SCIENTIA MARINA 86(3)
September 2022, e038, Barcelona (Spain)
ISSN-L: 0214-8358
https://doi.org/10.3989/scimar.05259.038

Physical and chemical tagging methods for the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea)

Pedro M. Santos [1], Bernardo Ruivo Quintella [2,3], David Jacinto [2], Ana Gomes [1], Carolina Saldanha [1], Sílvia Lourenço [1,4], Patrícia Mega Lopes [5], Maria João Correia [5], David Mateus [2], Teresa Cruz [2,6], Ana Pombo [1,4], José Lino Costa [3,5]

[1] MARE – Marine and Environmental Sciences Centre, Politécnico de Leiria, 2520-630 Peniche, Portugal.
(PS) (Corresponding author) E-mail: pedro.c.santos@ipleiria.pt. ORCID-iD: https://orcid.org/0000-0003-1117-1801
(AG) E-mail: anasofsangomes@gmail.com. ORCID-iD: https://orcid.org/0000-0002-6565-9370
(CS) E-mail: c.a.saldanha97@gmail.com. ORCID-iD: https://orcid.org/0000-0002-3896-0790
(SL) E-mail: silvia.lourenco@ipleiria.pt. ORCID-iD: https://orcid.org/0000-0003-4426-0894
(AP) E-mail: ana.pombo@ipleiria.pt. ORCID-iD: https://orcid.org/0000-0003-1397-9206
[2] MARE – Marine and Environmental Sciences Centre, Laboratório de Ciências do Mar, Universidade de Évora, 7521-903 Sines, Portugal.
(BRQ) E-mail: bsquintella@fc.ul.pt. ORCID-iD: https://orcid.org/0000-0002-0509-4515
(DJ) E-mail: djacinto@uevora.pt. ORCID-iD: https://orcid.org/0000-0001-7475-9579
(DM) E-mail: djrm@uevora.pt. ORCID-iD: https://orcid.org/0000-0003-1583-9733
(TC) E-mail: tcruz@uevora.pt. ORCID-iD: https://orcid.org/0000-0002-8242-475X
[3] Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal.
(JLC) E-mail: jlcosta@fc.ul.pt. ORCID-iD: https://orcid.org/0000-0002-3843-4635
[4] MARE – Marine and Environmental Sciences Centre, ESTM, Politécnico de Leiria, 2520-641 Peniche, Portugal.
[5] MARE – Marine and Environmental Sciences Centre, Faculdade de Ciências, Universidade de Lisboa, 1600-548 Lisboa, Portugal.
(PML) E-mail: patricia.ls.tuca@gmail.com. ORCID-iD: https://orcid.org/0000-0003-4114-007X
(MJC) E-mail: mjcorreia@fc.ul.pt. ORCID-iD: https://orcid.org/0000-0003-3764-1381
[6] Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, 7002-554 Évora, Portugal.

Summary: The sea urchin *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata: Echinoidea) is an important economic resource in Europe, but intense harvesting has led to the collapse of several natural populations. Echinoculture, associated with restocking and stock enhancement practices, is an alternative to this problem. In this procedures, reliable individual identification through tagging is a valuable source of information. However, very few studies address the effect of tagging methods on *P. lividus* and the tagging of marine invertebrates still presents several challenges: decreased growth, high mortality rates and low tag retention rates. Under laboratory conditions, the present study evaluated the effectiveness of three tagging methods (passive integrated transponders [PIT-tags], coded wire tags [CWTs] and calcein) on wild *P. lividus* for 60 days in terms of total wet weight, total weight gain (mg ind.$^{-1}$ day^{-1}), survival and tag retention. The final total wet weight was significantly higher in the untagged (control) group than in the PIT-tagged group. Survival rate was 100% for the PIT-tag, calcein and control groups, and 97% for the CWT group. Tag retention differed significantly according to the tagging method: 100% in the calcein group, 76.7% in the PIT-tag group and 38.0% in the CWT group.

Keywords: echinoderm; calcein; PIT-tag; coded wire tag; restocking; aquaculture; ecological studies.

Métodos físicos y químicos de marcaje del erizo de mar *Paracentrotus lividus* (Echinodermata: Echinoidea)

Resumen: El erizo de mar *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata: Echinoidea) representa un recurso económico relevante en Europa, pero su intensa explotación ha llevado al colapso de varias poblaciones naturales. La acuicultura de erizos de mar, asociada a las prácticas de repoblación y mejora del stock son alternativas a este problema. En estos procedimientos, una identificación individual fiable, a través de métodos de marcaje, representa una valiosa fuente de información. Sin embargo, muy pocos estudios abordan el efecto de los métodos de marcaje en *P. lividus* y el marcaje de invertebrados marinos aún presenta varios desafíos: disminución del crecimiento, altas tasas de mortalidad y bajas tasas de retención de las marcas. En condiciones de laboratorio, el presente estudio evaluó la eficacia de tres métodos de marcaje (*passive integrated transponders* - PIT-tags, *coded wire tags* – CWT y calceína) en *P. lividus* silvestre, durante 60 días, en términos de peso húmedo total, ganancia de peso (mg ind.$^{-1}$ día^{-1}), supervivencia y retención de etiquetas. El peso húmedo total final fue significativamente mayor en el grupo sin marcar (control), en comparación con los individuos marcados con PIT-tags. La tasa de supervivencia fue del 100% para los grupos PIT-tag, Calceína y Control, y del 97% para el grupo CWT. La retención de etiquetas fue significativamente diferente según el método de etiquetado: 100% en el grupo Calceína, 76,7% en el grupo PIT-tag y 38,0% en el grupo CWT.

Palabras clave: equinodermo; calceína; PIT-tag; coded wire tag; repoblación; acuicultura; estudios ecológicos.

Citation/Como citar este artículo: Santos P.M., Quintella B.R., Jacinto D., Gomes A., Saldanha C., Lourenço S., Lopes P.M., Correia M.J., Mateus D., Cruz T., Pombo A., Costa J.L. 2022. Physical and chemical tagging methods for the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea). Sci. Mar. 86(3): e038. https://doi.org/10.3989/scimar.05259.038

Editor: X. Turon.

Received: December 28, 2021. **Accepted:** May 19, 2022. **Published:** August 22, 2022.

INTRODUCTION

In a context of steady decline of marine fishery resources, the aquaculture sector, associated with restocking and stock enhancement practices, offers an important mitigation (Agatsuma 2020, FAO 2020). However, to efficiently evaluate the success of stock enhancement programmes and ecological studies, one must first identify and develop a cost-effective tagging method, because it is essential for reared individuals to be successfully distinguished from the wild population (Bell et al. 2006, Bartley and Bell 2008). These techniques enable the study of growth and survival rates, population abundance, predator-prey interactions, population maturity, movement processes and patterns, habitat use and responses to environmental changes (Boada et al. 2015, Gianasi et al. 2015, Agatsuma 2020). An efficient tagging method must be easily identifiable in the field and in aquaculture facilities**,** cause little tissue damage (avoiding infections), have a low impact on behaviour and growth, and have a relatively high retention rate (Lauzon-Guay and Scheibling 2008, Cipriano et al. 2014, Gianasi et al. 2015, Searcy-Bernal et al. 2016).

Approximately 75000 t of sea urchins are harvested annually, and Chile, USA, Japan, Canada, Russia, Mexico, Philippines, Peru, Korea and New Zealand are the main harvesting countries (Sun and Chiang 2015, Stefánsson et al. 2017, Agatsuma 2020). Sea urchin gonads have long been regarded as a highly-valued gastronomic delicacy, primarily in Japan, which is the main importer and accounts for 90% of world trade (Stefánsson et al. 2017). The sea urchin *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata: Echinoidea) is the most consumed echinoid species in Europe. It is an important economic resource, particularly in France, Spain, Italy, Ireland, and to a lesser extent in Portugal (Stefánsson et al. 2017). Following the global trend of intense harvesting of edible sea urchins, the increasing market demand for *P. lividus*, particularly since the 1970s, has resulted in the collapse of several natural populations in Europe (Andrew et al. 2002, Bertocci et al. 2014). Furthermore, overfishing of their predatory fish potentially leads to population outbreaks, transforming abundant marine forests into barren grounds (Boudouresque et al. 2020).

Various tagging methods have been tested in these organisms, in the context of the growing sector of commercial aquaculture of sea urchins and restocking actions (de la Uz et al. 2018, Agatsuma 2020), and also in ecological studies (Mattison et al. 1976, McClanahan and Muthiga 1989, Boada et al. 2015). Physical tags inserted during the test and chemical marking with fluorochromes have been studied since the 1960s, while the use of decimal coded wire tags (CWTs) and passive integrated transponder (PIT) tags in sea urchins has only been reported since the late 1990s (Ebert 2013). More specifically, external methods such as T-bar anchor tags are often used in fish mark-recapture studies enabling individual identification, but they are relatively large and may affect sea urchin behaviour (Sandford et al. 2020). Other techniques, such as painting the sea urchin spines with nail polish (Agatsuma et al. 2000, Cipriano et al. 2014) or with antifouling paint, do not provide individual identification, may have low durability and affect survival (Cipriano et al. 2014). Beads glued to the spines have shown low retention rates (Cipriano et al. 2014). PIT-tags, developed in the 1980s and used with increasing frequency in aquaculture, are glass-encapsulated biocompatible microchips with an electromagnetic coil (Gibbons and Andrews 2004). The microchips provide a unique individual alphanumeric code, which is read noninvasively by radio frequency identification, avoiding the capture of the animal. These relatively small and weightless devices have been used for subcutaneous, intramuscular and body cavity implantation (Rogers-Bennett et al. 2003, Acolas et al. 2007) and have a reliable long-term operational durability (Gibbons and Andrews 2004, Woods and James 2005). However, the reliability of this tagging method for *P. lividus* still needs to be further evaluated with regard to its effect on growth (Cipriano et al. 2014). Given the unsuitability of PIT-tags for the tagging of smaller sea urchins (Woods and James 2005), internal CWTs, commonly used as a simple fish-tagging method (Sandford et al. 2020), have been tested in sea urchins, although by very few authors, using different species (Kalvass et al. 1998, Sonnenholzner et al. 2011, de la Uz et al. 2018). A CWT is a small magnetized stainless-steel wire that is easily identified with a magnetic field detector and is etched with a number sequence that allows batch identification or millions of individual codes. However, individual code identification requires microscope use, with a 20-40x magnification (Martin 2011) and the sacrifice of the animal, which inhibits identification in the field and

repeated readings over time (Cieciel et al. 2009, Gianasi et al. 2015, Sandford et al. 2020). Fluorochrome chemical markers, including tetracycline and calcein, have been extensively used in several taxa as a long-term marking process (Gorzelak et al. 2017, Li et al. 2020). In the tagging process, individuals are exposed to fluorochrome, which binds irreversibly to calcium ions and is incorporated in the carbonate structure of ossicles of growing animals during the process of biomineralization. Consequently, under epifluorescence microscopy, the stained calcified structures, such as the Aristotle's lantern in sea urchins, fluoresce bright green (Ellers and Johnson 2009, Haag et al. 2013, Johnson et al. 2013). Calcein tagging is an affordable, simple and effective method for mass tagging of invertebrates to study their biology, particularly individual growth, life history and population structure, although individual identification is not achieved (Ellers and Johnson 2009, Gianasi et al. 2015, Jacinto et al. 2015). Moreover, calcein tagging is in general less toxic, more intensely fluorescent and more absorbable than tetracycline (Monaghan 1993). It is approved for application in aquaculture-reared animals destined for human consumption (Purcell et al. 2006), so it is safer than physical internal tags for consumers.

The tagging of marine invertebrates is often invasive and still presents several challenges, such as high mortality rates, low tag retention rates and altered behaviour and growth, particularly for animals with a small body size and a morphological structure like that of sea urchins (Ebert 1965, 2013, Lauzon-Guay and Scheibling 2008, Rodríguez-Barreras and Wangensteen 2016). The effects of invasive tagging on survival can also be further aggravated by abiotic and biotic factors, such as adverse water temperature and salinity, or predation (McClanahan and Muthiga 1989, Boada et al. 2015). Furthermore, since each tagging method is highly species-specific, its characteristics must be considered within the scope of the desired goal (Lauzon-Guay and Scheibling 2008, de la Uz et al. 2018). So far, few studies have addressed the impact and success of multiple tagging methods on *P. lividus*. In this study, a preliminary trial was conducted to test external (T-bar, nail polish, antifouling paint and beads), internal (PIT-tags and CWTs) and chemical marks (calcein) on wild *P. lividus* reared in the laboratory over 60 days with regard to survival and tag retention. Furthermore, in the main trial, taking into consideration the results of the preliminary experiment, the effect on survival, total wet weight, total weight gain (TWG) and tag retention of two physical tags (PIT-tags and CWTs) and one chemical tag (calcein) was also tested in wild individuals reared in the laboratory.

MATERIALS AND METHODS

Preliminary trial

A 60-day preliminary trial was first held in order to evaluate tagging techniques and choose the three best options for the main trial regarding tag retention and animal survival. A total of 220 wild *P. lividus* were harvested in the intertidal zone of Porto Batel (Peniche, Portugal; 39°19'08.5"N 9°21'24.1"W) in December 2019. Seven tagging methods were selected: T-bars, nail polish, antifouling paint, glued beads, PIT-tags, CWTs and calcein. Individuals were separated into seven groups of 20 to 30 mm test diameter and four groups with 35 to 45 mm diameter. Each group contained 20 individuals allocated to a grid cage. The groups were kept separated, submerged in a 1000 L tank with a recirculating aquaculture system (RAS). Because of the low diameter of the injection needle (see section "Tagging procedures"), the external non-invasive marks (nail polish, antifouling paint and glued beads) and CWTs were tested only in individuals of 20 to 30 mm. Both size classes were considered in the tests using PIT-tags and calcein and in the control to assess possible differences between them caused by the higher degree of potential physical/chemical stress. Only individuals of 35 to 45 mm were marked with T-bars because this technique is more invasive. Further details of this preliminary experiment, including the marking techniques and the results regarding survival of the organisms and tag retention rates, are detailed in Table 1. According to the results obtained in this first trial, in terms of survival and tag retention, three methods were selected for the main trial: CWTs, PIT-tags and calcein.

Main trial

Experimental setup and collection of sea urchins

Wild individuals of *P. lividus* (20-30 mm horizontal test diameter) were collected from intertidal rock pools of Porto Batel (Peniche, Portugal; 39°19'08.5"N 9°21'24.1"W) in July 2020. They were transported to the Marine and Environmental Sciences Centre (MARE, Polytechnic of Leiria) in isothermal boxes and were acclimatized for one week in RAS and fed ad libitum with *Ulva* spp. The experimental design was composed of four RAS and each system consisted of three 40 L holding tanks and a 70 L sump tank supplied with sand- and UV-filtered natural seawater. Each system was equipped with aeration, mechanical and biological filtration, a protein skimmer (Bubble Magus C3.5, Jiyang Aquarium Equipment Co., Ltd., Jiangmen, China) and a water pump (Reef-Pump 2000, TMC Iberia, Portugal). During the trial, to monitor the seawater quality, temperature, pH, salinity and dissolved oxygen were measured every two days with a YSI Professional Plus multiparameter meter (YSI Inc., Yellow Springs, OH, USA). These parameters were kept at 21.9±0.4°C, 8.2±0.1, 33.1±0.5 and 92±1%, respectively, using the same methodology as Santos et al. (2020a). Ammonia, nitrite and nitrate were monitored every two days with API® Test Kits (Mars Fishcare, Inc., Chalfont, Pennsylvania, USA) and kept within optimal values for marine species.

Before the tagging procedures (T1), all individuals (n=120) were measured (horizontal test diameter) with a vernier calliper (Insize, code 1205-150S, INSIZE Co., Ltd., Zamudio, Spain; ±0.05 mm accuracy), briefly dried with absorbent paper and weighed (total wet

Table 1. – Preliminary trial: details of the tagging techniques, retention rates of the tags and survival of the sea urchins of the species *Paracentrotus lividus* submitted to different tagging methods and reared for 60 days.

Method	Tagging technique	Survival	Tag retention
Nail polish	Applied to 5 dry spines	90%	0%
Antifouling paint	Applied to 5 dry spines with a fine paintbrush	100%	0%
Glued beads	2 mm beads glued to the top of 5 spines with a non-toxic super-glue	100%	0%
Coded wire tags	See "Tagging procedures"	95%	74%
PIT-tags (20-30 mm)	See "Tagging procedures"	95%	63%
PIT-tags (35-45 mm)			94%
Calcein (20-30 mm)	See "Tagging procedures"	100%	100%
Calcein (35-45 mm)			
Control (20-30 mm)	-	100%	-
Control (35-45 mm)			
T-bars	External plastic T-bar tags inserted through a drilled hole in the aboral region of the individual (approximately 1 mm diameter)	5%	100%

weight) using an electronic precision balance (Kern PCB 2500-2, Kern & Sohn GmbH, Balingen, Germany; accuracy of 0.01 g).

Tagging procedures

The individuals were separated into groups of 30 to be used in each tagging procedure and in the control (untagged). The stainless-steel CWT tags (0.25 mm in diameter and 1.1 mm in length) (Northwest Marine Technology®, Inc. [NMT], Shaw Island, Washington, USA) were inserted manually into the 30 individuals with the automated wire tagging machine Mark IV Tag Injector (NMT), which cuts the wire tag and injects it with a 0.57 mm diameter needle in a single operation. The injection needle was adjusted to a fixed position and it was inserted in each individual through the peristomial membrane into the coelomic cavity. For each sea urchin, the presence of the magnetic tag within the coelom was confirmed using a portable V-Detector sampling detector (NMT), which detects a small change in the magnetic field when a CWT is present.

A second group of 30 sea urchins was PIT-tagged using Biomark HPT8 tags (Biomark, Inc., Boise, Idaho, USA) with 8.4 mm length and 1.4 mm diameter and an operating frequency of 134.2 kHz, providing a unique identification number for each individual. The PIT-tags were carefully inserted into the coelomic cavity through the peristomial membrane using a plastic MK165 syringe with an N165 needle (length=5.1 cm; nominal outer diameter=1.65 mm) (Biomark, Inc., Boise, Idaho, USA) under binocular magnifying glass. The PIT-tags were scanned with the portable Biomark HPR Plus™ automatic reader.

For the chemical marking, the 30 sea urchins were evenly distributed into three 30 L continuously aerated tanks containing a calcein solution of filtered seawater at 100 mg calcein L^{-1} and kept for 24 h. The calcein solutions were prepared by diluting calcein disodium salt (CAS 108750-13-6, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) in distilled water using a 250 mL beaker on a magnetic stirrer. After a 24 h tagging period, the sea urchins were gently washed and kept in filtered seawater for another 24 h. Afterwards, before the sea urchins were transferred to the recirculating aquaculture systems, all individuals were checked for the fluorescent mark in the calcified structures, mainly in the visible portion of the Aristotle's lantern. For this procedure, a UV-FL-1 Dive Light™ and yellow filter glasses (NightSea LLC, California, USA) were used.

Rearing trial

The individuals were immediately distributed into the tanks after the tagging procedures. Four treatments (calcein, PIT-tags, CWTs and control), each with three replicates, randomly assigned among the 12 tanks, were carried out over 60 days from July to September 2020. A total of 30 individuals were randomly allocated for each treatment at the beginning of the trial (T1), with 10 individuals per tank, corresponding to a density of 1.6±0.1 g L^{-1}. The size range of the individuals was uniformly distributed among the tanks, with no significant differences in test diameter and total weight between the tanks or treatments ($p>0.05$). The initial global test diameter was 24.1±0.2 mm (see Fig. A1 of the appendices for details of data distribution). The initial individual total wet weight in each group was 5.89±0.27 g (PIT-tags), 6.35±0.38 g (CWTs),

6.15±0.28 g (calcein) and 6.59±0.52 g (control) (see Fig. A2 of the appendices for details of data distribution). A 12:12 h light:dark photoperiod was adopted. Sea urchins were fed with a commercial extruded diet with algae-based ingredients (Sparos Lda., Olhão, Portugal), specifically formulated for *P. lividus* (Lourenço et al. 2021). The pellets (size=1.8 cm) were administered ad libitum every two days.

At the end of the rearing period (T2), all individuals were briefly dried with absorbent paper and weighed (total wet weight of the individual, ±0.01 g), with the same procedure as that used in T1 (see Fig. A3 of the appendices for details of data distribution). Test diameter increment was not taken into account because of the relatively short length of the trial, the slow growth rates of sea urchins (Lourenço et al. 2021) and the linear measurements, which are commonly biased in sea urchins (Ebert 2004, 2017). Conversely, weight is a more reliable measure of global growth (Ellers and Johnson 2009). The presence of the tags was assessed according to the methodologies described above. See Figure A4 of the appendices for an illustration of the three tagging and detection methods.

As a measure of growth, TWG (mg ind.$^{-1}$ day^{-1}) was computed as follows (adapted from Shpigel et al. 2004):

$$TWG = \frac{(W_{final} - W_{initial})}{t}$$

where W_{final} and $W_{initial}$ represent the final and initial average total wet weight per tank (mg), respectively, and t represents total time in days.

Retention rate (R) (adapted from Pennock et al. 2016), expressed as the percentage of sea urchins that retained the tag at the end of the trial, was computed as follows:

$$R = \left(\frac{N_{tagged}}{N_{live}} \right) x100$$

where N_{tagged} and N_{live} represent the number of tagged individuals and the number of live individuals, respectively.

Survival (S) rate was expressed as the percentage of live individuals at the end of the trial.

Statistical analysis

The results were expressed as mean ± standard error (se) and a significance level of α=0.05 was used for the statistical tests. A one-way permutational multivariate analysis of variance (PERMANOVA) (Pseudo-$F_{(\text{degrees of freedom, residual degrees of freedom})}$ = value; p-value), with treatment (4 levels: PIT-tags, CWTs, calcein and control) as a fixed factor, was performed to test for significant differences in total wet weight at the beginning and at the end of the trial, with 30 sea urchins per treatment, except in the CWT group, which had 29 individuals in the end. A one-way PERMANOVA using 999 permutations, with treatment (4 levels) as a fixed factor, was performed to test for significant differences in TWG, using three mean values (from each tank) per treatment. The data from the three replicates in each treatment were pooled together in these tests. Homogeneity of univariate dispersion was analysed using the PERMDISP test. Analyses were based on Euclidean distances of untransformed data. Unrestricted permutation of raw data and Type III sums of squares were applied. Pair-wise a posteriori compari-

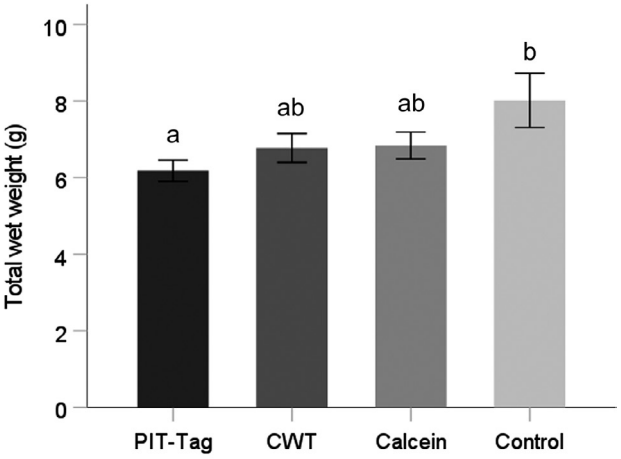

Fig. 1. – Individual total wet weight (mean ± se) of *Paracentrotus lividus* at the end of a 60-day rearing in the laboratory after tagging with PIT-tags, coded wire tags (CWTs) and calcein, including an untagged control (n=30 individuals per treatment, except the CWT group, n=29). Note: bars sharing the same letter are not significantly different (p>0.05).

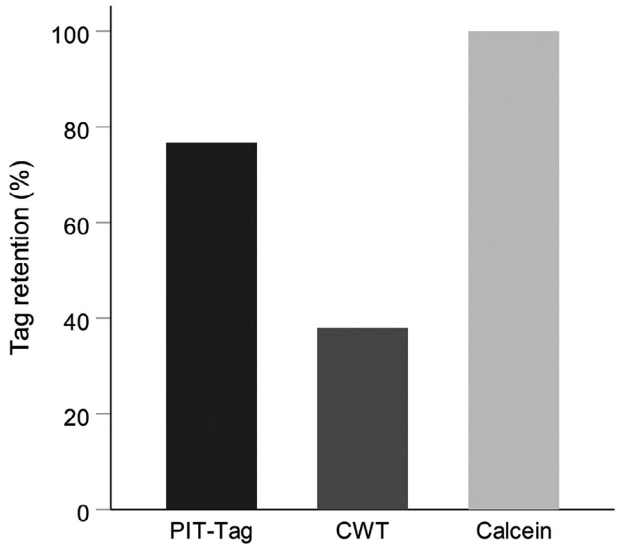

Fig. 2. – Retention (%) of PIT-tags, coded wire tags (CWTs) and calcein used to tag *Paracentrotus lividus* after a 60-day experimental period in the laboratory (n=30 individuals per treatment, except the CWT group, n=29). A significant association between tagging method and tag retention was detected (p<0.05).

sons were conducted when applicable. The Pearson chi-square test (χ^2 (degrees of freedom) = value; p-value) was used to assess a possible association between the tagging method and sea urchins' survival, as well as between the tagging method and the tag retention. Post-hoc z-tests for independent proportions, with Bonferroni-adjusted p-values, were performed in cases of statistical significance. PERMANOVA was carried out using PRIMER 6 (version 6.1.13) and PERMANOVA+ add on (version 1.0.3) (PRIMER-E Ltd., Plymouth, United Kingdom). The Pearson chi-square tests were performed using IBM® SPSS™ Statistics for Windows, version 27 (IBM Corporation, Armonk, New York, U.S.).

RESULTS

Total wet weight

At the end of the trial, the individual total wet weight for each group was 6.18±0.28 g (PIT-tags), 6.77±0.38 g (CWTs), 6.84±0.35 g (calcein) and 8.02±0.71 g (control) (Fig. 1). Total wet weight at the end of the trial was significantly affected by the tagging method [$F_{(3, 115)}$=2.81, p=0.039]. Significant differences were only found between the PIT-tag and control groups (p=0.023). TWG for each group was the following: 4.9±2.6 mg ind.$^{-1}$ day^{-1} (PIT-tags), 7.7±3.0 mg ind.$^{-1}$ day^{-1} (CWTs), 11.5±6.5 mg ind.$^{-1}$ day^{-1} (calcein) and 23.8±12.3 mg ind.$^{-1}$ day^{-1} (control). TWG was not significantly affected by treatment [$F_{(3, 8)}$=1.34, p=0.320].

Survival

Survival rate was 100% for the PIT-tag, calcein and control groups, and 97% for the CWT group. No significant association between tagging method and survival was detected among treatments [$\chi^2_{(3)}$=3.03, p=0.340].

Tag retention

The tag retention rate was 100% in the calcein group, 76.7% in the PIT-tag group and 38.0% in the CWT group, as represented in Figure 2. A significant association between tagging method and tag retention was detected among treatments [$\chi^2_{(2)}$=28.63, p<0.001], with calcein presenting a significantly higher retention rate than the CWT group (p<0.001).

DISCUSSION

Total wet weight

The three tagging methods tested in the main trial promoted no significant differences in TWG during the 60-day trial. Similar results regarding growth between tagged and control animals were also reported by Hagen (1996), Rodríguez-Barreras and Sonnenholzner (2014) and Rodríguez-Barreras and Wangensteen (2016), who tested PIT-tags on *Strongylocentrotus droebachiensis*, *Tripneustes ventricosus* and *Echinometra lucunter*, respectively. Kalvass et al. (1998), testing PIT-tags and CWT on *Strongylocentrotus franciscanus*, and de la Uz et al. (2018), testing CWTs on *P. lividus*, also found no significant tagging effect on growth. Sonnenholzner et al. (2011), who tested tetracycline, CWTs and PIT-tags on *Strongylocentrotus purpuratus* under laboratory conditions, also reported similar results. However, these studies only presented test diameter growth. The results of the present study are also supported by the experiments carried out by Ellers and Johnson (2009) with polyfluorochrome marking, including calcein, and confirm the absence of a negative impact of calcein on growth. However, in the present study, untagged sea urchins (control) showed a significantly higher total wet weight at the end of the experiment than the PIT-tag group. On the other hand, Woods and James (2005) found no significant differences in total weight between PIT-tagged and control treatments in *Evechinus chloroticus* reared for five months. However, the use of a much higher size class (78 mm) might have contributed to a lower physiological stress caused by the tagging method. Unlike the final individual total wet weight, TWG corresponds to the weight increment obtained during the trial per replicate tank, hence the distinct statistical output between the two parameters. Overall, the similar pattern observed in total wet weight and TWG is consistent with the level of invasiveness of each tagging method, as the control group showed the highest values, followed by calcein (which did not involve physical intrusion), CWTs and, finally, PIT-tags. T.A. Ebert (2013) reported a possible reduced growth using invasive tags such as CWTs and PIT-tags, and this finding is supported by Lauzon-Guay and Scheibling (2008), who obtained a lower total weight in PIT-tagged *S. droebachiensis* in a field experiment. The results of Kalvass et al. (1998) also suggest a lower test diameter increase in PIT-tagged sea urchins than in individuals injected with tetracycline. In fact, PIT-tagging may have an inhibitory impact on feeding intake, thus affecting growth (Lauzon-Guay and Scheibling 2008), and also on invertebrate behaviour (Wilson et al. 2011), and its effects in *P. lividus* should be further investigated in future studies. The lower TWG in the group marked with calcein than in the control group agrees with the results obtained by Russell and Urbaniak (2004), who observed a temporary decrease in the growth rate of juvenile *S. droebachiensis* tagged with calcein. This decrease might be explained by the stress to which the individuals are submitted during the marking process, by changes in biomineralization or by sub-lethal toxicity, which directly affects growth (Purcell and Blockmans 2009). In fact, Purcell and Blockmans (2009) refer to the fluorochrome concentration of 100

mg L^{-1} as mildly toxic for use in sea cucumbers, being detrimental to growth and behaviour. Ellers and Johnson (2009) also reported a decrease in growth during the first month after marking with different fluorochromes, and it is well-known that growth, including somatic and gonadal growth, is directly influenced by the physiological condition of the animal (Delorme and Sewell 2016). In conditions of physiological compensation or depression, there is a decrease in the total energy available for production, so growth might be reduced (Delorme and Sewell 2016, Harianto et al. 2018). Dworjanyn and Byrne (2018) reported that the sea urchin *Tripneustes gratilla* showed lower somatic and gonadal growth when exposed to higher physiological stress levels. The presence of invasive tags, such as PIT-tags, in the coelomic cavity, as well as the tagging process, might represent a physiological challenge for the sea urchin's immune system (Cipriano et al. 2014). The mass of the PIT-tags used in this study (approximately 30 mg) represented only 0.5% of the tagged sea urchins' mean weight. Since sea urchins have a relatively sedentary behaviour, the relationship between the tag and the body mass might not be a relevant factor in this study. Nevertheless, the relatively small PIT-tags used may have attenuated their deleterious effects on *P. lividus* growth, supporting previous recommendations to favour smaller tags whenever possible (Lauzon-Guay and Scheibling 2008). The possibility of a reduced growth performance in tagged sea urchins must be considered particularly in aquaculture operations, in which low growth rates already represent a production bottleneck (Lourenço et al. 2021). It should also be noted that the use in the present study of a dry formulated feed containing algae-based ingredients most likely promoted growth in the sea urchins (Cyrus et al. 2013, Santos et al. 2020b) and might have contributed to the regenerative processes after tagging. This study is also the first to describe the effect of the three methods on TWG in *P. lividus*.

Survival

In the main trial, all treatments resulted in 100% survival, with the exception of the CWT group (97%), contrary to what is suggested by T. A. Ebert (2013) regarding the potential negative effects on survival of invasive tags. The results obtained, particularly regarding PIT-tags and CWTs, clearly demonstrate the remarkable tissue regenerative capacities of sea urchins. Their peristomial membrane is mainly composed of mutable fibrillar-collagenous tissues, which are vital elements to enable a faster regeneration process (Barbaglio et al. 2012, Brown and Caldwell 2017). The absence of a significant detrimental effect on survival with internal tags is also documented by Hagen (1996), Duggan and Miller (2001), Woods and James (2005), Lauzon-Guay and Scheibling (2008), Sonnenholzner et al. (2011), Rodríguez-Barreras and Sonnenholzner (2014), Gianasi et al. (2015), Rodríguez-Barreras and Wangensteen (2016), de la Uz et al. (2018) and Grosso et al. (2021). In the scope of PIT-tagging, the present study also presents new advances, because it achieved 100% survival in smaller PIT-tagged sea urchins (20-28 mm) than those used in other related studies. By contrast, Cipriano et al. (2014), Rodríguez-Barreras and Sabat (2015) and Tourón et al. (2022) reported approximate mortality rates of 60%, 20% and 10%, respectively, in sea urchins also tagged with 8 mm PIT-tags. Furthermore, although sea urchins exhibit regenerative test processes (Candia Carnevali 2006), the adult calcification rates are relatively low (Mos et al. 2016) and might explain the high mortality rates, particularly in smaller individuals tagged with external tagging methods involving test perforation, as exemplified in the preliminary study with the individuals marked with T-bars (Duggan and Miller 2001, Clemente et al. 2007, Rodríguez-Barreras and Sabat 2015). Consequently, despite the easy identification that it enables for field studies, this method should only be applied in short-term experiments (de la Uz et al. 2018). However, the effect of the perforated orifice diameter on survival should be further investigated in future studies, as openings of less than 1 mm would likely result in a faster healing process, and thus in a reduced mortality (McClanahan and Muthiga 1989, Boada et al. 2015, Tourón et al. 2022). External tagging methods may also increase predation rates in the field, particularly for T-bars (Rodríguez-Barreras and Sabat 2015). The survival rates obtained in this study with calcein (100%) are similar to those obtained by Ellers and Johnson (2009), corroborating the viable use of calcein as a non-toxic marker if used in suitable concentrations (Fox et al. 2018). Nonetheless, the toxicity levels vary greatly between taxa and size classes and according to abiotic factors (e.g. temperature) and the duration of the administration (Moran 2000, Purcell and Blockmans 2009).

Tag retention

The assessment of the retention rates clearly shows that calcein marking stands out as the most reliable tagging method, as all individuals clearly displayed a fluorescent stain in the visible part of the Aristotle's lantern under UV light, resulting in 100% retention rate. Ellers and Johnson (2009) also report a 100% marking rate in *S. droebachiensis* after calcein immersion baths in either 0.75 mg L^{-1} or 75 mg L^{-1}. Similarly, Russell and Urbaniak (2004) reported a total marking success in the same species marked with calcein at approximately 45 mg L^{-1}. Rodríguez et al. (2016) also achieved 100% of tagged *P. lividus* using calcein concentrations of only 10 and 20 mg L^{-1} to mark smaller individuals (5-10 mm). In contrast, Dumont et al. (2004), four days after a 21 mg L^{-1} calcein bath applied to *S. droebachiensis* (>20 mm in test diameter), only obtained 71.4% of marked individuals, which might be explained by the relatively low

Table 2. – Summary of the main characteristics of PIT-tags, coded wire tags (CWT) and calcein used to tag sea urchins and recommendations on their use.

Tag	Individual identification	Growth studies	Animal sacrifice	Field identification	Recommended minimum size
PIT-tags	✓	✓	X	✓ (see described method)	20 mm (possibly less - further studies needed)
CWTs	✓	✓	X (only for individual identification)	✓	20 mm (possibly less - further studies needed)
Calcein	X	✓	X (only for growth evaluation)	✓ (see described method)	not applicable

calcein concentration, given the size of the sea urchins, and a seasonal effect on the calcification rates (Ebert et al. 2008).

According to these results, a lower concentration of the calcein solution could probably have been used in the present study. However, the intensity level of the fluorescent mark must be considered while using the described detection method, especially for identification in the field. Ellers and Johnson (2009) indicate very low visibility of the low-dose marks, and Purcell and Blockmans (2009) report an enhanced mark in sea cucumbers at 100 mg L^{-1} in comparison with 50 mg L^{-1}, although the efficiency of calcein tagging by immersion may vary according to the species studied (Rodríguez et al. 2016) and age and growth rates (Purcell et al. 2006). Purcell et al. (2006) hypothesized that sun exposure attenuates the intensity of the mark in sea cucumbers, which might not be a relevant issue for sea urchins because the visible part of the Aristotle's lantern is not directly exposed to sunlight. The detection method used in this study (UV light with appropriate filter glasses) and described for the first time for sea urchins offers an important practical advantage by allowing marked sea urchins to be identified in the field (Shao et al. 2017), especially in low light conditions. Therefore, for identification purposes only, contradicting Rodríguez et al. (2016) and Tourón et al. (2022), this method avoids the sacrifice of the animals for microscopy analysis (Purcell and Blockmans 2009, Rodríguez et al. 2016).

Regarding PIT-tagging, the associated retention rates are generally high in a wide range of aquatic animals, particularly in fish (Zakęś et al. 2019) but also in sea turtles (Omeyer et al. 2019), cephalopods (Estefanell et al. 2011), crustaceans (Sato et al. 2013), abalones (Searcy-Bernal et al. 2016) and sea cucumbers (Gianasi et al. 2015), although the retention is variable between species (Gianasi et al. 2015, Rodríguez-Barreras and Sabat 2015, Rodríguez-Barreras and Wangensteen 2016, Rodríguez-Barreras et al. 2017, Omeyer et al. 2019). Similarly to the present study, Tourón et al. (2022) obtained retention rates of 83% with 8 mm PIT-tags inserted in *P. lividus* with an average test diameter of 20 mm. However, these authors only achieved approximately 10% with 11.4 mm PIT-tags from another brand. In other studies, retention rates above 90% with PIT-tags were obtained in *P. lividus* (Cipriano et al. 2014, Grosso et al. 2021), *S. droebachiensis* (Hagen 1996, Lauzon-Guay and Scheibling 2008), *S. purpuratus* (Sonnenholzner et al. 2011), *S. franciscanus* (Palleiro-Nayar et al. 2009), *T. ventricosus* (Rodríguez-Barreras and Sonnenholzner 2014) and *E. chloroticus* (Woods and James 2005). While most of these authors used PIT-tags with approximately 12 mm length, the present study used 8.4 mm tags. Furthermore, although the above authors report higher retention rates than those of the present study (77%), the sea urchins tested belong to higher size classes with test diameters greater than 60 mm. Most of those studies covered a broad sea urchin size range, and a relatively narrow range (20-28 mm) was used herein for PIT-tagging, thus strengthening the results. In fact, some of the reported retention rates should be analysed with caution, considering the respective survival rates according to the size classes. Individual size is one of the main factors affecting PIT-tagging success in sea urchins (Lauzon-Guay and Scheibling 2008, Gianasi et al. 2015), depending on the ratio between PIT-tag size and test diameter (Hagen 1996, Larsen et al. 2013). Cipriano et al. (2014) and Rodríguez-Barreras and Sonnenholzner (2014) reported 60% mortality in the smaller class of PIT-tagged sea urchins (20 mm and 40 mm, respectively). Furthermore, the PIT-tag detection method applied overcomes a recurrent issue in the identification process, particularly in the field (Duggan and Miller 2001, Lauzon-Guay and Scheibling 2008), by allowing underwater antennas to be used to rapidly collect individual data without animal sacrifice. This facilitates the individual study of sea urchins' movement, behaviour, growth and survival in the wild, also representing an additional advantage for aquaculture and stock management practices. In tidal pools, a portable antenna can be connected to the HPR Plus reader and automatically read the mark, as used in this study, and in subtidal surveys the same system could be coupled with an extension cable.

The low retention rate of CWT obtained in this study (38%) was also documented to occur in fishes and sea cucumbers (Guy et al. 1996, Purcell et al. 2006, Cieciel et al. 2009). In sea urchins, Sano et al. (2001) obtained tag losses greater than 40% using a size class similar to the one used in the present study. By contrast, de la Uz et al. (2018) achieved a retention rate of 80% in a similar

size class of *P. lividus* using an injection needle with the same diameter and CWTs twice the length of the ones used in the present study. Sonnenholzner et al. (2011) also obtained high retention rates for CWTs in *S. purpuratus* (<22 mm) using slightly longer tags. Given the 0.57 mm opening made by the injection needle, a 1 mm difference in the tag length may, indeed, significantly affect tag loss probabilities. Internal tag retention rates are influenced by several factors, including species behaviour, individual size, life-history traits, tag size, angle and zone of insertion, and improper tagging techniques (Guy et al. 1996, Gianasi et al. 2015, Omeyer et al. 2019, D'Arcy et al. 2020). The encapsulation or rejection of internal tags is well described in fish (Gheorghiu et al. 2010), sea cucumbers (Purcell et al. 2006) and starfish (Olsen et al. 2015), and the main reason for internal tag loss in sea urchins is probably their exit through the opening made by the needle in the first days after injection, since the lesion is expected to heal within a few days (Sonnenholzner et al. 2011). High tag loss (83%) in sea urchins was reported to occur in the first day (Sonnenholzner et al. 2011) or in the first month after tagging (de la Uz et al. 2018). In particular, experiments with PIT-tags report tag loss in the first five days (Lauzon-Guay and Scheibling 2008). In this context, a tagging needle with a nominal outer diameter that is as low as possible should always be favoured, thus promoting higher tag retention (D'Arcy et al. 2020).

In future studies, not only the healing of the wound should be monitored: a topical substance that accelerates the healing process, such as iodine-based solutions, may also be tested in sea urchins, as suggested by Gibbons and Andrews (2004). The tagging material should also be disinfected (Zakęś et al. 2019). Nonetheless, the period for complete healing of the lesion may vary among species and individual size, as is described for fish species (Navarro et al. 2006).

CONCLUSIONS

The results from the present study, summarized in Table 2, confirm the suitability of PIT-tag implantation in smaller sea urchins (Tourón et al. 2022) without greatly affecting retention rates, contradicting the suggestions of Rogers-Bennett et al. (2003) and Ellers and Johnson (2009) that sea urchins smaller than 30 mm and 25 mm, respectively, cannot survive PIT-tag implantation. The relatively high price and time-consuming tagging process of PIT-tags (Woods and James 2005, Purcell et al. 2006) may limit mass tagging, but the detection method applied in the present study offers practical advantages for researchers, particularly in the field.

CWTs are a cheaper tagging method for *P. lividus*, with insignificant negative effects on survival and growth in the short term, but the low retention rates may compromise their use for research purposes. In the future, larger CWTs (>2 mm in length) should be given preference to minimize tag losses. However, although this method allows detection in the field with portable equipment, the process of individual identification is not as practical as the PIT-tag detection method mentioned above. It demands the collection of the animals, their sacrifice and the facilities to perform individual identification under a microscope. Internal tags may also involve potential issues when sea urchins are to be used for human consumption, because of the risk of accidental tag ingestion (Gheorghiu et al. 2010, Zakęś et al. 2019).

Regarding calcein tagging, this study is the first that tests its effect on survival, weight gain and tag retention in a small size class of *P. lividus*, and it also describes an innovative detection method. Calcein immersion showed the most promising results in all the assessed parameters, despite precluding individual identification. It is a fast method for tagging large numbers of small sea urchins without significantly affecting survival or growth. Finally, the overall success of this tagging experiment in terms of growth, survival and tag retention may be significantly different when sea urchins are kept in the field (Lauzon-Guay and Scheibling 2008, Boada et al. 2015, Rodríguez-Barreras and Sabat 2015, Searcy-Bernal et al. 2016). Furthermore, a potential reduced growth performance in the long term can be a disadvantage, particularly in field conditions. For longer experiments aiming to perform estimates on age and growth and in the context of commercial echinoculture, it is not advisable to use invasive tagging methods, particularly PIT-tags, and calcein is a more reliable choice.

ACKNOWLEDGEMENTS

This work was supported by Fundação para a Ciência e Tecnologia (FCT) through the strategic project UIDB/04292/2020 (grant number) granted to MARE and by the Operational Programme MAR2020 through the project "Ouriceira Aqua" (grant number MAR-02.01.01-FEAMP-0004) and the project "Ouriceira Mar" (grant number MAR-01.03.02-FEAMP-0012). A.P. was supported through the Scientific Employment Stimulus Programmes (grant number CEECINST/00051/2018).

REFERENCES

Acolas M.L., Roussel J.M., Lebel J.M., Baglinière J.L. 2007. Laboratory experiment on survival, growth and tag retention following PIT injection into the body cavity of juvenile brown trout (*Salmo trutta*). Fish. Res. 86: 280-284. https://doi.org/10.1016/j.fishres.2007.05.011

Agatsuma Y. 2020. Stock enhancement of regular sea urchins. In: Lawrence J.M. (ed), Sea Urchins: Biology and Ecology. Academic Press, San Diego, CA, pp. 299-313. https://doi.org/10.1016/B978-0-12-819570-3.00017-2

Agatsuma Y.U., Nakata A.K., Matsuyama K.E. 2000. Seasonal foraging activity of the sea urchin *Strongylocentrotus nudus* on coralline flats in Oshoro Bay in south-western Hokkaido, Japan. Fish. Sci. 66: 198-203. https://doi.org/10.1046/j.1444-2906.2000.00035.x

Andrew N., Agatsuma Y., Ballesteros E., et al. 2002. Status and management of world sea urchin fisheries. Oceanogr. Mar. Biol. 40: 343-425. https://doi.org/10.1201/9780203180594.ch7

Barbaglio A., Tricarico S., Ribeiro A., et al. 2012. The mechanically adaptive connective tissue of echinoderms: Its potential for bio-innovation in applied technology and ecology. Mar. Environ. Res. 76: 108-113. https://doi.org/10.1016/j.marenvres.2011.07.006

Bartley D.M., Bell J.D. 2008. Restocking, stock enhancement, and sea ranching: Arenas of progress. Rev. Fish. Sci. 16: 357-365. https://doi.org/10.1080/10641260701678058

Bell J.D., Bartley D.M., Lorenzen K., Loneragan N.R. 2006. Restocking and stock enhancement of coastal fisheries: Potential, problems and progress. Fish. Res. 80: 1-8. https://doi.org/10.1016/j.fishres.2006.03.008

Bertocci I., Dominguez R., Machado I., et al. 2014. Multiple effects of harvesting on populations of the purple sea urchin *Paracentrotus lividus* in north Portugal. Fish. Res. 150: 60-65. https://doi.org/10.1016/j.fishres.2013.10.010

Boada J., Sanmartí N., Selden R.L., et al. 2015. Evaluating potential artifacts of tethering techniques to estimate predation on sea urchins. J. Exp. Mar. Bio. Ecol. 471: 17-22. https://doi.org/10.1016/j.jembe.2015.05.011

Boudouresque C.F., Blanfuné A., Pergent G., et al. 2020. Impacts of Marine and Lagoon Aquaculture on Macrophytes in Mediterranean Benthic Ecosystems. Front. Mar. Sci. 7: 218. https://doi.org/10.3389/fmars.2020.00218

Brown L.R., Caldwell G.S. 2017. Tissue and spine regeneration in the temperate sea urchin *Psammechinus miliaris*. Invertebr. Reprod. Dev. 61: 90-96. https://doi.org/10.1080/07924259.2017.1287779

Candia Carnevali M.D. 2006. Regeneration in Echinoderms: repair, regrowth, cloning. Invertebr. Surviv. J. 3: 64-76.

Cieciel K., Pyper B.J., Eckert G.L. 2009. Tag retention and effects of tagging on movement of the giant red sea cucumber *Parastichopus californicus*. North Am. J. Fish. Manag. 29: 288-294. https://doi.org/10.1577/M07-194.1

Cipriano A., Burnell G., Culloty S., Long S. 2014. Evaluation of 3 tagging methods in marking sea urchin, *Paracentrotus lividus*, populations under both laboratory and field conditions. J. Aquac. Res. Dev. 5: 276. https://doi.org/10.4172/2155-9546.1000276

Clemente S., Hernández J.C., Brito A. 2007. An external tagging technique for the long-spined sea urchin *Diadema aff. antillarum*. J. Mar. Biol. Assoc. United Kingdom. 87: 777-779. https://doi.org/10.1017/S0025315407053349

Cyrus M.D., Bolton J.J., De Wet L., Macey B.M. 2013. The development of a formulated feed containing *Ulva* (Chlorophyta) to promote rapid growth and enhanced production of high quality roe in the sea urchin *Tripneustes gratilla* (Linnaeus). Aquac. Res. 45: 159-176. https://doi.org/10.1111/j.1365-2109.2012.03219.x

D'Arcy J., Kelly S., McDermott T., et al. 2020. Assessment of PIT tag retention, growth and post-tagging survival in juvenile lumpfish, *Cyclopterus lumpus*. Anim. Biotelemetry. 8: 1-9. https://doi.org/10.1186/s40317-019-0190-6

Delorme N.J., Sewell M.A. 2016. Effects of warm acclimation on physiology and gonad development in the sea urchin *Evechinus chloroticus*. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 198: 33-40. https://doi.org/10.1016/j.cbpa.2016.03.020

Duggan R.E., Miller R.J. 2001. External and internal tags for the green sea urchin. J. Exp. Mar. Bio. Ecol. 258: 115-122. https://doi.org/10.1016/S0022-0981(01)00213-1

Dumont C., Himmelman J.H., Russell M.P. 2004. Size-specific movement of green sea urchins *Strongylocentrotus droebachiensis* on urchin barrens in eastern Canada. Mar. Ecol. Prog. Ser. 276: 93-101. https://doi.org/10.3354/meps276093

Dworjanyn S.A., Byrne M. 2018. Impacts of ocean acidification on sea urchin growth across the juvenile to mature adult life-stage transition is mitigated by warming. Proc. R. Soc. B Biol. Sci. 285: 20172684. https://doi.org/10.1098/rspb.2017.2684

Ebert T. 2004. Shrinking sea urchins and the problems of measurement. In: Heinzeller T., Nebelsick J. (eds), Echinoderms: München. Balkema, London, pp. 321-326. https://doi.org/10.1201/9780203970881.pt4

Ebert T.A. 1965. A technique for the individual marking of sea urchins. Ecology. 46: 193-194. https://doi.org/10.2307/1935273

Ebert T.A. 2013. Growth and survival of postsettlement sea urchins. In: Lawrence J.M. (ed), Sea Urchins: Biology and Ecology. Academic Press, San Diego, California, pp. 83-117. https://doi.org/10.1016/B978-0-12-396491-5.00007-1

Ebert T.A. 2017. A Problem in Measuring Sea Urchins. Ann. Mar. Biol. Res. 4: 1026.

Ebert T.A., Russell M.P., Gamba G., Bodnar A. 2008. Growth, survival, and longevity estimates for the rock-boring sea urchin *Echinometra lucunter lucunter* (Echinodermata, Echinoidea) in Bermuda. Bull. Mar. Sci. 82: 381-403.

Ellers O., Johnson A.S. 2009. Polyfluorochrome marking slows growth only during the marking month in the green sea urchin *Strongylocentrotus droebachiensis*. Invertebr. Biol. 128: 126-144. https://doi.org/10.1111/j.1744-7410.2008.00159.x

Estefanell J., Socorro J., Afonso J.M., Roo J., Fernandez-Palacios H., Izquierdo M.S. 2011. Evaluation of two anaesthetic agents and the passive integrated transponder tagging system in *Octopus vulgaris* (Cuvier 1797). Aquac. Res. 42: 399-406. https://doi.org/10.1111/j.1365-2109.2010.02634.x

FAO. 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action. FAO, Rome, Italy, pp. 244.

Fox E., Meyer E., Panasiak N., Taylor A.R. 2018. Calcein staining as a tool to investigate coccolithophore calcification. Front. Mar. Sci. 5: 326. https://doi.org/10.3389/fmars.2018.00326

Gheorghiu C., Hanna J., Smith J.W., et al. 2010. Encapsulation and migration of PIT tags implanted in brown trout (*Salmo trutta* L.). Aquaculture 298: 350-353. https://doi.org/10.1016/j.aquaculture.2009.10.004

Gianasi B.L., Verkaik K., Hamel J.F., Mercier A. 2015. Novel Use of PIT Tags in Sea Cucumbers: Promising Results with the Commercial Species *Cucumaria frondosa*. PLoS One. 10: e0127884. https://doi.org/10.1371/journal.pone.0127884

Gibbons J.W., Andrews K.M. 2004. PIT tagging: Simple technology at its best. Bioscience 54: 447-454. https://doi.org/10.1641/0006-3568(2004)054[0447:PTSTAI]2.0.CO;2

Gorzelak P., Dery A., Dubois P., Stolarski J. 2017. Sea urchin growth dynamics at microstructural length scale revealed by Mn-labeling and cathodoluminescence imaging. Front. Zool. 14: 1-17. https://doi.org/10.1186/s12983-017-0227-8

Grosso L., Rakaj A., Fianchini A., et al. 2021. Integrated Multi-Trophic Aquaculture (IMTA) system combining the sea urchin *Paracentrotus lividus*, as primary species, and the sea cucumber *Holothuria tubulosa* as extractive species. Aquaculture 534: 736268. https://doi.org/10.1016/j.aquaculture.2020.736268

Guy C.S., Schultz R.D., Clouse C.P. 1996. Coded Wire Tag Loss from Paddlefish: A Function of Study Location. North Am. J. Fish. Manag. 16: 931-934. https://doi.org/10.1577/1548-8675(1996)016<0931:CWTLFP>2.3.CO;2

Haag N., Russell M.P., Hernandez J.C., Dollahon N. 2013. Assessing fluorochrome-staining efficacy in the green sea urchin, *Strongylocentrotus droebachiensis* (Muller, 1776). Cah. Biol. Mar. 54: 625-631.

Hagen N.T. 1996. Tagging sea urchins: a new technique for individual identification. Aquaculture 139: 271-284. https://doi.org/10.1016/0044-8486(95)01156-0

Harianto J., Nguyen H.D., Holmes S.P., Byrne M. 2018. The effect of warming on mortality, metabolic rate, heat-shock protein response and gonad growth in thermally acclimated sea urchins (*Heliocidaris erythrogramma*). Mar. Biol. 165: 96. https://doi.org/10.1007/s00227-018-3353-8

Jacinto D., Penteado N., Pereira D., Sousa A., Cruz T. 2015. Growth rate variation of the stalked barnacle *Pollicipes pollicipes* (Crustacea: Cirripedia) using calcein as a chemical marker. Sci. Mar. 79: 117-123. https://doi.org/10.3989/scimar.04135.08B

Johnson A.S., Salyers J.M., Alcorn N.J., et al. 2013. Externally visible fluorochrome marks and allometries of growing sea urchins. Invertebr. Biol. 132: 251-269. https://doi.org/10.1111/ivb.12024

Kalvass P.E., Hendrix J.M., Law P.M. 1998. Experimental analysis of 3 internal marking methods for red sea urchins. Calif. Fish Game. 84: 88-99.

de la Uz S., Carrasco J.F., Rodríguez C., López J. 2018. Evaluation of tagging and substrate refuges in release of juvenile sea urchins. Reg. Stud. Mar. Sci. 23: 8-11. https://doi.org/10.1016/j.rsma.2018.02.008

Larsen M.H., Thorn A.N., Skov C., Aarestrup K. 2013. Effects of passive integrated transponder tags on survival and growth of juvenile Atlantic salmon *Salmo salar*. Anim. Biotelemetry 1: 1-7. https://doi.org/10.1186/2050-3385-1-19

Lauzon-Guay J.S., Scheibling R.E. 2008. Evaluation of passive integrated transponder (PIT) tags in studies of sea urchins: caution advised. Aquat. Biol. 2: 105-112. https://doi.org/10.3354/ab00040

Li H., Lei Y., Li T., Jian Z. 2020. Effects of calcein incorporation on benthic foraminiferal community under various concentrations and incubation durations. Mar. Micropaleontol. 157: 101874. https://doi.org/10.1016/j.marmicro.2020.101874

Lourenço S., Cunha B., Raposo A., et al. 2021. Somatic growth and gonadal development of *Paracentrotus lividus* (Lamarck, 1816) fed with diets of different ingredient sources. Aquaculture 539: 736589. https://doi.org/10.1016/j.aquaculture.2021.736589

Martin R.A. 2011. Evaluating a novel technique for individual identification of anuran tadpoles using coded wire tags. Herpetol. Conserv. Biol. 6: 155-160.

Mattison J.E., Trent J.D., Shanks A.L., Akin T.B., Pearse J.S. 1976. Movement and feeding activity of red sea urchins (*Strongylocentrotus franciscanus*) adjacent to a kelp forest. Mar. Biol. 1977 391. 39: 25-30. https://doi.org/10.1007/BF00395589

McClanahan T.R., Muthiga N.A. 1989. Patterns of predation on a sea urchin, *Echinometra mathaei* (de Blainville), on Kenyan coral reefs. J. Exp. Mar. Bio. Ecol. 126: 77-94. https://doi.org/10.1016/0022-0981(89)90125-1

Monaghan J.P. 1993. Notes: Comparison of Calcein and Tetracycline as Chemical Markers in Summer Flounder. Trans. Am. Fish. Soc. 122: 298-301. https://doi.org/10.1577/1548-8659(1993)122<0298:NCOCAT>2.3.CO;2

Moran A.L. 2000. Calcein as a marker in experimental studies newly-hatched gastropods. Mar. Biol. 137: 893-898. https://doi.org/10.1007/s002270000390

Mos B., Byrne M., Dworjanyn S.A. 2016. Biogenic acidification reduces sea urchin gonad growth and increases susceptibility of aquaculture to ocean acidification. Mar. Environ. Res. 113: 39-48. https://doi.org/10.1016/j.marenvres.2015.11.001

Navarro A., Oliva V., Zamorano M.J., et al. 2006. Evaluation of PIT system as a method to tag fingerlings of gilthead seabream (*Sparus auratus* L.): Effects on growth, mortality and tag loss. Aquaculture 257: 309-315. https://doi.org/10.1016/j.aquaculture.2006.02.072

Olsen T.B., Christensen F.E.G., Lundgreen K., et al. 2015. Coelomic transport and clearance of durable foreign bodies by starfish (*Asterias rubens*). Biol. Bull. 228: 156-162. https://doi.org/10.1086/BBLv228n2p156

Omeyer L.C.M., Casale P., Fuller W.J., et al. 2019. The importance of passive integrated transponder (PIT) tags for measuring life-history traits of sea turtles. Biol. Conserv. 240: 108248. https://doi.org/10.1016/j.biocon.2019.108248

Palleiro-Nayar J.S., Sosa-Nishizaki O., Montaño-Moctezuma G. 2009. Estimación de la tasa de crecimiento corporal del erizo rojo *Strongylocentrotus franciscanus* en cautiverio y en el Arrecife Sacramento en la Bahía El Rosario, Baja California, México. Cienc. Pesq. 17: 21-28.

Pennock C.A., Frenette B.D., Waters M.J., Gido K.B. 2016. Survival of and tag retention in Southern Redbelly dace injected with two sizes of PIT tags. North Am. J. Fish. Manag. 36: 1386-1394. https://doi.org/10.1080/02755947.2016.1227403

Purcell S.W., Blockmans B.F. 2009. Effective fluorochrome marking of juvenile sea cucumbers for sea ranching and restocking. Aquaculture 296: 263-270. https://doi.org/10.1016/j.aquaculture.2009.08.027

Purcell S.W., Blockmans B.F., Nash W.J. 2006. Efficacy of chemical markers and physical tags for large-scale release of an exploited holothurian. J. Exp. Mar. Bio. Ecol. 334: 283-293. https://doi.org/10.1016/j.jembe.2006.02.007

Rodríguez-Barreras R., Sabat A.M. 2015. Evaluation of three tagging methods in the sea urchin *Diadema antillarum*. J. Mar. Biol. Assoc. United Kingdom. 95: 1255-1260. https://doi.org/10.1017/S0025315415000302

Rodríguez-Barreras R., Sonnenholzner J. 2014. Effect of Implanted PIT-Tags on Growth, Survival, and Tag Retention in the Sea Urchin *Tripneustes ventricosus*. Caribb. J. Sci. 48: 132-137. https://doi.org/10.18475/cjos.v48i3.a02

Rodríguez-Barreras R., Wangensteen O.S. 2016. Assessing the reliability of two tagging techniques in the echinoid *Echinometra lucunter*. Reg. Stud. Mar. Sci. 5: 51-54. https://doi.org/10.1016/j.rsma.2016.01.006

Rodríguez A., Hernández J.C., Clemente S. 2016. Efficiency of calcein tagging on juveniles of the sea urchins *Diadema africanum* and *Paracentrotus lividus*. Mar. Ecol. 37: 463-469. https://doi.org/10.1111/maec.12287

Rodríguez-Barreras R., López-Morell J., Sabat A.M. 2017. Effectiveness of two tagging devices in the sea cucumber *Holothuria (Halodeima) grisea*. Mar. Freshw. Res. 68: 563-567. https://doi.org/10.1071/MF15473

Rogers-Bennett L., Rogers D.W., Bennett W.A., Ebert T.A. 2003. Modeling red sea urchin (*Strongylocentrotus franciscanus*) growth using six growth functions. Fish. Bull. 101: 614-626.

Russell M.P., Urbaniak L.M. 2004. Does calcein affect estimates of growth rates in sea urchins? In: Heinzeller T., Nebelsick J.H. (eds), Echinoderms: München - Proceedings of the 11th International Echinoderm Conference, 6-10 October 2003, Munich, Germany. Taylor & Francis Group, London, pp. 53-57. https://doi.org/10.1201/9780203970881.ch10

Sandford M., Castillo G., Hung T.C. 2020. A review of fish identification methods applied on small fish. Rev. Aquac. 12: 542-554. https://doi.org/10.1111/raq.12339

Sano M., Seki T., Omori M., Taniguchi K. 2001. A tagging method for the sea urchin *Strongylocentrotus nudus* (A. Agassiz) with Coded Wire Tag. Nippon Suisan Gakkaishi. 67: 23-29. https://doi.org/10.2331/suisan.67.23

Santos P.M., Albano P., Raposo A., et al. 2020a. The effect of temperature on somatic and gonadal development of the sea urchin *Paracentrotus lividus* (Lamarck, 1816). Aquaculture 528: 735487. https://doi.org/10.1016/j.aquaculture.2020.735487

Santos P.M., Ferreira S.M.F., Albano P., et al. 2020b. Can artificial diets be a feasible alternative for the gonadal growth and maturation of the sea urchin *Paracentrotus lividus* (Lamarck, 1816)? J. World Aquac. Soc. 51: 463-487. https://doi.org/10.1111/jwas.12656

Sato T., Yoseda K., Abe O., et al. 2013. Growth of the coconut crab *Birgus latro* estimated from mark-recapture using passive integrated transponder (PIT) tags. Aquat. Biol. 19: 143-152. https://doi.org/10.3354/ab00517

Searcy-Bernal R., Montaño-Moctezuma G., Anguiano-Beltrán C., et al. 2016. Preliminary Studies on the Use of Pit Tags to Evaluate Abalone Restocking. J. Shellfish Res. 35: 677-683. https://doi.org/10.2983/035.035.0314

Shao N., Chen J., Hu J., et al. 2017. Visual detection of multiple genetically modified organisms in a capillary array. Lab Chip. 17: 521-529. https://doi.org/10.1039/C6LC01330A

Shpigel M., McBride S.C., Marciano S., Lupatsch I. 2004. The effect of photoperiod and temperature on the reproduction of European sea urchin *Paracentrotus lividus*. Aquaculture 232: 343-355. https://doi.org/10.1016/S0044-8486(03)00539-8

Sonnenholzner J.I., Montaño-Moctezuma G., Searcy-Bernal R. 2011. Effect of three tagging methods on the growth and survival of the purple sea urchin *Strongylocentrotus purpuratus*. Panam. J. Aquat. Sci. 5: 414-420.

Stefánsson G., Kristinsson H., Ziemer N., et al. 2017. Markets for sea urchins: a review of global supply and markets. Intern. Matis Rep. Skýrsla Matís.

Sun J., Chiang F.S. 2015. Use and Exploitation of Sea Urchins. In: Brown N.P., Eddy S.D. (eds), Echinoderm Aquaculture. John Wiley & Sons, Inc., Hoboken, New Jersey, pp. 25-45. https://doi.org/10.1002/9781119005810.ch2

Tourón N., Paredes E., Costas D. 2022. Comparison of the Effectiveness of Different Tags in the Sea Urchin *Paracentrotus lividus* (Lamarck, 1816). In: Hufnagel L. (ed), Biodiversity of Ecosystems. IntechOpen, London, pp. 1-11. https://doi.org/10.5772/intechopen.102594

Wilson C.D., Arnott G., Reid N., Roberts D. 2011. The pitfall with PIT tags: marking freshwater bivalves for translocation induces short-term behavioural costs. Anim. Behav. 81: 341-346. https://doi.org/10.1016/j.anbehav.2010.10.003

Woods C.M.C., James P.J. 2005. Evaluation of passive integrated transponder tags for individually identifying the sea urchin *Evechinus chloroticus* (Valenciennes). Aquac. Res. 36: 730-732. https://doi.org/10.1111/j.1365-2109.2005.01277.x

Zakęś Z., Rożyński M., Demska-Zakęś K. 2019. Effect of PIT tagging on hematology and plasma composition of juvenile pikeperch (*Sander lucioperca* (L.). Aquac. Int. 27: 971-981. https://doi.org/10.1007/s10499-019-00357-7

APPENDICES

Fig. A1. – Boxplot representing the initial test diameter of *Paracentrotus lividus* at the beginning (T1) of a 60-day rearing in the laboratory using PIT-tags, coded wire tags (CWTs), calcein and an untagged control. The boxplot represents the minimum, maximum, median, first quartile and third quartile of the data set.

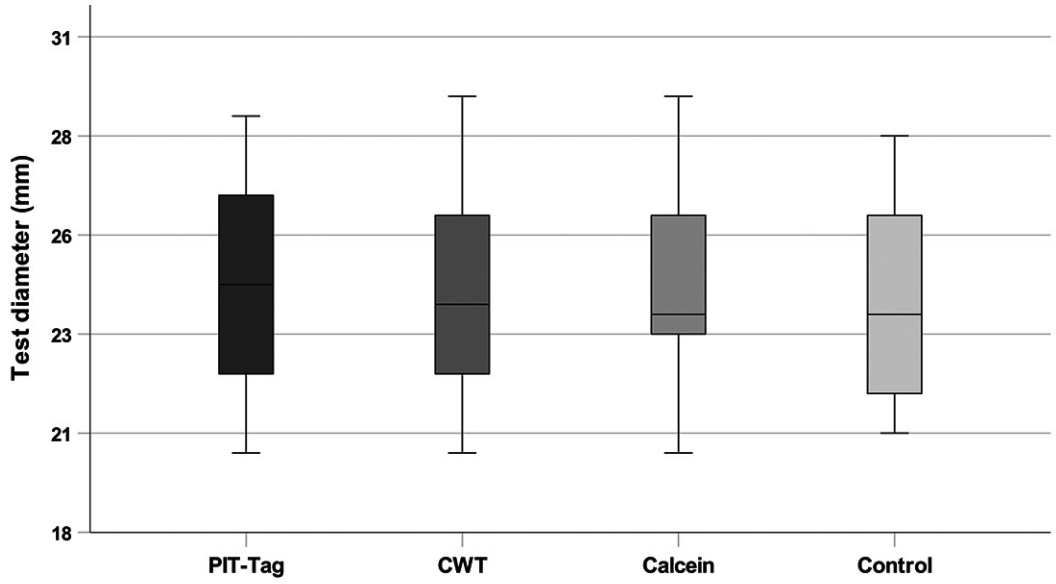

Fig. A2. – Boxplot representing the initial total wet weight of *Paracentrotus lividus* at the beginning (T1) of a 60-day rearing in the laboratory using PIT-tags, coded wire tags (CWTs), calcein and an untagged control. The boxplot represents the minimum, maximum, median, first quartile and third quartile of the data set and the dot represents an outlier.

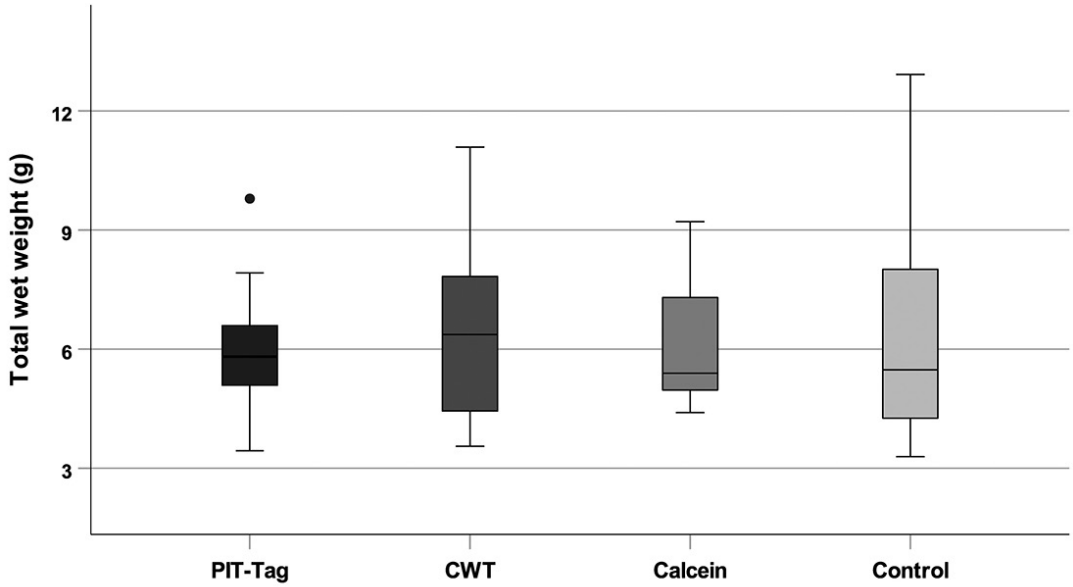

Fig. A3. – Boxplot representing the final total wet weight of *Paracentrotus lividus* at the end (T2) of a 60-day rearing in the laboratory using PIT-tags, coded wire tags (CWTs), calcein and an untagged control. The boxplot represents the minimum, maximum, median, first quartile and third quartile of the data set and the dot represents an outlier.

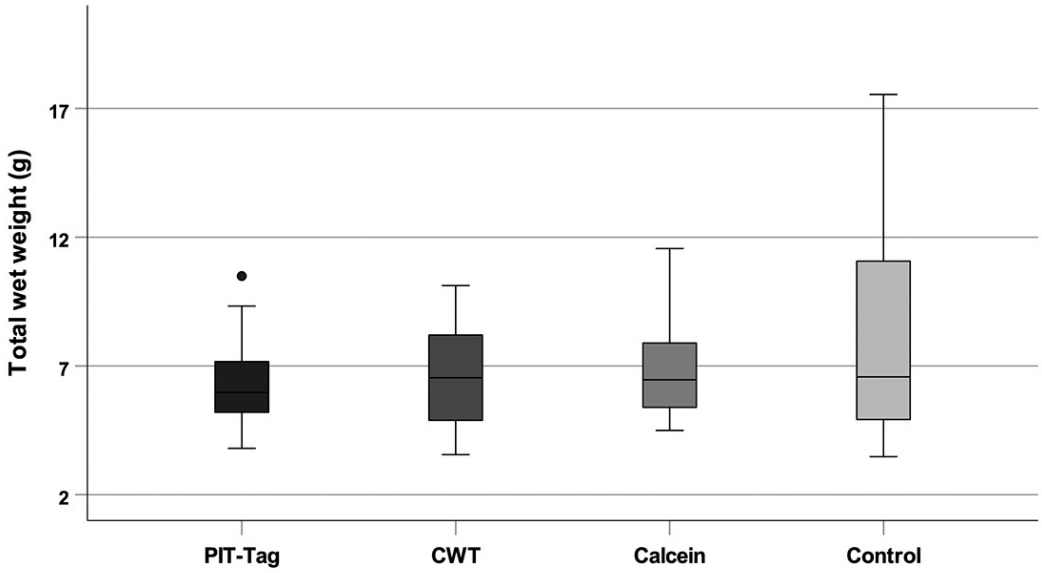

Fig. A4. – Photographs illustrating the PIT-tags, coded wire tags (CWT) and calcein used to tag *Paracentrotus lividus* for a 60-day experimental period in the laboratory. A, detail of the implantation of a Biomark HPT8 PIT-tag using a MK165 syringe with an N165 needle. B, the portable Biomark HPR Plus™ automatic reader used to detect PIT-tags in the coelomic cavity of the sea urchins. C, detail of the Mark IV Tag Injector, showing the CWT injection needle. D, the portable V-Detector (NMT), used to assess the presence of CWTs in the coelomic cavity. E, detail of the calcein bath (100 mg calcein L^{-1}) applied to *P. lividus* for 24 h. F, *P. lividus* tagged with calcein immersion and maintained in a recirculating aquaculture system for 60 days. A green, fluorescent stain is visible on the exposed part of the Aristotle's lantern (arrow) under UV light and yellow filter glasses.