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Importance of Gamete Quality in Ecotoxicological Application: Natural versus Bred Population in *Paracentrotus lividus*

Sartori Davide, Lera Samantha, Silvia Giuliani,
Simona Macchia, Lorenzo Morroni,
David Pellegrini and Andrea Gaion

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Abstract

Several approaches have been tested to respond to the depletion of wild stocks, from the production of seeds to the setting up of closed echinoculture systems, starting with fertilization of eggs with the consequent development to adult sea urchins. Hence, in the last years, our research group has focused on the assessment of a feasible and sustainable strategy aimed to ensure a rapid and effective gonadal growth of healthy gametes in recirculating aquaculture system (RAS) to employ in ecotoxicological application. In order to compare the health of obtained gametes with wild populations, the effectiveness of diets was evaluated with different biological parameters, such as fertilization and embryo-development test, and with histological analysis of gonads to appraise the stage of maturation. Moreover, the information regarding different breeding conditions of adults and genetic variability should be combined with the analysis of larval settlement and its requirements, demonstrating the importance of these parameters for the possible closure of the echinoculture cycle in RAS. Results achieved so far in terms of gonadal development and health of gametes have provided evidence of success in overcoming natural gaps between reproductive events in natural populations and an efficient and standardize breeding condition in RAS.

Keywords: *Paracentrotus lividus*, gonadal growth, sexual maturation, diets, recirculating aquaculture system

1. Introduction

The commercial fisheries and destructive harvesting methods employed to meet market demand, caused, in the last decades, a dramatic depletion of *Paracentrotus lividus* in Europe, especially in the Mediterranean, leading to a complete disappearance of urchins from areas of former abundance. This scenario was exacerbated by its worldwide recognition as one of the most reliable bioindicator species, and its gametes have been used for biological assays in marine pollution monitoring.

Several approaches have been tested to respond to the depletion of wild stocks, from the production of seeds (juveniles for out-planting) [1] to the induction of gonadal growth (GG) in organisms belonging to natural populations [2, 3], but the most challenging strategy could be the setting up of closed echinoculture systems, starting with fertilization of eggs with the consequent development to adult sea urchins [4, 5].

The reproductive cycle of echinoids has been extensively studied and documented since the early 1930 [6, 7] and several studies have been carried investigating different light/dark regimes, temperature, and supply of artificial diets to ensure a rapid gonadal growth and to promote an effective maturation of gametes of *P. lividus*.

The aim of the present chapter is to present the most effective breeding conditions to promote gonadal growth and sexual maturation in *P. lividus*. Particular emphasis will be put toward the work carried out by our research group in the assessment of a feasible and sustainable strategy aimed to ensure a rapid and effective gonadal growth of healthy gametes in recirculating aquaculture system (RAS) with pellets, macroalgae, and formulated diets rich in carotenoids (*Spinacia oleracea* and *Zea mays*), which have been demonstrated to be crucial for various biological function, especially during egg production and development [8–11].

In order to compare the health of obtained gametes with wild populations, the effectiveness of diets was evaluated with different biological parameters, such as fertilization and embryo-development test, and with histological analysis of gonads to appraise the stage of maturation.

The results obtained by our group demonstrated the good feasibility of a low-cost, easy to standardize and sustainable diet in rearing vulnerable species such as *P. lividus* in RAS.

2. Gonadosomatic growth and sexual maturation in *Paracentrotus lividus* natural population

Different factors can be significant for the somatic growth (SG) of *P. lividus*, such as water temperature, nutritional aspects of nutriment, and gonadal development [12]. Among these, previous research demonstrated that seasonal variations of growth rate seem to be predominantly related to water temperature with a dilatory effect in response to low temperatures; in fact, Le Gall [13] reported the absence of growth in the population of sea urchins in the English

Channel when temperature was between 4 and 7°C. From the literature on this topic, growth is noted to increase proportionally with increasing temperature between 7 and 18°C, with the optimum condition predominantly between 18 and 22°C above this condition, growth slows to a halt completely when temperature exceeds 28°C [14–17]. In the Mediterranean Sea, growth peaks during spring, when the water temperature is between 12 and 18°C, while sporadically occurs in autumn and is practically absent in winter [14–17]. As a result of these premises, it can be estimated that urchins of 2 cm in diameter are around 2 years old and it takes approximately 4–5 years for them to reach 4 cm in diameter [12, 16, 18–21].

P. lividus has an annual reproductive cycle. According to some authors, these species present a single spawning event [22, 23], while others support the hypothesis that in a year may occur two reproductive events [24–26]. The reproductive cycle of *P. lividus* has been studied in detail by several authors and is known as the cycle of many echinoids, which is influenced by various environmental factors such as temperature [22, 23], photoperiod [17, 22, 23], hydrodynamics conditions [27], and trophic availability [23, 25–27].

Gonadosomatic index (GSI) is defined as the ratio between the mass of gonads and the mass of the whole organisms [23]; generally, the highest GSIs are reached by bigger individuals, with size tests ranging from 40 to 70 mm, rather than individuals belonging to the 20–40 mm class size [28, 29]. Previous studies on the gonadal growth (GG) of *P. lividus* in the Mediterranean and in the Atlantic Ocean reported the presence of two growth peaks whose timing can vary considerably even in populations of neighboring adjacent areas: indicatively the first peak takes place in spring followed by the second in late-summer/autumn [22, 29, 30].

Both field and *in vitro* studies seem to confirm the correlation between SG and GG with food availability [21, 31] and high rate of organic matter ingestion [32]. Gonadal development in particular seems to be enhanced by temperatures comprised between 18 and 22°C [17], even if some contrasting data were obtained from *in situ* studies reporting developed gonads in well-fed subtidal populations both in open sea and in lagoon environments [12, 22, 33–35]. High GSIs have been measured in populations with either low or high densities: the former [36] as a consequence of a low competition for food, the latter where the substrate is populated by a few algal species [23]. On the basis of these information, it can be noted that individuals of *P. lividus* invest greatly in reproductive strategy in conditions of scarcity of food. Although, GG, could be supported by high supply of algal fragments or food of high nutritional value transported by current flow.

P. lividus has separate sexes and there is no sexual dimorphism, yet, hermaphroditism cases have been observed for this species [22, 37]. *In vitro*, sexual maturity is reached in individuals of size ranging between 13 and 20 mm and/or after 5 months [38, 39]; however, sexual maturity can be reached in natural populations over longer periods of time.

2.1. Gamete spawning in *Paracentrotus lividus*

According to Fenaux [25], although the production of gametes takes place up to a temperature of 8°C, spontaneous spawning is not possible under 13.5°C. Thus, the reproductive period at subtropical latitudes takes places, from autumn to spring, until temperatures remain

below 20–22°C. Along the French Mediterranean coasts, two main reproductive events were observed: one between May and June and the other in September/October [25]. In contrast with this scenario, data in the literature report for Italian populations of *P. lividus* in one single spawning period from October to June [22, 23, 40].

Generally, during the spawning events, male and female of *P. lividus* aggregate simultaneously release their gametes in the water [41]. Although these episodes do not always involve all individuals of a population, the homogenized suspension of sperms and eggs can trigger and encourage the release of gametes by other sea urchins located in remote places [42]. In both cases of single or double spawning periods throughout the year, water temperature seems to play a key role in determining the start of the event. While two spawning events have been registered, the first episode occurs when the temperature reaches 14–16°C and the second when the temperature returns to these values after the summer [22, 25, 43, 44]. It has been hypothesized that the first release can also be prompted by lengthening of photoperiod (about 15 h of daylight) rather than the temperature, while the end of spawning events seems to be controlled exclusively by temperature [30, 45]. Even though the presence of one or two spawning periods can be observed within the same region among different habitats [46], according to Lozano et al. [23], the natural emission of gametes would occur exclusively during spring/early summer. It should be taken in consideration, though, that the presence of larvae and postmetamorphosis individuals (1 mm in diameter) in October would seem to reveal the presence of a spawning event in late summer. Boudouresque and Verlaque [47], in apparent contrast with the pace dictated by the above reported variables (water temperature, photoperiod, habitat), reported that spawning can occur almost year-round, although not profusely. This adaptation can be a strategy to facilitate the dispersal of larvae and ensure greater reproductive success of the species.

2.2. Getting started: An overview on formulated diets to enhance gonadal growth and sexual maturation

To date, several studies have been conducted to identify the most suitable breeding condition for *P. lividus*, and frequently the results shown in these studies are not in accordance. Diverse formulated diets are available to promote GG, some formulated diets of particular interest among them are reported in **Table 1**.

Basuyaux and Blin [54, 55] tested six different diets (three of which were algal-based and the other three being based on maize) to enhance SG of the sea urchin *P. lividus* in a semi-closed system; their study reported the best growth rates with a mixed diet (*maize* and *Palmaria palmata*) and the lowest results obtained with the administration of algal-based diets. These results find confirmation in the data described by Garmendia et al. [56] after 60 days of rearing with carrot and algae (*Gelidium sesquipedale*). Frantzis and Grémare [32] rearing over a 6-month period sea urchin with macrophytes achieved the highest ingestion and gonadal production with *Rissoella verruculosa*, which is frequently reported among the most preferred species of seaweed for *P. lividus* despite the presence of repellent and toxic metabolites such as brominated compounds in it [57, 58].

	Tested species	Composition	Reference
Texas A&M formulation	<i>Lytechinus variegatus</i>	Three different formulation based on protein content: final protein concentrations vary from 9 to 31% dry weight (as fed basis)	[48]
NIWA feed	<i>Evechinus chloroticus</i>	<ul style="list-style-type: none"> Protein 40.8% by dw; Carbohydrates 26.2% by dw 	[49]
NIFA feed	<i>Evechinus chloroticus</i>	<ul style="list-style-type: none"> Protein 24.5% by dw; Carbohydrates 49.6% by dw 	[49]
Lawrence diet	<i>Loxechinus albus</i> , <i>Strongylocentrotus franciscanus</i> and <i>Strongylocentrotus purpuratus</i>	<ul style="list-style-type: none"> Total crude protein 12–24% by wt 15–20% by wt; Total carbohydrate 30–60% by wt 40–55% by wt; Total crude fat 3–9% by wt. 4–7% by wt 	[50]
Micciche' et al.	<i>Paracentrotus lividus</i> , <i>Loxechinus albus</i> , <i>Strongylocentrotus granularis</i> , <i>Strongylocentrotus intermedius</i>	<ul style="list-style-type: none"> Egg albumen 45–55% by wt; <i>Lactuca sativa</i> 30–40% by wt; Fish flour 4–6% by wt. 	[51]
Ross Island Salmon Ltd. feed	<i>Strongylocentrotus droebachiensis</i>	<ul style="list-style-type: none"> Rockweed meal (<i>Ascophyllum nodosum</i>) 22.84% by dw; Soybean meal 27.92% by dw; Dulse powder (<i>Palmaria palmata</i>.) 10.00% by dw; Lecithin 1% by dw; Canola oil 2% by dw 	[52]
St. Andrews Biological Station feed	<i>Strongylocentrotus droebachiensis</i>	<ul style="list-style-type: none"> Rockweed meal (<i>Ascophyllum nodosum</i>) 8.40% by wt; Soybean meal 45.00% by wt; Lecithin 2% by dw; Canola oil 4% by wt. 	[53]
Pliva-Kalinovica-Zagreb factory diet	<i>Paracentrotus lividus</i>	<ul style="list-style-type: none"> Crude protein 12.6–22.4% by dw; Fat 7.6% by dw; Crude fiber 1.8–2.4% 	[54, 55]

Table 1. Formulated diet of particular interest to promote gonadal growth with their major component.

Studying the effect of light regime and temperature in rearing condition, indications reported in the literature are once again not consistent: McCarron [59] showed that darkness supports higher SG than the photoperiod treatment whereas other works exhibited that the most suitable rearing condition to enhance gonadal growth were short photoperiod (9 h daylight) and temperature ranging between 18 and 24°C [17, 45]. In addition to these results, for a short rearing period, Fabbrocini et al. [60] found that nutrition appears to be the key factor in the induction of GG.

Comparing the results obtained with “vegetal” and “animal” diet, Fernandez and Boudouresque [61] suggested that the latter resulted in the highest absorption rate, and consequently the best GG; on the contrary, the highest ingestion rate was obtained with vegetable feed. With regard to absorption, results showed that it was negatively correlated with ingestion and carbohydrate level content of the food. These data were confirmed by further works and substantially the prepared diets, characterized by high protein content, guaranteed a better GG in comparison with vegetable diet or low protein content diet [62–65]. Luis and co-worker [66] studying the effect of plant-based diet on spawning performance of *P. lividus* in captivity recorded that a mixed diet maize and seaweed gave better results (79% of the tested urchins) if compared to pure maize diet (50%) and the pure seaweed diet (36%) in terms of consistency of spawning throughout the year.

In conclusion, when the reproductive conditions (organisms in mature or premature stage) allow short breeding periods, the synchronization of the gonads to the emission stages can be induced simply by controlling the diet, without the need for altering the photoperiod and stressed the animals with unnatural photoperiod.

2.3. Breeding condition to promote sexual maturation in RAS

Interest in cultivation of sea urchins has increased over the last 2 decades as a consequence of the depletion of wild stocks [1, 22, 47, 62, 67, 68]. In this context, breeding of *P. lividus* for restocking wild populations in addition to its use for human consumption is an aspect to consider for future developments. An additional reason for that is since 1980, it was recognized worldwide among the most reliable bioindicator species [69], and its gametes have been extensively used for biological assays [70–89]. Therefore, the development of rearing techniques for this species is a current issue for both production of gametes for ecotoxicological application and restocking depleted natural populations due to the growing market demand of roe; a request that otherwise natural populations are unable to meet [83].

Hence, in the last years our research group has focused on the assessment of a feasible strategy aimed to ensure a rapid and effective GG of healthy gametes of *P. lividus* in RAS.

2.3.1. Maintenance of mature stage in *Paracentrotus lividus* in RAS

Our first goal was focused on the maintenance in RAS over a 4-month period of *P. lividus* specimens in mature stage collected during spawning period. After initial 5 days acclimatization fasting period, individuals were reared in the recirculating aquaculture system at 14°C temperature and photoperiod of 10 h L: 14 h D testing two different diets. The first diet was composed of 50% (in volume) maize kernel that had been previously crushed with a blender into grains of a few millimeters and the remaining 50% (in volume) by a mixture of fresh seaweed (*Dyctiopteris* sp., *Padina Pavonica*, *Dyctiota* sp., *Ulva lattuga*, *Halopteris scoparia*, *Flabellia petiolata*, *Laurencia* sp., *Corallina elongata*, *Codium* sp.), collected from the same sampling site of sea urchin. The second diet consisted of a mixture in equal proportion of maize and freshly chopped spinach leaves. Spinach, such as maize, is a vegetable with a high nutritional value and high content of carotenoids [83]. Several studies have demonstrated that spinach leaves

contain natural antioxidants with potential biological activities [90–92]. Indeed polyphenols are now widely accepted as physiological antioxidants that have significant activities and capacity to protect critical macromolecules against the numerous degenerative diseases linked to free reactive oxygen species (ROS) [93–95].

Before being administered, the ingestion rates of every single element of diets were assessed in order to establish the most suitable feed for sea urchins in RAS (**Table 2**). Results showed that in order of preference, the most appreciated food were maize, *Dyctiopteris* sp., spinach, and *Codium* sp.

Considering that brown algae together with *Posidonia oceanica* leaves are among the main components of adult *P. lividus* diet [96–98], with regard to the brown alga *Dyctiopteris* sp. results confirmed this expectation. On the contrary, it is not as clear as the poor desirability for the other brown algae administered. The low appreciation of red algae, with the exception of *Laurencia* sp., could be related to the presence of brominate substances as reported by Codomier et al. [57] for the red alga *Asparagopsis armata*. Among the seaweed provided, *P. lividus* clearly did not show any preference toward algae that have a coriaceous consistency due to the presence of precipitates of calcium carbonate in the structure of the alga (*Corallina elongata*, *Padina pavonica*, and *Flabellia petiolata*). The poor desirability toward *Halopteris scoparia* could be due to the presence of phenolic compounds, which act as a deterrent for *P. lividus* [99]. However, in contrast with what reported in literature [47], *P. lividus* used in our experiments did not ingest important quantities of the green alga *Ulva lactuca* (ingestion rate [g/day + sd]: 0.030 ± 1.06 by dw, see **Table 2**). As already mentioned above, among the red algae, an appreciable consumption has been registered for *Laurencia obtusa*; these data seem to be in contrast with those reported in Boudouresque and Verlaque [47].

Species	Ingestion rates (g/day dw)
<i>Codium</i> sp.	0.137 ± 0.32
<i>Corallina elongata</i>	0.025 ± 0.68
<i>Dyctiopteris</i> sp.	0.141 ± 0.45
<i>Dyctiota</i> sp	0.076 ± 1.16
<i>Flabellia petiolata</i>	0.030 ± 2.08
<i>Halopteris scoparia</i> (= <i>Stypocaulon scoparium</i>)	0.035 ± 2.13
<i>Laurencia</i> sp.	0.115 ± 0.89
<i>Zea mays</i> (crushed Kernel)	0.281 ± 0.93
<i>Padina pavonica</i>	0.085 ± 0.56
<i>Spinacia oleracea</i>	0.139 ± 1.18
<i>Ulva lactuca</i>	0.030 ± 1.06

Table 2. Ingestion rates by for the macrophyte, maize, and spinach tested. Ingestion rates are expressed in terms of dry weight per day. The values are mean and standard deviation.

The consumption of spinach resulted to be comparable to the “most preferred algae”; however, the consumption of maize was significantly higher than those recorded for macrophytes. This unusual feeding behavior could be partly explained by the higher content of carbohydrates and proteins in maize with respect to algae and spinach. Indeed, these two ingredients exhibit a very similar biochemical composition, characterized by high water content and moderate presence of proteins and lipids. Maize is a primary source of energy supplement and can provide up to 30% protein, 60% energy, and 90% starch in animal diets [100] and is an important source of carotenoids. Echinenone and (60R)- β -carotene-4-one are the major carotenoids in both ovaries and testes [101, 102]; in gonads, echinenone accounts for approximately 50–60% of the total pigment [11, 103] and it is metabolized from dietary β -carotene. It is transported to or stored into gonads in much greater concentrations than other carotenoids, where it may play a role both in production of eggs, development, and immunological modulation [9, 102, 104, 105].

To evaluate the quality of gametes and embryos obtained from organisms maintained into mature stage in RAS, fertilization, and embryo-development tests by means of reference toxicant were performed every month during treatment with gametes collected by reared individuals.

Diets tested in these trials, in combination with a 10 h L:14 h D light regime and a water temperature of 14°C, have ensured the maintenance of animals into a mature prespawning stage for a 4-month period [83]. This result permitted to overcome the summer months during which, at our latitude, it is not possible to obtain gametes from organisms belonging to wild populations [40]. EC_{50} values obtained, both for fertilization and embryo-development tests during the experiments, were consistent with the laboratory control chart and those reported in the literature for different species of echinoderms, including *P. lividus* [10, 78, 82, 106–109]. It is worth reporting that the gametes obtained by tested individuals were comparable to those of natural populations in terms of response to the reference toxicant copper(II) nitrate ($Cu(NO_3)_2 \times 3H_2O$) [83].

2.3.2. Reliable breeding condition to promote sexual maturation in RAS

P. lividus has an annual reproductive cycle, although according to some authors these species presents a single spawning event [22, 23], whereas others support the hypothesis that two reproductive events may occur in a year [24–26]. The reproductive cycle of *P. lividus* has been well studied, and much research has been carried out to determine all its phases in relation to temperature [22, 23, 29, 30] photoperiod [17, 22, 23], hydrodynamics conditions [27], and trophic availability [23, 25–27].

After having identified the suitable conditions to maintain *P. lividus* in mature stage (stages III–V) [22] in RAS, our research group focused on the analysis of fast reliable breeding conditions to promote GG and sexual maturation in *P. lividus* in order to have a continuous production of gametes for scientific research and ecotoxicological tests. Different diet treatments to enhance gametes maturation were tested by our research group in a previous study [83], focusing in particular on the reliability of a maize and spinach diet (MSD), a macroalgae diet (MD), and a diet based on a commercial pellet normally used in aquaculture for warm-water species (Classic K®, PD). The biochemical composition of pellet Classic K® (Hendrix S.p.A) employed in our study is shown in **Table 3**.

Composition	%
Protein	43.0
Crude fat	11.5
Crude fiber	3.2
Ash	8.0
Phosphorus	0.8
Digestible energy (MJ/kg)	14.8

Table 3. Biochemical composition (%) of pellet Classic K® (Hendrix S.p.A.).

The MD and the MSD were the same, previously employed in the maintenance of mature stage in *P. lividus* reared in RAS.

Before being fed *ad libitum* with three chosen diets, sea urchins were starved for 2 months in order to promote the reabsorption of gonads and get them in phase regarding their reproductive cycle [30]. Specimens were kept in aquaria with $12 \pm 1^\circ\text{C}$ water temperature and exposed to a photoperiod 12 h L:12 h D completely deprived of food [4]. To promote the maturation of adult *P. lividus*, diets have been tested for 9 weeks in combination with a photoperiod of 10 h L:14 h D and water temperature of 16°C . In order to evaluate the effectiveness of the different diets, multiple biological data were analyzed, in particular: gonad index (GI), histological examination of gonadic tissue; analysis by using harmonic generation (HGM) and two-photon photon (2PF) microscopy; fertilization and embryo-development test with a reference toxicant ($\text{Cu}(\text{NO}_3)_2 \times 3\text{H}_2\text{O}$).

The second and third harmonic generation microscopy (SHG-THG) and the 2PF are nonlinear microscopy techniques, which base their optical resolution on the interaction of the wavelength of light with matter.

The THG and SHG techniques are a nonfluorescent multiphoton technique of laser scanning microscopy that allow to acquire signals with submicron spatial resolution without the use of fluorescent markers [111]. In copepods and zebrafish, these techniques have been shown to be able to reveal the onset of cell death mechanisms (apoptosis) [111–113]. In particular, the signal of the SHG can reveal the distribution of collagen fibers and striated muscle myosin [114], while THG microscopy is more versatile than SHG microscopy and can highlight, through the discontinuity of the refractive index, the morphology of cell membranes and of lipid vesicles [115]. The microscope used for this work is based on a femtosecond laser Cr-forsterite, which operates around 1230 nm. This laser is able to penetrate deep into the tissue causing little damage compared with the common Ti: sapphire laser used in fluorescence microscopy (700–1000 nm). The laser was mounted on an Olympus BX51 microscope, and plutei obtained from gametes of reared organisms in recirculating aquaculture system were observed with a 60× immersion objective and numerical aperture (NA) of 1.2 (**Figure 1**) at the Molecular Imaging Center, National Taiwan University, Taiwan.

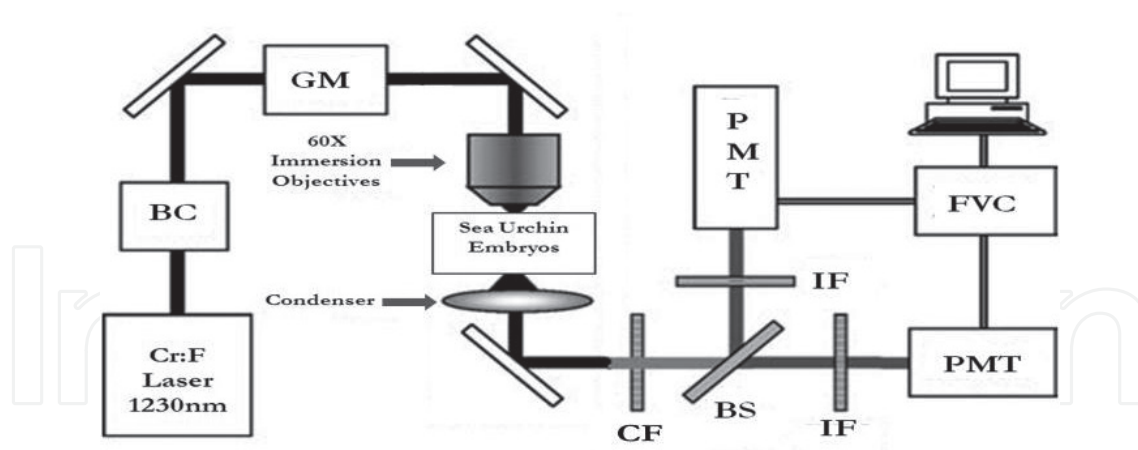


Figure 1. Schematic diagram of the microscope: BC, beam collimator; GM, galvanometric mirrors; CF, color filter; BS, beamsplitter; IF, interference filter; PMT, photomultiplier tube; FVC, fluoview control unit [110].

Tested diets were successful in stimulating gametogenesis and ensuring the production of healthy gametes in short time (3 weeks); among these, MSD gave the best results in terms of GI values. According to histological analysis, only MSD and PD diets were suitable to guarantee a rapid transition of sea urchins from an inactive phase (stage VI-Spent) to an active phase of gametogenesis (stages II–V) [22]. These results confirmed that photoperiod, temperature, and diet allowed to maintain the constant presence of mature *P. lividus* in the rearing tanks and overcome summer period during which sea urchins belonging to natural population are unable to produce gametes [40].

EC₅₀ values obtained from fertilization and embryo-development tests with reference toxicant (**Figure 2a** and **b**) were consistent with those reported in the literature, demonstrating the good quality of gametes [10, 72, 82, 106, 109]. In particular, sperm obtained from animal reared with MD diet seems to be more sensitive toward copper(II) nitrate if compared with gametes obtained from other diets or belonging to natural population. This aspect reveals that the high energetic value of PD diet (protein = 43.0% and crude fat = 11.5%) and the richness in carotenoids and antioxidant in MSD diet proved to be important factors in the ability of sperm to cope with toxicity resulting from exposure to the reference metal [83]. The effect of the biological richness of MSD in terms of energy source, carotenoids, and antioxidants can be seen in the high EC₅₀ value; indeed after a 9-week treatment, plutei demonstrated to have a resistance against copper(II) nitrate comparable with the wild population, higher than plutei bred with MD or PD diet (**Figure 2b**); these data were further confirmed by histological evidence [116].

Indeed, as previously demonstrated, thanks to the combination of the high nutritional value due to carbohydrates content and fairly good presence of protein in maize [117] with the remarkable richness in carotenoids and polyphenols of spinach [90, 118], MSD holds the essential chemical compounds to protect biological tissues against oxidative processes [119, 120].

Nevertheless, both field and *in vitro* studies seemed to confirm that somatic and GG ensued when food availability and organic matter ingested was high [21, 31, 32], although other physical parameters such as light regime and temperature can positively affect it [59].

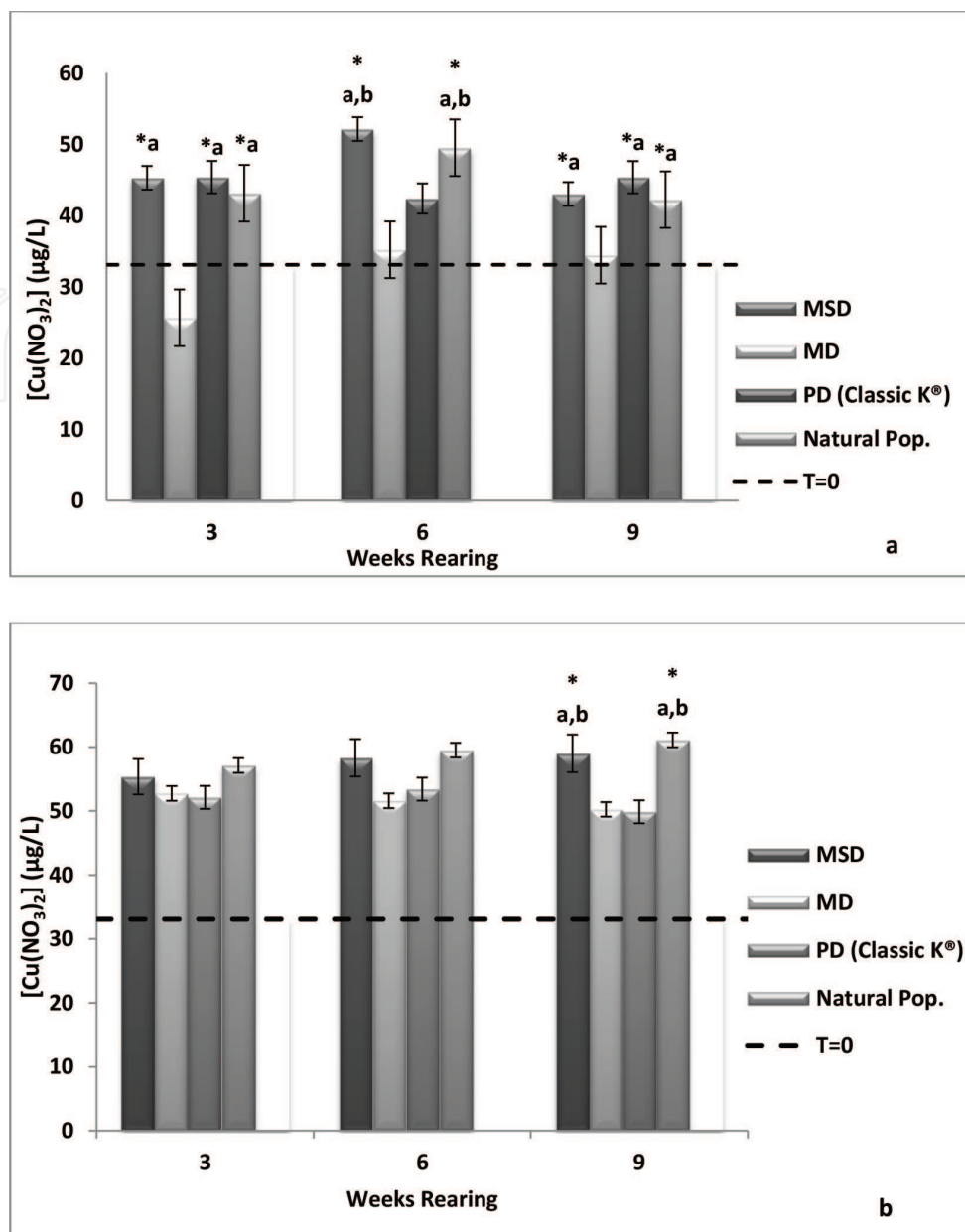


Figure 2. EC_{50} ($\mu\text{g/L}$) values of reference toxicant $[\text{Cu}(\text{NO}_3)_2 \times 3\text{H}_2\text{O}]$ obtained with fertilization (a) and embryo development (b) tests performed on *Paracentrotus lividus* reared in a recirculating aquaculture system (RAS) with artificial diets maize and spinach (MSD), pellet Classic K® (PD) and macroalgae (MD). The EC_{50} values obtained are compared with those obtained from *P. lividus* belonging to a natural population (Natural Pop.). EC_{50} ($\mu\text{g/L}$) values obtained at $T = 0$ for the wild population are reported. The values are mean and standard deviation [116]. “Note: *a= statistically sigfootnificant with respect to MD diet; *b statistically significant with respect to PD diet”.

This chapter reviews innovative techniques established by our research group with regard to sea urchin plutei developed from gametes of reared organisms. Results concerning bred organisms were compared with those obtained from organisms belonging to the natural population in order to evaluate and assess possible events of cell death or abnormalities development biological damage linked with the rearing conditions and diets employed.

The evaluation of effects induced by rearing conditions by means of HGM microscopy techniques highlighted differences between plutei from the three diets. In particular, plutei obtained from the MSD diet presented an increase in fluorescence signal, both with 2PF and THG technique that we could hypothesize be related to apoptotic [121] or autophagy event [122, 123] (**Figure 3**). On the contrary, natural larvae or larvae obtained after PD or MD diets did not show any increase in fluorescence signal. Another factor to consider when evaluating MSD diet is that plutei showed a loss of THG signal in the skeleton, potentially due to incorrect tissue organization in the skeletal structures, phenomenon previously observed in *P. lividus* plutei exposed to HgCl_2 [121]. Considering these data, although feasible both for GG and gametes maturation in short rearing period, MSD could lead to the generation of plutei unstable for ecotoxicological and echinoculture application. Further investigations are needed with regard to this topic, considering that programmed cell death is a physiological process aimed to prepare the tissues of *P. lividus* larvae before metamorphosis [124].

The HGM microscopy technique applied in this study allowed the observation of abnormalities in the development of sea urchin plutei obtained from *P. lividus* kept in RAS with different diets. This technique is certainly a valuable and promising tool for applications in ecotoxicological studies, as confirmed by other *in vivo* studies with other model organisms such as zebrafish embryos (*Danio rerio*) [111, 112]. The same apoptotic body in the zebrafish hindbrain was positively stained through the fluorescent marker acridine orange. In a study conducted on nauplii of *Acartia tonsa* authors reported that the strong fluorescent signal detected with the 2PF and with the THG was associated with the onset of apoptosis in the digestive system of copepods, data confirmed by the classical staining technique of TUNEL

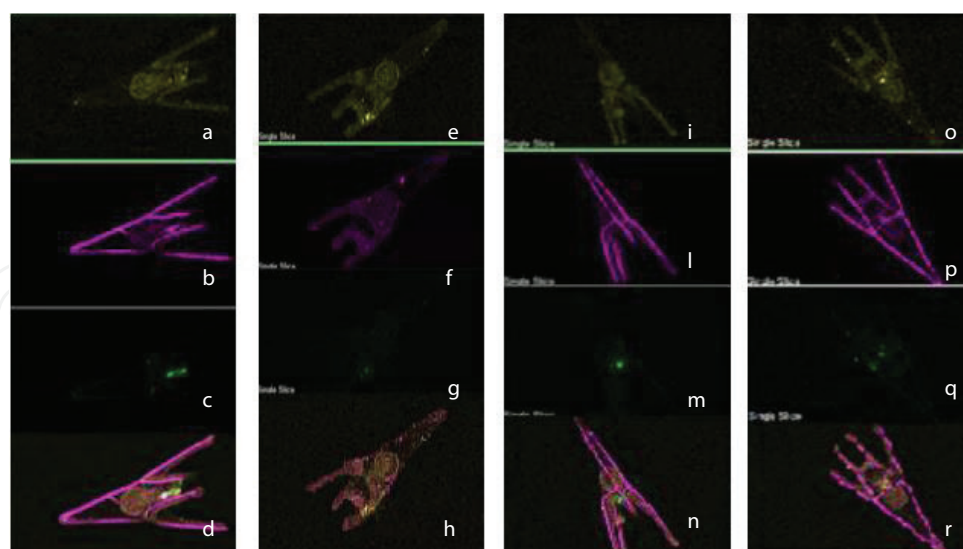


Figure 3. Sea urchin plutei obtained from different diets observed with light microscopy, two photon fluorescence (2PF) microscopy (a, e, i, o), third (THG) (b, f, l, p), and second (SHG) harmonic generation microscopy (c, g, m, q). The images obtained merging the THG, 2PF, and SHG signals are presented in images d, h, n, r. Plutei observed are obtained from *Paracentrotus lividus* belonging to natural population (a–d) and reared with maize and spinach (e–h), pellet Classic K® (i–n) and macrophytes (o–r).

[113]. In our experience, the noninvasive nature of the SHG and THG technique has permitted a three-dimensional observation of the cellular structures of sea urchin pluteus allowing the observation of morphological changes, in the complex development processes related to the rearing condition. In addition, these techniques provided important results without the use of fluorescence markers, overcoming the common phenomena of photodamage, phototoxicity, and photobleaching linked to the use of fluorescent probes.

3. Further development

Results achieved so far in terms of gonadal development and health of gametes have provided evidence of success in overcoming natural gaps between reproductive events in natural populations and an efficient and standardize breeding condition in RAS. In the past few years, different authors published results originating from different cultivating conditions, considering various environmental parameters as well as diets. Raposo et al. [125], for example, tested the efficacy of three artificial diets (A, green macroalgae *Codium* sp.; B, solid mix diet, with macroalgae *Codium* sp., carrot and cabbage; and C, maize and spinach) during 80 days to promote gonadal growth and the maturation of *P. lividus* gametes. In their experiments, a temperature of $20 \pm 1^\circ\text{C}$ was set during the dietary treatment, whereas in our trials this temperature caused spawning in specimens compromising the continuous collection of gametes. Sanja et al. [126] tested the efficacy of four different diets in a semiclosed recirculating system. In this experiment, temperature and salinity of seawater were not kept constant but allowed to vary according to natural environment; in particular, they ranged between 15 and 23°C and 32 – 37‰ , respectively. Colak et al. [127] opted for a temperature of 22°C and a salinity of 38‰ and a diet based on pellet to cultivate urchins for histological analysis. The variability of results combined by the different environmental conditions of the tanks suggests that different populations of *P. lividus*, sampled by diverse areas and in different periods of the year, can respond in dissimilar ways to aquaculture conditions. To confirm this hypothesis, we compared the toxicological responses of three different populations toward three contaminants; these populations were distributed in a radius of ca. 10 km and the sampling activity was performed in five different times of the year [128]. Results showed a high variability of responses, even if considering a small-scale variation of populations. This result suggests that further analyses are required in order to assess the variability of a larger distribution of populations and the contribution of the genetic variability. Therefore, genetic analyses will be performed on the urchins belonging to these populations, with the expected results useful to comprehend the genetic variability and its correlation with the results previously obtained.

Finally, the information regarding different breeding conditions of adults and genetic variability should be combined with the analysis of larval settlement and its requirements. Indeed, Colin et al. [129] demonstrated that settlement rates of competent urchin larvae were significantly correlated with different substrates. Brundu et al. [130] combined the analysis of two different settlement substrates with four larval dietary treatments on the survival and growth

of the larvae, demonstrating the importance of these parameters for the possible closure of the echinoculture cycle in RAS.

Author details

Sartori Davide^{1*}, Lera Samantha², Silvia Giuliani¹, Simona Macchia¹, Lorenzo Morroni¹, David Pellegrini¹ and Andrea Gaion³

*Address all correspondence to: davide.sartori@isprambiente.it

1 Italian National Institute for Environmental Protection and Research—ISPRA, Piazzale dei Marmi, Livorno, Italy

2 Agenzia Regionale per la Protezione Ambientale della Toscana—ARPAT, Lucca, Italy

3 South Devon College, Vantage Point, Paignton, UK

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