t*-test, after testing the data for homoscedasticity with the *F* test ($\alpha = 0.05$) and normality with the Shapiro-Wilk test ($\alpha = 0.05$).

3 Results

The calcein marking was 100% successful, and over the three months of the experiment, all the sea urchins observed in the shelters presented the calcein mark, either at Cavalinho or at São Sebastião rocky shores. The UV portable light and filter glasses were efficient to detect the fluorescent mark in the tagged sea urchins, at variable weather conditions (sunny and cloudy days) (Fig. 1).

In the first two monitoring weeks, broken tests and full tests of dead sea urchin were observed inside the shelters, resulting on 4% and 1% (respectively in Cavalinho and in São Sebastião) confirmed deaths at the beginning of the study. The number of marked sea urchins observed in the area showed a significant decline over the monitoring period. At the end of the three-month study, only 59 and 61 marked (7.0% and 7.6%, respectively) sea urchins were observed and recaptured from the shelters, respectively from São Sebastião and Cavalinho (Fig. 2). The decline in the number of sea urchins includes the individuals lost from the study site due to the severe sea conditions that caused the disappearance of 16 shelters over the three-month period (10 at São Sebastião and 6 at Cavalinho). In the shelters remaining in the study area, it was observed an average reduction of 10% to 20% in the number of marked sea urchins from one survey to the next, during the first monitoring month and a half (every 2 days, weekly and fortnightly). The number of sea urchins drastically declined in the final survey, when 40% of specimens have disappeared from the shelters at São Sebastião, relative to the observations registered one month before, and 70% in Cavalinho. After the three months experience, the percentage of sea urchins observed inside the 12 remaining shelters, relative to the starting number, varied between 5% and 35%, with an average of $17 \pm 8\%$. During the three-month monitoring surveys, only five marked sea urchins were observed outside the shelters, at a distance inferior to 1 m, which represented less than 0.05% of the sea urchins monitored outside the shelters.

The analysis of sea urchins' growth during the experiment (~3 months) was conducted using the sample of the

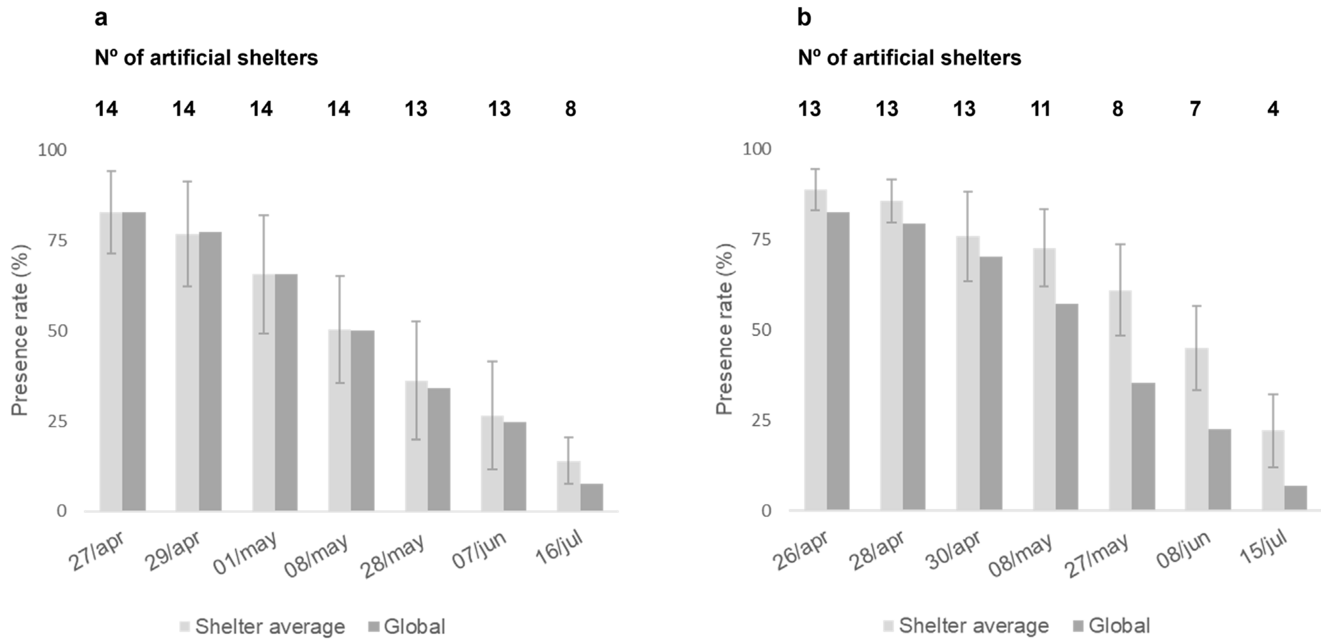

Fig. 2. Monitoring results of the sea urchin release experiment at Cavalinho (a) and São Sebastião (b): mean and standard deviation of shelter presence rate (%) and global presence rate (%). On the top the number of shelters in each monitoring day is presented.

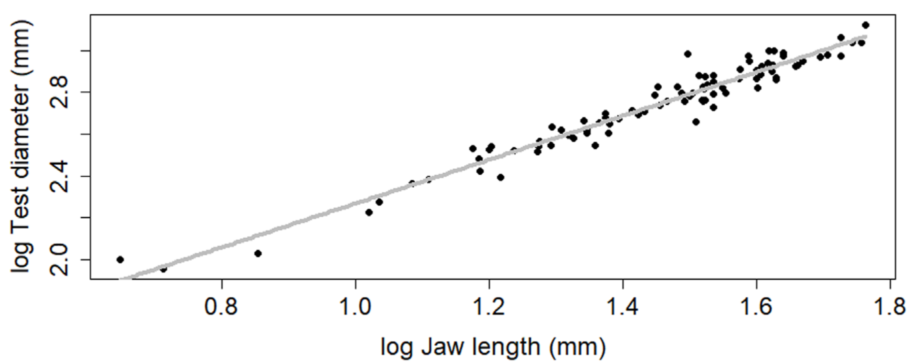

Fig. 3. Relationship between the jaw length and the test diameter (log-log plot) for the recovered *Paracentrotus lividus* marked with calcein ($n = 94$). The grey line represents the linear model ($R^2 = 0.951$, $n = 94$, $p < 0.01$).

individuals recovered inside the shelters, at the end of the experiment. The observation of the jaws with the stereomicroscope confirmed that all recovered sea urchins (94 individuals) were marked with calcein. The following equation describes the relationship between *TD* and *J* (Fig. 3):

$$TD = 3.396 * J^{1.046} \ (R^2 = 0.951,\ n = 94,\ p < 0.01)$$

The mean growth rate was not significantly different between sites ($t = 0.68$, df = 16, p-value = 0.505), and was globally estimated to be 0.470 ± 0.181 mm month^{-1} in test diameter (mean ± SD) during the three-month experience.

4 Discussion

Improving the external visibility of marked sea urchin is essential to monitor and study stock enhancement operations. This pilot experiment showed that the 48 hr bath with highly concentrated calcein (150 mg L^{-1}), resulted in externally visible marks on juvenile *P. lividus*, detectable on the animal spines with UV portable lights and filter glasses for, at least, a period of 3 months. These findings provide a useful methodology to monitor sea urchin releases, without sacrificing the animals or manipulate them in the field. Although dead sea urchins were observed in the interior of the shelters, either due to predation, manipulation stress, disease or natural death, the mortality rates in the first days were very low (1% to 4%) and validate the minimal impact of the marking method, as well as of handling and transportation, on the survival of sea urchins. The growth rates estimated are in line with the growth described for the species in the wild, specifically in the north and northeast Spain (5–9 mm in the first year of life) (Ouréns et al., 2013), although results should be interpreted with caution due to the lack of independence of data (sea urchins growing in the same shelter). Nevertheless, it is reasonable to assume that the effects of handling, marking and transportation had little influence in the feeding behaviour of individuals.

Although predation is one of the greatest obstacles to the survival of released hatchery-reared sea urchin juveniles (de la Uz et al., 2018), to our knowledge there are no studies on the use of protection or acclimatization methods to reduce the animal's vulnerability to predators in releasing experiments. Captive-born sea urchins suffer the negative effects of captivity when introduced in the natural environment, and the use of acclimatization methods can decrease their vulnerability to predation, waves and currents (Brundu et al., 2020). In fact, the disappearance of the sea urchins after one week when released without shelters during preliminary experiment, contrast with the presence of more than 50% of sea urchins in the releasing sites after the same period, when protection was used. Therefore, the shelters used in this study showed that providing protection to the sea urchins can potentially increase their survival, given them more time to acclimate to a novel environment in a safe place.

The percentage of sea urchins present at the releasing sites after the three months is potentially higher than 17% since individuals were observed in the vicinity of the structures. It is possible that some dispersed out of the monitoring area, either naturally, via waves and currents, or forced by the drag of shelters due to strong hydrodynamics. In highly hydrodynamic rocky shores as São Sebastião, improvements on shelters or on their fixation to the seafloor must be performed, to avoid such events. Marked sea urchins may have gone unnoticed outside the structures during the surveys, although monitoring covered an area that potentially incorporates the species home range. The foraging activity of *P. lividus* in a 24-h period has been described to vary between 6 and 220 cm (Hereu, 2005) and seems more dependent on size and food availability than on the presence of predators (Barnes and Crook, 2001). Therefore, since the study sites offered abundant food for the released specimens, and the monitoring covered 2 m around the shelters, it seems reasonable to assume that sea urchins may have come out of shelters during the night, when they are more active (Hereu, 2005), and were either predated or dragged outside the study area during the foraging activity.

This pilot study confirmed the benefit of using protection to provide a period of acclimation when sea urchins are translocated and released in the new environment, and the efficiency of calcein marking for the assessment of reseeding operations. However, it was not possible to assess the impact of the sea urchins release on population density due to the reduced number of individuals used in the experiment, that hampered the recapture of individuals outside the shelters. Therefore, in future experiments, an increase in the number of released individuals must be considered, to assess the efficiency of this solution for stock enhancement. In these operations, a potential risk that must be minimized concerns the acclimatization method used, that can potentially provide refuge and/or preys to young octopus, an abundant species in Portuguese coastal areas (Almeida et al., 2022). Experiments using reared sea urchins should also be developed to evaluate the efficiency of shelters in reducing the negative effects of captivity before the release into the wild. Finally, the ecological viability of reseeding must be monitored and assessed, to anticipate declines in the algal biomass due to sea urchin grazing activity or changes in the interactions with other herbivores.

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