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Original Article

Paracentrotus lividus roe enhancement by a short-time rearing in offshore cages using two agar-based experimental feed

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Abstract: In this study, we tested the effect of two agar-based biocomposites (differing for the fish meal source), on the *Paracentrotus lividus* gonad cycle progression and biochemical composition, in an offshore pilot scale trial. The purpose of this study was to identify a feeding regime for *P. lividus* that would allow adequate gonadal growth without accelerating the gamete maturation, in order to extend the market period. The purpose was pursued through the use of agar as a binding agent in the manufacturing of biocomposites to be used as feed for *P. lividus* and the realization of special offshore cages. The gonad index, histology, biochemical composition of fatty acids (FA) and free amino acids (FAA) and volatile molecule fingerprint were evaluated. Both the gonad index and the histological analysis showed a slower gametogenesis progression in the gonads of caged sea urchins with respect to the field collected ones, consistent with our previous results obtained testing a similar feed composition in a recirculating system. The amount and the relative proportions of FA and FAA in the gonads of wild and cultured gonads were similar, while the analysis of the volatile substances of the gonads of the sea urchin conducted using the electronic nose, shows that they vary as a result of the different feeding treatments. These results show that *P. lividus* sea urchins can be housed, under this feeding regime during the recovery phase of the gonads, without acceleration of the maturation of the gametes and thus in conditions to allow the extension of the market period. Moreover, the here tested agar-based biocomposites make it possible to reduce the management costs of the rearing system as they need to be replaced only once a week, and its environmental impact as they guarantee a limited nutrient dispersion in the water.

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Introduction

Once abundant in the Mediterranean Sea, the *Paracentrotus lividus* is now an endangered sea urchin species that requires specific conservation actions to prevent its total disappearance. Overfishing for commercial purposes is one of the most crucial causes of *P. lividus* rarefaction (Secci, 2014). Indeed, the edible gonad or roe of *P. lividus,* is consumed in many Mediterranean countries and thus has a high-market demand (Matsiori et al., 2012; Pais et al., 2012). The paucity of the *P. lividus* natural populations could be partially restored by the acceleration of farming development. Sea urchin aquaculture activity is characterized by a rapidly expanding sector (FAO, 2018) accompanied by increasing demand for artificial feed specially-designed to meet the nutritional needs of the species. Despite that a formulated diet frequently leads to gonads with poor quality in terms of texture, firmness, color, and taste (Luo et al., 2014; Walker et al., 2015), it has been shown that a prepared diet, with respect to the natural one, increases both growth and yield of the gonads (Otero-Villanueva et al., 2004; Pearce et al., 2004; Schlosser et al., 2005; Azad et al., 2011).

The relationship between the feed composition and the growth rate of a given aquatic species is crucial for

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the optimization of the rearing conditions. Most importantly, nutrient loss and feed softness should be prevented. Binding agents have been employed to improve the water stability of aquatic animal feed and given that binders often account for a consistent part of the formulated feed, the effect of these substances on the ingestion, digestion, and growth should not be overlooked (Volpe et al., 2008, 2012, 2014, 2015; Coccia et al., 2010). Gelatine and polysaccharides of marine origin (such as alginates and agar) are among the most popular binders to manufacture feed for sea urchins (Fernandez and Pergent, 1998; Mortensen et al., 2004; Daggett et al., 2005; Kennedy et al., 2007; Barker et al., 1998; Akiyama et al., 2001; Pearce et al., 2002; Fabbrocini et al., 2011; 2012, 2015). Interest has also been devoted to binders, such as guar gum, corn starch (Pearce et al., 2002) and pectins (Fabbrocini et al., 2015). Generally, the presence of the binder produces an increase in the gonad weight compared to the natural diet, albeit with variations depending on the percentage of inclusion (see Paolucci et al., 2012 for review).

Among the biopolymers studied for applications as a binder of aquatic animal feeds, agar has shown to have characteristics favorable for use as a binder in the preparation of diets formulated for sea urchin *P. lividus* (see Paolucci et al., 2012 for review). Agar alone or in combination with other biopolymers produces biocomposites with suitable consistency and resistance into the water. In a recent study, it has been shown that agar manages to maintain a good consistency after prolonged immersion in seawater (over 15 days), and it is not degraded by the sea urchin grazing activity (Fabbrocini et al., 2015). Bio-composites with agar as a binder are appreciated by *P. lividus* and are completely consumed within three days, thus reducing the amount of food wasted to a negligible level and increasing the rate of ingestion (Fabbrocini et al., 2012, 2015). In *P. lividus* reared in laboratory conditions, biocomposites made with agar have been shown to induce a slightly slower progression of the reproductive cycle compared to a commercial feed (Fabbrocini et al., 2015).

The gonads of the sea urchin consist of nutritive phagocytes and gametes (Walker et al., 2001). The gonads with the best characteristics in terms of firmness, consistency, color, and taste are obtained when the ratio between nutritive phagocytes (abundant during recovery and growth phases) and gametes (abundant during the mature and generation phases) is in favor of the former (Böttger et al., 2006; Osako et al., 2007). Thus, the purpose of this study is to slow down the maturation of gametes to obtain gonads with a ratio between phagocytes and gametes in favor of the former. The aim was achieved using artificial diets based on agar as a ligand administered in urchins in offshore cages located along the south-eastern Italian coast during the recovery period.

Materials and Methods

Sea urchin collection: Sea urchins were collected by a scuba diver in the early September (beginning of the recovery phase) 2013, along the South East Italian coast around Ugento (Lecce, Apulia Region). At this site, the *Posidonia oceanica* was abundant as the main feed item of sea urchins. 20 sea urchins were analyzed immediately after the collection and measured for test diameter (TD), body weight, gonad weight and gonad maturation phase (this initial sample is defined as T0), and provided the initial baseline. A caliper and an ordinary weighing scale with 5 gr calibrations were used in measuring TD and individual weight, respectively.

Cages and stocking density: Each cage (1x1x0.25 m) was formed by a frame of steel bars covered with plastic wire with a mesh size of 3 cm. A removable tray containing the biocomposite was placed on the bottom of the cages. The biocomposite was covered by a plastic wire with a mesh size of 10 cm that allowed the sea urchins to graze on it. Six experimental cages were randomly arranged in two lines of three cages each. A block of cement on both sides of the lines fixed them on the bottom. 30 adult sea urchins with size range 35-40 mm were placed in each cage. The cages were sited at 700 m offshore and 12 m of depth in the coastal strip of Ugento (Ionio Sea, 39.883462, 18.100411).

Diet formulation and manufacturing: Sea urchins

within the cages were fed two formulated diets containing 23% protein, 50% carbohydrate, 5% fat and 2% agar as a binder. The two diets differed for the source of the fish meal, one included anchovy flour as fish meal (diet FM), the other included krill flour instead of fish meal (diet KM). The diets were made of 10% dried nutrients dissolved in 2% agar solution (Volpe et al., 2018). Briefly, 2 gr of agar (Sigma-Aldrich Co., St. Louis, MO, USA) were dissolved in 90 ml of distilled water in a water bath placed on a stirring plate until boiling, then left to cool down at a temperature comprised between 40 and 50°C. Nutrients (10 gr) were ground to a particle size <250 μm, mixed with 10 ml of distilled water at RT to obtain a creamy mixture, that was slowly mixed with the agar solution. The final mixture, defined as biocomposite, was then poured into a rectangular container (tray) (1x1x2 cm) and allowed to cool down at RT. Ingredients, nutrient composition, amino acid and fatty acid content of the two formulated diets or biocomposite, are reported in Table 1 (after Volpe et al., 2018).

Roe enhancement trial: The roe enhancement trial lasted four weeks, commenced in the beginning of September and ended in the beginning of October 2013. Sea Urchins were field collected by a scuba diver and placed in offshore cages at a density of 30 specimens/cage. There were six cages in total. Three cages were intended to sea urchins fed with the diet FM and three cages intended to sea urchins fed with the diet KM. The two diets were offered to sea urchins in the form of biocomposite, made of nutrients and agar. The biocomposite was poured into a tray and made solidify as reported above. Thereafter, the trays containing the biocomposite were transported to the trial site and placed on the bottom of the cages. Previous studies (Volpe et al., 2018) showed that sea urchins fed on the biocomposite and that only traces of biocomposite were present after a week. The tray was removed from the bottom of the cage and replaced with another tray containing the biocomposite with a weekly frequency. The sea urchins were fed *ad libitum*. At the end of the trial, the sea urchins were sacrificed and histological, biochemical, and olfactory analyses were carried out on the gonads. Moreover, ten sea urchins were collected again in the same area, as a field control; this group is referred to as T4.

Gonad index and gonadal stage: Sea urchins were allowed to drip for approximately 5 min, weighed (0.2 mg accuracy) and then dissected; the gonads were extracted and fresh-weighed for evaluation of the Gonad Index (GI):GI = gonad wet weight (g) / sea urchin wet weight (g) × 100. Histological analysis was performed as reported by Fabbrocini et al. (2012). Gonadal stage was assigned according to Spirlet et al. (2000): stage I (spent with relict gametes), stage II (spent empty), stage III (recovery), stage IV (growing), stage V (premature) and stage VI (mature), stage VII (spawning); stage VIII (post-spawned).

Free Amino Acid analysis: Free Amino Acid (FAA) analysis of the gonads of the sea urchins was performed according to Volpe et al. (2018). Briefly, the extraction of the gonads was carried out with 0.1% formic acid for 1 hour. The extract was centrifuged at 18000xg for 60 minutes and the supernatant filtered with 0.5 and 0.45 μm Millipore filters. The filtrate was analyzed with a cation exchange column (100 x 6 mm AG50WX8-H + Bio Rad). The mobile phase was 3.0 M NH3. The eluted fractions were evaporated to dryness, recovered with 0.1 M HCl and filtered through a 0.45 um Millipore filter. HCl was removed and the dried samples were reconstituted with a lithium citrate buffer (0.2 M, pH 2.2) for analysis. AA analysis was performed by reverse phase (RP) HPLC, using a Waters chromatograph model 2690 equipped with fluorescence detector model 474. Quantification was made using the peak area of the fluorescence emission intensity by excitation at 350 nm and recording fluorescence emission at 395 nm. The retention time of the amino acids was used for the identification. Amino acids were quantified by comparison of the corresponding peak area with the respective calibration curve.

Lipid and fatty acid analysis: The Soxhlet method, using diethyl ether as a solvent, was employed to analyze the lipids of the sea urchin gonads, according to the methods of analysis used for chemical control of food of the National Institute of Health, 1996

Table 1. Composition of the feed formulation, free amino acid composition (mg/100gr) and amino acid composition (mg/100gr) of the two manufactured diets. Diet FM contained anchovy flour as fish meal. Diet KM contained krill flour as fish meal. The analysis was carried out on three samples of each diet (n=3). Values are reported as mean±SEM (after Volpe et al., 2018).

	Diet FM	**Diet KM**
Ingredients	**% in the diet**	
Anchovy meal	19	-
Krill meal	-	19
Pea flour	27	27
Corn flour	25	25
Kelp (dry)	25	25
Agar	2	2
Fish oil	1.8	1.8
Vitamin premix[1]	0.1	0.1
Mineral premix[2]	0.1	0.1
Nutrient composition (gr/100gr)		
Total protein	23.06±2.12	22.02±1.99
Total carbohydrate	49.77±4.32	49.67±4.66
Total fat	6.95±0.56	7.04±0.52
Free amino acid composition (mg/100gr)		
Aspartic acid	nd	5.5±0.4
Glutamic acid	8.1±0.7	15.5±1.1
Serine	3.0±0.3	4.7±0.4
Asparagine	56.8±4.9	nd
Glycine	1.8±0.2	9.4±0.8
Histidine	3.6±0.3	nd
Alanine	4.5±0.3	16.3±1.4
Tyrosine	nd	4.0±0.3
Valine	3.1±0.3	5.6±0.5
Cysteine	40.4±0.4	39.8±3.5
Isoleucine	19.6±1.6	17.0±1.5
Leucine	nd	18.4±1.6
Lysine	17.3±1.5	35.5±3.1
Phenylalanine	4.9±0.3	6.0±0.5
Amino acid composition (mg/100gr)		
Aspartic acid	6.68±0.73	6.07±0.69
Glutamic acid	5.25±0.64	5.30±0.55
Serine	2.98±0.37	2.33±0.27
Glycine	4.30±0.51	2.71±0.30
Glutamine	1.94±0.18	2.83±0.29
Histidine	2.22±0.25	1.82±0.20
Threonine	2.94±0.30	2.38±0.28
Arginine	3.60±0.40	4.36±0.49
Alanine	4.32±0.49	3.06±0.35
Proline	1.56±0.18	1.37±0.16
Tyrosine	3.38±0.37	2.12±0.22
Valine	3.72±0.39	3.05±0.31
Cysteine	0.81±0.09	0.74±0.09
Methionine	1.79±0.19	1.76±0.19
Isoleucine	2.80±0.27	2.60±0.29
Leucine	6.32±0.67	5.72±0.61
Lysine	3.39±0.34	3.68±0.40
Phenylalanine	3.33±0.39	3.30±0.39
Tryptophan	0.35±0.38	nd

1 Zoofast, Italy **2** Mineral premix contains (mg kg−1)Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; I, 0.1; and antioxidant (BHT), 100. Vitamin premix contains (mg kg−1) E, 30; K, 3; thiamine, 2; riboflavin, 7; pyridoxine, 3; pantothenic acid, 18; niacin, 40; folacin, 1.5; choline, 600; biotin, 0.7 and cyanocobalamin, 0.02.

(Reports ISTISAN 96/34), as reported by Volpe et al. (2018). The fatty acid profile as fatty acid methyl esters (FAMEs), was analyzed by gas chromatography, according to the AOAC method 996.06 (AOAC, 1997), using a SP-2560 (100 m x 0.25 mm x 0.20 μm, Supelco) capillary column in a split mode (ratio 1:100). The operating conditions started with the oven temperature of 140°C, for 5 min. The temperature linearly increased to 260°C (4°C/min) and was held for the remaining time of analysis. The retention times of the standard mixture FAMEs (Fatty Acid Methyl Esters) were employed to identify the fatty acid composition of the sample. Data were recorded and processed by the ChromQuest 5.0

Table 2. Total and gonad weight, GSI and gonad stage values are reported. Wild T0= sea urchins caught and analyzed at the beginning of the roe enhancement trial. Wild T4= sea urchins caught and analyzed at the end of the roe enhancement trial. Diet FM=sea urchins fed the diet containing anchovy as fish meal and analyzed at the end of the roe enhancement trial. Diet KM=sea urchins fed the diet containing krill as fish meal and analyzed at the end of the roe enhancement trial. N=10. Values are reported as mean ±SEM (P<0.01; one-way ANOVA followed by Bonferroni's test). Superscript letters indicate significant differences among groups.

Group	Total weight (gr)	Gonad weight (gr)	GSI	Stage
Wild T0	53.37±6.46	0.60±0.12^{c}	1.14±0.30^{c}	II-III
Wild T4	52.51±5.32	3.27±1.01^{a}	6.08±1.21^{a}	V-VI
Diet FM	63.95±6.78	2.05±0.76^{b}	3.18±0.65^{b}	IV-V
Diet KM	63.64±6.34	2.48±0.93^{b}	3.84±0.70^{b}	IV-V

Table 3. Free Amino Acid concentrations in the gonads of wild and cultured sea urchins. Wild T4=sea urchins caught and analyzed at the end of the roe enhancement trial. Diet FM=sea urchins fed the diet containing anchovy as fish meal and analyzed at the end of the roe enhancement trial. Diet KM= sea urchins fed the diet containing krill as fish meal and analyzed at the end of the roe enhancement trial. N=6. Values are reported as mean ±SEM (P<0.01; one-way ANOVA followed by Bonferroni's test). Superscript letters indicate significant differences among groups.

	Wild (T4)	Diet FM	Diet KM
Asp	3.4±0.20^{b}	5.47±0.55^{a}	0.014±0.01^{c}
Glu	28.47±1.82^{c}	65.83±3.75^{a}	52.07±6.41^{b}
Ser	43.5±0.89^{b}	87.87±6.88^{a}	97.87±8.66^{a}
Gly	859.80±31.86^{b}	1397.63±102.70^{a}	1059.77±74.01^{b}
Gln	86.77±4.40^{b}	296.9±14.75^{a}	307.03±6.65^{a}
His	31.37±1.90^{c}	75.43±2.45^{a}	62.3±3.30^{b}
Thr	17.8±2.05^{c}	48.5±3.20^{a}	28.73±3.10^{b}
Arg	147.77±15.80^{b}	355.8±15.05^{a}	333.47±22.23^{a}
Ala	95.3±4.95^{c}	222.03±9.76^{a}	188.87±11.30^{b}
Pro	54.53±4.15^{b}	49.4±3.92^{b}	71.7±1.45^{a}
Tyr	18.3±1.85^{b}	60.6±3.60^{a}	57.77±2.40^{a}
Val	6.7±0.40^{c}	29.8±4.25^{a}	15.63±0.80 ^{b}
Met	1.4±0.20^{b}	6.1±0.40^{a}	4.53±1.11^{a}
Ile	6.23±0.65^{b}	11.6±1.37^{a}	7.3±0.40^{b}
Leu	7.57±1.06^{c}	30.53±3.26^{b}	17.43±0.91^{a}
Lys	184.83±13.86^{c}	386.43±12.30^{a}	314.1±13.36^{b}
Phe	7.57±0.47^{c}	28.67±1.07^{a}	18.47±1.81^{b}

software (Thermo, Rodano, Italy).

Volatile molecule fingerprint: The volatile molecule fingerprint of gonads was analyzed by a commercial portable electronic nose (PEN 3), including the Win Muster software for data mining, Airsense Analytics Inc. (Schwerin, Germany) as reported in Laurienzo et al. (2013) and Volpe et al. (2018). The analyses were performed using recording data in the steady state.

Statistical analysis: Data are expressed as mean±SD. One-way ANOVA was followed by Bonferroni's test. The data were tested for normality with Shapiro-Wilks test and for homogeneity of variance with Cochran's test; P-values <0.01 were considered significant. The Win Muster software was used. The Correlation Matrix showed the discrimination power measuring the severability of classes. The discrimination indexes (DI) were in the range of 0 and 1, as previously reported (Volpe et al., 2014). Values lower than 0.5 show a low severability, values ≥0.5 indicate a severability of samples. Values of discrimination indexes ≥0.950 are significant.

Results

Gonad index and gonadal stage: *Paracentrotus lividus* gonad weight and GSI at the beginning (T0) and at the end of the trial (T4) and after four weeks of formulated diet (diet FM and diet KM) are reported in Table 2. Significant differences (P<0.01) in the gonad weight and GSI between wild urchins and formulated diet fed urchins, were detected. The histological evaluation of the gonads (according to Spirlet et al., 2000) showed that a certain progression of the reproductive cycle occurred. On T0 sea urchins were at the beginning of the gametogenesis (stages II-III): a thin layer of early developing gametes became to be evident along the acinal wall, while nutritive phagocytes created a

Table 4. Free Amino Acid concentrations in the gonads of wild and cultured sea urchins. Wild T4= sea urchins caught and analyzed at the end of the roe enhancement trial. Diet FM=sea urchins fed the diet containing anchovy as fish meal and analyzed at the end of the roe enhancement trial. Diet KM=sea urchins fed the diet containing krill as fish meal and analyzed at the end of the roe enhancement trial. N=6. Values are reported as mean ±SEM (P<0.01; one-way ANOVA followed by Bonferroni's test). Superscript letters indicate significant differences among groups.

Component (Acid Metil Ester), Area %	Wild (T4)	Diet FM	Diet KM
Caprylic C8:0	0.54±0.10	0.57±0.03	0.61±0.04
Tridecanoic C13:0	0.17±0.03^{a}	0.01^{b}	0.15±0.01^{a}
Myristic C14:0	4.84±0.60	4.80±0.02	5.28±0.11
Pentadecanoic C15:0	0.77±0.09	0.84±0.01	0.78±0.08
Palmitic C16:0	16.10±1.10	16.59±0.15	16.82±0.12
Palmitoleic C16:1	1.99±0.02^{b}	2.05±0.10^{b}	2.58±0.07^{a}
Heptadecanoic C17:0	0.62±0.05^{a}	0.62±0.01^{a}	0.47±0.03^{b}
cis-10-Heptadecenoic C17:1	0.66±0.06^{a}	0.47±0.04^{b}	0.69±0.07^{a}
Stearic C18:0	5.75±0.71	5.86±0.03	6.19±0.04
Elaidic C18:1 n9t	0.51±0.02^{a}	0.40±0.03^{b}	0.18±0.03^{c}
Oleic C18:1 n9c	1.35±0.50	1.12±0.12	1.40±0.04
Linolelaidic C18:2 n6t	0.38±0.01^{b}	0.56±0.03 ^{a}	0.16±0.01^{c}
Linoleic C18:2 n6c	2.24±0.20	2.46±0.06	2.57±0.07
Arachidic C20:0	0.24±0.03^{a}	0.01^{c}	0.18±0.01^{b}
γ-Linolenic C18:3 n6	0.37±0.04^{c}	0.58±0.05^{a}	0.49±0.03^{b}
cis-11-Eicosenoic C20:1	1.99±0.06^{b}	2.03±0.08^{b}	2.63±0.02^{a}
Linolenic C18:3 n3	6.19±0.31^{b}	7.08±0.12^{a}	4.40±0.04^{c}
cis-11.14-Eicosadienoic C20:2	2.55±0.21^{b}	2.90±0.07^{a}	3.07±0.02 ^{a}
Behenic C22:0	0.10±0.01^{b}	0.01^{c}	0.24±0.01^{a}
cis-8.11.14-Eicosatrienoic C20:3 n6	0.48±0.02^{b}	0.49±0.01^{b}	0.62±0.02^{a}
Erucic C22:1 n9	1.95±0.11^{a}	1.48±0.04^{b}	1.87±0.07^{a}
cis-11.14.17- Eicosatrienoic C20:3 n3	2.99±0.12^{a}	3.11±0.10^{a}	2.27±0.03^{b}
Arachidonic C20:4 n6	9.51±0.90^{ab}	9.28±0.07^{b}	10.83±0.09^{a}
cis-5.8.11.14.17-eicosapentanoic C20:5 n3	12.57±1.23	13.32±0.06	13.77±0.07
cis-4.7.10.13.16.19-Docosahexaenoic	4.33±0.35	5.98±0.06^{a}	3.67±0.05^{c}

mesh-work in the center of acini (stage II, spent empty); a thicker meshwork of nutritive phagocytes filled the acini and a layer of developing gametes was present along the acinal wall in the recovery specimens (stage III). At the end of the trial, sea urchins fed both the formulated diet (FM and KM groups) appeared to belong to the stages IV-V (growing and premature): developing primary oocytes surrounded by nutritive phagocytes were observed in the ovary, and columns of developing spermatocytes were present in the testes of growing sea urchins; while developing gametes were clearly observed, and mature oocytes or spermatozoa began to accumulate in the center of the ozone of the premature specimens. On the contrary, the gonads of wild urchins (T4) proved to have progressed faster in the gametogenesis; in fact, in addition to premature (stage V) ones, also mature sea urchins were observed (stage VI), in which gonads were packed with mature gametes, and few developing gametes could be observed.

Free Amino Acids (FAA) in the gonads: Almost all the essential amino acids were detected in the gonads of both wild (T4) and cultured urchins (diet FM and diet KM). There were no qualitative differences in the FAA profiles among groups. The most represented FAA were glycine, glutamine, arginine, alanine, and lysine. Glycine was also the dominant amino acid in both the wild and cultured urchins. There was a significant increase in the concentration of all amino acids in the gonads of cultured sea urchins with respect to the wild sea urchins, with the exception of isoleucine and proline (only in the gonads of sea urchins fed on the FM diet) (Table 3).

Fatty Acids: The composition of the FA of the gonads of wild and cultured sea urchins is reported in Table 4. The most represented FA were Myristic acid (C14:0), Palmitic acid (C16:0) and Stearic acid (C18:0) among the saturated fatty acids (SFAs), Palmitoleic acid (C16:1), Oleic acid (C18:1n9c), cis-11-Eicosenoic acid (C20:1) and Erucic acid (C22:1n9) among the monounsaturated fatty acids (MUFAs) and Linolenic (C18:3n-3), Linoleic (C18:2n-6),cis-11,14,17-

Table 5. Correlation Matrix among the *Paracentrotus lividus* groups. Wild= urchins caught and analyzed at the end of the roe enhancement trial (T4). Diet FM= urchins fed the diet containing anchovy as fish meal and analyzed at the end of the roe enhancement trial. Diet KM=urchins fed the diet containing krill as fish meal and analyzed at the end of the roe enhancement trial. The numbers indicate the DI. Values of DI ≥ 0.95 are significant.

	Diet FM	Diet KM	Wild (T4)
Diet FM	0.000		
Diet KM	0.747	0.000	
Wild (T4)	0.550	0.632	0.000

Eicosapentanoic acid (C20:5n-3, EPA), Arachidonic acid (C20:4n6, AA), cis-11,14-Eicosadienoic (C20:2), cis-11,14,17-Eicosatrienoic (C20:3n-3), and cis-4,7,10,13,16,19-Docosahexaenoic (C22:6n3) among the polyunsaturated fatty acids (PUFAs). Significant differences were limited to cis-11-Eicosenoic that was higher in the KM diet fed sea urchins, linolenic acid that was higher in the FM diet fed sea urchins and lower in the KM diet fed sea urchins with respect to the wild sea urchins, cis-11,14-Eicosadienoic that was higher in cultured sea urchins, Arachidonic acid that was lower in the FM diet fed sea urchins and higher in the KM diet fed sea urchins with respect to the wild sea urchins, while the DHA showed the opposite trend, showing a higher concentration in FM diet fed sea urchins with respect to KM diet fed and wild sea urchins.

Volatile molecule fingerprint: The PCA analysis of volatile substances of the sea urchin gonads (Fig. 1) evidenced separated groups of wild sea urchins (T4), diet FM and diet KM fed sea urchins, although there were no significant differences among wild sea urchins (T4) and diet FM and diet KM fed sea urchins as shown by the DI values of the CM reported in Table 5.

Discussions

In this study, we present the results of an offshore trial carried out on adult sea urchins *P. lividus* fed on two agar-based formulated diets or biocomposites for four weeks. The trial took place at the beginning of the recovery phase that in this area takes place in September-October (Fabbrocini and D'Adamo, 2010). The goal of the study was to evaluate the ability of two

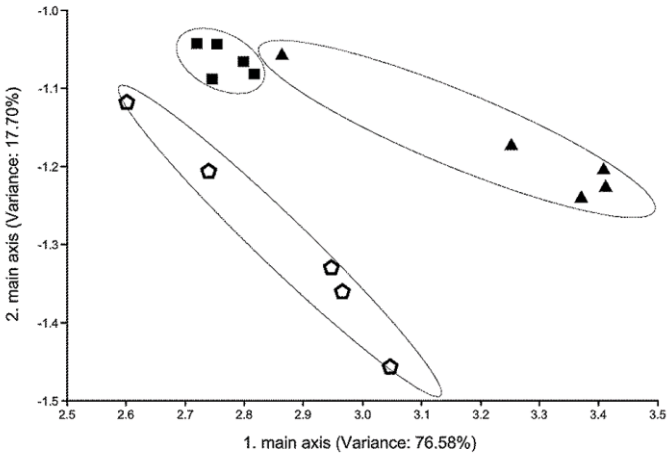

Figure 1. PCA analysis of the volatile substances of the gonads of wild and cultured urchins. Square = Wild T4 (sea urchins caught and analyzed at the end of the roe enhancement trial). Triangle = Diet FM (sea urchins fed the diet containing anchovy as fish meal and analyzed at the end of the roe enhancement trial). Pentagon = Diet KM (sea urchins fed the diet containing krill as fish meal and analyzed at the end of the roe enhancement trial).

agar-based formulated diets to stimulate gonadal growth without accelerating the gamete maturation. The results presented here confirm previous studies carried out in a laboratory-scale trial, showing that *P. lividus* fed on agar-based formulated diets, underwent a slow progression of the reproductive cycle (Fabbrocini et al., 2015). As reported in this study, under the here tested feeding regime, the histological analysis shows a slower gametogenesis progression in the gonads of caged sea urchins with respect to the field collected ones. In fact, reared sea urchins were in growing or premature phase, without differences among diets, while mature gonads were observed in wild sea urchins. It has been widely and scientifically demonstrated that the diet contributes crucially to the development and growth of sea urchin gonads (Otero-Villanueva et al., 2004; Pearce et al., 2004; Schlosser et al., 2005; Azad et al., 2011; Zupo et al., 2018). Specifically, in the case of *P. lividus*, the induction of gonad maturation was obtained in RAS (Recirculating Aquaculture Systems), using the commercial pelletized feed with a high protein content (46% dry weight; Fabbrocini and D'Adamo, 2010; 2011). In a previous study, sea urchins fed for three months with agar-based formulated diets with the same composition used in this study showed a IGS

higher than the individuals captured in the wild in the same period (Volpe et al., 2018). On the contrary, the same formulated diets administered here did not accelerate the growth of the gonads. We may hypothesize that agar may affect the nutrient absorption, although in this study the amount and the relative proportions of FAA and FA in the gonads of wild and cultured gonads were similar and in some instances, even higher concentrations of FAA and PUFAs were detected in cultured sea urchin gonads. Moreover, some authors report that agar may prolong the time that the nutrients remain in the intestine, thus favoring their absorption (Sanaka et al., 2007). In an interesting feeding trial carried on tilapia fingerlings, the authors report that a negative growth was observed with agar at 35% of proteins in the diet, while a positive growth was observed with agar and 24% of proteins in the diet (Shiau and Liang, 1994), indicating that the addition of agar may affect either negatively or positively the body growth, depending on the percentage of protein in the diet. We do not know if this may be the case of *P. lividus* and surely the topic requires further studies.

Proteins are the main components of the *P. lividus* gonads. The protein content of the gonads is linked to the reproductive cycle, with an inverse relationship between the protein reserve and gametogenesis (Arafa et al., 2012). This may explain why we found a higher amount of amino acids in cultured sea urchins with respect to the wild ones, whose reproductive cycle was more advanced. We may hypothesize that the slower advancement of the reproductive cycle in the cultured sea urchins accompanies to reduced employment of proteins and, as a consequence, high amount of amino acids in the gonads. Such a hypothesis seems to be sustained by a study carried out by Osako et al. (2007), showing that the free amino acid composition in the gonads of the sea urchin, *Anthocidaris crassispina* is the result of a combination of internal and external factors, such as the gonadal maturation and the diet, respectively.

In this study, Palmitic, Linolenic EPA and ARA are the most represented fatty acids in the *P. lividus* gonads. Such composition is in general agreement with the literature (Martinez-Pita et al., 2010; Arafa et al., 2012; Sialiani et al., 2016; Rocha et al., 2019) and with a previous study carried out during the recovery period using the same formulated diets used in this study (Volpe et al., 2018). This is not surprising since the nutritional composition of the sea urchin gonads is dependent on the diet (Lourenco et al., 2018) and dietary lipids play a key role in characterizing the fatty acid profile (Martinez-Pita et al., 2010; Carboni et al., 2013; Marsh et al., 2013). However, it has been observed that in *P. lividus*, the endogenous production of fatty acids is independent of dietary intake (Kabeia et al., 2017), since echinoderms are able to carry out all the desaturation reactions necessary to convert the precursors C18 PUFA ALA (18: 3n-3) and LA (18: 2n-6) in EPA (20: 5n-3) and ARA (20: 4n- 6), which would explain the presence of ARA in the gonads, but not in the diets.

During the gonadal growth, nutrients are accumulated in the nutritive phagocytes, and are gradually transferred from the phagocytes to the developing gametes during gametogenesis. Thus, phagocytes decrease their size in favor of eggs and spermatozoa that progressively fill the gonad (Walker et al., 2001). The quality of the gonads is determined by taste, color, consistency, and compactness, which are in turn strongly influenced by the biochemical composition that is by the content of lipids, carbohydrates, carotenoids and proteins (Fernandez, 1997; Lawrence, 2007). The content of carbohydrates and proteins greatly influence the consistency and compactness, while the protein and lipid content determine the flavor of the gonads (Siikavuopio et al., 2007).

We used the electronic nose for the evaluation of volatile molecule fingerprint. The operating principle of the electronic nose is clearly different from that of the commonly used analytical instruments (GC, GC-MS, HPLC, HPLC-MS, etc.). In fact, it does not analyze the volatile fraction of the food, largely responsible for the perception of the aroma, separating and identifying the various components, but gives an overall assessment. The analysis of the volatile substances of the gonads of the sea urchin conducted

using the electronic nose shows that they vary as a result of the different feeding treatments. The composition of protein and lipid profile, on the other hand, varies greatly between the different species of sea urchins and is strongly influenced by diet and physiological processes such as the reproductive phase (Fernandez, 1997; Fernandez and Pergent, 1998; Lawrence, 2007). This could explain the differences detected by the electronic nose in this study and indicate that they may be the result of the differences in the biochemical composition of the gonads, especially FAA and FA.

The here tested biocomposites were made from 2% agar and 10% nutrients and already tested for their good consistency, poor water absorption, and limited dispersion of nutrients in both lab tanks and offshore cages (Paolucci et al., 2015; Volpe et al., 2018). Differently from a previous study (Volpe et al., 2018), here the biocomposite covered the bottom of the cage, and the sea urchins could continuously graze on it, as they do in their natural environment (Bouduresque and Verlaque, 2013). After one week, only traces of the biocomposite were present on the bottom of the cage indicating that the sea urchins were actively feeding. The replacement of the biocomposite took place only once a week, reducing the management costs of the echinoculture system. This, together with the fact that there was a limited dispersion of nutrients in the water (Fabbrocini et al., 2015; Paolucci et al., 2015), is an evident advantage as represented by the combination of the biocomposite and the offshore cage with the sliding bottom.

Conclusion

These results show that *P lividus* sea urchins can be housed, under this feeding regime during the recovery phase of the gonads, without acceleration of the maturation of the gametes and thus in conditions to allow the extension of the market period. Moreover, the here tested agar-based biocomposites make it possible to reduce the management costs of the rearing system as they need to be replaced only once a week, and its environmental impact as they guarantee a limited nutrient dispersion in the water.

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