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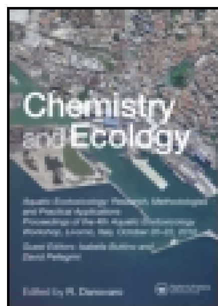

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Gametes and embryos of sea urchins (*Paracentrotus lividus*, Lmk., 1816) reared in confined conditions: their use in toxicity bioassays

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Sea urchins of the species *Paracentrotus lividus*, collected along the Southern Adriatic coast at the end of their reproductive season, were reared in a recirculating aquarium under controlled temperature and feeding conditions in order to induce off-season maturation of gametes to be used in toxicity bioassays. After 14 weeks of rearing, the gonad index was calculated, histological analyses of the gonads were performed, their fertilisation ability was measured and the EC_{50} for copper in embryotoxicity bioassays was evaluated. The sperm motility pattern (based on the percentages of total motile and rapid sperm, together with sperm velocity parameters) was assessed using a computerised analyser. All specimens showed a significant increase in gonad yield with respect to field-collected animals and were in pre-spawning or spawning stages. The motility parameters, fertilisation ability and EC_{50} level of the collected gametes were comparable to those of field-matured specimens. In conclusion, the sea urchins reared under experimental conditions were characterised by faster gonad maturation than the field population, producing viable gametes and larvae whose biological response in toxicity bioassays was similar to that of field-reared specimens of *P. lividus*.

Keywords: sea urchin; *Paracentrotus lividus*; aquaculture; gonad index; histological analyses; computerised sperm motility analyses; fertilisation; embryotoxicity test

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1. Introduction

Echinoderm gametes and larvae are among the most widely used biological tools for the quality assessment of coastal waters [1–5]. The availability of gametes and larvae characterised by high quality and low biological variability, regardless of the seasonal reproductive cycle, is a key factor in guaranteeing the reliability and reproducibility of bioassays [6,7].

Off-season broodstock conditioning may be a useful strategy to achieve this goal. Food availability, temperature and photoperiod are important triggers of the gametogenic cycle in both *Paracentrotus lividus* and other sea urchin species in natural conditions [8–10]. Much research has been carried out into the induction of alterations to the gametogenic cycle by manipulating the physical factors known to be involved in the sea urchins' reproductive cycle while rearing the

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animals in captivity [11–14]. The effects of diet composition on gonad yield and gonad quality factors [15–17] and the effects of feeding regime and rearing system structure on juvenile growth [18–21] have been widely investigated.

However, because these studies have focused on the production of sea urchins for aquaculture purposes, the experimental rearing protocols are often not laboratory-scaled, but are conducted in high-volume aquaria, which often require long cleaning times and a continuous seawater supply.

However, if the aim of the conditioning procedure is the production of gametes and embryos for laboratory purposes, i.e. ecotoxicological or developmental biology research [22–24], such production needs to be carried out in accordance with rearing protocols, and in laboratory-scaled closed rearing systems that can be easily managed even at some distance from the sea [6,18]. In addition, if high-quality gamete production, and not simply gonad yield, is the main goal of the rearing procedure, great care must be taken with the diet, as gamete quality is known to be greatly influenced by broodstock feeding conditions [25,26].

The aim of this study was to optimise a rearing protocol for the conditioning of adult *P. lividus* in a laboratory-scaled recirculating aquarium, evaluating the effects of rearing conditions on the biological response of the obtained gametes and embryos.

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2. Materials and methods

2.1. *Sea urchin collection*

Sea urchins (*P. lividus*) were hand-collected with the aid of Scuba-diving equipment in July 2009 from the rocky seabed near Termoli (41°54′N; 16°10′E) on the southern Adriatic coast of Italy at a depth of <2 m. Sea urchins were immediately placed in a cooler, wrapped in a wet cloth to insulate them from the ice, and carried to the laboratory within 1 h.

2.2. *Experimental design*

On arrival in the laboratory, the sea urchins were measured (horizontal diameter) by calliper (0.05 mm accuracy) and adult specimens (with a test diameter of between 35 and 45 mm excluding spines) were selected for the experimental trials. In accordance with our standardised procedure, sea urchins that spawn during transfer to the laboratory are discarded, however, this did not occur in this case.

Twenty randomly selected specimens were immediately sacrificed to determine the t0 gonad index, reproductive stage, motility parameter values, fertilisation ability and EC_{50} level for copper in embryotoxicity assays.

The remaining sea urchins were slowly acclimatised to the rearing temperature of the experimental aquarium in a small tank, with no additional aeration. Specimens were continuously checked so that spawning sea urchins could be immediately removed, preventing them from inducing spawning in the others. However, in this case no spawning event occurred; and indeed no viable gametes were recorded in the t0 evaluations (see Section 3.3).

Selected sea urchins were then placed in a recirculating aquarium and acclimatised for 5 days to the confined rearing conditions. At the end of the 5-day acclimatisation period, injured individuals were discarded and 20 specimens were selected for the trial.

The sea urchins were reared for up to 14 weeks in a closed rearing system consisting of two rectangular interconnected tanks (100 L each) with an external filter, denitrator, protein skimmer, UV steriliser, refrigerator and centrifugal pump that recirculated natural seawater (from the Tremiti islands Marine Protected Area, southern Adriatic Sea) at 50 L·min^{-1}.

Aeration in the tanks provided additional water movement and air supply to the sea urchins. The abiotic parameters were measured daily by multiparametric probe (YSI 6920) and kept at constant temperature (18 ± 1 °C), salinity (36 ± 1‰), pH (8.00–8.20) and dissolved oxygen (>90%). Temperature and salinity ranges reflected field conditions, as recorded during sampling. Because the rearing system was adjacent to the windows, the entire experiment was kept in a natural photoperiod (from 16 h L : 8 h D to 12 h L : 12 h D). As only one tank was used in this trial, the other was isolated from the water circuit; therefore, the stock density was 0.2 individuals·L^{-1}.

The sea urchins were fed *ad libitum* on pelletised (2.5 × 2.5 × 5 mm) formulated feed (Classic K®, hendrix SpA, Mozzecane – VR, Italy). Twice a week the tanks were cleaned and uneaten feed was removed and replaced; feed was supplied at a rate of 2% of sea urchin biomass·day^{-1}. The approximate nutritional values of the formulated feed are shown in Table 1. These rearing conditions were found to be optimal in a previous 4-week *P. lividus* rearing trial [27].

Table 1. Composition of the commercial formulated feed (Classic K®, hendrix, SpA); proteins of animal origin account for less than 5%.

	Dry mass (%)
Crude protein	46.5
Crude fat	10.5
Crude fiber	2.4
Ashes	9.5

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2.3. *Evaluation parameters*

Evaluation of the gonad index (GI) and histological analysis of gonads were performed on the reared specimens (Reared 14-wk) and compared with those of specimens collected in the field at t0 (Field t0) and at the end of the trial (Field 14-wk). Because no or few viable gametes were recovered from Field t0 and Field 14-wk specimens, in November, 20 sea urchins (Field Control) were again collected in the same area as the previous ones, in order to compare the quality of gametes from laboratory-reared specimens (Reared 14-wk) and naturally maturing animals (Field Control).

2.3.1. *Gonad index*

Twenty sea urchins from each group (Reared 14-wk, Field t0, Field 14-wk) were allowed to drip for ~5 min, weighed (0.2 mg accuracy) and then dissected; the gonads were extracted and fresh weighed for the GI evaluation:

$$\text{GI} = \text{gonad wet weight (g)}/\text{sea urchin wet weight (g)} \times 100$$

2.3.2. *Histological evaluation*

One of the five gonads of each animal was fixed in 10% formalin and embedded in paraffin; 7-μm sections were obtained by microtome, placed on slides and stained with Mayer's haemalum and eosin. The slides were observed by microscope and each was assigned a reproductive stage using the nomenclature in Byrne [8]: stage I (recovery), stage II (growing), stage III (premature), stage IV (mature), stage V (spawning) and stage VI (spent).

2.3.3. *Gamete evaluation*

Gamete evaluation analyses were performed on Reared 14-wk specimens and Field Control specimens. Each individual was sexed by examining the extruded gametes. An aliquot of sperm or eggs from each animal was diluted in 34‰ artificial seawater (ASW) [28] and observed under a microscope for a preliminary evaluation of sperm motility and egg morphology.

For the assessment of fertilisation ability, pools of male and female gametes were created. Dry sperm was diluted in ASW at a ratio of 1:1000, added to ASW-washed eggs at a ratio of sperm to eggs of 15,000:1 and incubated in 10 mL polystyrene multiwell dishes until fertilised eggs (30 min, 18 °C, in the dark) and plutei larvae were obtained (72 h, 18 °C, in the dark). Samples were preserved in concentrated buffered formalin, and the percentages of fertilised eggs and plutei with normal development were scored by observing 200 eggs (or larvae, respectively) for each sample [29]. Six replicates for each experimental group were performed.

The sperm motility pattern was evaluated by computerised analyser on individual semen samples (nine males for each group). Dry sperm was diluted in ASW at a ratio of 1:1000; sperm movement was recorded using a 100 frame$\cdot$s^{-1} camera (Basler, 782 × 582 resolution) attached to a microscope (Nikon Eclipse E600) with a phase-contrast objective (10 × 10 magnification) and connected to a computerised motion analysis system, the Sperm Class Analyzer® (SCA, Microptic, s.l., Spain). The SCA acquisition parameters were set as follows: maximum area $= 400\,\mu m^2$ minimum area $= 50\,\mu m^2$; frame rate $= 100 \cdot s^{-1}$; total captured images = 100.

For each semen sample, six motility records were taken in six different microscopic fields; each record consisted of the mean of three replicates, each analysing 250–500 sperm tracks.

The following motion parameters were also assessed:

(1) Sperm motility classes: Percentages of total motile, rapid (velocity $>100\,\mu m \cdot s^{-1}$), medium ($45\,\mu m \cdot s^{-1} <$ velocity $<100\,\mu m \cdot s^{-1}$) and slow ($10\,\mu m \cdot s^{-1} <$ velocity $< 45\,\mu m \cdot s^{-1}$) sperm. Spermatozoa with a velocity of $< 10\,\mu m \cdot s^{-1}$ were recorded as static.
(2) Curvilinear velocity (VCL; $\mu m \cdot s^{-1}$): the velocity of the sperm head along its real curvilinear track, as perceived in two dimensions under the microscope.
(3) Straight-line velocity (VSL; $\mu m \cdot s^{-1}$): the velocity of the sperm head along its linear track between its initial and final positions.
(4) Average path velocity (VAP; $\mu m \cdot s^{-1}$): the velocity of the sperm head along its spatial average trajectory.

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2.3.4. *Embryotoxicity tests*

Embryotoxicity tests were performed using ASW for the negative control and geometrically scaled copper solutions as reference toxicants for the positive controls, as described in Losso et al. [4]. Briefly, fertilised eggs were incubated in 10-mL polystyrene multiwell dishes containing the test solutions at a final density of 200$\cdot$mL^{-1} until plutei larvae were obtained (72 h, 18 °C, in the dark). Samples were preserved in concentrated buffered formalin, and the percentages of plutei with normal development were scored by observing 200 larvae for each sample.

2.3.5. *Statistical analyses*

At the end of the rearing trial, the statistical differences in the evaluation parameters among the experimental groups (t0, reared and controls) were determined [30]. Prior to analysis, percentage data were arcsine-transformed and tested for normality using Shapiro–Wilk's test and for homogeneity of variance using Cochran's test. The data on gonad indices (t0, Reared 14-wk and Field 14-wk groups) did not pass the homogeneity of variance test, so non-parametric Kruskal–Wallis

analysis of variance (ANOVA) on ranks was performed. No quantitative analysis of histological preparations was conducted.

The statistical significance of the differences in sperm motility data and percentages of fertilised eggs and normally developed plutei larvae between Field Control and Reared 14-wk specimens were determined using one-way ANOVA. The data on total motile and rapid spermatozoa and on velocity (VCL, VSL and VAP) did not pass the homogeneity of variance test and were therefore analysed using non-parametric Kruskal–Wallis ANOVA on ranks.

The EC_{50} values (95% confidence limits) for copper in the embryotoxicity tests were calculated using the Trimmed Spearman–Karber statistical method [31].

All analyses were performed with the StatSoft, Inc. (2008) STATISTICA data analysis software system, version 8.0.

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3. Results

Results are shown in Tables 2–3 and in Figures 1–3.

3.1. *Gonad index*

As reported in Table 2, the GI increased greatly in reared sea urchins ($H_{2,60} = 42.106, P < 0.001$), and was significantly higher than both Field t0 and Field 14-wk samples ($P < 0.001$).

3.2. *Histological evaluation*

The histological analyses (Figure 1) explain the GI values. In the Field t0 sea urchins, ~40% of specimens were in spent phase (stage VI). Unspawned gametes were still present, but no

Table 2. Gonad indices (±SD, n = 20/group) of reared *P. lividus* (Reared 14-wk) and of *P. lividus* collected in the field at the beginning (Field t0) and at the end of the rearing trial (Field 14-wk).

	GI
Field t0	2.16 ± 1.54
Reared 14-wk	14.02 ± 2.55
Field 14-wk	6.70 ± 4.07

Table 3. Percentages (mean values ± SD) of Fertilization (FEC) and of Normal Developed Plutei Larvae (NPL), and EC_{50} levels ($\mu g \cdot g^{-1}$ copper) of reared *P. lividus* (Reared 14-wk) and of *P. lividus* collected in the field during the spawning season (Field control). At the beginning (Field t0) and at the end of the rearing trial (Field 14-wk) tests were not performed as no or few gametes were recovered.

	FEC	NPL	EC_{50}
Field t0	–	–	–
Reared 14-wk	85.7 ± 4.4	80.7 ± 2.9	47.9 ± 5.8
Field 14-wk	–	–	–
Field control	83.3 ± 4.4	80.5 ± 3.7	48.9 ± 5.0

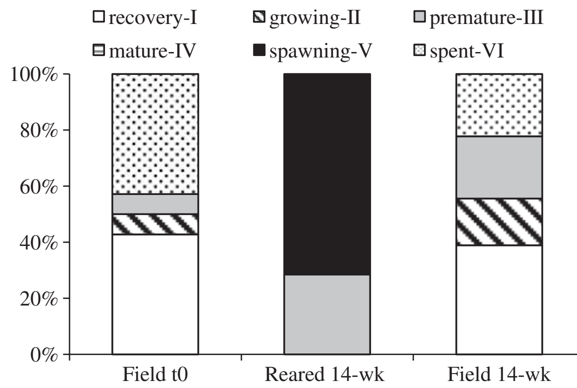

Figure 1. Reproductive condition of *P. lividus* gonads ($n = 30$ per group) at t0 (field t0), after 14 weeks of feeding (reared 14-wk), and in field-collected specimens at the end of the trial (field 14-wk). Gonadal stages are classified according to Byrne [8].

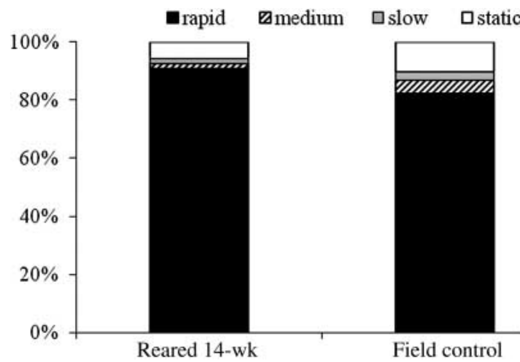

Figure 2. Relative percentages of spermatozoa motility classes in 14-wk reared *P. lividus* and specimens collected in the field during the spawning season (Field Control). Mean ± SD. Rapid (velocity > 100 μm·s^{-1}); medium (45 μm·s^{-1} < velocity < 100 μm·s^{-1}); slow (10 μm·s^{-1} < velocity < 45 μm·s^{-1}); static (velocity < 10 μm·s^{-1}).

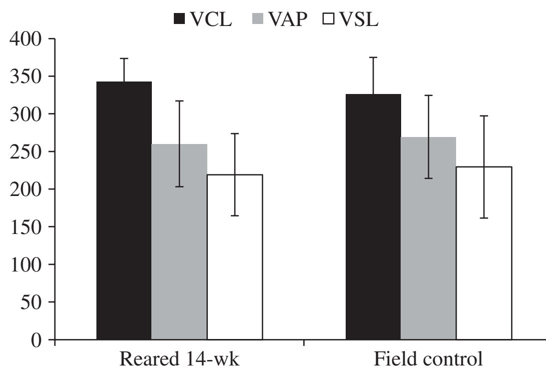

Figure 3. Curvilinear velocity (VCL; μm·s^{-1}), average path velocity (VAP; μm·s^{-1}), and straight-line velocity (VSL; μm·s^{-1}) of spermatozoa from 14-wk reared *P. lividus* and specimens collected in the field during the spawning season (Field Control). Mean ± SD.

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developing gametes were observed along the ascinal wall. A similar percentage of sea urchins was in the recovery phase (stage I): nutritive phagocytes were forming a meshwork across the ascini; in some cases unspawned gametes were still present and in the female gonads ova in the process of lysis were sometimes observed. Gonads containing developing gametes (stages II–IV) accounted for only 20% of the analysed specimens.

After 14 weeks of rearing under experimental conditions, ∼70% of sea urchins were in spawning phase (stage V), with gonads full of mature gametes; both male and female gonads had ascini with spaces vacated by shed gametes, while gametogenesis to replace the spawned gametes continued. The remaining 30% were in premature phase (stage III), with developing gametes along the ascinal wall and the centre of the ascini increasingly filled with mature gametes.

By contrast, in the Field 14-wk collected samples ∼60% of sea urchins were in a less advanced phase of the reproductive cycle (stages I–II), with a thick meshwork of nutritive phagocytes across the ascini and a thin layer of developing gametes along the ascinal wall. Premature gonads (stage III) accounted for only 20%, while ∼20% were still in the spent phase (stage VI).

3.3. *Gamete evaluation*

In both Field t0 and Field 14-wk sea urchins, no or few viable gametes were recovered. Therefore, the quality of gametes in Reared 14-wk specimens was compared with that of gametes in sea urchins matured in the field and collected during the following reproductive season (Field Control).

Figure 2 shows the relative percentages of spermatozoa belonging to the different motility classes in terms of their velocity ($10\,\mu m{\cdot}s^{-1} <$ slow $< 45\,\mu m{\cdot}s^{-1} <$ medium $< 100\,\mu m{\cdot}s^{-1} <$ rapid) recorded by the SCA® system. Rapid sperm accounted for ∼80% and total motile sperm ∼85–90% in both Reared 14-wk and Field Control sea urchins. However, the percentages of both rapid sperm and total motile sperm were significantly higher in reared sea urchins ($H_{1,108} = 41.038, P < 0.001$ and $H_{1,108} = 24.776, P < 0.001$, respectively). Velocity parameter values are shown in Figure 3. The recorded curvilinear velocities (VCL) ranged from 250 to $390\,\mu m{\cdot}s^{-1}$, average path velocities (VAP) from 200 to $350\,\mu m{\cdot}s^{-1}$ and straight-line velocities (VSL) from 100 to $300\,\mu m{\cdot}s^{-1}$, with no significant differences between reared and wild-caught specimens.

As shown in Table 3, no differences were recorded between laboratory and field-matured sea urchins in the percentages of either fertilised eggs or normally developed plutei larvae. Moreover, the EC_{50} level for copper recorded in the embryotoxicity test carried out on gametes from reared sea urchins fell in the range recorded for the sea urchins matured under environmental conditions in the same sampling area.

4. Discussion and conclusions

This study describes a method for culturing the sea urchin *P. lividus* in a closed-circuit laboratory-scaled system. The rearing conditions tested in this study accelerated sea urchins' gonad growth in terms of both GI and gamete maturation. Conditioned sea urchins were all in late maturation phases (pre-mature to spawning). By contrast, most specimens collected in the field at the end of the trial were in earlier maturation phases (spent to growing), as expected on the basis of their reproductive cycle in the Mediterranean area [10], specifically along the mid-to-low Adriatic coast where the specimens used in this study were sampled [32]. Specimens collected at t0 (July) were mainly in spent or recovery phase, again consistent with what has been reported for this area [10,32].

Although temperature and photoperiod are important triggers of the gametogenic cycle in reared sea urchins [11,13,33], it has been extensively demonstrated that in confined conditions gonad

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growth mainly depends on dietary regime, in terms of both the quality and quantity of the feed [21,34,35]. Protein content seems to be a key factor in artificial diets.

Indeed, sea urchins fed on relatively protein-rich prepared feed experience a considerably greater increase in their gonad yield than those fed on low-protein diets [35–37]. Moreover, varying dietary content and using plant or animal protein sources affects the biochemical composition of gonads [38] and gonad production [39]. In addition, prepared feed, such as the one used in this trial, is an extruded product in which carbohydrates are heated, possibly making them more digestible than algal carbohydrates [40]. Furthermore, prepared diets usually contain vitamin and mineral supplements, and are consistent in quality, whereas acroalgal nutritional values may fluctuate widely depending on the season and the sampling site [37,41]. The feed provided in this experiment (Table 1) has a significantly higher protein content than the algae of the *Ulva* genus (∼25–27%) [42,43] that grow on the sea bed where the sea urchins used in this trial were collected. Moreover, it seemed to be highly palatable to the reared sea urchins, which is a key concern in developing suitable artificial diets, as it in turn influences sea urchins' growth [44].

Besides the increase in GI, Reared 14-wk sea urchins provided viable gametes and embryos, whose sperm motility, fertilisation ability and sensitivity to reference toxicants in embryotoxicity bioassays were comparable with those of Field Control organisms collected in the same area during the spawning period.

Computer-assisted sperm motility analysis can be considered a promising tool for rapid and easy-to-perform toxicity bioassays [24,45]. It provides a quantitative and objective assessment of sperm motility parameters which are not manually observable but have been shown to be positively correlated with fertilisation capacity [45,46]. The motion parameters of the reared sea urchins' sperm, recorded in this study by SCA®, showed levels comparable with those recorded in wild-caught specimens of *P. lividus* and other sea urchin species [24,47–49].

The effects of toxicants on the fertilisation capacity of sea urchin gametes and larval development have been widely investigated [1,4,50]. Our results confirm the effectiveness of the experimental rearing conditions, because the percentages of fertilised eggs and normally shaped plutei larvae, as well as the effects of copper on larval development, are similar to those reported in the literature for wild collected *P. lividus* [50,51].

The effects of broodstock rearing conditions on gamete quality have been studied in many sea urchin species. Prolonged starvation periods have been found to significantly reduce fertilisation ability in reared *P. lividus* [27]. By contrast, in *Strongylocentrotus droebachiensis* high food quality and quantity have been found to enhance the production of nutritive phagocytes [52–54], while a high-protein diet results in higher egg quality and larval survival than kelp-fed specimens [26]. *Arbacia lixula* populations feeding on more abundant macroalgae foods have been found to produce eggs with higher protein and lipid levels than those having access to lesser quantities of algae [25].

Given that proteins and lipids are the most important energy reserves in the eggs of echinoderms, and that their concentrations are strongly dependent on the broodstock feeding regime, it may be assumed that the feeding regime tested here was of sufficient nutritional quality to support gamete development.

In a previous series of experiments [27], *P. lividus* specimens collected at the end of their reproductive cycle (May) and kept under the same rearing conditions tested here were able to immediately reactivate gamete production, with no need to subject them to the stress of altering the photoperiod, and at the end of the 4-week trial viable gametes were recovered from almost 65% of the sea urchins. It may therefore be assumed that these combined feeding, temperature and photoperiod conditions can support not only out-of-season continuation of gametogenesis activity during a short rearing period, but are also enough to guarantee, over a longer rearing period (14 weeks), the sexual maturation of specimens in spent or recovery conditions.

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Regarding the duration of the conditioning trial, according to Shpigel et al. [11], *P. lividus* specimens took 120 days to reach the mature stage from the spent one, with only 20% of *P. lividus* in the growing phase after 90 days of rearing. It should be pointed out that sea urchins were fed on prepared pellets containing less protein than in our experiment (21 vs 46.5%). In addition, unlike our rearing protocol, sea urchins were starved before beginning the experiment; it has been demonstrated in *Lytechinus variegatus* [55,56] that after starvation, stomach and intestinal tissues must undergo histological and functional reorganisation before they resume the transfer of nutrients to the gonads. These differences could be the reason why our conditioning protocol proved to be faster, considering the similar rearing temperature and the fact that we did not manipulate the photoperiod.

In conclusion, the sea urchins reared under the experimented conditions experienced faster gonad maturation than the field population, producing viable gametes and larvae whose biological response in toxicity bioassays was similar to that of field specimens of *P. lividus*. In addition, it should also be noted that the closed rearing system described here is laboratory-scaled and easy to manage at some distance from the sea, while the formulated feed is not expensive, has no need of manipulation and produces a negligible amount of waste.

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