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Somatic growth and gonadal development of *Paracentrotus lividus* (Lamarck, 1816) fed with diets of different ingredient sources

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ABSTRACT

Sea urchins' gonads are a delicacy highly appreciated worldwide. In Europe, *Paracentrotus lividus* is one of the most valuable edible sea urchin species and a desired target for aquaculture. One of the challenges of echinoculture is to increase the sea urchins' growth rate during the on-growing phase and reducing the production cycle required to obtain sea urchins of commercial size (test diameter ≈ 50 mm). The present study aimed to evaluate the growth and gonad development of *P. lividus* urchins fed with three dry diets for 15 weeks. The diets were formulated with ingredients of three different sources: an algae-based, a fishmeal-based and a cereals-based diet. The somatic growth was assessed by biometric indicators like the linear and specific growth rates. The gonadal development was assessed by the gonadal somatic index (GSI), gametogenesis level, proximate composition and fatty acids profile. The results obtained showed high growth rates in all the three diets tested (0.44% *per* day). The sea urchins fed with the cereals diet presented the highest GSI (8.22%) with higher proportion of gonads in growing and premature stages. The proximate composition of the sea urchins' gonads was significantly affected by diet and sea urchins' sex, particularly the lipid content. Concomitantly, fatty acids (FA) profile of the gonads was influenced by both diet and sea urchins' sex with saturated and polyunsaturated FA playing an important role in this differentiation.

1. Introduction

The sea urchins' gonads – commercially called roe or "uni" – are highly prized food products which market value depends on their organoleptic qualities (Monfort, 2002; Stefánsson et al., 2017). The growing gastronomic interest in sea urchin roe, the high market value and the decline of the major sea urchin fisheries (Andrew et al., 2002) have contributed to the intensification of applied research for the aquaculture production of edible sea urchins (echinoculture) (McBride, 2005). The main goal of the echinoculture is to bridge the gap between the demand and the supply of roe (Pearce, 2010), providing edible sea urchins with excellent quality gonads to the market throughout the entire year. To achieve this goal, the research has been following two parallel paths: the enhancement of gonads quality of wild captured sea urchins; and the development of an economically viable full-cycle production. In both cases, it is essential to develop artificial feeds cost effective that meet the species nutritional requirements for: successful production of offspring; to increase the somatic growth rates during the on-growing phase; and to produce sea urchin's roe of high market quality (Pearce et al., 2002; McBride, 2005; Schlosser et al., 2005; Bouderesque and Verlaque, 2013).

The European sea urchin *Paracentrotus lividus* (Lamarck 1816) is the most valuable and harvested edible sea urchin in Europe (Pais et al., 2007; Machado et al., 2019). It is mainly explored in France, Italy and Spain (Monfort, 2002; Stefánsson et al., 2017) where the whole sea urchin can be sold at approximately 5€ Kg^{-1} and the fresh cleaned roe can reach 60 to 80 € Kg^{-1} (Carboni et al., 2012). *Paracentrotus lividus* is a common species in the rocky pools and shallow subtidal reefs in the NE Atlantic, Macaronesia region and Mediterranean Sea (Alves et al., 2001; Bouderesque and Verlaque, 2013; Hernández et al., 2013). This species

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is considered a slow grower (Frantzis et al., 1988; Bouderesque and Verlaque, 2013) with a maximum gonad yield - measured as gonadal somatic index, GSI - of adult sea urchins varying between 15% (Machado et al., 2019) and 18% (Rocha et al., 2019). In nature, the *P. lividus* can take up to 5 years to attain > 45 mm test diameter (TD), with a maximum growth rate between 5.8 and 7.7 mm year^{-1} (Turon et al., 1995). The species attains first maturity (when 50 % of the urchins of a specific population are mature) with variable TD, between 20 and 36 mm (Machado et al., 2019; Ouréns et al., 2013). Indeed, the growth rate, size-at-maturity and maximum test diameter depend on food availability, nutritional quality, and local hydrodynamics (Jacinto et al., 2013; Lozano et al., 1995; Turon et al., 1995). In controlled environmental conditions as those in aquaculture, the feeding regime and the diet nutritional quality assume the predominant role. The nutritional value and the feed type of diets offered to sea urchins have also a high impact in food intake and in feed conversion ratio (Fernandez and Boudouresque, 2000; Spirlet et al., 2001; Prato et al., 2017). Like other sea urchins', *P. lividus* regulates the feed intake to meet the physiologic needs independently of the protein or energetic level of diet (Heflin et al., 2016; Lourenço et al., 2020).

The impact of feed type, quantity and quality in the somatic growth of edible sea urchins have been addressed for other sea urchin species including *Loxechinus albus* (Molina 1782), *Lytechinus variegatus* (Lamarck, 1816), *Tripneustes gratilla* (Linnaeus 1758), *Pseudechinus huttoni* (Benham 1908) and *Strongylocentrotus droebachiensis* (Müller 1776) by applying variable feeding strategies including exclusive macroalgae feeding (Frantzis and Grémare, 1992; Poorbagher et al., 2010; Castilla-Gavilán et al., 2019), by combining macroalgae and extruded pelleted diets (Otero-Villanueva et al., 2004; Eddy et al., 2012; Cárcamo, 2015; Cyrus et al., 2015), and by using exclusively dry feeds and extruded pelleted diets (Fernandez and Pergent, 1998; Mcbride et al., 1998; Fernandez and Boudouresque, 2000; Akiyama et al., 2001; Otero-Villanueva et al., 2004; Kennedy et al., 2005; Hammer et al., 2006a, 2006b, 2010; Dworjanyn et al., 2007; Poorbagher et al., 2010; Eddy et al., 2012; Heflin et al., 2013, 2016; Cárcamo, 2015; Cyrus et al., 2015; Vizzini et al., 2018).

The single use of artificial diets can be advantageous because these can potentially deliver predictable growth performances based in cost-effective feeds, using market available ingredients with a uniform nutritional composition. Moreover, the viable full-cycle production of sea urchins will benefit of a reduction of the time expended during the on-growing phase by increasing the growth rates of pre-mature sea urchins. The present study aimed to evaluate the effect of diets' ingredient sources in the somatic growth of *P. lividus* during the on-growing phase. To verify this effect, it was conducted a growth trial of 15 weeks using three new dry diets with identical proximate composition formulated specifically for *P. lividus* with practical ingredients of different sources: animal-based, algae-based and vegetables-based sources. The sea urchins' growth was assessed with growth performance and feed consumption indicators. The gametogenic stage, the proximate composition and the fatty acids profile of the gonads was also evaluated to determine the effect of diets in this primordial storage organ in the sea urchins.

2. Materials and methods

2.1. Sea urchins' collection

Paracentrotus lividus urchins with an initial test diameter (TD_i) 17.6 ± 3.5 mm were manually collected during low tide in the rocky pools of an intertidal beach near Peniche (Portugal, 39º 19.345' N, 9º 21.548' W) and transported to MARE aquaculture laboratory in isothermal boxes filled with seawater. The sea urchins were carefully cleaned, the fragments of algae and small stones removed from the spines, and randomly placed in 40 L glass tanks at a density of 5g L^{-1} for a fasting period of two weeks to ensure nutritional consistency among individuals. During the fasting period, the seawater temperature, pH, salinity, and dissolved oxygen (DO) average values were 18.82 ± 0.86 ºC, 7.68 ± 0.32, 33.94 ± 1.08 ppt and 7.03 ± 0.74 mg L^{-1}, respectively. The seawater dissolved nitrite (NO_2^-) and dissolved ammonia (NH_3) were 0.04 ± 0.11 mg L^{-1} and 0.22 ± 0.36 mg L^{-1}, respectively.

Following the fasting period, a sample of 20 sea urchins were randomly selected, weighed (baseline body weight, BW_b ± 0.01 g), measured with a calliper (baseline test diameter, TD_b ± 0.1 mm), dissected and their gonads weighed (GW_b ± 0.001 g) to establish the baseline condition for the sea urchins subject to the growth trial.

2.2. Experimental diets

Three experimental diets of identical proximate composition were specifically developed for *P. lividus* by SPAROS, Lda. (Olhão, Portugal) using different ingredients of commercial sources. The animal-based diet (fishmeal) included fish meal, squid meal and krill meal. The algae-based ingredients (algae) included *Phaedactylum*, *Spirulina*, *Chlorella*, *Tetraselmis*, *Laminaria* and *Schizochytrium* powder. The vegetable-based diet (cereals) included soybean meal, soy protein and wheat gluten. To meet some of the specific nutritional requirements of *P. lividus*, the three experimental diets were supplemented with vitamin C (750 mg Kg^{-1}) and E (1800 mg Kg^{-1}), monocalcium phosphate, carbonates, silicates and β-carotene (10 mg Kg^{-1}). The diets were presented to the sea urchins in the form of round and flat pellets with a particle size of approximately 1.8 cm and 0.66 g. The ingredients composition of the three experimental diets used is detailed in Table 1.

Concerning their proximate composition, the three experimental diets presented a crude protein content of approximately 30% DM, a crude fat content of 9% DM and a carbohydrates content of 45% DM (Table 2). Due the diversity of ingredients sources, the diets fatty acid (FA) profiles were different among diets. The cereals diet presented higher content in saturated fatty acids (SFA) and monounsaturated FA (MUFA) (around 49% and 38% respectively) and the lowest content of polyunsaturated FA (PUFA), with values below 12%. The algae and fishmeal diets showed a more equitable distribution of the different types of FA presenting about 40% SFA, 30% MUFA and 30% PUFA. The palmitic acid (C16:0) was the most abundant SFA in all diets, followed by myristic (C14:0) and stearic (C18:0) acids. The oleic acid (C18:1n9) was the major MUFA detected in all diets although palmitoleic acid (C16:1n7) also appeared in appreciable amounts. It was observed a prevalence of omega-6 PUFAs in all diets, being the linoleic acid (C18:2n6) the most representative. The algae diet contained the highest amount of omega-3 PUFAs (about 10% of total FA), presenting, in decreasing order, eicosapentaenoic acid (C20:5n3, EPA), docosahexaenoic acid (C22:6n3, DHA) and alpha-linolenic acid (C18:3n3, ALA).

To evaluate the diets stability in water, triplicate samples with 5 g of pellets were immersed in seawater for 48 h at environmental temperature. The pellets were then removed and dried at 105 °C for 24 h and weighed to determine mass losses as the difference between the initial weight of sample and its weight after immersion and drying.

2.3. Growth trial

The rearing system consisted of three recirculating aquaculture systems (RAS) with three 40 L holding tanks used as diets replicates, randomly assigned to guarantee that each system contained one replicate of each experimental diet. Each system was equipped with a 70 L sump tank, an air-cooled water chiller (Frimar F200, ®Fernando Ribeiro, Ltd. Barcarena Portugal), mechanical filtration, biological filtration, a water pump (Hailea HX-6530, Guangdong, China) and a protein skimmer (Bubble Magus C3.5, Aquarium Equipment Co., Jiyang China). The sea urchins (n = 135) with average BW_i 5.66 ± 2.36 g were randomly distributed by the nine tanks (N_{tank} = 15) with an initial biomass of 5 g L^{-1} (density = 98 g m^{-2}). The system flow rate was 150 L h^{-1} in all tanks corresponding to an exchange rate of 3.75 per hour. Temperature, pH, dissolved oxygen (DO) and salinity were registered

Table 1
Ingredients composition of the three experimental diets used in the used in the growth trial.

Ingredients (%)	Experimental Diets		
	Algae	Fishmeal	Cereals
Fish gelatin [a]	2.00	2.00	2.00
Corn gluten [b]	15.00	15.00	15.00
Corn meal [c]	26.70	36.40	35.00
Macroalgae mix[d]	15.00	15.00	15.00
Fish meal [e]		4.00	
Squid meal [f]		3.50	
Krill meal [g]		3.00	
Spirullina [h]	4.00		
Phaeodactylum [i]	5.00		
Chlorella [j]	4.00		
Tetraselmis [k]	1.00		
Laminaria powder [l]	5.00		
DHA-Rich algae (Schizochytrium) [m]	1.00		
Soy protein concentrate [n]			4.00
Wheat gluten [o]			4.00
Soybean meal [p]			3.00
Fish oil [q]	4.70	4.50	5.40
Arachidonic acid oil [r]	0.50	0.50	0.50
Soy lecithin [s]	1.00	1.00	1.00
Vitamin & Mineral Premix [t]	2.00	2.00	2.00
Vitamin C [u]	0.10	0.10	0.10
Vitamin E [v]	0.20	0.20	0.20
Spinach powder [w]	2.50	2.50	2.50
Antioxidant[x]	0.20	0.20	0.20
Monocalcium phosphate[y]	2.50	2.50	2.50
Calcium carbonate[z]	5.00	5.00	5.00
Calcium silicate[α]	2.00	2.00	2.00
β-carotene 10% [β]	0.10	0.10	0.10
L-Taurine[ε]	0.50	0.50	0.50

[a]Pharma grade bloom 240: 92% CP, LAPI Gelatine SPA, Italy; [b] Corn gluten: 61% CP, 6% CF, COPAM, Portugal.
[c] Corn meal 9% CP, 4% Ribeiros Comércio e Industria de Cereais, Lda Portugal; [d] Ocean Feed 11% CP, 0.6% CF, Ocean Harvest, Ireland; [e] Fishmeal Super Prime, 66.3% CP, 11.5% CF, Pesquera Diamante, Peru; [f] Super prime without guts: 82% CP, 3.5% CF, Sopropêche, France; [g] krill meal 61% CP, 17% CF, Aker Biomarine, Norway; [h]*Arthrospira platensis*, 72% CP, 1% CF, Sopropêche, France; [i]*Phaedactylum tricornutum*, 38% CP, 15% CF Allmicroalgae, Portugal; [j]*Chlorella vulgaris*, 63% CP, 9% CF, Allmicroalgae, Portugal; [k]*Tetraselmis chuii* 23% CP, 6% CF, Allmicroalgae, Portugal; [l] Algovert®, *Laminaria digitata*: 8% CP, 0.6% CF, Setalg, France; [m] ALL-G rich, 10% CP, 63% CF, Alltech, Ireland; [n] Soycomil P: 65% CP, 1% CF, ADM, The Netherlands; [o] Vital, 80% CP, 6% CF Roquette, France; [p] Soybean meal 44% CP, 4%; CF Ribeiro & Sousa, Portugal; [q] Sopropêche, France, 98% CF; [r]Arachidonic acid oil 40%, 98% CF Huatai Biopharma, China; [s] Lecico P700IPM, LECICO GmbH, Germany; [t] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alphatocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassiumiodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middling's; [u] Rovimix C35, DSM Nutritional Products, Switzerland; [v] Rovimix E50, DSM Nutritional Products, Switzerland; [w] Seah International, France; [x] Antioxidant powder, Paramega PX, Kemin Europe NV, Belgium; [y] Monocalcium phosphate: 22% phosphorus, 16% calcium, Fosfitalia, Italy; [z] PREMIX Lda, Portugal; [α] ZEOFREE 600, Evonik, Germany; [β] Rovimix ® β-carotene, DSM Nutritional Products, Switzerland; [ε] 98% Tau, ORFFA, The Netherlands.

daily with handheld multiparameter probe (YSI Inc, Ohio, USA) and maintained in the three systems at 19 ± 1.2 °C, a pH of 7.8 ± 0.3, a salinity of 33.9 ± 1.1 ppt and DO of 7.0 ± 0.7 mg L^{-1}. Ammonia and nitrites were also monitored daily with a bench photometer (Hanna HI 83203, Rhode Island, USA), and kept within optimal values for the sea urchins (Basuyaux and Mathieu, 1999; Repolho et al., 2011).

Table 2
Proximate composition and fatty acids profile of the three experimental diets used in the growth trial.

	Experimental diets		
Proximate composition	Algae	Fishmeal	Cereals
Dry matter, DM (%)	97.74 ± 0.21	96.27 ± 1.07	95.13 ± 0.12
Moisture (%)	2.26 ± 0.21	3.73 ± 1.07	4.87 ± 0.12
Ash (% DM)	16.37 ± 0.37	13.79 ± 0.16	13.09 ± 0.21
Crude fat (% DM)	9.80 ± 0.89	8.95 ± 0.11	9.39 ± 0.47
Crude protein (% DM)	33.34 ± 0.15[a]	32.23 ± 0.44[b]	31.32 ± 0.40[c]
Carbohydrates (% DM)	44.62 ± 1.40	45.7 ± 1.87	45.85 ± 1.7
Gross Energy[1] (kJ/g DM)	18.63	18.63	19.31
Fatty acids (% Total FA)			
C14:0	5.73 ± 0.11	5.94 ± 0.13	8.35 ± 0.25
C15:0	0.46 ± 0.14	0.48 ± 0.02	0.59 ± 0.02
C16:0	25.67 ± 0.54	25.91 ± 0.12	32.15 ± 0.13
C17:0	0.90 ± 0.09	0.62 ± 0.20	0.43 ± 0.01
C18:0	5.00 ± 0.55	5.83 ± 0.13	6.66 ± 0.36
C20:0	0.49 ± 0.03	0.35 ± 0.04	0.38 ± 0.05
C22:0	0.37 ± 0.02	0.35 ± 0.10	0.00 ± 0.00
C23:0	0.21 ± 0.17	0.09 ± 0.03	0.06 ± 0.05
C24:0	0.23 ± 0.17	0.10 ± 0.06	0.04 ± 0.03
∑ SFA	39.06 ± 0.41	39.67 ± 0.22	48.66 ± 0.18
C15:1n5	0.13 ± 0.09	0.11 ± 0.02	0.15 ± 0.01
C16:1n7	5.95 ± 0.23	5.16 ± 0.06	6.66 ± 0.11
C17:1n7	0.40 ± 0.04	0.49 ± 0.02	0.15 ± 0.03
C18:1n9 (*cis* + *trans*)	21.67 ± 0.47	26.82 ± 0.04	29.74 ± 0.23
C20:1n9	0.61 ± 0.11	0.84 ± 0.08	0.81 ± 0.04
C24:1n9	0.24 ± 0.06	0.11 ± 0.02	0.03 ± 0.03
∑ MUFA	28.99 ± 0.58	33.53 ± 0.05	37.55 ± 0.38
C18:2n6 (LA)	16.55 ± 0.39	17.67 ± 0.07	10.73 ± 0.05
C18:3n6	0.53 ± 0.05	0.27 ± 0.01	0.04 ± 0.03
C18:3n3 (ALA)	1.67 ± 0.03	1.09 ± 0.05	0.43 ± 0.02
C20:4n6 (ARA)	2.43 ± 0.11	1.74 ± 0.06	0.00 ± 0.00
C20:5n3 (EPA)	6.27 ± 0.11	3.55 ± 0.11	0.29 ± 0.25
C22:6n3 (DHA)	2.26 ± 0.43	1.13 ± 0.06	0.00 ± 0.00
∑ PUFA	29.70 ± 0.27	25.44 ± 0.15	11.48 ± 0.28
∑ n6	19.51 ± 0.28	19.68 ± 0.01	10.76 ± 0.02
∑ n3	10.20 ± 0.34	5.76 ± 0.16	0.72 ± 0.27
n6/n3	1.91 ± 0.09	3.42 ± 0.09	17.00 ± 7.99

[1] Gross energy was estimated theoretically by converting the average % of crude protein, % crude fat and % carbohydrates present in each diet in calories, namely 1 g crude protein = 5 to 5.80 cal, 1 g crude fat = 9.30 to 9.50 cal and 1 g carbohydrates = 3.90 to 4.20 cal (Merrill and Watt, 1973) accordingly with experimental diets major ingredients sources.

The growth trial lasted for 15 weeks (103 days). During this period, the sea urchins were fed each 48 h, with an initial ration of 0.5% the tank biomass and adjusted accordingly to the presence of feed remains to assure that all sea urchins were fed to satiation. The feed remaining in each tank was dried at 105º C for 24 h (Memmert, Schwaback, Germany) and weighed (± 0.01 g) (Sartorius, TE1245) to further determination of feed intake.

At the end of the trial, all urchins were individually weighed (BW_f ± 0.01 g) and measured with a calliper (TD_f ± 0.1 mm). The sea urchins were dissected by a circular incision around the peristomal membrane and the gonads removed, blotted on a clean paper towel, and weighed individually (final gonad weight, GW_f ± 0.1 mg). One gonad per individual was fixed during 48 h in 4% neutralized formaldehyde and stored in 70 % ethanol for gonad gametogenic analysis. The remaining gonads were frozen at -80 ºC and freeze-dried for further chemical analyses.

For field control, it was collected, in the same site of the original group, a sample of 20 sea urchins with TD identical to the sea urchins at the end of the growth trial. The biometric parameters and gonads collection were conducted as described before for the urchins from baseline and growth trial samples.

2.4. Sex ratio and gonadal development

Sea urchin's sex and gametogenic stage were determined by

histological analysis of their gonads. The fixed gonads were dehydrated in automatic tissue processor (Leica® TP1020, Wetzlar Germany) with a sequential submersion in increasing ethanol grade solutions followed by xylene for clearing and impregnated with paraffin wax at 60 °C. The gonads were then sectioned transversely to the longitudinal axis with a thickness of 5 μm using a rotary microtome (Accu-Cut® SRM^{TM} 200, Sakura Finetek Europe BV, Netherlands). The sections were then stained with Harri's haematoxylin and eosin Y and observed under an optical microscope Leica® DM 2000 LED equipped with a Leica® MC170 5MP HD camera and Leica Application Suite V4.4.0 software (Leica Microsystems GmbH, Wetzlar, Germany). The gametogenic stage of each gonad was classified into six stages (Byrne, 1990): recovery (stage I), growing (stage II), premature (stage III), mature (stage IV), partly-spawned (stage V) and spent (stage VI). Individual maturity stages data were pooled by sex by diet to determine maturity stages proportions for further statistical analysis.

2.5. Chemical analysis

Freeze-dried gonads' samples were ground and pooled in males and females' samples by tank guaranteeing triplicate samples by sex and by diet. All the chemical analyses were conducted in duplicates.

The soluble protein content was determined by adapting the method described by Lowry et al. (1951) to maximize the protein extraction yield. Freeze-dried samples (≈ 30 mg) were homogenized with 10 mL of 0.1 M NaOH by sonication (Digital Sonicator Branson 250, Connecticut, USA) and maintained at 4°C overnight. The samples were then centrifuged at 3000 × G for 10 min (Eppendorf Centrifuge 5810 R, Billerica, US) and the supernatant collected and conveniently diluted (1:10 v:v). For colour development, 1 mL of Lowry solution (500 μL of 2% Na_2CO_3 + 400 μL of 0.1 M NaOH + 50 μL of 1% $KNaC_4H_4O_6.4H_2O$ + 50 μL of 0.5% $CuSO_4.5H_2O$) was added to 300 μL of samples, BSA standards or 0.1 M NaOH (for blank). After incubation at 37 °C for 5 minutes, 100 μL of 50% Folin-Colciteau phenol reagent were added and the samples were again incubated during 60 min. Then, 200 μL aliquots (triplicates) were transferred to 96 x 96 flat bottom microplates and the absorbance was read at 750 nm by spectrophotometry (Synergy H1 Hybrid Reader Biotek ® Winooski, USA). Protein concentration was obtained by interpolation of the values of samples absorbance in standard BSA (VWR chemicals, Leuven, Belgium) calibration curve (33.3 - 66.7 - 100 - 133 - 167 - 233 μg mL^{-1}). The protein content of sea urchins' gonads was expressed as percentage of dry mass (% DM).

The gonads' carbohydrates content was determined following the phenol-sulphuric acid method (Dubois et al., 1956) with the adaptations suggested by Masuko et al. (2005). The freeze-dried samples (≈ 30 mg) were hydrolysed with 8 mL of H_2SO_4 1M for 60 min in a 90 °C water bath. After cooling, the mixture volume of hydrolysate was adjusted to 10 mL. For colour reaction, 200 μL of each hydrolysate was mixed with 500 μL of H_2SO_4 97% and heated for 15 min at 90 °C. Then, 100 μL of 5% phenol was added to the mixture and stirred. Triplicate aliquots of 200 μL were transferred to 96 x 96 flat bottom microplate and the absorbance was read at 490 nm. Carbohydrates concentration was obtained by interpolation of samples absorbance in increasing D-glucose (VWR chemicals, Leuven, Belgium) concentrations calibration curve (0.0 - 0.04 - 0.08 - 0.12 - 0.16 and 0.2 mg mL^{-1}). The amount of total carbohydrates in experimental diets was expressed as % DM of glucose.

Total lipids were extracted by the Bligh and Dyer (1959) method and quantified by UV-vis spectrophotometry according De Coen and Janssen (1997). Freeze-dried samples (≈ 30 mg) were homogenised with solution of chloroform: methanol: water (0.5:0.5:0.5 mL) and centrifuged at 2000 × G and 4°C for 10 minutes for phase separation. The lower organic phase was recovered and diluted 1:3 with chloroform. Afterwards, 100 μL of diluted sample were mixed with 500 μL of 97% sulfuric acid and heated at 200 °C for 10 minutes. After cooling, 1.5 mL of ultrapure water were carefully added and 300 μL aliquots (in triplicate) were transferred to 96 x 96 flat bottom microplate. Samples absorbance was read at 375 nm. The calibration curve was prepared with tripalmitin (ACROS Organics^{TM}) standard solutions in chloroform (0.0 - 0.6 - 1.3 -1.6 -1.9 - 2.6 mg mL^{-1}), treated as described for the diluted samples. The results of lipid content were expressed as percentage of dry matter of gonads (% DM).

The FA's content of the gonads lipidic fraction was converted into FA methyl esters (FAME) and analysed by gas chromatography (GC) following Fernandez et al. (2015). The freeze-dried samples (≈ 50 mg) were homogenised with 2 mL of acid methanol (2% sulfuric acid) and heated at 80 °C water bath for 2 h. Afterwards, 1 mL of Mili-Q water and 2 mL of n-hexane were added to the mixture, stirred, and centrifuged at 1500 × G during 5 min to phase separation. Finally, the upper organic phase was recovered into 2mL vials and analysed in a gas chromatograph (Finnigan Ultra Trace) equipped with a Thermo TR-FAME capillary column (60 m × 0.25 mm ID, 0.25 μm film thickness), an auto sampler (AS 3000, Thermo Electron Corporation) and a flame ionization detector (FID) under the conditions described by Silva et al. (2017): The temperatures of injector (splitless) and the detector were 250 and 280 °C, respectively. Helium (1.5 mL min^{-1}) was used as carrier gas. Air and hydrogen were supplied to the detector at flow rates of 350 and 35 mL min^{-1}, respectively. Standard mixtures (SUPELCO 37, PUFA Nº1 from Marine source and PUFA Nº3 from Menhaden oil, SUPELCO, Bellefonte, Pa., U.S.A.) were used to identify the FAME's in samples. The concentration of each FA was expressed as % of total FA.

2.6. Calculations

2.6.1. Growth performance

The effect of the three experimental diets in sea urchins' somatic growth and gonad development was evaluated for both females and males by tank replicate through the determination of the following parameters:

Weight Gain (WG, g) = BW_f -BW_i

Diameter Gain (DG, mm) = TD_f - TD_i

Average Body Weight (ABW, g) = (BW_f + BW_i) / 2

Linear Growth Rate (mm day^{-1}) = (TD_f - TD_i) / N_{days}

Specific Growth Rate (% day^{-1}) = (log (BW_f) – log (BW_i)) / N_{days}

Average Gonad-somatic index (GSI_{tank}, %) = ($\sum$ (individual GW / individual BW)) / N_{tank}

Where:

BW_i is the average of initial body weight of the sea urchins in each replicate tank;

BW_f is the average of final body weight of the sea urchins in each replicate tank;

TD_i is the average of initial test diameter of the sea urchins in each replicate tank;

TD_f is the average of final test diameter of the sea urchins in each replicate tank;

N_{tank} represents the number of sea urchins in the tank by the end of growth trial;

N_{days} represents the number of trial days.

2.6.2. Feed efficiency

The sea urchins' feed efficiency by experimental diet was evaluated for both females and males by the determination of:

Feed Consumption (FC, g) = $\sum_{tank}$ (dry weight of feed offered each feeding event – dry weight of feed remaining at the end of 48h). Food losses due to the pellets' instability in the seawater were subtracted to food weigh provided to the sea urchins at each feeding event prior to FC calculations.

Voluntary Feed Intake (VFI, g/ 100g ABW/day) = 100 x (FC / ABW/ N_{days})

Feed Conversion Ratio (FCR) = FC / WG

Dry matter intake (DMI, g/ 100 g ABW/ day) = 100 x (FC x diet % DM) / ABW / N_{days})

Protein Efficiency Ratio (PER) = WG / FC x Diet Protein %

Protein intake (PI, g / 100 g ABW/ day) = 100 x ((FC x Diet Protein %) / ABW/ N_{days})

Fat intake (FI, g / 100 g ABW/ day) = 100 x ((FC x Diet Fat %) / ABW/ N_{days})

Carbohydrates intake (GI, g / 100 g ABW/ day) = 100 x ((FC x Diet Glycogen %) / ABW / N_{days})

2.7. Statistical analysis

All statistical tests were performed in R environment (R version 3.6.2). The growth performance, feed efficiency, gonad proximate composition and fatty acids profile results are expressed as mean ± standard deviation (std) of the three replicate tanks by dietary group (n = 3). The effects of experimental diets (three levels: algae, fishmeal and cereals) in the feeding efficiency indicators were tested through one-away ANOVA with a randomized block design. The effects of both experimental diet and sea urchin sex in the growth performance indicators were analysed by two-away ANOVA using a mixed effects model with a randomized block design. The experimental diet (three levels: algae, fishmeal and cereals) was treated as fixed effects factor and sea urchins' sex (two levels: F and M) was treated as random effects factor. Both factors were treated as orthogonal. Prior to formal analysis, the variables distribution was checked for normality and for homoscedasticity using the Shapiro-Wilk normality test and Levene test, respectively. Whenever the assumptions of data normality and homoscedasticity did not hold, the main factors and interaction effect were tested using the aligned rank transform analysis of variance (ART). ART function computes a separate aligned ranked response variable for each effect of the model followed by classic ANOVA on each of the aligned ranked responses (Wobbrock et al., 2011). In case of statistically significant differences, *post-hoc* multiple pairwise comparisons were performed with Tukey HSD test and with pairwise Wilcoxon rank sum test for the parametric and the non-parametric test, respectively. The sex ratio within experimental diets treatment was determined as the ratio between the number of males and the total number of sea urchins by treatment, and the balance of the sex ratio by binomial test. The effect of the experimental diet in the gonad development level was evaluated by chi-square association test on the maturity stages proportion data by treatment (including experimental diets and wild samples) and by sea urchins' sex.

The FA content data were normalized by log 10 +1 transformation, followed by Shapiro-Wilk and Levene tests of each FA to check for normality and homoscedasticity, respectively. Due to collinearity the fatty acids C14:0, C14:1n5, C16:1n7 and C20:5n3 were removed from the analysis. The normalized FA matrix was then used to evaluate the main effect of the single factors diet and urchin sex and the interaction effect of both factors through MANOVA. A principal component analysis (PCA) was then conducted in the FA correlation matrix to evaluate which fatty acids were more influential in separating the different samples groups by diet and by sea urchins' sex. In all cases, significant differences were considered when $p < .05$.

3. Results

3.1. Growth performance and nutrients utilization

The stability test conducted on the experimental diets' pellets showed that, after 48 h immersed in seawater, the pellets from algae diet lost 21.4 ± 1.0% of their mass, the pellets from cereals diet lost 19.5 ± 0.2%, and the pellets from fishmeal diet lost 18.7 ± 0.8% of their mass. The mass losses were significantly lower for the fishmeal diet (F = 9.47, df = 2, $p = .01$).

The three experimental diets were well accepted by the sea urchins, although with different VFI among groups ($p < .05$, Table 3). The group fed with the algae diet presented significantly higher VFI (0.50 ± 0.05 g /100 g ABW/day), while the sea urchins fed with fishmeal and cereals diets presented similar VFI (algae diet = 0.39 ± 0.06 g /100 g ABW/day and cereals diet = 0.45 ± 0.03 g /100 g ABW/day, Table 4). Despite the differences observed in the feed intake, the FCR was identical between dietary groups and for both females and males (Table 3), with an average of 1.11 ± 0.44 for all dietary treatments (Table 4). PER was different among dietary groups ($p < .05$, Table 3) with the sea urchins fed with the fishmeal diet presenting a higher protein efficiency (4.45 ± 1.51) when compared with groups fed with the algae and cereal diets (Table 4). While the DM intake was identical among diets ($p > .05$), Table 3), protein, lipids and carbohydrates intake were significantly different among the dietary groups ($p < .05$ for protein, lipids and carbohydrates intake, Table 3), with the sea urchins fed with algae diet presenting the highest intake of all the macronutrients evaluated (Table 4).

During the trial, four sea urchins died (one urchin from the group fed with algae diet; another from the group fed with fishmeal diet, and two urchins from the group fed with the cereals diet), resulting in a mortality rate of 1.36 % without differences between treatments. The average linear growth rate of the sea urchins fed the three experimental diets was of 0.07 ± 0.01 mm day^{-1} independently of dietary groups ($p > .05$, Table 3) or sea urchin's sex ($p > .05$, Table 3). The weight gain attained during the growth trial was also identical among dietary groups ($p > .05$, Table 3) and between sexes ($p > 0.05$, Table 3) averaging 3.21 ± 1.03 g representing an average specific growth rate of 0.44 % *per* day with marginal differences among dietary groups ($p = .05$, Table 3).

Table 3
Two-way ANOVA analyses examining the effects of diet and sea urchin sex on the growth performance of sea urchins fed with the experimental diets for 15 weeks[1].

Indicator	Source	df	MS	F	p
Feed Efficiency					
Voluntary feed intake	**Diet**	**2**	**0.18**	**7.74**	**0.005**
	Residuals	15	2.35×10^{-3}		
Feed conversion ratio	**Diet**	**2**	**0.51**	**3.43**	**0.06**
	Residuals	15	0.15		
Protein Efficiency Ratio	**Diet**	**2**	**6.92**	**5.65**	**0.01**
	Residuals	15	1.22		
DM Intake	Diet	2	9.0×10^{-3}	0.34	0.72
	Residuals	15	0.03		
Protein Intake	**Diet**	**2**	$\mathbf{2.6 \times 10^{-3}}$	**10.58**	**0.001**
	Residuals	15	2.5×10^{-5}		
Lipid Intake	**Diet**	**2**	$\mathbf{3.0 \times 10^{-4}}$	**14.57**	**< 0.001**
	Residuals	15	2.0×10^{-5}		
Carbohydrates Intake	**Diet**	**2**	$\mathbf{3 \times 10^{-3}}$	**6.3**	**0.01**
	Residuals	15	4.8×10^{-4}		
Growth Performance					
Weight gain	Diet	2	1.92	1.83	0.20
	Sex	1	6.0×10^{-4}	0.001	0.98
	Diet x Sex	2	0.85	0.81	0.47
	Residuals	12	1.05		
Diameter gain	Diet	2	4.31	3.13	0.08
	Sex	1	0.30	0.22	0.65
	Diet x Sex	2	1.46	1.06	0.38
	Residuals	12	1.38		
Linear growth rate	Diet	2	8.1×10^{-4}	3.13	0.08
	Sex	1	2.9×10^{-5}	0.22	0.65
	Diet x Sex	2	2.8×10^{-4}	1.06	0.37
	Residuals	12	1.5×10^{-3}		
Specific growth rate	Diet	2	5.2×10^{-2}	3.90	0.05
	Sex	1	1.1×10^{-4}	0.008	0.93
	Diet x Sex	2	1.1×10^{-2}	0.83	0.46
	Residuals	12	1.3×10^{-2}		
Gonad somatic index	**Diet**	**2**	**17.89**	**5.59**	**0.02**
	Sex	**1**	**21.06**	**6.58**	**0.02**
	Diet x Sex	2	9×10^{-2}	0.03	0.97
	Residuals	12	3.20		

[1] df represents the degrees of freedom for each factor and factors interaction, MS represents the residuals mean squares computed for each factor main effect and factor interaction determined by type II two-way ANOVA. F represents the F-test, and *p* represents the significance level.

Table 4
Growth performance, somatic indices and nutrients intake of sea urchins fed with the experimental diets for 15 weeks[1].

	Algae		Fishmeal		Cereal	
	Female	Male	Female	Male	Female	Male
Weight gain (g)	3.00 ± 1.28	3.15 ± 0.70	3.52 ± 1.58	4.16 ± 0.83	3.15 ± 0.92	2.32 ± 0.37
Diameter gain (g)	7.11 ± 1.37	6.71 ± 0.63	7.90 ± 1.72	8.69 ± 1.09	7.33 ± 0.88	6.17 ± 1.03
Linear growth rate (mm/day)	0.07 ± 0.01	0.06 ± 0.006	0.08 ± 0.02	0.08 ± 0.01	0.07 ± 0.008	0.06 ± 0.01
Specific growth rate (% weight/day)	0.40 ± 0.13	0.43 ± 0.09	0.50 ± 0.18	0.58 ± 0.09	0.40 ± 0.12	0.40 ± 0.31
Gonad index (GI)	6.52 ± 2.47^{ab}	4.09 ± 1.61^{ab}	6.20 ± 0.68^{b}*	4.23 ± 0.91^{b}	9.29 ± 0.87^{a}	7.20 ± 2.91^{a}
Voluntary feed intake (g/100 g urchin / day)	0.51 ± 0.07^{a}	0.50 ± 0.03^{a}	0.40 ± 0.07^{b}	0.38 ± 0.05^{b}	0.43 ± 0.04^{ab}	0.46 ± 0.02^{ab}
Feed conversion ratio	1.37 ± 0.58	1.19 ± 0.28	0.89 ± 0.45	0.67 ± 0.20	1.13 ± 0.49	1.44 ± 0.28
Protein efficiency ratio	2.47 ± 1.07^{b}	2.60 ± 0.61^{b}	4.09 ± 1.90^{a}	4.85 ± 1.30^{a}	3.12 ± 1.09^{b}	2.27 ± 0.46^{b}
DM intake (g /100 g urchin/ day)	0.50 ± 0.07^{a}*	0.16 ± 0.01^{a}	0.39 ± 0.07^{b}*	0.12 ± 0.02^{b}	0.41 ± 0.04^{ab}*	0.14 ± 0.007^{ab}
Protein intake (g/100 g urchin/day)	0.17 ± 0.02^{a}	0.17 ± 0.01^{a}	0.13 ± 0.02^{b}	0.12 ± 0.02^{b}	0.14 ± 0.01^{b}	0.14 ± 0.007^{b}
Lipids intake (g/ 100 g urchin/ day)	0.05 ± 0.007	0.05 ± 0.003	0.04 ± 0.006	0.03 ± 0.005	0.04 ± 0.004	0.04 ± 0.002
Carbohydrates intake (g/ 100 g urchin/day)	0.23 ± 0.03^{a}	0.22 ± 0.02^{a}	0.18 ± 0.03^{b}	0.17 ± 0.02^{b}	0.20 ± 0.02^{ab}	0.21 ± 0.01^{ab}

[1] Values are presented as mean ± standard deviation, SD ($n = 3$). Values in the same row with different superscripts differ significantly ($p < .05$). Absence of superscripts indicates no significant differences between treatments.

Conversely, both diet ($p < .05$, Table 3) and sea urchin's sex ($p < .05$, Table 3) had an effect in the GSI, with the males fed with algae diet presenting the lowest gonad yield (4.09 ± 1.61%) and the females fed with cereals diet presenting the highest gonad yield (9.29 ± 0.87%) (Fig. 1A).

3.2. Gonadal gametogenic analysis

The sex ratio (M:F) was unbalanced towards males in the group fed with algae diet ($\chi^2 = 27$, $p = .07$) and balanced in the groups fed with fishmeal diet ($\chi^2 = 22$, $p = .02$) and with cereals diet ($\chi^2 = 22$, $p = .007$).

It was observed a significant association between the proportional distribution of maturity stages and the samples origin (field control vs reared urchins, $\chi^2 = 52.97$, $p = .02$) (Fig. 1B) particularly due to a higher proportion of stage II females in the field control and an higher proportion of stage IV females in the group fed with the algae diet. In the growth trial group, no significant association was observed between experimental diet and the maturity stages distribution ($\chi^2 = 29.61$, $p = .25$). However, within each dietary group was possible to identify a clear association between the maturity stages distribution and sea urchins' sex. In the group of sea urchins fed with algae diet there was a strong association between this diet and stages IV and V females ($\chi^2 = 39.44$, $p = .03$). For the sea urchins' groups fed with the fishmeal and cereal diets, no significant association was observed between sex and any maturity stage (fishmeal: $\chi^2 = 29.45$, $p = .25$; cereals: $\chi^2 = 25.88$, $p = .41$).

3.3. Gonads proximate composition and FA profiles

The proximate composition of the sea urchins' gonads was affected by both diet and sea urchins' sex. The soluble protein content varied among dietary groups ($p < .05$, Table 5), with the group fed with the algae diet presenting the highest content of soluble proteins (53 % DM, Table 5). The gonads' carbohydrates content also varied among dietary groups ($p < .05$, Table 5) with the sea urchins fed with the cereals diet presenting higher level of carbohydrates (15 % DM, Table 5). The lipidic content of the sea urchins' gonads was not affected by the experimental diets ($p > .05$, Table 5), but it was significantly affected by the sea urchins' sex ($p < .05$, Table 5), with the females presenting in all cases a significantly higher lipidic content (averaging 12.21 ± 1.43 % DM) when compared with males (averaging 8.13 ± 0.92 % DM) (Fig. 2).

The MANOVA results showed that the FA profile in the gonads was affected by the diet ($F_{app} = 24.42$, df = 38, $p < .001$), by the urchin's sex ($F_{app} = 25.82$, df = 19, $p = .001$) and by the interaction of both factors ($F_{app} = 4.58$, df = 38, $p = .002$). The sea urchins fed with fishmeal and cereals diets tended to accumulate more SFA and MUFA, while the sea urchins fed with the algae diet were richer in PUFA (Table 6). The $\sum$ n6/n3 index was significantly affected by both diet (F = 20.10, df = 2, $p < .001$) and sea urchin's sex (F = 25.26, df = 1, $p < .001$). The urchins fed

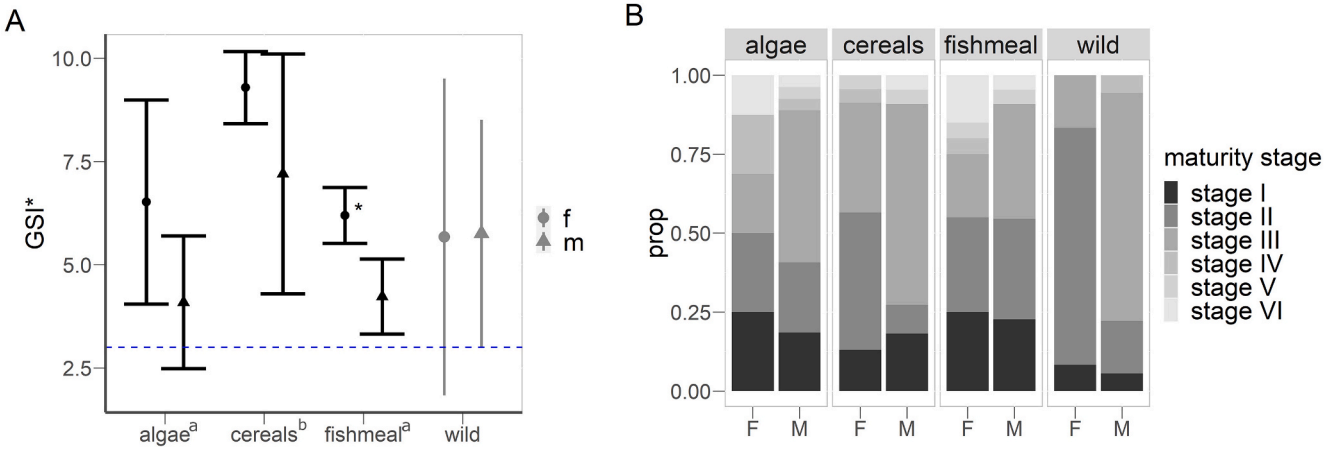

Fig. 1. Average gonad somatic index (GSI, A) and proportional distribution of gonad gametogenic stages (B) of the sea urchins fed with the three experimental diets in the growth trial of 15 weeks and field control. The dashed line indicates the initial GSI. Error bars indicate standard deviation from the average ($n = 3$). F represents the females and M the males. Different superscripts [a,b] with diets names in the x axis indicate statistical significant differences between experimental treatments. * indicates statistically significant differences between females and males within experimental treatment ($p < .05$).

Table 5
Gonad proximate composition of *Paracentrotus lividus* juveniles fed with the experimental diets for 15 weeks[1].

	algae	fishmeal	cereals	ANOVA		
				Diet (df = 2)	Sex (df = 1)	Diet x sex (df = 2)
Soluble Protein (% DM[1])	52.83 ± 11.78[a]	34.61 ± 10.41[b]	40.15 ± 4.69[ab]	F = 5.29 (p = .03)	F = 3.43 (p = .09)	F = 0.27 (p = .77)
Carbohydrates (% DM[1])	11.90 ± 1.39[ab]	9.73 ± 0.48[b]	15.11 ± 4.18[a]	F* = 22.05 (p = .007)	F* = 2.30 (p = .20)	F* = 1.29 (p = .37)
Lipids (% DM[1])	10.11 ± 2.21	10.49 ± 2.58	9.95 ± 2.76	F = 0.20 (p = .83)	F = 34.52 (p < .001)	F = 0.02 (p = .98)

[1] DM stands for dry matter; Values are presented as mean ± standard deviation, SD (n = 3). df represents the degrees of freedom for each factor and factors interaction, F represents the F-test, and F* the approximation to F-test obtained by non-parametric ART ANOVA. p represents the significance level. Values in the same row with different superscripts differ significantly (p < .05). Absence of superscripts indicates no significant differences between treatments.

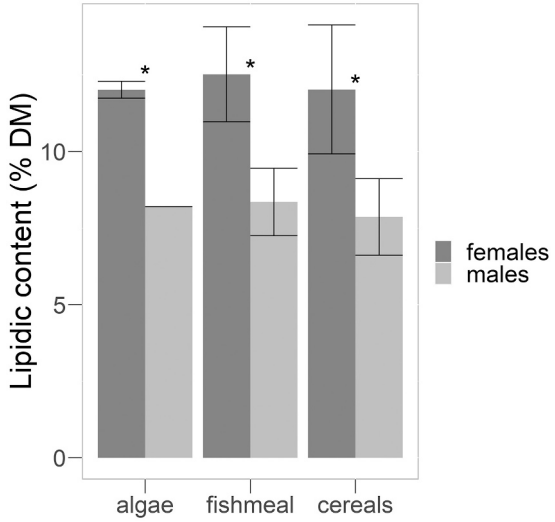

Fig. 2. Average gonad lipidic content of the sea urchins fed with the three experimental diets in the growth trial of 15 weeks. Error bars indicate standard deviation from the average (n = 3). * indicates statistically significant differences between females and males (p < .05).

with fishmeal and cereal diets presented higher index, resulting of the higher contribution of n6 PUFA for the FA acids profile. Overall, the females presented higher ∑ n6/n3 in the three dietary treatments.

The PCA showed that most of the variability observed among the groups of fatty acids profiles is explained by diets (PC1 62 % of variance explained) and then by sex (PC2 20 % of variance explained) allowing a clear distinction between females and males fed with the different diets (Fig. 3). The gonads of females fed with the algae diet were characterized by a higher content of the erucic acid (C22:1n9) and the heptadecenoic acid (C17:1n7), while males fed with the same experimental diet had a higher content of the PUFA C20:4n6 (ARA), DHA and the saturated heneicosylic acid (C21:0). The gonads of the males fed with the fishmeal diet presented a relatively high level of linoleic acid (18:2n6) and C22:1n9. The gonads of females fed with cereals diet were characterized by a higher content of C16:0 and the males fed with the same diet showed relatively higher level of ecosenoic acid (C20:1n9) and ecosadienoic acid (C20:2n6).

4. Discussion

The three experimental diets analysed promoted both somatic and gonadal growth of *P. lividus* urchins for 15 weeks. The average specific growth rate recorded was 0.44 % *per* day and the average GSI was 6.25% for all dietary treatments. The growth trial results indicate that, while the growth performance was not affected by the diets sources, the gonad yield and the maturity level were significant affected by these, with sea urchins fed with the cereals diet presenting the highest GSI (8.25 ± 2.23%) with highest percentage of gonads in growing and premature stages. Considering the 30 months of on-growing phase estimated by Grosjean et al. (1998) for the full cycle production of *P. lividus*, this study comprises a relatively short growth trial. However, the results obtained showed that the ingredients sources can have a, even though, subtle effect in the maturation level of gonads which balance between the storage and gametogenesis is crucial to promote somatic growth rate reducing gametogenesis. In longer trials, this effect of terrestrial plants based-diet may be enhanced by combining it with other environmental conditions favourable to somatic growth, like seawater temperatures close to that experienced by the urchins during winter (Kelly, 2001) and a long day photoperiod (Shpigel et al., 2004). Additionally, in a RAS system supported by well water source, it would be necessary to have an additional source of inorganic carbonate ions to support the high demand required for urchin test growth (Grosjean et al., 1998).

In nature, the sea urchins are predominantly herbivorous species and macroalgae are their primary diet (Lawrence et al., 2013). However, a macroalgae restrict diet offers low levels of protein and energy to support the high growth rates and gonad yield required for a profitable production (Pearce et al., 2002; Eddy et al., 2012; Prato et al., 2018). On the other hand, nutritively rich diets tend to promote the early maturation of gonads, reducing growth rates and gonad yield (Kennedy et al., 2005). For this reason, it is important to find a diet formulation for sea urchins during on-growing phase, that could enhance the somatic growth without precociously mature gonads. The experimental diets evaluated in the present study, meet the dietary protein and energetic content known to promote high growth rates and high gonad yield in *P. lividus* (Lourenço et al., 2020), and, by uniquely varying the ingredients in the experimental diets it was possible to evaluate the impact of feed sourcing in the production cycle of *P. lividus*.

The feed intake was relatively high since the beginning of the growth trial showing that all diets were well accepted by the sea urchins. Overall, the voluntary feed intake of the sea urchins fed with algae diet was higher relatively to the feed intake of the groups fed with the other two diets, suggesting that macroalgae based ingredients can promote higher feed consumption due to palatability factors. Nevertheless, no differences were found in the FCR among dietary groups, being inferior to those observed for *P. lividus* adults fed with other dry diets (Shpigel et al., 2004). These differences in the FCR showed that premature sea urchins are more efficient in the nutrients' utilization in relation to sea urchins that already initiate the reproductive phase.

The sea urchin's growth was evaluated by both the linear and the specific growth rates. The specific growth rate varied between 0.44% day^{-1} and 0.58% day^{-1}, with the sea urchins fed with the fishmeal diet presenting a, yet not significant, tendency to a higher growth rate. These results were higher to those reported by Cook and Kelly (2007) and consistent with the study conducted by Spirlet et al. (2001) which reported growth rates ranging between 0.18 and 0.39% day^{-1} for sea urchins of 10 to 25 mm test diameter fed with extruded diets based in solely soy-bean and in mixture of soya-bean and fish protein ingredients. The observed linear growth rate was similar across the three treatments, resulting in an average increase of test diameter by 0.07 mm day^{-1}. The previous studies evaluating the effect of pelleted diets in the growth of *P. lividus* urchins did not evaluate the linear growth rate. However, these

Table 6
Fatty acids profile (% Total FA)[1] of *Paracentrotus lividus* juveniles fed with the experimental diets for 15 weeks. Different superscripts indicate statistically significant differences between diets and between interaction groups (diets x sex). * indicates differences between females and males (*p*-value < .05).

	Algae		Fishmeal		Cereals		ANOVA F (*p*)		
	F	M	F	M	F	M	Diet (df = 2)	Sex (df = 1)	Diet x Sex (df = 2)
C14:0	8.84 ± 0.42^{c}*	7.83 ± 0.19^{c}	12.07 ± 0.95^{b}*	10.13 ± 0.44^{b}	14.12 ± 0.24^{c}*	11.43 ± 2.35^{c}	41.59 (< 0.001)	24.54 (< 0.001)	0.75 (0.48)
C15:0	0.72 ± 0.13*	0.69 ± 0.11	0.72 ± 0.03*	0.89 ± 0.03	0.75 ± 0.07*	0.88 ± 0.23	2.75 (0.08)	4.35 (0.05)	1.73 (0.20)
C16:0	21.11 ± 0.89^{b}	20.85 ± 1.23^{b}	27.61 ± 0.52^{a}	24.13 ± 0.94^{c}	29.52 ± 0.31^{a}	24.98 ± 2.18^{a}	62 (< 0.001)	38.07 (< 0.001)	5.90 (0.01)
C17:0	0.32 ± 0.04^{a}	0.25 ± 0.04^{a}	0.20 ± 0.02^{ab}	0.23 ± 0.02^{ab}	0.22 ± 0.12^{b}	0.18 ± 0.03^{b}	6.03 (0.01)	1.09 (0.31)	2.03 (0.15)
C18:0	3.49 ± 0.15^{a}	3.65 ± 1.70^{a}	3.04 ± 0.54^{a}	3.43 ± 0.70^{a}	2.65 ± 0.96^{b}	1.69 ± 1.24^{b}	6.67 (0.005)	1.19 (0.29)	1.68 (0.21)
C21:0	1.22 ± 0.14^{a}*	2.60 ± 0.40^{a}	0.93 ± 0.14b*	1.63 ± 0.24^{b}	0.31 ± 0.12^{b}*	0.82 ± 0.21^{b}	103.96 (< 0.001)	103.96 (< 0.001)	2.06 (0.15)
C22:0	0.33 ± 0.06^{a}	0.22 ± 0.10^{a}	0.22 ± 0.03^{a}	0.19 ± 0.08^{a}	0.16 ± 0.05^{b}	0.22 ± 0.07^{b}	4.07 (0.03)	0.54 (0.47)	3.90 (0.03)
∑SFA	36.04 ± 1.13^{c}	36.07 ± 0.69^{c}	44.78 ± 0.79^{a}	40.63 ± 0.94^{b}	47.73 ± 1.40^{a}	40.20 ± 3.19^{b}	53.91 (< 0.001)	43.35 (< 0.001)	10.41 (< 0.001)
C14:1n5	0.43 ± 0.07^{b}*	0.28 ± 0.02^{b}	0.47 ± 0.05^{b}	0.43 ± 0.04	0.72 ± 0.04^{a}	0.64 ± 0.19^{a}	37.60 (< 0.001)	8.15 (0.01)	12.05 (0.32)
C16:1n7	2.74 ± 0.11^{b}	2.08 ± 0.38^{b}	3.21 ± 0.25^{b}*	2.59 ± 0.16^{b}	4.47 ± 0.28^{a}	3.13 ± 0.57^{a}	40.82 (< 0.001)	51.95 (< 0.001)	1.62 (0.22)
C17:1n7	0.20 ± 0.03^{a}	0.20 ± 0.03^{a}	0.09 ± 0.02^{b}	0.10 ± 0.06^{b}	0.03 ± 0.05^{c}	0.02 ± 0.04^{c}	37.65 (< 0.001)	0.01 (0.90)	0.03 (0.97)
C18:1n9 (*cis* + *trans*)	4.50 ± 0.17	2.39 ± 0.25	5.31 ± 0.24	4.41 ± 2.81	4.08 ± 0.82	2.48 ± 0.70	6.28 (0.01)	63.09 (< 0.001)	0.52 (0.60)
C18:1n7	0.67 ± 0.04^{b}*	1.18 ± 0.11^{b}	0.87 ± 0.06^{b}*	1.06 ± 0.54^{b}	1.47 ± 0.14^{a}*	1.88 ± 0.33^{a}	16.44 (< 0.001)	7.55 (0.01)	0.96 (0.40)
C20:1	5.14 ± 0.56*	6.33 ± 0.33	5.98 ± 0.15*	6.98 ± 0.51	5.70 ± 0.35*	6.76 ± 0.86	6.18 (0.01)	31.63 (< 0.001)	0.26 (0.77)
C20:1n9	7.43 ± 0.26^{c}	7.04 ± 0.29^{c}	8.26 ± 0.31^{b}	8.32 ± 0.98^{b}	9.90 ± 0.78^{a}*	8.84 ± 0.41^{a}	29.49 (< 0.001)	4.82 (0.04)	1.64 (0.21)
C22:1n9	1.18 ± 0.14^{a}	1.19 ± 0.27^{a}	1.20 ± 0.22^{a}	1.37 ± 0.32^{a}	0.71 ± 0.31^{b}	0.69 ± 0.21^{b}	18.06 (< 0.001)	0.19 (0.67)	0.28 (0.76)
∑ MUFA	22.30 ± 0.29^{b}	20.67 ± 1.47^{b}	25.39 ± 0.40^{a}	25.25 ± 1.52^{a}	27.06 ± 0.74^{a}*	24.44 ± 1.09^{a}	45.20 (< 0.001)	15.82 (< 0.001)	3.22 (0.06)
C18:2n6 (LA)	10.59 ± 0.32^{b}*	6.59 ± 0.65^{b}	8.83 ± 1.16^{b}*	6.39 ± 1.29^{b}	4.17 ± 0.37^{a}*	3.28 ± 1.62^{a}	40.56 (< 0.001)	18.65 (< 0.001)	0.553 (0.58)
C18:3n6	1.03 ± 0.16^{a}	0.74 ± 0.07^{ab}	0.61 ± 0.08^{b}	0.67 ± 0.12^{b}	0.30 ± 0.08^{d}	0.56 ± 0.18^{cb}	33.25 (< 0.001)	2.12 (0.16)	11.38 (< 0.001)
C18:3n3 (ALA)	2.81 ± 0.09^{a}	2.96 ± 0.49^{a}	1.87 ± 0.11^{bc}	2.50 ± 0.56^{c}	1.77 ± 0.23^{b}	3.02 ± 0.71^{c}	5.54 (0.01)	21.67 (< 0.001)	4.11 (0.03)
C20:2n6	0.99 ± 0.27^{c}	1.05 ± 0.21^{c}	2.98 ± 0.64^{b}	3.40 ± 0.44^{b}	6.11 ± 1.23^{a}	5.79 ± 0.24^{a}	252.32 (< 0.001)	0.47 (0.50)	0.94 (0.41)
C20:3n6	1.54 ± 0.06^{c}	1.51 ± 0.20^{c}	1.21 ± 0.01^{cb}	1.61 ± 0.20^{c}	0.98 ± 0.04^{b}	2.40 ± 0.35^{a}	2.11 (0.14)	117.95 (< 0.001)	45.00 (< 0.001)
C20:4n6 (ARA)	11.35 ± 0.88^{ad}	13.95 ± 1.09^{a}	7.44 ± 0.15^{b}	10.08 ± 0.73^{de}	5.41 ± 0.82^{c}	9.81 ± 1.84^{e}	48.67 (0.001)	73.35 (< 0.001)	6.08 (0.01)
C20:5n3 (EPA)	7.70 ± 0.30^{a}*	10.03 ± 0.05^{a}	3.40 ± 0.21^{b}*	5.66 ± 0.22^{b}	2.50 ± 0.54^{b}*	5.58 ± 2.08^{b}	41.53 (< 0.001)	43.43 (< 0.001)	2.32 (0.12)
C22:5n3	0.30 ± 0.04^{b}	0.53 ± 0.02^{a}	0.10 ± 0.03^{c}	0.18 ± 0.04^{cd}	0.04 ± 0.02^{ce}	0.16 ± 0.08^{fc}	114.49 (< 0.001)	65.13 (< 0.001)	3.34 (0.05)
C22:6n3 (DHA)	1.42 ± 0.43^{b}	2.63 ± 0.34^{a}	0.31 ± 0.10^{cef}	0.39 ± 0.06^{dc}	0.13 ± 0.02^{ef}	0.31 ± 0.25^{fd}	160.52 (< 0.001)	19.59 (< 0.001)	5.83 (0.01)
∑ PUFA	37.73 ± 1.01^{a}	39.98 ± 0.80^{a}	26.72 ± 0.68^{b}	30.86 ± 0.94^{c}	21.40 ± 1.77^{d}	30.91 ± 3.48^{c}	93.47 (< 0.001)	72.18 (<0.001)	14.06 (< 0.001)
∑ n6	25.51 ± 0.80^{a}	23.84 ± 0.17^{a}	21.06 ± 0.64^{b}	22.14 ± 0.82^{ab}	16.96 ± 1.88^{c}	21.84 ± 0.94^{ab}	46.85 (< 0.001)	23.51 (<0.001)	20.77 (< 0.001)
∑ n3	12.23 ± 0.22^{a}*	16.15 ± 0.81^{a}	5.67 ± 0.10^{b}*	8.72 ± 0.67^{b}	4.45 ± 0.72^{b}*	9.07 ± 2.99^{b}	48.46 (< 0.001)	47.74 (< 0.001)	2.36 (0.12)
n6/ n3	2.09 ± 0.03^{b}*	1.48 ± 0.08^{b}	3.72 ± 0.11^{a}*	2.55 ± 0.23^{a}	3.92 ± 0.88^{a}*	2.69 ± 1.02^{a}	20.10 (< 0.001)	25.26 (< 0.001)	0.21 (0.82)

Fatty acids profile (mean ± SD % Total FA, N = 3) of Paracentrotus lividus juveniles fed with the experimental diets for 15 weeks. Different superscripts indicate statistically significant differences between diets and between interaction groups (diets x sex),* indicates differences between females and males (p-value < .05).

results are in line with the results obtained by Castilla-Gavilán et al. (2019), which obtained linear growth rate between 0.05 mm day^{-1} and 0.08 mm day^{-1} by feeding 11 mm TD *P. lividus* with different macroalgae, and are higher to the growth rates by Vizzini et al. (2018) who fed *P. lividus* juveniles of 16 mm TD with lettuce discards in sea-cages; and also higher to the results reported by Frantzis and Grémare (1992), that fed 14-16 mm TD juveniles with a collection of different macroalgae.

Sea urchins' gonads have two complementary functions acting as reproductive and storage organs. The interaction between these two functions has a high influence in the gonads structure and size. Moreover, the gonad development is highly influenced by feed ingestion and nutrients quality (Kennedy et al., 2007). In this study, the initial GSI was 2.99% and at the end of the growth trial the highest GSI was verified in sea urchins fed with cereals diet, with an average of 8.22%, suggesting that the cereals diet promote high gonad growth. Overall, the GSI results were better than those observed by Vizzini et al. (2018). However, they were relatively below those observed by Fernandez and Boudouresque (2000) who obtained a GSI between 10% and 14% in 20 to 25 mm

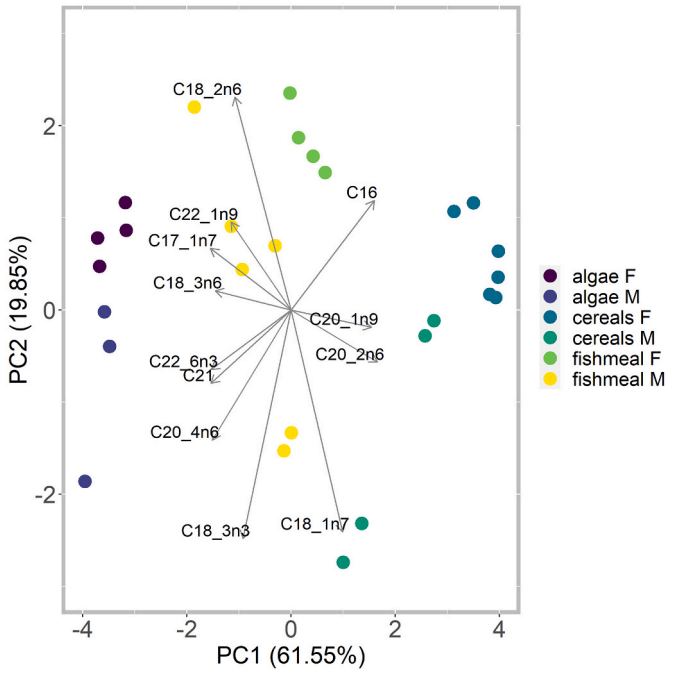

Fig. 3. Biplot of the principal component analysis to the fatty acids profile of the female and male gonads of sea urchins fed with the three experimental diets for 15 weeks. F represents the females and M represents the males.

juveniles fed with diets with ingredients of vegetable, animal and mixed sources, and also bellow the GSI obtained by Spirlet et al. (2001) feeding the sea urchins with soya-bean and mixed pellets. The relatively low GSI observed could be related with some degree of biogenic acidification in the rearing system. The biogenic acidification occurs due to reduction of rearing system pH, reduced calcite saturation state and increasing ρCO_2 which are critical when rearing calcified organisms (Stumpp et al., 2012; Mos et al., 2015). Despite the low initial density (98 g m^{-2}) and relatively high exchange rate (3.75 *per* hour), the acidified characteristics of the seawater used (well seawater) and the hypothetical accumulation of CO_2 in the system could have a negative impact in the gonad growth due to unbalanced carbonate chemistry within the RAS system as reported for *T. gratilla*. In their study, Mos et al. (2016) showed that even that the sea urchins' survival and consumption rates were not affected, the gonad growth can decrease up to 67 % due to biogenic acidification.

Nevertheless, the high specific growth rates observed in urchins fed with the three diets were obtained at the expenses of increasing gonad weight. This precocious increase gonad production, as result of high dietary protein content, may have a negative effect in the further production yield because it inhibits a further growth in diameter (Kelly et al., 1998; Kennedy et al., 2005; Cook and Kelly, 2007) with urchins obtaining smaller maximum size and potential lower GSI (Fernandez and Pergent, 1998; Martínez et al., 2003; Sánchez-España et al., 2004). Precocious gonad growth has been observed in other species juveniles when fed with formulated feeds, including *L. variegatus* (Hammer et al., 2004), *Psammechinus miliaris* (Kelly et al., 1998), *P. depressus* (Akiyama et al., 2001) and *Loxechinus albus* (Olave and Bustos, 2001). The GSI results were also affected by the sea urchins' sex with females presenting tendentially higher GSI than males, even though this difference being statistically significant only for the group fed with the cereals diet.

Despite the unbalanced sex ratio observed in the group fed with algae diet, our results indicate a tendency to the precocious maturation of the gonads in both females and males, when compared with the gonad development of sea urchins of the same size from wild populations (see Fig. 1B). The sea urchins fed with the three diets presented a relatively high proportion of gonads in mature, spawning, and post-spawned stages (the maturity proportions range between 9% and 31%) when the wild population of similar size in May (by the end of the growth trial) presented only 5.6% of the individuals within these maturity stages. Nevertheless, a high percentage of individuals were in the growing and premature stages, that are characterized by an increase of the gonad size due to the nutrient storage in the nutritive phagocytes (Byrne, 1990; Walker et al., 2005) particularly in the females (Rocha et al., 2019). Noteworthy, the prevalence of the growing and premature gonadal stages can also be associated to the rearing temperature. During the growth trial, the seawater was maintained near 18 ºC. The results obtained by Santos et al. (2020) for the effect of rearing temperature in *P. lividus* gonadal development showed that the sea urchins reared at 18 ºC tended to present a slower progression in the gametogenic cycle. This reduction in the gonadal development can, in fact, be beneficial for somatic growth and maximum size to attain, if the growth rates are not affected.

The protein content was the main gonad component followed by the carbohydrates (measured as glycogen) and lipids. The proximate composition of the gonads of the *P. lividus* was affected by the experimental diets, indicating that even though the proximate composition of the experimental diets was identical, the ingredient sources used had an impact in the sea urchins' gonads composition. Moreover, the sea urchins' sex also presented itself as an influential factor. In fact, the echinoids' gonad composition is influenced by both endogenous (e.g. sea urchin sex and maturity stage) or exogenous (e.g. nutrients sources) factors (Arafa et al., 2012; Siliani et al., 2016; Volpe et al., 2018). The results showed that the sea urchins fed with algae diet stored more protein in their gonads in agreement with protein intake and partially with SGR. Previous studies have shown a correlation between increasing protein consumption and increasing gonad growth (Fernandez and Boudouresque, 2000; Fernandez and Pergent, 1998; Agatsuma, 2000; Akiyama et al., 2001; Hammer et al., 2004; Hammer et al., 2006b, 2012; Heflin et al., 2012). However, the experimental diet with higher intake levels did not resulted in a higher gonad production as would be expected from previous studies (Akiyama et al., 2001; Eddy et al., 2012).

The carbohydrates were identified as a primary source of energy for gonad growth and gametogenesis in sea urchins (Montero-Torreiro and Garcia-Martinez, 2003; Marsh et al., 2013). The carbohydrate level in all three diets ranged between 12 and 15 % DM, with the gonads of sea urchins fed with the cereals diet containing higher levels of carbohydrates, despite the lower specific growth rates, confirming the hypothesis that, for itself, dietary carbohydrate level is a poor predictor of sea urchin growth (Heflin et al., 2012). Moreover, the gonad carbohydrates content was identical to the lower levels observed by Montero-Torreiro and Garcia-Martinez (2003) in wild populations. Noteworthy, a relatively low carbohydrates level in the gonad is balanced with a high level of protein with implications in the gonads structure due to cell differentiation (Mol et al., 2008; Montero-Torreiro and Garcia-Martinez, 2003; Rocha et al., 2019; Veracchia et al., 2012).

The lipids are important energy reserves, with a structural function that is fundamental for somatic growth and gonad production (Kennedy et al., 2007). The gonad lipidic content was identical in the three experimental groups and slightly lower to those observed in wild populations (Montero-Torreiro and Garcia-Martinez, 2003). Nevertheless, females had a higher lipid content when compared to males, regardless the dietary treatment. It is possible that *P. lividus* females are more efficient into converting feed into gonad reserves compared to males independently of diet or rearing conditions (Baião et al., 2019; Rocha et al., 2019).

The FA are important structural and physiological components of cell membranes, and their concentrations in natural or formulated diets affect the growth and development of gonads (Sargent et al., 2002). Our results confirm that both experimental diets and sea urchin sex have a significant impact in the gonad fatty acid profile as already confirm in other studies (Cook et al., 2000; Galloway et al., 2015; Prato et al., 2018). In fact, the sea urchins fed with algae and fishmeal diets showed a higher content in polyunsaturated FA, particularly ARA and EPA,

particularly evident in the males group fed with the algae diet. De la Cruz-Garcia et al. (2000) already reported the PUFAs as to be the most abundant FA class, accounting for 44.7%, followed by SFAs 35% and MUFAs 20.3% of the total FAs. On the other hand, the sea urchins fed with the cereals diet, particularly females, were richer in saturated FA, mainly C14:0 and C16:0. The FA C16:0 is, in fact, an important component of the lipid fraction in the *P. lividus* gonads. It is synthesized in plant chloroplasts leading to the biosynthesis of LA and ALA through desaturation and elongations mechanisms, and later to ARA, EPA, and DHA through the "Δ8 pathway" (Angioni and Addis, 2014; Kabeya et al., 2017). The sea urchins fed with algae diet presented a significant higher content of DHA in relation to the other dietary groups. In their study, Cook et al. (2000) hypothesized about the role of DHA in the sea urchins growth, however in the presented study we did not found any correlation between the higher levels of this fatty acid and neither somatic growth or gonad development. These differences in PUFA gonad content between experimental groups had an impact in the n6/n3 index. In comparison with the wild sample, this index was clearly unbalanced towards the n6 PUFA due to the high content of C18:2n6, C20:2n6 and ARA, particularly in the females. In the present study, the high content of C18:2n6 is correlated with the rich source of SFA, particularly C16:0 provided in the cereals diet. This could indicate that arachidonic acid metabolic pathway is particularly active in females in growing and premature gonadal stages (Unuma et al., 2003; Wang et al., 2019). In fact, Prato et al. (2017) have already observed a n6/n3 ratio particularly high in the gonads of adult *P. lividus* fed with dry feeds prepared with different levels of soybean meal, wheat, rapeseed meal, krill and macroalgae. These high levels of n6 PUFA in modern aquaculture products was addressed by Grigorakis (2007) showing that these are related with higher levels of terrestrial plant-originating C18:2n6. Nevertheless, the three diets produced gonads with high level of PUFA which have important benefits for human health by preventing heart and vascular system diseases as well diabetic conditions (Garaffo et al., 2011).

5. Conclusion

Our results showed that the three experimental diets promoted high growth rates of *P. lividus* during on-growing phase independently of ingredients sources. Nevertheless, these higher growth rates were followed by a significant and differentiated level of gonads maturation due the interaction of diets sources and sea urchins' sex. The cereals-based diet promoted a significant increase of GSI in association with gonads in the initial developmental stages, while the sea urchins fed with algae diet indicated a precocious maturation of the gonad. The present study results showed that while the diets nutritional content is key to promote the growth independently of diets sources, the later can have a key role into controlling the gonad maturation process. Finally, the formulation of feeds for sea urchins rearing using ingredients of terrestrial plants sources can provide the protein, SFA and MUFA requirements to somatic and gonad growth.

This was a short growth trial when comparing with the long on-growing phase estimated to the full-cycle production of *P. lividus*. To maintain growth rates as high as those obtained here in longer trials in RAS are still challenging. Besides optimal nutritional conditions, the rearing conditions including the characteristics of sourced seawater in RAS, its pH and the content inorganic forms of carbonate ions is crucial to maintain urchins' high growth rates at longer term. Considering that closed and semi-closed systems are energetically more expensive and require more labour hours, it would be interesting to compare the production efficiencies obtained in such systems with flow-through systems, which can be energetically more efficient.

Main achievements

- The sea urchins' somatic growth is independent of diet sources
- Both diet sources and sea urchins' sex affect gonad growth and proximate composition
- The females fed with the cereals diet stored high levels of LA and the males fed with algae diet stored high levels of ARA and EPA
- The cereals diet showed good results for on-growing phase providing high growth rates and low level of precocious gonad maturation

Declaration of Competing Interest

None.

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