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Technical note

Long-term maintenance of the sea urchin *Paracentrotus lividus* in culture

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ABSTRACT

The common sea urchin *Paracentrotus lividus* (Lamarck, 1816) is an important commercial species in the Mediterranean Sea for the consumption of its gonads (roe). This species has also long been used as an animal model in developmental biology and as an indicator in the assessment of environmental quality. In recent decades, the exploitation of this marine resource has become increasingly intensive, causing the depletion of wild stocks. The ripple effect observed in the laboratory use of this species has been the growing difficulty in finding valiant mature animals in the wild. We focused on the long-term maintenance of wild *P. lividus* and on the essential question of diet to maintain the animals and improve gonad development. The use of practical ration blocks which are nutrient-rich and show stability, easy storage and handling, resulted reduction in labor requirement and time for feeding streamlining the feeding practice. A significantly higher gonad production and a prolonged period of reproduction were obtained compared to wild caught individuals over the same period of time.

1. Introduction

The common sea urchin *Paracentrotus lividus* (Lamarck, 1816) is a regular edible echinoid, which is very widespread throughout the Mediterranean coasts and in the north eastern Atlantic, from Scotland to southern Morocco (Tortonese, 1965; Boudouresque and Verlaque, 2013). Over the years, several laboratories have chosen this species as an animal model. Molecular biology and eco-toxicology studies, which require the use of gametes and embryos at various stages of development (Giudice, 1973; Pagano et al., 1986; Pagano et al., 1993; Privitera et al., 2012), have been added to the classic studies on fertilization and development (Monroy, 1986). One of the basic requirements demanded by an experimental model is its availability throughout the year.

P. lividus living along the Italian coasts has a single reproductive period, which generally lasts from October to June with a peak from December to March. Gonads vary in size and gametogenetic state according to this annual cycle. These seasonal fluctuations lead to a limited availability of gametes at certain times of the year, which is a major limitation to using this model system in biological experimentation.

Sea urchins are also a valuable resource for the high commercial value of gonads (roe), and there is an international demand for the production of marketable quality gonads. *P. lividus* gonads are esteemed as a luxury sea food by Mediterranean countries. Due to its importance in research as an animal model and in aquaculture as seafood, much research has been carried out on this species to determine all the phases of the reproductive cycle and relate them to environmental characteristics (Byrne, 1990; Lozano et al., 1995; Spirlet et al., 1998; Sanchez-Espana et al., 2004; Sellem and Guillou, 2007; Garmendia et al., 2010). Three factors are universally cited as important to the reproductive cycle: diet, photoperiod and temperature. Copious work has been produced on the modification of the gametogenic cycle through experimental manipulation while rearing the sea urchins in confinement, to obtain gonads with features that increase their commercial value (Lawrence et al., 1997; Walker and Lesser, 1998; Spirlet et al., 2000; Shpigel et al., 2004; Shpigel et al., 2005; Kirchhoff et al., 2010; McCarron et al., 2010; Marsh et al., 2013; Sartori et al., 2015). This field is still underdeveloped because of each species of sea urchin has its own environmental or chemical cue (Kirchhoff et al., 2010). Food appears to play a pivotal role in the regulation of the reproductive cycle and it has been attested that the gonadic growth is strongly correlated with the availability, quantity and quality of food (Fernandez et al., 1995; Boudouresque and Verlaque, 2013 and ref. therein). Several studies have shown that sea urchins fed with high rations of good quality food improve their reproductive capacity. Therefore, one of the critical aspects in maintaining productive individuals in the laboratory is the determination of an optimal or at least efficient feeding regime.

This study was at first addressed towards the enhancement of the research status of *P. lividus*, improving their use as laboratory animal. A coveted result in this latter direction is control the reproductive cycle, maintaining individuals in a “ready to spawn” condition. This allows us to quickly obtain gametes (on demand) for their application in different

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fields, such as ecotoxicology and developmental biology. Thus, the objective of this paper is to describe a simple system and management focused mainly on the feeding practice to induce and control gonadal growth in a rapid timespan.

2. Methods

2.1. Sea urchin collection

Sea urchins were hand collected by scuba-diving from a rocky site of the gulf of Naples, along the southern Tyrrhenian coasts of Italy. A group of 100 *P. lividus* was collected in September 2013 (week 0) from a natural population and reared in a culture system for 18 weeks (rearing test period). Other groups of 10 individuals were collected from the field population at intervals of 6 weeks (week 6; week 12; week 18) to establish the population condition in the wild and for comparison with cultured population. Sea urchins were chosen to be relatively uniform in size (diameter 40.6 ± 1.5 mm, mean ± SD), and presumably in age, to minimize variation in growth potential, feed consumption potential and initial gonad weight. Specimens with mean diameters above 40 mm normally correspond to adult stages.

Captured animals were placed in a cooler and were carried to the laboratory under moist conditions within 2 h. In the laboratory, the sea urchins were measured and acclimatized for 1 week to confined rearing conditions before starting the feeding regime in the culture system.

2.2. Culture system

The culture system (Fig. 1) was addressed to the long-term maintenance of sea urchins and was tested during this study (rearing test period). It is still running without major changes at the Marine Resources for Research Facility of the Stazione Zoologica of Naples.

Sea urchins were held in suspended baskets (50 cm × 35 cm × 25 cm) in a recirculating system that received low flows of make-up seawater (2–3%) for compensating water losses associated with routine tank cleaning. Our system consisted of 2 square tanks (500 L), each containing up to 4 suspended baskets. Stocks up to 50 sea urchins can be maintained in a single basket. A centralized Life Support System (LSS) maintain optimal sea water conditions. This consisted of a reservoir equipped with cartridge filter, protein skimmer, ultraviolet sterilizer and refrigerator; a centrifugal pump recirculated natural seawater at a rate of 7.5 L min^{-1} to each tank. Aeration in the tanks provided additional water movement and air supply for the urchins.

Dissolved oxygen (> 90% saturation), pH (8.0 ± 0.1), and salinity (38.0 ± 0.2) were measured 3 times a week by a multi-parameter probe (YSI-85, USA). Seawater temperature, which was recorded daily, was 16 ± 1 °C and the photoperiod was set for 12 light: 12 dark. Ammonia, nitrite, nitrate and phosphate concentration were checked every week by a spectrophotometer (HACH USA, DR/2500) and values matches parameters required for a healthy recirculation system (Huguenin and Colt, 2002). Twice a week the tanks were cleaned removing uneaten food and fecal pellets by siphoning.

Table 1
Ingredients and the ratio at which they were mixed to prepare the Ration Blocks of Food (RBF) and proximate nutrient analysis (per g dry matter). Energetic level of food was calculated as gross energy by burning sample of wet food in a bomb calorimeter.

Ingredients	Ration (%)
Algae	38
Mussels	25
Corn	17
Supplements	12
Agar-agar	8
Nutrients	% dry matter
Carbohydrate	21
Crude protein	20
Crude fat	1.5
Crude fibre	9
Minerals	14
Ash	20
Gross Energy (MJ Kg^{-1})	12

2.3. Feeding practice

We drew up a mixed diet based on animal meal (seafood) and vegetable meal (natural algae) and used this to formulate a prepared "ready to use" food. We produced a compound combining dry powdered ingredients with agar–agar as binder (modified by Nagai and Kaneko, 1975), to form a moist pellet with sea water. The resulting mixture was molded before solidifying in Plates 1 cm thick, from which Ration Blocks of Food (RBF) were cut by hand. Diet ingredients included mussel meal, corn, natural macro-algae (*Ulva lactuca*) and microalgae (*Spirulina platensis*), fish oil and mineral supplement (calcium carbonate) (Table 1). Nutritional analysis was conducted at the Laboratory "ANALISIS" of Angri (Salerno; Italy). The formulated food was analyzed in duplicate to determine its crude protein, fat, moisture, ash, fibre, carbohydrate and gross energy contents (Table 1) using protocols according to the regulation of the DM 18/03/09 (Directive 2008/100/CE).

We shaped RBF weighing ~1 g to feed sea urchins in culture twice a

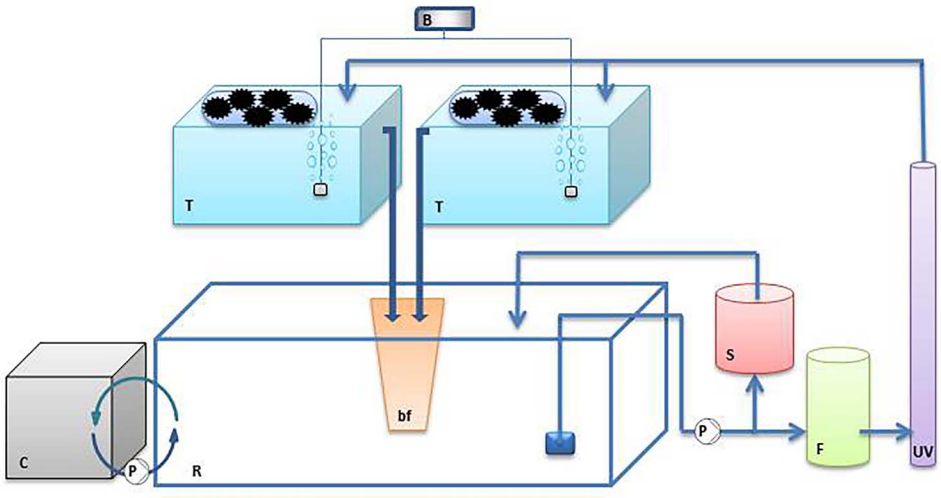

Fig. 1. Schematic diagram of sea urchins culture system. **T** = Tanks; **C** = Chiller Unit; **R** = Reservoir; **bf** = bag filter; **S** = Protein Skimmer; **F** = Cartridge Filter; **UV** = Ultraviolet sterilizer; **B** = Blower; **P** = Centrifugal Pump.

week. The weekly amount of RBF was provided at a ratio of 3–5% of the wet sea urchin biomass. We found that this quantity corresponded to rations which were always completely eaten.

2.4. Evaluation parameters

Sea urchins were monitored over time and some indicators were selected for assessing the effectiveness of the applied feeding practice in the culture system. We chose as indicators in this study the following parameters: biometrical measures; spawning tests as measure of the ability to spawn, *i.e.* to reproduce; gonad index values, as gonadal growth. Histological analysis was performed for assessing the maturation state of gonads. The rearing test started in September and lasted 4 months, covering the season from pre- to reproductive periods in the gulf of Naples. At intervals of 6 weeks, 10 sea urchins in long-term maintenance (LM) were randomly selected from the culture system and tested. A group of 10 sea urchins (wild) was also harvested from wild populations as the first sampling after 6, 12 and 18 weeks. They were tested to access the population condition in the wild and for comparison with cultured population.

2.4.1. Biometrical measures

Test diameters without spines were measured in millimeters with a plastic Vernier caliper and the wet weights in grams were obtained using a precision balance (Mettler Toledo PL 202S).

2.4.2. Spawning test

The spawning test aimed to provide an indirect assessment of the reproductive ability and maturation state of cultivated sea urchins. Gonad maturation is assumed as directly proportional to the ease in spawning and inversely proportional to the inducing stimulus, as mentioned for other sea urchins (Scheibling and Hatcher, 2013). The practical procedure consisted in the application of progressively more invasive stimuli, as described by Cirino and Toscano (2012). An increasing value for the related positive spawning was assigned to the stimuli in the following sequence: manual shacking of the animal, with value 1; electrical shock at low voltage, with value 2; intracoelomic injection of potassium chloride (1 mL of KCl 0,5 M), with value 3. Negative spawning was assigned value 4.

2.4.3. Gonad index value

The Gonad Index (GI) values derived from the ratio between the gonad weight and the total body weight and indicated the relative gonadal growth during the different stages of the sea urchins reproductive cycle. Coupled with the histological analysis of gonads, the GI values gave us a measure of the growth trend and level of maturity reached. For GI calculation and histological analysis, individuals were sacrificed and the gonads were extracted and freshly weighed for the following GI evaluation

$$GI = [\text{gonad wet weight } (g\,kg^{-1})/\text{sea urchin wet weight } (g\,kg^{-1})] \times 100$$

2.4.4. Histological analysis

A single gonad from each tested animal was fast fixed in Bouin's fluid for 12–24 h at 4 °C, dehydrated in an ethanol series, vacuum-embedded in paraffin wax and then sectioned at 7 μm. They were mounted on slides and stained with alcian periodic acid Schiff reagent (AB/PAS) method. Finally, the sections were dehydrated through an ethanol series, cleared in xylene, mounted, observed and photographed. Histological analysis followed the classification schemes of Byrne (1990) and Spirlet et al. (1998) in which successive stages (between spent and mature/spawned gonads) are recognized for gametogenesis during the reproductive cycle. Gonads were classified in 6 stages.

2.5. Statistical analysis

Mean and standard deviation of the gonad index and reproductive ability (spawning test) were calculated for 10 sea urchins (captured in wild or cultured in lab) at each time, i. e. on 6th, 12th and 18th weeks. Student *t-test* was used to compare means. All the statistical analysis was performed using Microsoft Excel software.

3. Results

3.1. Growth and survival

Diameter size of the sea urchins did not change over the 4 months of experiment. No sea urchins died during this period.

3.2. Feeding practice

The Ration Blocks of Food (RBF) showed to be a very efficient way for feeding practice. It was observed that RBF was easy to prepare, manage, and stock, very attractive to sea urchins and stable in water even after freezing. Preparation is very rapid and it can be perfectly fractioned and weighed according to the needs. RBF can be easily handled and stored frozen, which did not affect water stability and attractiveness. In addition, RBF is extremely versatile and can be used for different experimental purposes by changing the composition.

RBC are readily consumed by the sea urchins. Attraction and ingestion, which are the usual means of evaluating the response of sea urchins to food (Lawrence et al., 2013), were clearly evident in the behavioral response following the introduction of the blocks of food in tank. Sea urchins moved towards food, extending and waving their tube feet in order to find it. Blocks were always ingested within a few hours. The conservation of rheological properties of RBF was verified after 50 h in sea water, that is a long time to be consumed by the animals. No rotten or melted blocks were observed after that time.

3.3. Reproductive ability and gonad growth

At the starting point (week 0) almost all tested animals gave negative results in the spawning test (80% value 3–4; 20% value 1) (Fig. 2). At the end of the rearing period test (week 18), all tested LM animals in culture gave positive outcomes in the spawning tests (100%). After 6 weeks, 60% of the LM sea urchins spawned following manual shacking (value 1) or electrical shocking (value 2). All of the tested sea urchins spawned after shacking (value 1) from the 12th week (Fig. 2a). In Fig. 2b the Wild controls showed the expected seasonality, with a growing increase in reproductive ability throughout the rearing test period (20% value 1 at week 0; 40% value 1 at week 6; 60% value 1–2 at week 12; 60% value 1 at week 18).

The GI values of animals entering the culture system (week 0) ranged between 4.6 and 6.4 (mean value 5.3 ± 0.62) (Fig. 3). The LM sea urchins in culture showed very rapid gonad growth, increasing GI values during the 4 months. The final average value of the GIs of these animals after 18 weeks was 19.1 ± 1.8 SD (8.7 ± 1.7 SD at week 6; 13.8 ± 1.5 SD at week 12). GI values of wild sea urchins collected as controls during all the rearing period attested a steady decline of gonadal mass (2.7 ± 0.5 SD at week 6; 1.8 ± 0.3 SD at week 12; 1.5 ± 0.7 SD at week 18), which significantly differ from GI of cultured sea urchins ($N = 10$; $P < 0.001$).

3.4. Gonad development

Gonad histology of sea urchins at week 0 revealed the reproductive status of animals in an advanced recovery stage (Fig. 4a; b). The ovary showed clusters of oocytes at different maturation stages along the acinal wall, the nutritive phagocites (NPs) forming a dense meshwork all over the acinus and some ova already visible.

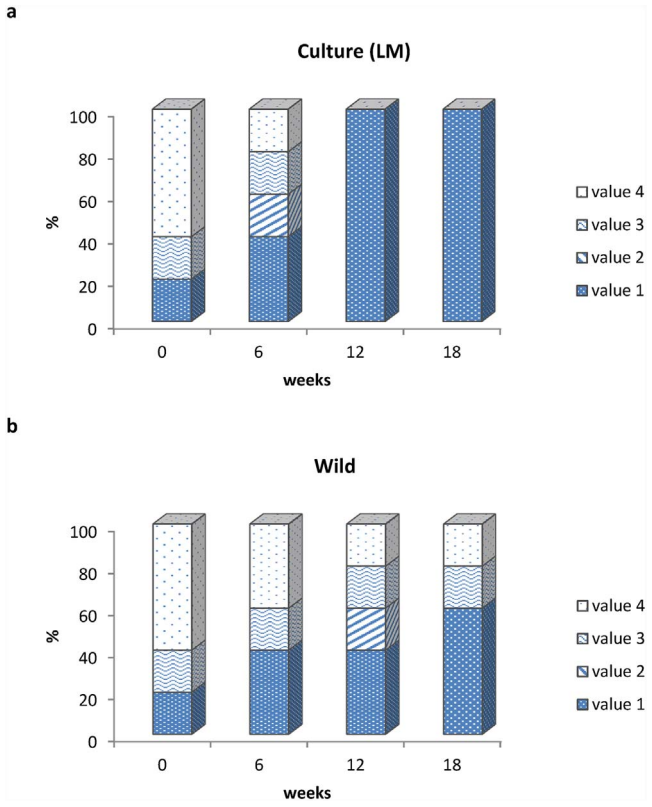

Fig. 2. Reproductive ability (%) of the cultured long-term maintenance (a) and of wild (b) sea urchins. Spawning tests were used to assess the readiness to release gametes of sea urchins. At stimuli progressively more invasive correspond increasing values for the related positive spawning, from the value 1 for individuals promptly spawning to the value 4 for individuals not spawning.

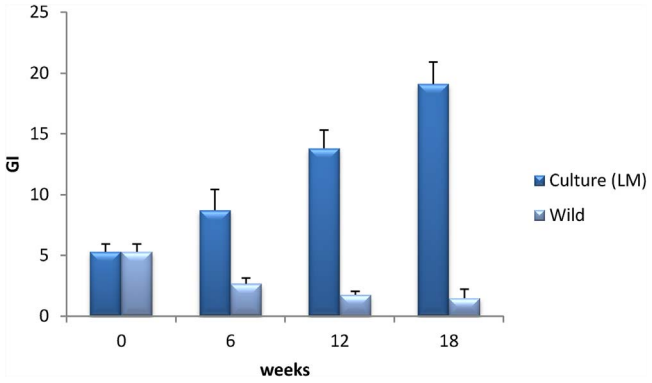

Fig. 3. Gonad Index (%) of the sea urchins under culture conditions (LM) compared with the wild sample ones (Wild). Values are the mean ± SD for each sampling points.

Gonads of wild sea urchins shown in Fig. 4c (week 6) and Fig. 4e (week 12) continued in the maturation process showing a progressive rise in the amount of big oocytes and ova in the acina and the simultaneous detriment of the NP mesh. In January (week 18) it was observed most cases of mature gonads, full of advanced developing stages with residual trace of NP (Fig. 4g).

Gonads of LM sea urchins maintained in the culture system over the rearing test period (Fig. 4d, w6; f, w12; h, w18) were histologically ranked between the *growing stage* and the *premature stage* described by Byrne (1990) and Spirlet et al. (1998). The gonads of examined individuals were permanently in an "*active growing up stage*", which enabled animals to replace promptly released eggs. The NP mesh was largely present and indicated that the vitellogenesis was active.

The relative frequency of reproductive status of *P. lividus* gonads under culture conditions were compared with wild conditions during the rearing test, at each time interval (week 0; 6; 12; 18) (Fig. 5). From week 12 onward, all sea urchins maintained in culture were mature. At the same time, wild individuals showed different frequencies of the maturation stages and lower frequencies of mature sea urchins (20% at week 12 and 70% at week 18).

4. Discussion

The physical conditions in our culture system were maintained quite stable for the whole rearing test time (T = 16 °C ± 1, typical of cold months in the gulf of Naples; photoperiod 12light: 12dark, typical of middle season) to minimize the influence of environment on the growth and maturation of gonads. In wild populations of *Paracentrotus lividus*, as in most temperate echinoids, gonads undergo an annual cycle. In particular, sea urchins living along the Italian coasts have a single reproductive period which generally lasts from October to June, when temperature begins to raise. The peak of maturity is observed from December to March, when the percentage of mature individuals can reach up to 100%. Before and after this central period, most animals show gonads in pre-reproductive (*growing stage* and *premature stage* of Byrne, 1990) or post-reproductive phases (*partly spawned stage* and *spent stage* of Byrne, 1990). Regardless of the minor differences in the descriptions of reference authors (Byrne, 1990; Spirlet et al., 1998), histological analysis of gonads reveals that gonads accumulate reserve material during the growing phase. Spawning typically takes place during the maturation phase, after which relict gametes are re-absorbed by nutritive phagocytes and gonads become virtually devoid of sexual cells. The variation in size and gametogenic state of sea urchin gonads during the reproductive cycle is related to the relative abundance of nutritive phagocytes (NP) that support the development of sexual cells *versus* the amount of the gametes themselves (Marsh et al., 2013).

According to our evidence, the sea urchins collected and examined in September (week 0; Fig. 4a; b), had begun their breeding season and would have likely reached maturity as of December. The analysis of the following results from the wild sea urchins collected as controls over the test periods (week 6 and week 12) confirmed the ongoing process of the reproductive cycle (Fig. 4c; e). At the same time, GIs started to drop as usual at the onset of gametogenesis, decreasing until the end of the spawning season (Fig. 3). The histology of gonads from the tests at week 18 showed in most cases mature gonads, supporting the positive results for the reproductive activity (spawning tests; 60% value 1) (Fig. 4g). The low value of GIs (mean 1.5 ± 0.7 SD at week 18) from these sea urchins was a sign that these gonads were going towards a *spent stage* after the complete resorption of tissues. One third of the wild sample tested at that time confirmed this hypothesis showing gonads in spent stage (Fig. 5). The comparison between the reproductive patterns shown by cultured (LM) and wild sea urchins at the end of the rearing period (week 18) gave clear evidence of the differences in the maturation process of their gonads and in the future fate after spawning of the gametes.

The high GI values of LM sea urchins maintained in the culture system (from 8.7 ± 1.7 SD at week 6–19.1 ± 1.8 SD at week 18) were largely ascribable to the increased amount of the NP mesh indicating active vitellogenesis. The histological analysis showed that the gonads of LM females in culture were in an "*active growing up stage*", characterized by continuous vitellogenesis and oocyte maturation (Fig. 4d; f; h). This condition enabled LM animals to replace promptly released eggs, giving them a permanent potential reproductive ability. Moreover, the possibility of obtaining gametes inducing spawning without the need for an invasive stimulus (Fig. 2a LM), makes it possible to re-utilize animals for permanent gonadic production in the laboratory, independent of any seasonal cycle of gonadic production in the field.

The high gonad production and the prolonged period of reproductive ability of cultured sea urchins (LM) suggested that they likely were in a good nutritional state. This is congruent with extensive

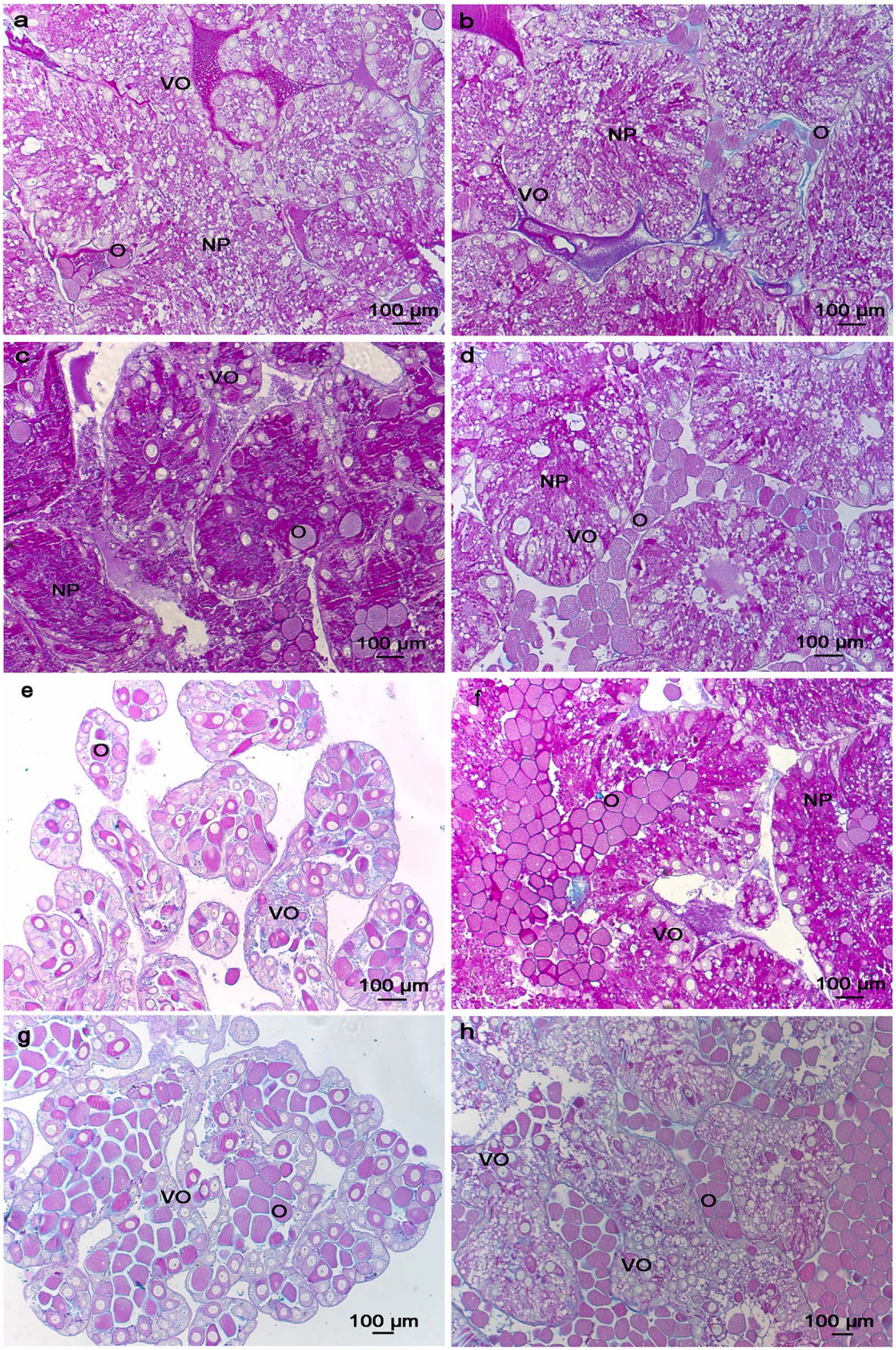

Fig. 4. Representative micrograph of histological sections of *Paracentrotus lividus* ovaries from wild and cultured sea urchins. Gonads in advanced recovery stage at beginning of the rearing period (week 0) (a; b). Ovaries in growing/premature stage (week 6) in wild sea urchins (c) and cultured ones (d). Ovaries in premature/mature stage (week 12) in wild sea urchins (e) and cultured ones (f). *P. lividus* mature ovaries (week 18) in wild sea urchins (g) and cultured ones (h). O = ova; NP = nutritive phagocytes; VO = vitellogenic oocytes.

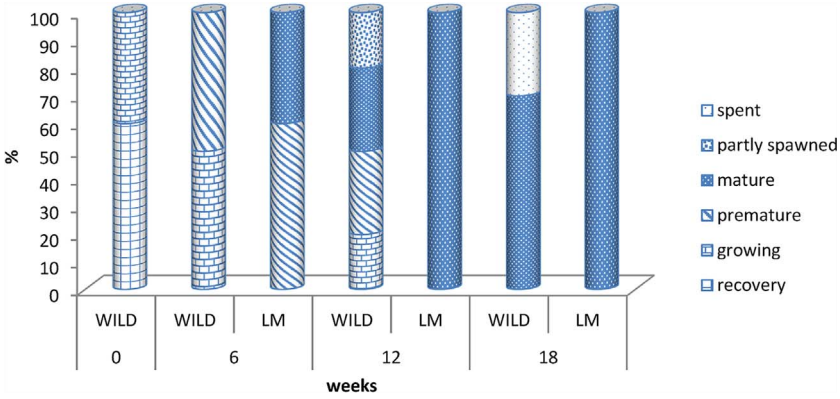

Fig. 5. Reproductive status of *P. lividus* gonads under culture conditions compared with wild conditions during the rearing test (week 0; 6; 12; 18). Histograms show the relative frequency of the 6 maturation stages observed in histological sections of the tested animals (classification according to Byrne, 1990). Representative histological patterns of the gonads are shown in Fig. 4.

literature attesting that sea urchins fed with high rations of good quality food improve their reproductive capacity, allocating food energy to increases in gonadal production (Fernandez and Boudouresque, 2000; Schlosser et al., 2005; Watts et al., 2013).

On this basis, copious work has been carried out on the diet of sea urchins attesting that formulated feeds, composed of animal meal (sea food) and vegetable meal (natural algae) appear to cover all the food needs of *P. lividus* (Lawrence et al., 1992; Fernandez et al., 1995; Fernandez and Pergent, 1998; Fernandez and Boudouresque, 2000; Spirlet et al., 2001; Schlosser et al., 2005; Fabbrocini and D'Adamo, 2010; Fabbrocini et al., 2012). Recent studies also confirm the benefits of a feeding regime of formulated feed and natural algae (*Ulva* sp.) for grow-out of sea urchins (Cyrus et al., 2014a; Cyrus et al., 2015).

Our experimental approach to nutrition of the cultured sea urchins was to consider the nutrient content of all feed ingredients in the diet and to plan the appropriate evaluation of outcomes. We chose natural raw materials as a source of nutrients to formulate the RBF diet focusing on the supply of the 3 important nutrients (proteins, carbohydrates and lipids). Natural food sources rich in these nutrients were chosen and mixed in order to obtain cost effective food formulation. The choice of ingredients and the ratio at which they were combined to formulate the prepared food was also done by considering the natural feeding habits of this species. *P. lividus* as a species is basically herbivorous and builds a calcareous exoskeleton, so it is reasonable assume that minerals are very important, which are likely supplied for the most part by algae and sea water. We have taken into account this special need adding minerals in the formulation of the prepared food ($CaCO_3$) and using seawater in the preparation of the agar–agar gel (Table 1). Moreover, in our food formulation we have considered the algal component as a major ingredient. Studies on the advantages of adding *Ulva sp*. in formulated food for the bioavailability of nutrients and mainly as a feeding stimulant have recently confirmed the importance of natural algae in the diet of sea urchins (Cyrus et al., 2014a; Cyrus et al., 2014b; Cyrus et al., 2015). Our outcomes showed that the nutrients ratio in the RBF diet was adequate both for gonadic growth and gamete production, providing balanced rations to livestock sea urchins.

Finally, the ration blocks have other advantages such as their constant quality, the decrease in labor requirement and time for feeding, and easy storage which reduce the seasonal disparity in feed availability. The concept of using ration blocks for feeding livestock animals is not new. It was introduced in feeding practices for ruminant production, revealing advantages for the entire supply chain (FAO, 2012). However, the present application for the raising of sea urchins is new and could also be a cutting edge practice with possible implications in the echinoculture field.

5. Conclusions

The aim of the present work was firstly to maintain productive individuals in the laboratory for research purposes. We found that ration blocks of food (RBF) feeding practices respond perfectly to the scientific use of *P. lividus* with wider perspectives. The food ration blocks can be customized according to scientific experimental needs. Blocks of different formulations can be made using different ingredients, including chemicals, feed additives and medicines, encouraging experiments in various fields of biology and ecology. Moreover, ration blocks were conceived as individual portions of food. By varying the ratio number of blocks per animal, they also make it possible to modulate the amount of food to be supplied according to the purposes of the maintenance program. The methods and results presented here are useful to a wider range of experts, including those interested in the laboratory applications of *P. lividus* and those involved in echinoculture and aquaculture research.

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Ethical statement

Paracentrotus lividus sea urchins were collected from locations that are not privately-owned nor protected in any way, according to the regulation of the Merchant Navy now called the Ministry of Agriculture, Food and Forestry (DPR 1639/68, 09/19/1980 confirmed on 01/10/2000). The Stazione Zoologica Anton Dohrn of Naples was approved by the Ministry for the Merchant Marine as an "Accredited Scientific Institution" and it is in compliance with all formalities according the Art. 27, 29, 30 of the DPR 1639/68. All animal procedures were in compliance with the guidelines of the European Union (directive 2010/63/U.E.).

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