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Roe enhancement of *Paracentrotus lividus*: Nutritional effects of fresh and formulated diets

Valerio Zupo[1,2] | Francesca Glaviano[2] | Marina Paolucci[3] | Nadia Ruocco[4,5,6] | Gianluca Polese[5] | Anna Di Cosmo[5] | Maria Costantini[4] | Mirko Mutalipassi[1]

[1]EMI Department, Benthic Ecology Centre, Stazione Zoologica Anton Dohrn, Ischia, Italy

[2]RIMAR Department, Unit MaRe, Stazione Zoologica Anton Dohrn, Napoli, Italy

[3]Department of Science and Technologies, University of Sannio, Benevento, Italy

[4]Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Napoli, Italy

[5]Department of Biology, University of Napoli Federico II, Naples, Italy

[6]CNR: Bio-Organic Chemistry Unit, Institute of Biomolecular Chemistry-CNR, Naples, Italy

Correspondence
Valerio Zupo, EMI Department, Benthic Ecology Centre, Stazione Zoologica Anton Dohrn, Villa Dohrn, Punta San Pietro, Ischia 80077, Italy.
Email: valerio.zupo@szn.it

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Abstract

Sea urchin gonads are exploited both for gastronomic and scientific purposes; thus, the production of large and mature gonads is essential. Natural populations of the Mediterranean Sea urchin *Paracentrotus lividus* are subject to increasing fishing efforts, due to continuous intensification of consumptions. Aquaculture practices may represent an answer, but the availability of artificial feeds accelerating the production of high-quality gonads in terms of size, taste, colour, firmness, maturity and viability of gametes is critical to boost the productions. The accessibility of cheap and effective feeds promoting the fattening of gonads and the quality of gametes is still a bottleneck slowing down the expansion of echinoculture practices. This investigation is aimed at enabling the development of this strategic sector, by comparing the dietetic effects of fresh foods and a commercial feed for aquaculture, to four newly formulated feeds. The protein contents of diets were strongly related to the GSIs. The abundance of fatty acids appeared inversely related to the viability of embryos and abnormalities of larvae. The features of an ideal diet for this sea urchin were defined, based on the results of experimental trials, and the need for increasing levels of plant-derived proteins during the grow-out period was demonstrated.

KEYWORDS
artificial diets, fats, gametes, gonadic index, proteins, sea urchin

1 | INTRODUCTION

Most sea urchins are typical plant feeders. They are the major consumers of huge standing stocks produced by macroalgae and seagrasses in various coastal environments (Lawrence, 1975). Their natural populations are important to control and keep constant the crops of many seagrasses (Boudouresque & Verlaque, 2007; Zupo, 1994), and their trophic role is usually played as large and abundant macroherbivores (Zupo, Alexander, & Edgar, 2017). Their natural diets are quite complex and contain, in addition to the main plant tissues, several items (Zupo, 1993) including small animal prey and various epiphytes, indispensable to complete the assortment of feeding principles ingested (Mazzella et al., 1992). Therefore, they could be considered as opportunistic herbivores (Zupi & Fresi, 1984). In addition, each species of sea urchin is adapted to local ecological conditions and evolved specific dietetic patterns (Lawrence, 2007).

The sea urchin *Paracentrotus lividus* (Lamark, 1816) is quite common throughout the Mediterranean Sea (Boudouresque & Verlaque, 2007), from the North Atlantic coasts of Ireland to southern Morocco (Bayed, Quiniou, Benrha, & Guillou, 2005; Symonds, Kelly, Caris-Veyrat, & Young, 2007). It is an important resource since the last century (Koehler, 1883), both as a marketable good (Devin, 2002; Williams, 2002) and an animal model for research in the life sciences (Buitrago et al., 2005; Yamabe, 1962). Its importance for

scientific investigations is also related to remarkable characters of embryos; in fact, it exhibits transparent eggs and embryos, allowing for a continuous monitoring of the division progressions, up to the development of a larva (Santella & Chun, 2011).

The progressive depletion of its natural stocks, due to overfishing (Barnes & Crook, 2001; Byrne, 1990; Le Direach, 1987), habitat destruction and natural diseases, reinforces the need for effective aquaculture practices (Mercurio & Sugni, 2016). In addition, its market has largely increased in the last three decades due to the intensification of requests (Pais et al., 2007; Williams, 2002). This evidence boosted investigations aiming at the definition of economically productive practices (Fernandez, 1996; Olave, Bustos, Lawrence, & Carcamo, 2001; Pearce, Daggett, & Robinson, 2002a; Sartori, Pellegrini, Macchia, & Gaion, 2016). Intensive aquaculture efforts produced a tremendous amount of information regarding the in vitro fertilization of eggs and the production of juveniles (Yokota, 2002), the best feeding practices for the grow out of adults (Mortensen, Siikavuopio, & Raa, 2004), the practices of caging (James, 2006a,b), as well as the stimulation of gonadal growth (Cook & Kelly, 2007; Pantazis, 2009; Spirlet, Grosjean, & Jangoux, 2000).

However, the economic effectiveness and therefore the feasibility of industrial plants able to produce marketable sized *P. lividus* are still hindered by the availability of excellent feeds, able to guarantee fast growth and gonadal maturation (Devin, 2002; Le Gall, 1990; Spirlet, Grosjean, & Jangoux, 1998). Researches in this field led to interesting and concrete, even if not conclusive, wet formulations (Lawrence, 2001) and dry feeds (Sartori et al., 2016; Woods, James, Moss, Wright, & Siikavuopio, 2008). While the interest in sea urchin aquaculture increased in the last decades, most research attention was devoted to the development of effective feeds for the grow out of adults (Agatsuma, 1998; Pearce, Daggett, & Robinson, 2003) promoting and accelerating the gonadal production (Fabbrocini, Volpe, Coccia, D'Adamo, & Paolucci, 2015; James & Heath, 2008; Lawrence & Lawrence, 2004; Pearce, Daggett, & Robinson, 2002b, 2002c; Pearce et al., 2002a).

Diets proposed for *P. lividus* (Bendich, 1994; Kawakami et al., 1998; Matsuno, 1991; Tsushlma, Kawakami, Mine, & Matsuno, 1997) contained plant carotenoid to improve the colour, maize for rapid fattening (Dado, 1999), spinach as antioxidant and other components useful to retard the chemical and physical degradation of feeds (Cabrita, Robles, & Herráez, 2008; Sartori et al., 2016). Notwithstanding such a prolonged and intense scientific production, the definition of the "perfect" diet for *P. lividus* is still to be attained and any detail adding information to this puzzling theme can be useful to reach well-devised aquaculture procedures (Fernandez, 1990).

Both ovaries and testes, commonly called "roe," are targets of economic interest. Thus, the quality of a feasible feed for adult sea urchins may be judged based on the size and quality of their gonads (Sartori, Scuderi, Sansone, & Gaion, 2015). Both the storage of nutrients in nutritive phagocytes involved in early maturation stages (Byrne, 1990) and the abundance of mature gametes in late maturation stages positively influence the size of sea urchin gonads (Woods et al., 2008). Diets for various species of echinoids have been devised allowing for their transfer to the aquaculture industry, in accordance with quality criteria in terms of taste, smell, firmness, and colour of gonads (de Jong-Westman, March, & Carefoot, 1995; de Jong-Westman, Qian, March, & Carefoot, 1995). Artificial diets were set for several species of echinoids (Cook, Bell, Black, & Kelly, 2000; Fernandez & Boudouresque, 2000; Lawrence, Plank, & Lawrence, 2003; McBride, Price, Tom, Lawrence, & Lawrence, 2004) and they promoted significant increases in gonadic yields.

Most of the mentioned studies rely on sea ranching, to respond to the reduction in productions from capture fisheries and the sharp decline in wild stocks of sea urchins. These techniques, however, can accelerate the harvest of natural populations. Environmental effects of sea ranching are far from being fully understood, and their actual sustainability should be carefully evaluated case by case (Mustafa, 2003). Nevertheless, the results of this study will be applicable to any culture condition, both for experimental or production purposes. Here, we aimed at investigating the gonadosomatic indices, the histological features of gonads and the quality of gametes and larvae produced by individuals reared under a range of dietetic regimes. To this end, we compared two different methods of feed preparation (using synthetic binders or flours), various levels of proteins and fatty acids, and three categories of diets represented by (a) newly developed dried formulations, (b) wet natural diets and (c) commercial pellets already tested for similar purposes (Fabbrocini et al., 2012, 2015). In particular, we compared the dietetic effects of common plant items that characterize the seabeds hosting the target sea urchins (Ortiz et al., 2006; Valente et al., 2006), that is the seagrass *Posidonia oceanica* and the macroalga *Ulva rigida*, to the effect of five formulated diets.

2 | MATERIALS AND METHODS

2.1 | Sampling of urchins

Sea urchins were collected by divers in fall (September) 2016 in the bay of Naples (40°77′N; 14°08′E) from algal mats, wrapped into humid paper sheets to avoid emission of gametes and kept in refrigerated containers up to the transfer into 1-m^2 flow-through seawater-aerated tanks kept at a temperature of 18°C, using an aquarium chiller. Each collected individual was measured (main diameter excluding spines) and weighed (wet weight). They were also inspected for their general health status, to avoid the introduction of individuals showing loss of spines, evident patches, slow movements, etc.; individuals showing such symptoms of stress or malformations were not used for further tests. A 12:12-hr light:dark photoperiod was imposed. The experimental conditions in the tanks hosting the animals were daily checked, by monitoring temperature, light intensity, oxygen concentration, redox potential and concentrations of total nitrogen, ammonia and nitrites.

TABLE 1 Feeding treatments investigated. Each treatment consisted of two replicates of 20 individuals

Treatment	Diet	Form	Ingredients
1	*Posidonia oceanica*	Fresh	Brown and green leaf tissues
2	*Ulva rigida*	Fresh	Freshly collected thalli
3	Hendrix Classic K	Dry pellet	Dehulled toasted soybean meal, wheat meal, fishmeal, sunflower meal, fish oil, soybean oil, rapeseed oil, dicalcium phosphate, vitamins A, D3, E and BHT
4	Test a	Dry pieces	Krill meal, pea flour, corn flour, *Fucus* powder, fish oils, *agar–agar*, vitamins, minerals
5	Test b	Dry pieces	Krill meal, pea flour, corn flour, *Fucus* powder, *agar–agar*, pomegranate oil, vitamins, minerals
6	Test c	Dry pieces	Carrots, *Brassica oleracea*, *Lactuca sativa*, *Ulva rigida*, boiled corn, *Mytilus* pulp, sunflower oil, fishmeal, wheat flour, Brewer's yeast
7	Test d	Dry pieces	*Brassica oleracea*, *Lactuca sativa*, *Ulva rigida*, corn oil, sunflower oil, fishmeal, soybeans, wheat flour, Brewer's yeast

2.2 | Outlining diets

A pelletized formulated feed (Classic K®; Hendrix SpA, Mozzecane—VR, Italy) was chosen as a positive control, because its use for feeding of sea urchins was previously tested and it demonstrated to provide rapid fattening of gonads, even faster with respect to individuals collected in the field (Fabbrocini & D'Adamo, 2010; Fabbrocini et al., 2012). This formulated feed contains vitamins and a considerable amount of proteins (Table 1), and this could be an important factor for gonad growth and maturation (Cook & Kelly, 2007; Jacquin et al., 2006; Schlosser, Lupatsch, Lawrence, Lawrence, & Shpigel, 2005). Therefore, it represented a positive control on the size and quality of gonads, as it is inexpensive, is available on the market and produces negligible amounts of wastes (Fabbrocini et al., 2012).

Fresh *U. rigida* and *P. oceanica* were chosen in their turn as natural dietetic items. These plants both characterize environments often populated by *P. lividus*. *Ulva rigida* is a tough green alga containing up to 250 g of proteins per kg of dry weight (Ortiz et al., 2006; Valente et al., 2006). It is considered a control feed in several feeding experiments on fish and invertebrates (Valente et al., 2006), and it is included in various diets for sea urchins (Frantzis & Grémare, 1993). Benthic macrophytes (both macroalgae and *Posidonia* leaves) were collected in shallow waters in the Bay of Napoli, reared in open cycle tanks and administered daily (Frantzis, Berthon, & Maggiore, 1988). Their supplies were renewed weekly to provide daily fresh feeds. The data obtained using these seaweed diets, whose effects on gonadal maturation are known (Frantzis, Grémare, & Vétion, 1992), have been compared to those obtained with our four experimental diets.

In addition, four newly formulated diets were tested and compared to the results yielded by the pelletized diet (available on the market) and the two fresh plant diets mentioned above. All formulated diets differed for the basic ingredients (Table 1), the proportions of proteins and fats (Table 2), as well as the preparation methods. In particular, Diets a and b contained krill meal as the main source of animal proteins and special oils to provide essential fatty acids (Table 1): They were prepared using synthetic binders to increase their stability in the water and then dried at low temperature. The first two formulated diets also differed for the source of fatty acids: The first (diet a) included fish oil, and the second (diet b) included pomegranate seed oil. Pomegranate oil is rich in PUFA (Siano et al., 2015). These compound diets were represented by moist rectangles (2 × 3 × 2 cm) of 100 g/Kg dry nutrients dissolved into 20 g/kg agar.

Biocomposite preparation was carried out according to Paolucci, Fasulo, and Volpe (2015), with some modifications. To obtain the biocomposites, two grams of agar–agar (Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved into 90 ml of distilled water on a stirring plate until boiling, then left cool down to a temperature of about 50°C. Nutrients (10 g) were ground down to a particle size lower than 250 μm and mixed with 10 ml of distilled water at room temperature to obtain a creamy mixture. The mixture was slowly added to the agar solution and then poured into rectangular containers

TABLE 2 Composition (g/kg) of feeds considered in this study

Feeding principle	Abundances (g/kg)						
Typology	Pellet	*Fresh items*		Biocomposites		Baked	
	Hendrix K[a]	*Posidonia oceanica*	*Ulva rigida*	Diet a	Diet b	Diet c	Diet d
Proteins	465	300–400	100–250	230.6	220.2	354.0	290.2
Crude fats	105	20–40	10–30	69.5	70.4	63.0	90.2
Fibres	24	280–320	50–60	20.2	30.2	60.3	42.0
Ashes	95	250–300	400–450	69.5	70.6	102.2	82.0

Notes. Data from Mathers & Montgomery (1997), Mensi et al. (2005), Shams El Din & El-Sherif (2013) and Peña-Rodriguez et al. (2011). Diets have been allotted according to the typology of their preparation and sources.
[a]Proteins of animal origin account for <5%.

(30 × 50 cm) and allowed to cool. At last, the biocomposites were collected, cut into small rectangles of 2 × 3 cm and stored into vacuum-sealed plastic bags at −20°C.

In a different way, Diets c and d were prepared using various flours to make them tougher and compact, then dried into an oven (150°C for 35 min) and finally freeze-dried to increase their stability. Diets c and d also contained brewer's yeast (to increase the toughness of the foils and the quantity of natural vitamins), as well as various sources of proteins (including soybeans, fish meals, mussel pulp) with the aim of sustaining the gonadal growth and maturation (Frantzis & Grémare, 1993). All formulated diets, after the preparation, were cut into 2 × 3 cm small pieces and kept at −20°C in dry containers, up to their use.

2.3 | Experimental procedures

Sea urchins were starved for 2 weeks prior to the start of the experiment and randomly allotted into groups of 20 individuals per 60-L tanks. Each feeding treatment was composed of two replicates of 20 individuals. Thus, seven feeding treatments were performed (Tables 1 and 2) into 14 tanks, each containing a group of 20 individuals. Feeds were daily administered ad libitum (Pearce et al., 2002a). A preliminary check of the actual consumption was permitted to evaluate the feeding rates in our experimental conditions, in order to avoid an accumulation of organic wastes that could generate water pollution. Faecal pellets produced and feed remains were carefully removed every morning, from each tank, and new feed portions were added.

The experiment lasted 90 days, and the results were recorded at 0, 45 and 90 days. At the end of the experiment (90th day), individuals were tested for their general health status by performing a "righting response test" (Bayed et al., 2005; Hyman, 1955). The temperature was slowly lowered to 16°C during the last 20 days, to facilitate the gonad maturation (Russell, 1998). Pools of five individuals were collected in each tank at 0, 45 and 90 days, and their gonads were dissected to evaluate the gonadosomatic indices (Keshavarz, Kamrani, Biuki, & Zamani, 2017). In addition, their gametes were collected and used for a fertilization test, followed by a morphological analysis of the larvae produced. The gonadosomatic index (GSI %) was calculated as:

$$\text{GSI \%} = \text{wet weight of gonads/wet weight of the sea urchin} \times 100.$$

The gonads collected at the 90th day, after the evaluation of GSIs, were fixed in Bouin's solution, included in paraffin blocks, sliced and haematoxylin-stained, to evaluate their maturation stages (Byrne, 1990).

2.4 | Analysis of results

The results of the mentioned seven diet treatments were used to rank the general health conditions of sea urchins, based on the righting response test and the fattening of gonads, evaluated by means of their gonadosomatic indices, histology analyses and fecundity tests. In addition, the diameter of each sea urchin, in each treatment, was recorded by means of a metal calliper at 15-day intervals, to evaluate the growth increments promoted by each diet. The righting response tests were performed according to Hyman (1955) and Lawrence and Cowell (1996) and consisted in the measurement of the time required by individual sea urchins to turn back over, once they were positioned with the aboral face down. The percentage of survivorships was periodically recorded.

Evaluation of fertilization rates and larval morphology analyses was conducted using gametes collected from individuals sacrificed at 45 and 90 days. Mature eggs were counted and fertilized with *P. lividus* sperms at a ratio of 100 sperms: 1 egg (Romano, Miralto, & Ianora, 2010). In particular, collected eggs were allowed to settle and washed three times with 0.22-μm filtered seawater (FSW); eggs were then diluted to a final concentration of 3,000 eggs/ml. Concentrated sperm was collected "dry" and stocked undiluted at +4°C. Sperm mix was diluted (10 μl in 10 ml SW) just before insemination, and its concentration was measured using a counting cell and adjusted by further dilutions, up to $3 \times e10^8$ sperms/ml. An aliquot of 100 μl of this solution was added to 100 ml of egg suspension. About 4 min after sperm addition, eggs were checked for successful fertilization and excess sperm was removed by washing embryos with FSW.

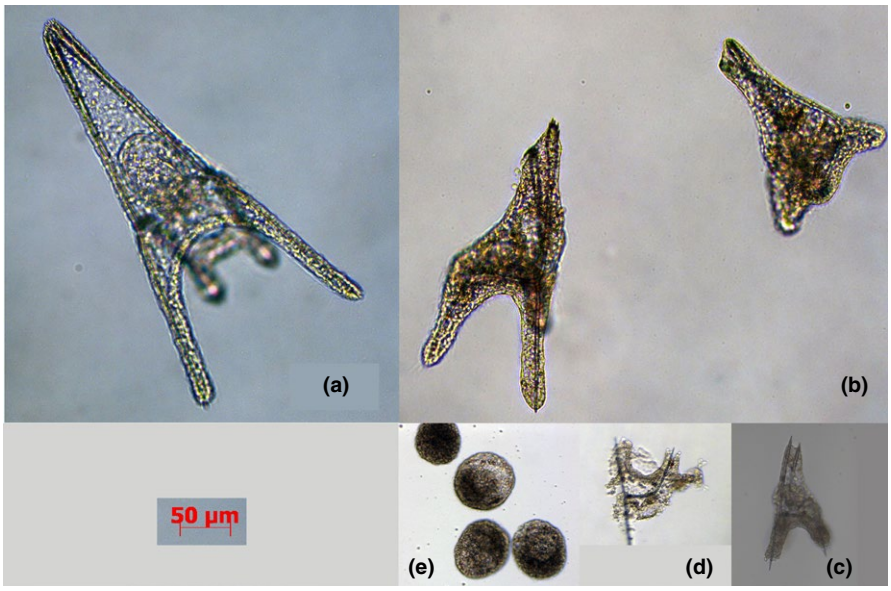

FIGURE 1 Shape of larvae and their possible abnormalities. (a) Normal pluteus; (b) deformities of the pluteus shape, with shortening of apical spiculae; (c, d) plutei deformities, abnormal growth of spicules and block of development; (e) blocked embryos and their blebbing

Multiwells containing 500 fertilized eggs were stored in a thermostatic chamber at 20°C and exposed to a 12:12-hr photoperiod. The test was ended after 48 hr, and *pluteus* larvae were fixed in paraformaldehyde and observed under the optical microscopy to assess (Figure 1) the percentage of abnormalities according to McEdward (1984) and Pagano et al. (1986). The significance of differences among treatments, for all of the above-mentioned records, was evaluated by one-way ANOVA. The significance of differences between the results obtained at 0, 45 and 90 days was assessed by means of *t* tests. Graphs and statistical analyses were computed using the software GraphPad Prism, version 6.00, for Macintosh (GraphPad Software, La Jolla, CA, USA, www.graphpad.com) and Statistica, version 10 (StatSoft Inc., Tulsa, OK, USA).

3 | RESULTS

3.1 | Health status and survival rates

Water quality parameters were kept consistent within the ranges usually measured in the field; any negative influence on survival and development of sea urchins should be excluded. The results of the righting response tests demonstrated good conditions of health of most individuals throughout the experiment. At the end of the test period (90th day), the average time to complete the righting tests was 1′ 22″ (±20″). Survival rates at 45 days (Figure 2a) ranged between 92% in the dietetic treatment with *U. rigida* and 100% in the dietetic treatment with *P. oceanica*. Other treatments exhibited intermediate values (Figure 2a). Survival rates decreased to 85% after 90 days in most formulated diets, and they were highest (Figure 2b) in the treatment with *P. oceanica* (100%) and *U. rigida* (89%). The differences in survival rates among diets were not significant (one-way ANOVA, $p > 0.05$), and the recorded mortality rates were due to individual variability.

3.2 | Size increments

Size increments during this short-term experiment were low, and they varied among diets. The size of animals, at the collection time, ranged between 38.03 and 40.33 mm (maximum diameter of thecae). The dietetic treatment with *P. oceanica* yielded the highest increments at 45 days (0.6 mm, corresponding to 1.0% of the initial size of thecae per month), while the lowest increments were produced by the treatments under the Diets a and b (Figure 3a), and they were, respectively, 0.22 and 0.20 mm, corresponding to 0.57% and 0.52% of the initial size of thecae per month. At the end of the experiment (Figure 3b), the dietetic treatment with the dry pellet yielded the highest size increments (1.38 mm in 90 days, corresponding to 1.17% of the initial size of thecae per month). The lowest increments were produced by the Diets a and b (Figure 3b), and they corresponded to about 0.4 mm in 90 days (0.34% of the initial size of thecae per month). The differences among diet treatments were significant (ANOVA, $p < 0.01$). Size increments were not constant during the experiment, but they were consistently proportional to the size of individuals (Figure 3). In fact, Diet c and *P. oceanica* produced a size increment of about 1.01% per month during the first 45 days and the same diets produced a size increment of 1.06% per month in the next 45 days.

3.3 | Gonadic indices

Sea urchins exhibited complex patterns of gonadic indices according to their diet treatments (Figure 4). Immediately after the collection, the first evaluation of GSI yielded an average value of 3.5% (±0.57%) and this represented the starting point of our experiments, to evaluate the effect of diet treatments. After 45 days of treatment, the formulated Diet d produced the best results (GSI 6.95%), followed by the Diet c (4.84%), while the formulated Diet b exhibited the worst performances for gonadic indices, with

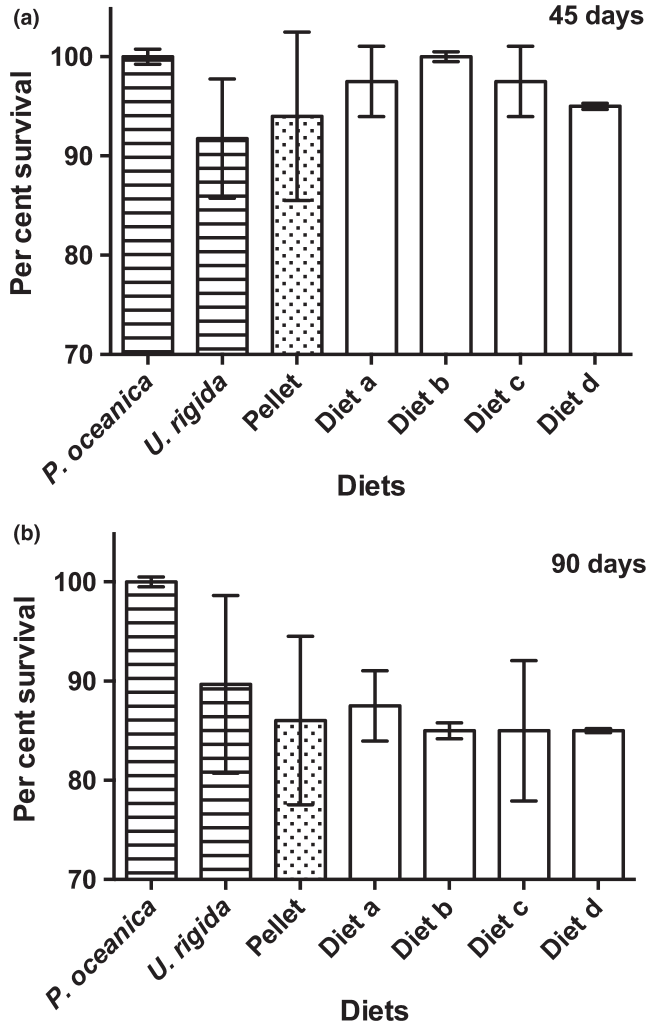

FIGURE 2 Survival rates obtained at 45 days (a) and 90 days (b) of the experimental treatments under various dietetic regimes. Fresh items are reported as horizontal banded bars. The control commercial pellet (Hendrix Classic K®) is indicated by a dotted bar. Four experimental diets evaluated in this study are indicated by white bars

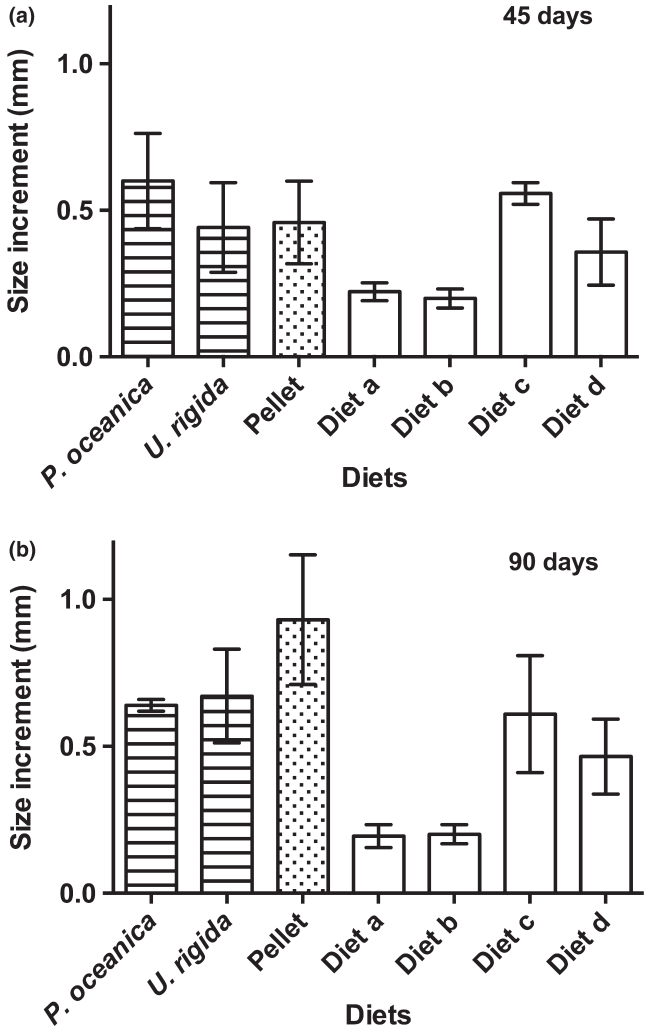

FIGURE 3 Size increments of theca diameter (in mm) in sea urchins measured at 45 days (a) and 90 days (b) of experiment. Fresh items are reported in horizontal banded bars. The control commercial pellet (Hendrix Classic K®) is indicated by a dotted bar. Experimental diets evaluated in this study are indicated by white bars

a reduction in the GSI down to 0.38% (Figure 4). The time progressions of GSIs were not homogeneous among diets, and they had contrasting trends, but the differences between the two testing periods were not significant for most diets (*t* test, $p > 0.05$). However, after 90 days of the experiments (Figure 4) the pellet prompted the highest gonadic indices (GSI 9.19%, $p < 0.01$), followed by the Diet c (5.30%; $p > 0.05$), *P. oceanica* (4.43%; $p > 0.05$) and Diet d (4.02%; $p > 0.05$). The lowest GSIs were obtained with *U. rigida* (2.13%), diet b (1.20%) and Diet a (0.43%). The overall differences in GSIs observed among dietetic treatments were significant (ANOVA, $p < 0.05$).

Variable levels of proteins characterized our dietetic treatments, fats and other feeding principles (Tables 1 and 2), and the protein abundance was significantly correlated to the GSI measured after 90 days of feeding (Figure 5) according to a linear relationships ($R^2 = 0.95$). In contrast, the contents in fats, fibres and ashes of various dietetic treatments (Tables 1 and 2) were not significantly related to the GSIs exhibited at the 90th day.

3.4 | Histological analyses

Gonadal stages were interpreted according to the reproductive cycles of *P. lividus* described by Byrne (1990). The analyses of gonads at various times of treatment indicated that they were in Stage V at Time 0 (partly spawned stage); the patterns of maturation were quite heterogeneous at 45 and 90 days (Figure 6); *U. rigida* treatment kept the reaching of Stage V (partly spawned stage) at 45 days (Figure 6a); the pellet treatment produced mature gonads (Stage IV) at the 90th day (Figure 6h); Diets b and d both conducted to Stage VI (spent stage) at the end of the experiment (Figure 6i, k); Diet c induced a growing stage (Stage II) at the end of the experiment (Figure 6j); and a recovering spent stage

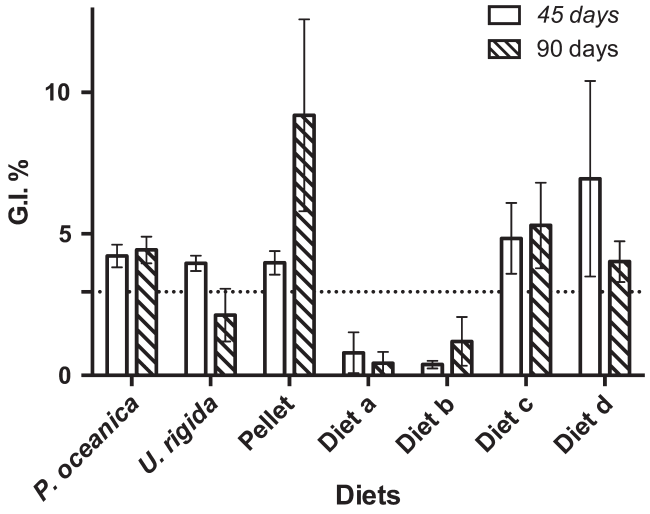

FIGURE 4 Gonadic indices recorded at 45 (white bars) and 90 (banded bars) days of the experiment under various dietetic treatments. The horizontal dotted line indicates the average GSI % measured at the start of the experiment

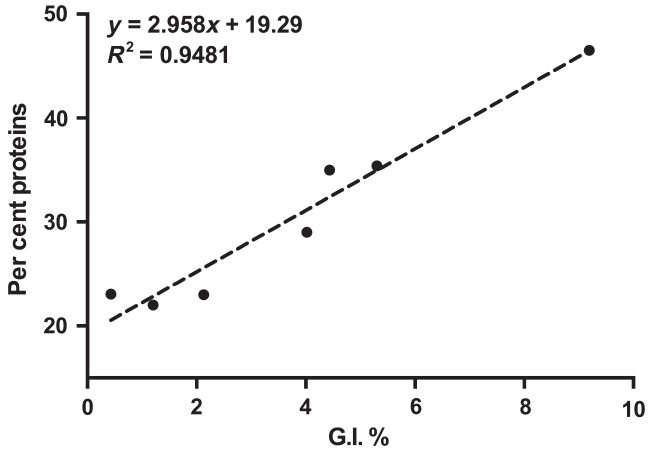

FIGURE 5 Relationship between protein contents of various dietetic treatments here tested and the gonadic indices (GSI %) recorded at 90 days of the experiment in each of them

was exhibited by *P. oceanica* samples at 45 days (Figure 6f), while oocytes were present in the ovaries at the 90th day (Figure 6l). Diet a was insufficient to allow gonadal growth, and, consequentially, the maturation stage could not be evaluated.

3.5 | Fertilization rates and larval morphology tests

The diet treatments produced no significant effects on the fertilization rates (Table 3) as all diets permitted rates of fertilized eggs ranging from 98% to 100% and these values were not different from controls (at $p > 0.05$). In contrast, the results of larval morphology tests were divergent with respect to the GSIs obtained under the considered dietetic regimes. In fact, the commercial pellet, exhibiting the highest GSI, also produced 85.47% of abnormal plutei, while the fresh diets, *P. oceanica* and *U. rigida*, produced 10.00% and 9.41% of abnormalities (ca. 90% and 91% of normal plutei), respectively (Figure 7). The percentages of abnormal plutei were higher in the Dietetic treatments c and d (14.06% and 27.46%, respectively), while no larvae were produced at all by individuals under the Dietetic regimes a and b (Figure 8), whose fertilized eggs developed into atretic embryos.

4 | DISCUSSION

The culture of sea urchins was conducted according to procedures devised by Sartori et al. (2016) and Azad, Pearce, and McKinley (2011), and the experimental conditions fitted the physiologic requirements of *P. lividus*, although the experiments were carried out in small aquaria. The righting response tests (Sherman, 2015) indicated that the survivorships exhibited sufficient health status at the end of the experimental period. Thus, the mortality rates observed at the end of experiments were due to stochastic events and the life in captivity conditions, as also confirmed by the absence of significant differences among treatments. *P. oceanica* and four artificial diets achieved higher survival rates both at 45 and 90 days, and none of diets (including the artificial diets and the pellet Classic K) produced mortalities higher than 15% at the end of tests. Similar survival rates were obtained by previous authors (Azad et al., 2011), adopting analogous experimental conditions. In addition, the resistance of animals to external stresses tends to increase during the development (Sartori & Gaion, 2016), thus assuring higher survival trends in older individuals.

The evaluation of size increments promoted by various diets was not the primary aim of this study, as previous research (Frantzis et al., 1992; Lawrence, 1975) revealed average size increments in the theca diameter of this species, both in nature and in the laboratory, comprised between 0.4 and 2 mm/month. The growth rates of this species were demonstrated to be slow (Boudouresque & Verlaque, 2007) and largely dependent on the amount and quality of feed ingested (Frantzis et al., 1988). However, our results were in the range reported for laboratory studies (Sartori et al., 2015), whose artificial diets promoted increases of the theca diameter of 0.5–1 mm/month and in agreement with those obtained in the field (Grosjean, Spirlet, & Jangoux, 1996). Thecae increments were slightly lower during the first 45 days of experiment, even when proportioned to the body size of animals. Thus, the period of acclimation to dietetic regimes produced slow growth, and succeeding periods yielded faster growth along with the maturation of gonads (Fabbrocini et al., 2012).

This study aimed at finding general rules for the formulation of artificial diets, able to maximize gonadic productions and maturation of gametes, as they represent the actual product of echinoculture practices (Fabbrocini & D'Adamo, 2010). In this perspective, the pellet Classic K confirmed its contribution in the production of large gonads (Sartori & Gaion, 2016), and after an acclimation period, when most feeds yielded similar results, it favoured gonadic indices as high as 9.19%, compared to the best performing formulated Diet c (GSI 5.3%) and the natural diet based on *P. oceanica* (GSI 4.4%). Other

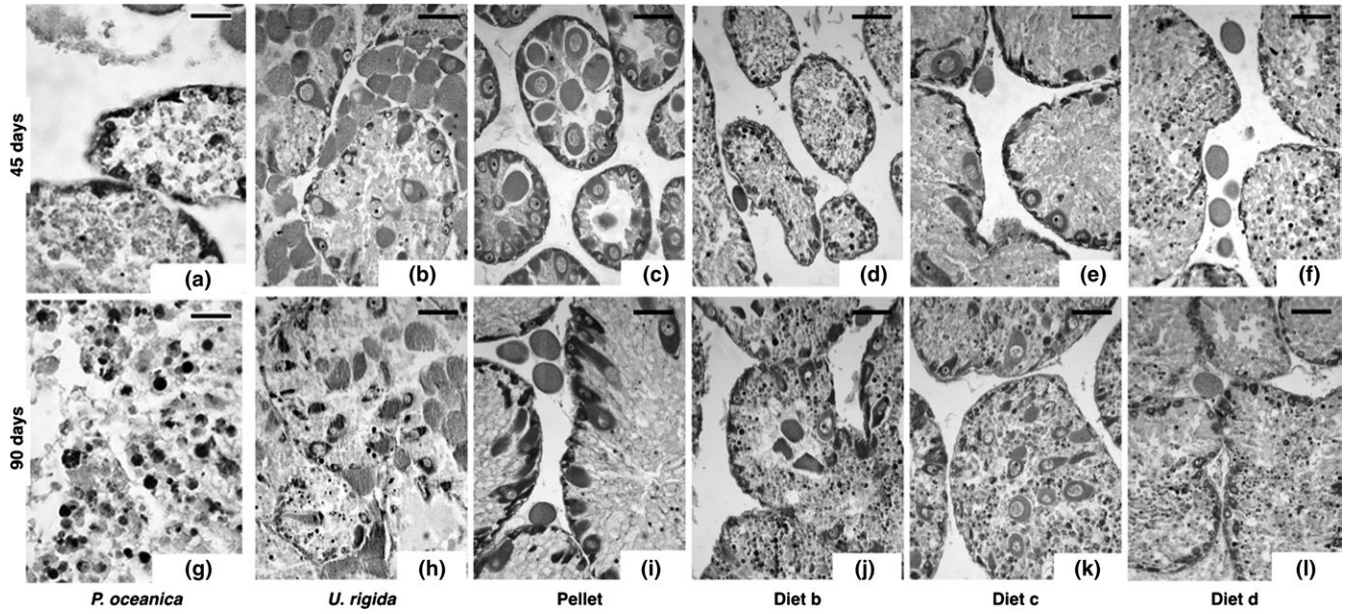

FIGURE 6 Ovaries of individuals after 45 days of culture (a–f) and 90 days of culture (g–l); a and g, under treatment with *Posidonia oceanica*; b and h, under treatment with *Ulva rigida*; c and i, under treatment with pellet Classic K feed; d and j, under treatment with formulated diet b; and e and k, under treatment with formulated Diet c; f and l, under treatment with formulated Diet d. Diet a did not produce evident gonadic masses to be histologically analysed. Scale bars correspond to 100 μm

TABLE 3 Average fertilization rates recorded using gametes produced within individual treatments and standard deviations (*SD*) among replicates

Treatment	Avg. fertilization rate	*SD*
Ulva rigida	99.2	1.35
Posidonia oceanica	100	1.55
Pellet	98.2	1.60
Diet A	100.0	0.13
Diet B	100.0	0.21
Diet C	97.9	2.05
Diet D	98.6	1.89

dietetic treatments, as in the case of *U. rigida* and the formulated Diet d, apparently produced slight reductions in the GSI, with respect to the check at 45 days. In fact, *U. rigida* yielded a GSI 3.95% at 45 days and 2.13% at 90 days. Therefore, Diet d yielded a G.I 6.95% at 45 days and 4.02% at 90 days. However, the differences between GSIs in the two test periods were not significant in several treatments and the most remarkable result was represented by the high efficiency of pellets, followed by the performances of Diets c and d.

It is interesting that a linear relationship was found between the protein contents of diets and the gonadic indices reached at the end of the experiment. This relationship is striking and quite significant, and it should be taken into account in the formulation of feeds for echinoculture practices. According to this relationship, the two formulated Diets a and b that contained the lowest percentage of proteins (230.6 and 220.2 g/kg, respectively), produced GSIs lower than 1.2%, both at 45 and 90 days. Also *U. rigida* that is considered as a control food for sea urchins and contained only 230 g/kg of proteins in our case, promoted a GSI of 2.13% at the 90th day. This result is comparable with the effect of the formulated Diet b. Results are in agreement with those by Fabbrocini et al. (2015), indicating that *P. lividus* fed on agar biocomposites for 14 weeks showed a modest increase in GSIs and a slight progression in the reproductive stages. This effect is not negative when echinoculture productions are directed to human consumption.

Previous research also took into account the role of fatty acids on body growth and gonad maturation. Several diets devised for various species of sea urchins, as *Strongylocentrotus* spp. (González-Durán, Castell, Robinson, & Blair, 2008) demonstrated the role of fatty acids to support their lipid metabolism. However, sea urchins demonstrated a clear ability for elongation and desaturation of shorter chain (18 C) polyunsaturated fatty acids to the longer chain (20 C) n-3 and n-6 HUFA (Castell et al., 2004). Thus, alternative sources of fatty acids may be feasible to obtain sufficient growth and gonadal maturation. This might explain why the dietary content of proteins was the most important factor to assure high GSIs. Our results indicate the absence of significant relationships between GSIs and the total contents of lipids in the tested diets, while there is a strong relationship between the gonadic indices and the diet contents of proteins.

The lipid contents of diets were coherent, in their turn, with the percentages of abnormal plutei. In fact, diets based on natural items (*P. oceanica* and *U. rigida*), containing lowest amounts of lipids, yielded the lowest rates of abnormalities and the commercial pellet, containing the highest percentages of lipids, yielded the highest rates of abnormal plutei. These data indicate that an ideal diet for *P. lividus* should contain relatively low quantities of lipids (as in

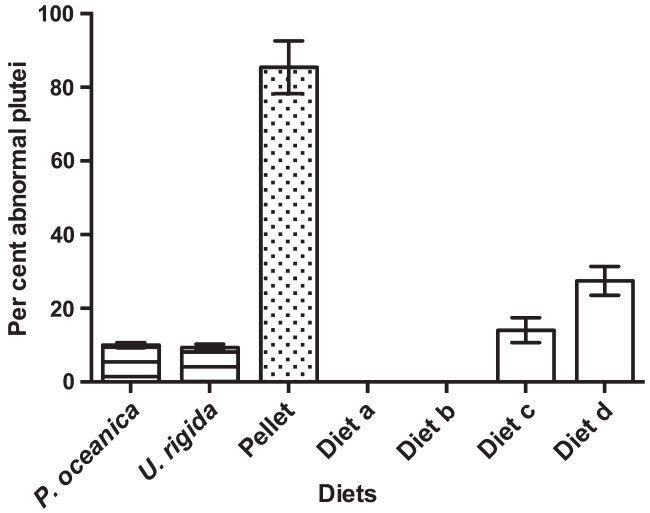

FIGURE 7 Percentage of abnormal plutei obtained from gametes collected, under each of the considered dietetic treatments, at the end of the experiment (90 days) and after in vitro fertilization. Diets a and b did not produce viable larvae

the case of Diets c and d, with 60 and 90 g/kg of fats, respectively) and high quantities of proteins to produce high gonadic indices (high weight of "roe") and low percentages of larval abnormalities. To a remarkable degree, the diet treatments had an effect on gonadic indices and influenced further larval development, but the fertilization was not influenced by the quality of feeds. In the same way, in various marine invertebrates the development of larvae was found to be more sensitive to maternal influences than the egg fertilization (Williams & Bentley, 2001).

Previous research mainly focused on the effects of diets on the abundance and taste of roe (Barker, Keogh, Lawrence, & Lawrence, 1998). However, modern applications of sea urchins for scientific purposes (Santella & Chun, 2011) and for the aims of ecological conservation (Abadie, Pace, Gobert, & Borg, 2018) claim for the need to assure good larval development and success of reproductive bursts. Similar studies performed on *Strongylocentrotus droebachiensis* (Pearce et al., 2002c) for a comparable experimental period, examined the effect of artificial diets with increasing levels of proteins (i.e., 190, 240 and 290 g/kg of their dry weight), and their results showed a positive effect of proteins on the gonadal increase

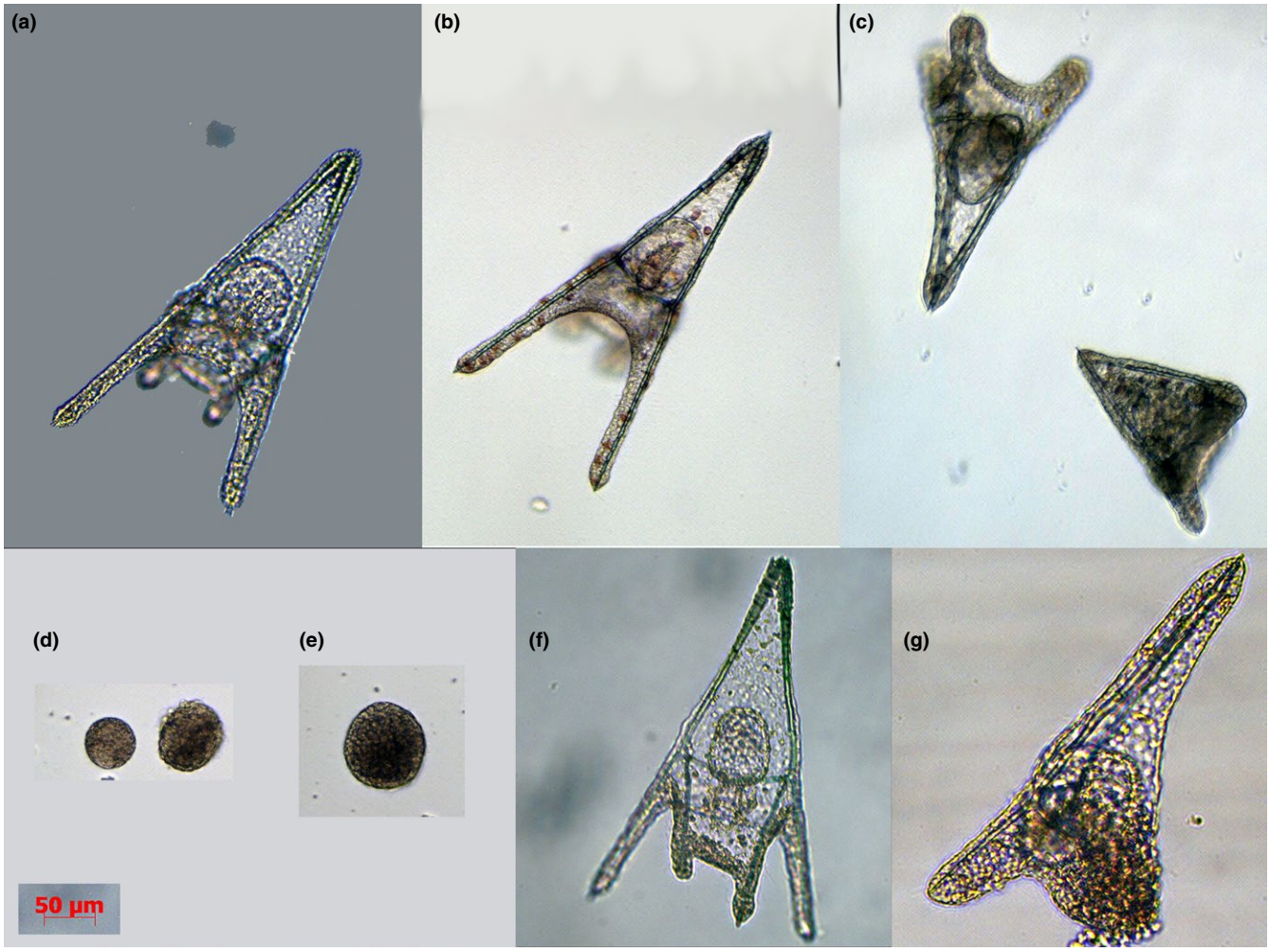

FIGURE 8 Larval shapes observed in: (a) *Posidonia oceanica* treatment; (b) *Ulva rigida* treatment; (c) pellet treatment; (d, e) diet treatments a and b, respectively, producing only atretic embryos showing blebbing; and (f, g) diet treatments c, d, respectively

of weight. As well, de Jong-Westman, March, et al. (1995) reported significantly higher gonad yields in adults of *S. droebachiensis* fed the prepared diets containing 200 g/kg protein (ground wheat, condensed fish soluble, and albumin) than in those given similar diets with only 100 g/kg of proteins.

Other investigations (Akiyama, Unuma, & Yamamoto, 2001) indicated, in contrast, that diets with protein concentration above 200 g/kg did not significantly alter somatic growth rates and GSI of various species of sea urchins (Akiyama et al., 2001; de Jong-Westman, Qian, et al., 1995; Kennedy, Robinson, Parsons, & Castell, 2000; McBride, Lawrence, Lawrence, & Mulligan, 1998). Nevertheless, the source of proteins may influence gonadic indices and various studies (e.g., Fernandez & Boudouresque, 1998, 2000) found that highest gonadic indices were achieved with diets exhibiting intermediate levels of animal proteins (about 100 g/kg of total proteins deriving from fish meals). In a consistent manner, GSIs were significantly lower in *P. lividus* fed the diets without animal proteins and in those with the highest level of animal proteins.

The dietetic effects herein demonstrated are modulated by ecological influences, as temperature, photoperiod and feed rations regulate the gonadic indices in various sea urchins (Garrido & Barber, 2001). The effects of environmental factors may be contrasting in different species, according to their life strategies and the features of their habitats. In the case of *P. lividus* that is a Mediterranean species with winter reproduction, high feed rations are necessary to produce gonadic tissues and long periods of starvation bring the gonads to a spent stage, due to the consumption of tissues to obtain energy (Sartori et al., 2016). In addition, conditions of high protein availability (predominant in winter, in local food webs) and low temperatures favour reproductive output in its populations. In fact, various vegetated ecosystems of the Mediterranean (e.g., *P. oceanica* meadows, one of the elective environments for this species) offer abundant resources for opportunistic herbivores and mesocarnivores in winter months (Zupo, 1993; Zupo et al., 2017). Thus, the winter reproduction of *P. lividus* is in accordance with its trophic requirements (abundance of protein-rich feeds, available as plant epiphytes) for the development of gonads.

Taking into account these considerations and the need to keep low the costs of feeding for productive purposes, a feed containing high proportions of carbohydrates (e.g., deriving from corn, wheat middling, soybean, algae) is suggested in the first phases of growth, up to the initial period of sexual maturation. The protein content of diets should be then increased up to the highest levels of 400–450 g/kg (assuring that most of it is provided by plant sources; Table 2), to facilitate the increase in gonadic tissues, while keeping relatively low (<90 g/kg) the abundance of lipids, to promote the production of viable gametes. In the case of aquaculture products directed to human consumption, a finishing may be considered, in the last period prior to collections, using agricultural wastes such as banana peels and pumpkins (Luo, Zhao, Chang, Feng, & Tian, 2014) to improve colour and taste of gonads.

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ORCID

Valerio Zupo http://orcid.org/0000-0001-9766-8784

Marina Paolucci http://orcid.org/0000-0002-1784-3843

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