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RESEARCH

Inclusion of *Dunaliella salina* in cold extruded and softly dried diets as a promising way to enhance the quality of *Paracentrotus lividus* gonads

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Abstract

The unicellular alga *Dunaliella salina* is a good source of β-carotene whereas the macroalga *Porphyra* sp. is rich in flavor-enhancing amino-acids such as glycine. Therefore, this study focused on the development of new dietary formulations for *Paracentrotus lividus,* including *D. salina, Porphyra* or glycine, aimed at improving roe color and flavour. Five isonitrogenous and isoenergetic diets were produced by cold extrusion and soft drying, meant to protect carotenoids. Sea urchins were fed these diets every 2 days in a recirculating aquatic system, at 18ºC. At the end of 8 weeks all diets proved equally effective in promoting growth and improving the gonadossomatic index. However, there were differences in the feed conversion ratio, which was lower in sea-urchins grown upon a diet based on commercial macroalgae mix and supplemented with *D. salina* at 1.5% (D2). Notable differences were found between males and females on carotenoids deposition in both gut and gonads, gonadal proximal composition, volatile compounds and anti-oxidant activity. This suggests sex-related differences in both nutrient assimilation and several metabolic processes. Gut carotenoids were influenced by both sex and diet, but gonads carotenoids were only affected by sex. All diets supplemented with *Dunaliella* improved gonad colour, resulting in redder and darker gonads when compared to a control (non-supplemented) diet. In particular D2 allowed to produce gonads with a higher colour saturation (C^*) and the lowest hue angle (h^*), comparable to a gourmet reference. Glycine supplementation and *Porphyra* led to an increase of sweet tasting amino acids in *P. lividus* roe. *Dunaliella salina and Porphyra* also increased umami tasting amino acids. The overall results point to D2 as the most promising formulation for *P. lividus* grow-out, as it allowed to obtain good colour roe at a lower feed conversion ratio.

Keywords Sea urchin roe · Carotenoids · Microalgae · Gonad quality · β-carotene

Introduction

Recent research has reinforced the idea that algae are valuable sources of both macro and micronutrients, including protein (Machado et al. 2020; Ferreira et al. 2021) and carotenoids (Generalic Mekinic et al. 2023). Due to this recognition and the increasing adoption of mass-production techniques worldwide, algae can be regarded as promising ingredients for aquaculture feeds, particularly for functional formulations, either for promoting health or for improving the quality of final products of fish, crustaceans or echinoids (Guedes et al. 2015; Generalic Mekinic et al. 2023).

Sea-urchin aquaculture has traditionally relied on kelp as a major feed source (Liu and Chang 2015). However, the increasing demand for sea-urchin roe worldwide calls for novel feed solutions (Sun and Chiang 2015). To reduce reliance on kelp and facilitate the expansion of sea-urchin aquaculture, several studies have explored alternative diets aimed at enhancing the quality of sea-urchin roe during grow-out (Lourenço et al. 2019). Research on *Paracentrotus lividus* has led to the development of pelleted feeds with dietary formulations that ensure high growth and good gonad yields, with protein being identified as a key nutrient (Carboni et al. 2015; Prato et al. 2017; Baião et al. 2019; Zupo et al. 2019; Lourenço et al. 2020). Using extruded diets, Baião et al. (2019) and Lourenço et al. (2020) reported optimal results for diets containing 30% DM of protein. Despite these recent

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efforts and achievements, important marketable traits such as color and taste still need to be refined to fully address consumer demands.

As in other aquatic animals, the color of sea-urchins roes is associated with carotenoid deposition which is in turn dependent on dietary carotenoid intake. Only autotrophs can de novo synthetize these molecules whereas heterotrophs obtain them directly from the diet or via bio-conversion of primary carotenoids (Maoka 2011). Sea-urchins obtain carotenoids from their diet, so their gut wall carotenoid profile largely reflects the dietary carotenoids profile (Haug et al. 2003; Symonds et al. 2007). Conversely, the pigment profile of their gonads does not seem to reflect the dietary carotenoid profile (Symonds et al. 2007). Echinenone is the primary carotenoid of sea-urchins gonads and is present only in heterotrophs (Maoka 2011). Its synthesis and deposition in animal tissues depends on the availability, uptake and bioconversion of β-carotene obtained from dietary sources (Tsushima 2007; Hagen et al. 2008; Maoka 2011). Challenges in enhancing gonad color partly arise from the complexity of bioconversion of dietary β-carotene into echinenone (Tsushima 2007), paleness being an usual issue due to a mismatch between gonad growth and carotenoid deposition (Plank et al. 2002). Moreover, female and male gonads exhibit different patterns of carotenoid deposition and depletion (Hagen et al. 2008), which may reflect different retention or synthesis capacities. Therefore, female and male gonads display slightly different colors (Baião et al. 2019, 2022b), yet consumers generally prefer orange roes regardless of sex (Baião et al. 2021a, b).

The dietary inclusion of synthetic carotenoids or natural carotenoid sources such as fruits and vegetables has been tested in several sea-urchin species, with variable and generally unsatisfactory results on gonads color, attributed to technical problems leading to carotenoids loss or degradation (Robinson et al. 2002; Shpigel et al. 2006; Suckling et al. 2011; Baião et al. 2022b). Although macroalgae are valuable sources of carotenoids and have classically been included in compound diets for sea-urchins, the use of microalgae as dietary supplements to optimize gonad color has not been sufficiently explored. The concentration of carotenoids in microalgae is generally much higher than in macroalgae, *Dunaliella salina* being amongst the microalgae with the highest concentrations of total carotenoids (Generalic Mekinic et al. 2023) and β-carotene in particular (Lafarga et al. 2020). Few studies have been published exploring the application of *Dunaliella* spp. in diets for sea-urchins, yet promising results have been reported for *Strongylocentrotus droebachiensis* and *P. lividus* (Robinson et al. 2002; Shpigel et al. 2006).

Beyond visual perception, flavor perception plays a crucial role in the acceptance of sea-urchin roes. Hence, the formulation of diets for sea urchins should aim at optimizing not only color but also other attributes such as odor and taste, i.e. the senses directly involved in flavor perception (Laing and Jinks 1996). Both volatile compounds and amino acids (AA) can influence flavor perception. Some volatile compounds result from carotenoids metabolism, through enzymatic and non-enzymatic processes like photo-oxygenation, (auto)oxidation and thermal degradation (Winterhalter and Rouseff 2001). These compounds serve important roles as flavor and fragrance substances and can be obtained naturally in both microalgae (e.g., *Dunaliella*) and macroalgae (e.g., *Porphyra*) (Winterhalter and Rouseff 2001). Amino acids such as methionine, cysteine and proline can also be perceived by human olfaction (Laska 2010). The taste of sea urchins is claimed to result from free amino acids, categorized based on their perceived impact on flavor, as either enhancing (desirable) or detracting (undesirable) amino acids (Hirano et al. 1978; Woods et al. 2007; Prato et al. 2018; Volpe et al. 2018). Although some differences exist between consumers depending on their background culture and expectations, flavor-enhancing amino acids are typically associated with sweetness (e.g., glycine), umami (e.g., glutamic and aspartic acid), a mild sourness (e.g., aspartic acid) or even slight bitterness (e.g., arginine and leucine) (Birch and Kemp 1989; Woods et al. 2007; Wang et al. 2020). On the other hand, flavor-detracting or undesirable amino acids are those perceived as strongly bitter or sour, such as valine. Therefore, the manipulation of dietary amino acid profile could have an impact on *P. lividus* gonad amino acid profile and taste.

This study aimed at a developing an extruded diet for *P. lividus* grow-out, formulated not only to ensure high growth and gonad yield, but also to maximize the deposition of dietary carotenoids and taste-associated amino-acids deposition, toward ultimate improvement of roe color and flavor. *Dunaliella salina* for being an important industrial source of β-carotene (Lafarga et al. 2020) was tested as a dietary supplement to improve color. *Porphyra* as a macroalga rich in sweet-taste and umami amino acids (Hwang et al. 2013) was in turn explored to replace a commercial macroalgae mix while glycine was used as a supplement to enhance the sweetness of *P. lividus* roes as per consumers' preferences. Cold extrusion and soft drying were used in the manufacture of feeds in an attempt to preserve the carotenoids' desired properties.

Materials and methods

The present study was performed by accredited scientists in laboratory animal science by the Portuguese Veterinary (1005/92, DGV-Portugal, following FELASA category C recommendations), being previously approved by CIIMAR ethical committee for Managing Animal Welfare (ORBEA),

in compliance with the European Union Directive 2010/63/EU and the Portuguese Decree Law n° 113/2013 on the protection of animals used for scientific purposes.

Experimental diets

Six extruded diets were formulated so as to contain 32% protein and 7% fat (DM basis) (Table 1). A basal diet (CTRL) was formulated using a blend of protein sources and including 15% of a commercially available macroalgae mix. Two diets with the same formula were supplemented with *D. salina* at 0.75% and 1.5% targeted at 150 or 300 µg g^{-1} of β-carotene DM, respectively, and hereafter denoted as D1 and D2. In two diets equally supplemented with *D. salina*, the macroalgae mix was totally replaced by *Porphyra* spp. as a good source of the amino-acids typically abundant in *P. lividus* roes (Dincer and Cakli 2007; Mol et al. 2008; Machado et al. 2020); these diets are hereafter denoted as D1P and D2P. A sixth diet, based on the basal formula, was supplemented with 1.2% of crystalline glycine, as an abundant amino acid in sea-urchins roes and believed to be a sweetness enhancer; this diet is hereafter denoted as GLY.

Diets were manufactured by SPAROS, Lda (Portugal): all ingredients were ground in a micropulverizer hammer mill (Hosokawa Micron, SH1, Germany), mixed in a paddle mixer (Mainca RM90, Spain) according to each specific formulation, humidified with water (250 g kg^{-1}) and compacted. Baião et al. (2022b) reported considerable losses of dietary carotenoids in pelleted diets manufactured by SPAROS (70 to 94% of degradation) and Valerio et al. (2020) recently reported thermal degradation kinetics of carotenoids. Hence the diets herein tested were extruded using a low-shear single screw extruder (ITALPLAST P150, Italy) at low temperature (< 30 °C), followed by soft drying in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France), at < 45ºC instead of 70ºC, in an attempt to preserve carotenoid properties. Dried feed pellets were subsequently sieved to produce the desired particle size (around 3 cm). The pellets of each diet were stable in water for 48 h.

Growth trial and sampling procedures

A growth trial was performed in the Fish Culture and Experimental Unit of CIIMAR (Matosinhos, Porto, Portugal) with wild adult sea urchins (*Paracentrotus lividus*), collected in the intertidal shores of Viana do Castelo (Portugal) prior to the sea urchin spawning season (Fernández-Boo et al. 2018; Rocha et al. 2019a). Sea urchins were kept in quarantine for 3 weeks in a 2000 L fiberglass tank, within a recirculating system; water parameters were kept similar to those prevailing in the collection site (i.e. 16 ± 1ºC, salinity 35‰). During the quarantine period, animals were slowly acclimated to 18ºC and fed the control diet.

After the acclimation period, sea urchins were fasted for 8 days to empty their gut content. Ten female and ten male sea urchins were then individually weighted and measured; gonads were collected to be weighed for gonadosomatic index (GSI) determination and sampled for further evaluation on maturation status (confirmed to be at stage I – recovery, 50% for females and 67% for males—and stage II – growing 50% for females and 33% for males). The remaining sea urchins were individually weighed and measured (initial average body weight of 52.32 ± 13.23 g; initial average test diameter of 5.0 ± 0.5 cm), and randomly placed inside 24 plastic mesh cages (30 × 27 × 17 cm) immersed in 55 L fiberglass tanks, at a stocking density of approximately 3.5 kg m^{-2}, as recommended by Siikavuopio et al. (2007). Each cage/tank held 18 urchins. All tanks were connected within a saltwater recirculating aquaculture system (RAS). The RAS was kept under the following conditions: water temperature of 18 ± 1 ºC, salinity of 35‰, flow rate of 4 L min^{-1}, 10 h light/14 h dark photoperiod and 8 mg L^{-1} of dissolved oxygen. Nitrogenous compounds and pH were regularly measured throughout the trial, and kept within the recommended levels for marine species (Blancheton 2000). The animals were adapted to the new tanks for 2 days before being fed the experimental diets. Diets were then randomly assigned to quadruplicate groups and sea urchins were hand-fed every 2 days for 8 weeks until apparent satiation, as previously described by Baião et al. (2019). Leftovers were collected before every new feeding, for further calculations of feed intake.

At the end of 8 weeks, all sea urchins were fasted for 8 days prior to sampling, to ensure complete gut clearance. All sea urchins from each tank were individually weighted and measured, and 8 individuals from each cage/tank were sampled for gut and gonad collection. After dissection, the gonads from all sampled sea urchins were weighed to determine GSI. The gut was collected from 6 sea-urchins from each cage/tank, weighed and stored for further analysis of carotenoid content. From the same 6 urchins, 3 males and 3 females: one gonad was divided into two portions—the larger portion to be used for color and texture instrumental analysis, and a small portion to be fixed for histological evaluation of the gametogenic stages; one gonad was collected for dry matter determination; two gonads were collected for protein and energy determination; and one gonad was collected for carotenoid analysis. From 2 other sea-urchins, one male and one female: one gonad was used for color, texture and histological; one gonad was collected for dry matter determination; one gonad was collected for carotenoid analysis and antioxidant determination; one gonad was collected for free amino-acids (FAA) determination; and one gonad was collected for volatile compounds analysis. To ensure enough quantity for the analytical procedures for protein, energy

Table 1 Formulation and proximate composition of the experimental diets

	CTRL	D1	D2	D1P	D2P	GLY
Ingredients (%)						
Squid meal[a]	5.00	5.00	5.00	5.00	5.00	5.00
Plant based glutens[b]	17.50	17.50	17.50	14.50	14.50	16.20
Plant based meals[c]	31.16	31.04	30.91	33.11	32.26	31.26
Krill oil[d]	1.00	1.00	1.00	1.00	1.00	1.00
Linseed oil[e]	1.00	1.00	1.00	1.30	1.30	1.00
Lecithin[f]	3.30	3.30	3.30	3.30	3.30	3.30
Vitamin and mineral premix[g]	2.00	2.00	2.00	2.00	2.00	2.00
Organic-based binder[h]	7.50	7.50	7.50	7.50	7.50	7.50
Mineral-based binders[i]	11.00	10.38	9.75	11.00	11.00	11.00
Laminaria[j]	5.00	5.00	5.00	5.00	5.00	5.00
Macroalgae mix[k]	15.00	15.00	15.00	-	-	15.00
Antioxidant[l]	0.54	0.54	0.54	0.54	0.54	0.54
Dunaliella salina[m]	-	0.75	1.50	0.75	1.50	-
Porphyra[n]	-	-	-	15.00	15.00	-
Glycine	-	-	-	-	-	1.20
Chemical Composition (%DM)						
DM	94.78	94.36	94.71	95.52	95.54	95.48
Ash	17.87	17.61	18.02	14.83	14.90	18.05
Protein	32.39	32.58	32.26	32.38	32.62	32.71
Energy $kJ.g^{-1}$	16.30	16.38	16.38	16.66	17.14	16.18
Lipids	7.33	7.35	7.59	7.92	8.17	7.44
Phosphorus	0.58	0.55	0.54	0.57	0.60	0.55
Total amino acids ($mg.g^{-1}$ DM)						
Arginine	22.23	25.91	25.35	22.29	21.86	23.11
Histidine	5.24	7.42	5.49	5.21	5.06	5.77
Lysine	15.65	22.57	17.37	14.47	14.30	14.35
Threonine	11.03	14.59	12.50	11.09	11.31	11.31
Isoleucine	15.18	17.78	14.98	14.18	13.55	15.40
Leucine	32.69	31.94	32.65	29.46	29.96	31.99
Valine	15.83	18.20	16.84	15.02	14.85	15.42
Methionine	7.90	9.24	8.07	7.46	7.53	8.05
Phenylalanine	15.06	16.75	15.08	14.03	14.02	15.55
Cystine	1.52	1.80	1.41	1.34	1.50	1.50
Tyrosine	11.19	12.24	10.80	11.20	10.46	11.82
Aspartic acid + Asparagine	23.46	25.95	23.44	21.80	22.10	21.80
Glutamic acid + Glutamine	76.46	67.28	64.87	59.49	60.27	69.18
Alanine	26.13	22.63	27.08	25.22	24.91	24.55
Glycine	31.06	28.13	32.77	29.84	32.37	41.31
Proline	47.22	38.10	46.00	40.11	40.08	44.67
Serine	16.91	20.26	16.51	16.38	15.84	16.78
Carotenoid content ($\mu g.g^{-1}$ DM)						
Lutein	1.89	17.82	38.86	71.10	71.56	1.71
Zeaxanthin	0.99	1.26	1.69	1.71	1.79	1.11
Echinenone	ND	ND	ND	ND	ND	ND
α-carotene	ND	10.34	23.72	16.57	21.65	ND
β-carotene[o]	-	48.70	119.77	76.80	99.58	-
Σ carotenes[p]	-	59.05	143.49	93.37	121.24	-
Σ carotenoids (HPLC)[q]	2.87	78.13	184.03	166.18	194.58	2.82
Expected β-carotene concentration[r]		142.66	284.26	150.05	290.93	
% of degradation of β-carotene[s]	-	65.86	57.86	48.82	65.77	-

Table 1 (continued)

[a] Super prime without guts: Sopropêche, France

[b] Wheat and corn gluten, COPAM, Portugal

[c] Micronized wheat; corn grain; starch, Ribeiros, Portugal

[d] SuperbaKrillTM oil, Solchem, Spain

[e] Henry Lamotte Oils GmbH, Germany

[f] Lecico P700IPM, LECICO GmbH, Germany

[g] Premix PV01 for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol 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^{-1} diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 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; excipiente wheat middlings

[h] Gelatin and plant gums, LAPI Gelatine SPA, Italy

[i] Zeolite, Kielseguhr, LIGRANA GmbH, Germany; monocalcium phosphate, Fosfitalia, Italy; calcium carbonate, PREMIX Lda, Portugal

[j] Algovert®, Laminaria digitata, Setalg, France

[k] Ocean feed shrimp—Seaweed mealTM, Ocean Harvest Technology, Ireland

[l] Antioxidant powder, Paramega PX, Kemin Europe NV, Belgium

[m] *Dunaliella* sp. Freeze-dried biomass powder, β-carotene content: 17.95 $mg.g^{-1}$ WW – Nutrally algae solutions, S.L., Madrid, Spain

[n] Biomass of *Porphyra* spp. collected in northern Portugal, at *Praia de Aguçadoura* rocky shore from July to September of 2019—[β-carotene] = 58.19 $\mu g.g^{-1}$ WW

[o] The concentration of β-carotene derivates are accounted for total β-carotene

[p] The concentration of carotenes is accounted for the concentration of β and α-carotene

[q] Concentration of carotenoids is accounted for the sum of the quantified carotenoids by HPLC

[r] Expected concentration of β-carotene in the diets considering the percentages of the micro and macroalgae and their β-carotene content

[s] Percentage of degradation calculated like: (Expected concentration of β-carotene in the diets – Actual c concentration of β-carotene in the diets) / Expected concentration of β-carotene in the diets

and carotenoid quantification, gonads within dietary treatments with similar GSI were pooled, keeping sexes separated. The sampling scheme is summarized in Fig. 1. All collected samples were snap frozen in liquid nitrogen and kept at −80ºC until analysis.

Growth performance was assessed based on the following parameters: Specific growth rate (SGR) = (Ln (final weight(g)) – Ln (initial weight(g))) / number of days × 100; Feed conversion ratio (FCR) = feed intake/ weight gain; Protein Efficiency Ratio (PER) = (final weight– initial weight) / ingested protein (g); Voluntary feed intake (VFI, %) = 100 × dry feed intake/average body weight / days of experiment; gonadosomatic index (GSI, %) = gonad weight/ total body weight × 100; Viscerosomatic index (%) = gut weight/total body weight × 100.

Proximate composition and free amino-acids profile analysis

The tested diets were ground and homogenized prior to proximate composition and amino acids analysis. Dry matter, ash, protein, energy, total lipids, carotenoid content and total amino acid content of the diets were assessed in duplicate. Dry matter analysis was performed at 105ºC for 24 h, ash by combustion in a muffle furnace (Nabertherm L9/11/B170, Germany; 500 °C for 5 h), crude protein (N × 6.25) using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, USA) and gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Germany) calibrated with benzoic acid. Total lipids were extracted and quantified using dichloromethane-methanol (2:1) by a gravimetric technique adapted from Folch et al. (1957).

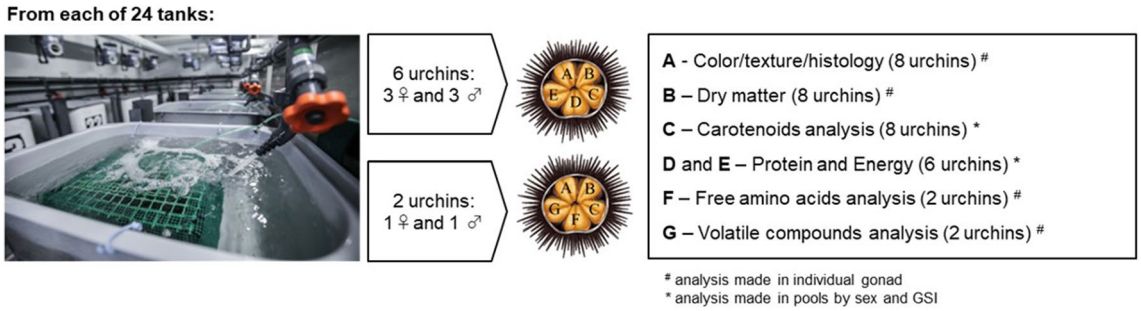

Fig. 1 Gonad collection of the sampled sea urchins and sample organization of analysis

Experimental diets were hydrolyzed (6 M HCl at 116ºC for 48 h in nitrogen-flushed glass vials) and pre-column derivatized with Waters ACccQ Flour Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analysis of total amino acid content was performed by ultra-high-performance liquid chromatography (UPLC) in a Waters Reversed-Phase Amino Acid Analysis System, using norvaline for internal standard. Tryptophan was not determined, as it is partially destroyed by acid hydrolysis. Instrument control, and data acquisition and processing were resorted to Waters Empower software.

Freeze–dried gonads were homogenized in a tissue homogenizer (Precellys, Bertin Instruments) using 12 metal beads, for 2 cycles of 20 s at 6300 rpm, and an interval of 30 s between cycles. Dry matter, protein and energy analysis were performed following the same methodology described for diets. Free amino acids were analyzed in individual gonads homogenized with HCl 0.1 M on ice, centrifuged at 1500 × *g* at 4 ºC for 15 min and the supernatant deproteinized by centrifugal ultrafiltration (10 kDa cut-off, 2500 g at 4ºC for 20 min); amino acids were then analyzed by UPLC as described above.

Carotenoid content of diets, gonads and gut

All diets were analyzed for carotenoid content and profile determination by high performance liquid chromatography (HPLC) with photodiode array (PDA) detection (Waters Alliance 2695, USA). Extractions were performed in duplicate using 500 mg of fresh ground feed, dulyhomogenized in 5 mL acetone. Two hundred microliters of 8-β-apocarotenal (Sigma Aldrich) (at a concentration of 170 µg mL^{-1}) and an antioxidant (BHT—butylated hydroxytoluene) (100 µL at a concentration of 0.05% m/v) were used as internal standards. Extraction was performed in a Precellys homogenizer, with 15 zirconium beads, for 6 cycles of 30 s at 8000 rpm with an interval of 45 s between cycles. The supernatant was then collected and filtered through a 0.45 µm PTFE filter. Samples were evaporated until complete dryness under a nitrogenous atmosphere, kept at −20ºC until analysis, and resuspended in acetone:acetonitrile (9:1) before injection. The stationary-phase was a 4 × 250 mm Purospher Star RP-18e (5 µm) column (Merck), and the mobile-phase consisted of ethyl acetate (solvent A) and acetonitrile/water (solvent B), at 9:1 (v/v). An overall flow rate of 1 mL min^{-1} was set with various volumetric ratios of solvents A and B along the elution time: 0–31 min (0–60% A); 31–36 min (60% A); 36–38 min (60–100% A); 38–43 min (100% A); 43–50 min (100–0% A); and 50–55 min (0% A). To maintain a constant temperature of 25 ºC, a column heater (Waters, USA) was used during the analyses. Spectra data from all peaks were collected within the range 250- 750 nm, and pigments were identified by comparing their retention times and the UV–visible photo-diode array spectra with the standards. Standards (HPLC grade) were purchased from Extrasynthese (lutein, zeaxanthin and β-carotene) and Sigma (echinenone). Calibration curves of each standard were produced, and results were afterward expressed as concentration of pigment per dry weight of feed (µg carotenoid g^{-1} DM) (Guedes et al. 2011). Gut and gonad carotenoid content and profile were ascertained via HPLC–PDA as described for diets, using 50–150 mg of freeze-dried and homogenized tissue in 10 mL of acetone. Carotenoid quantification was as described before.

Gonad total antioxidant capacity

Extracts were obtained from 50–150 mg of freeze-dried gonads pool and homogenized in 5 mL of acetone. The determination of gonad total antioxidant capacity was done via spectrophotometric assay using 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS•+) and 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazin-1-yl (DPPH•) as described by Amaro et al. (2018).

For quantification of antioxidant capacity, a calibration curve using a known antioxidant – Trolox, was established; hence, the antioxidant capacity was expressed as trolox equivalents (TE) per dry weight (DM) of gonad – (µgTE mg^{-1} DM).

Gonad color and texture instrumental analysis

Gonads color evaluation was performed as per to the concept put forward by the International Commission on Illumination (CIE, 1976) which describes color as a three-dimensional characteristic of appearance consisting of a lightness attribute (L*) and two chromatic attributes, hue and chroma. L* ranges from 0 for black to 100 for white. Hue (H◦) is determined by the dominant wavelength and is the name of a color as found in its pure state in the spectrum. Chroma (C*) refers to the saturation of a color and is a measure of how much grey and white light is mixed in with the 'pure' focal color. Color measurements were performed using a CR-400 colorimeter (Konica Minolta) with an aperture of 8 mm, at standard illumination D65. The equipment was firstly calibrated with a white plate as reference standard (Minolta Co., Ltd., Japan). Color parameters were measured by applying the apparatus onto the gonad, previously described for sea urchins (Robinson et al. 2002), and more specifically for *P. lividus* (Baião et al. 2019). Three replicate measurements were taken for each freshly collected gonad, and their averages determined for each measured parameter. The color parameters recorded by the colorimeter were: L* (lightness), a* (redness/greenness chromaticity) and b* (yellowness/bluish chromaticity); chroma and hue angle were calculated as $C^* = (a^{*2} + b^{*2})^{1/2}$ and $h^* = \tan^{-1}(b^*/a^*)$, respectively, as previously described by Choubert et al. (1997).

An instrumental analysis of resilience and firmness of the gonads of sea urchins was also performed using a TA.XT.plus texture analyzer, fitted with a 5 kg load cell and controlled by Exponent v6 software (Stable Micro Systems, UK). The measurements were made only once in each freshly collected gonad, due to the limited amount of sample. Both parameters were calculated using the Stable Micro Systems software, through a compression test using a 35 mm cylinder probe. Firmness is defined as the force (in grams) required to compress the sample by 25% of its initial height (at a 1.7 mm s^{-1}). Resilience is defined as the ability of the sample to recover its original height after being compressed, being calculated as: resilience (%) = post-test height/original height × 100.

Gonad volatile compounds analysis

Frozen gonad samples were thawed at room temperature and placed in a 20 mL glass vial with 1 mL of ultrapure water. The volatile compounds were extracted through headspace solid-phase microextraction (HS-SPME) using a CombiPAL automatic autosampler (Varian, USA) and a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber coating, following a method adapted from Barros et al. (2012). After a 5 min-period of incubation at 45ºC, the extraction was performed for 30 min at the same temperature under continuous stirring (250 rpm). All samples were randomly injected.

The analysis of volatile compounds was performed, upon chromatographic separation, following the procedures described by Baião et al. (2020) and similar equipment: a 436-GC model (Bruker Daltonics, USA) equipped with a SCION single quadrupole (SQ) mass detector and a Bruker Daltonics MS workstation software (version 8.2); a fused silica (Rxi-5Sil MS) capillary column (30 m × 0.25 mm internal diameter × 0.25 mm; Restek Corporation, USA); and high purity helium as the carrier gas (Gasin, Portugal). Peak areas were calculated from GC–MS chromatograms using MZmine 2.53 (Pluskal et al. 2010), after setting the following pre-processing parameters: retention time (RT) range: 2.55–25 min; m/z range: 50–200; MS data noise level: 1×10^4; m/z tolerance: 0.2; chromatogram baseline level: 1×10^4; and peak duration range: 0.03 – 0.5 min. All peak areas were normalized by the weight of the whole gonad, before proceeding to statistical analysis.

Gonad maturation

Sea urchin sex was determined during sampling by visual observation of the gametes expelled from the gonads and later confirmed via histological analysis. Gonads were fixed in formaldehyde for 24 h, and then stored in alcohol 70%. Fixed samples were dehydrated, embedded in paraffin and sectioned transversely to length axis, according to standard histological procedures. Two paraffin Sects. (7 µm thickness) of each gonad were stained with haematoxylin and eosin (H/E) and observed under a binocular microscope. The gametogenic stages of the ovary and testes were identified following the six stages of maturation described by Byrne (1990): recovery (stage I), with primary gametes (oocytes and spermatocytes) and nutritive phagocytes; growing (stage II) with clusters of primary gametes and packed nutritive phagocytes; premature (stage III) with gametes at all stages of development and reduced amount of nutritive phagocytes; mature (stage IV) with mature gametes (ova and spermatozoa) and few nutritive phagocytes; partly spawned (stage V) with loosely packed gametes and depletion of nutritive phagocytes; and spent (stage VI) with gonads empty of gametes.

Statistical analysis

All statistical analysis were conducted using software Statistica 13 and IBM SPSS Statistics 28. The results are expressed as mean ± standard deviation. All data were analyzed for normality (Kolmogorov–Smirnov's test) and homogeneity of variance (Levene's test). Whenever necessary, dependent variables were transformed to meet the assumptions underlying the selected statistical tests. When the output was significant, individual means were compared

using HSD Tukey's test. If data failed to meet the assumptions of ANOVA, then a Kruskal–Wallis (non-parametric) test was performed, followed by the Dunn's test with Bonferroni's correction to pinpoint significant differences between groups. In all cases, the minimum level of significance was set at $p < 0.05$. Principal Component Analysis (PCA) was performed to find the variables that differed significantly among dietary treatments. Pearson's correlations, considering a two-tailed analysis and significance levels of 0.05 and 0.01, were performed to assess significant correlations: between texture parameters, gonadal yield and proximate composition; between gut and gonad carotenoids and color parameters; and between gonads anti-oxidant status and gonads carotenoids and amino-acids.

Results

Dietary carotenoids and amino-acids

The dietary supplementation with *D. salina* and the replacement of a commercial macroalgae mix by *Porphyra* sp. were both effective in increasing the total carotenoid content in extruded diets for *P. lividus* (Table 1), mostly due to α- and β-carotene that were absent in the CTRL and GLY diets. Lutein was increased by 10- and 20-fold in D1 and D2 diets, respectively and over 37-fold in D1P and D2P diets when compared to the CTRL diet; GLY lutein content was close to the CTRL diet. When compared to the CTRL diet, zeaxanthin was increased by 27% and 71% in D1 and D2 diets, respectively, and by 73% and 81% in D1P and D2P diets, respectively, whereas GLY zeaxanthin content was close to that of the CTRL diet. Although dietary *D. salina* supplementation was effective in increasing the levels of α-carotene and mostly of β-carotene, the β-carotene levels detected in the diets after extrusion were much lower than those included in the mixture (targeted at 150 and 300 μg g^{-1} DM, in D1/D1P and D2/D2P diets, respectively), as shown in Table 1. A degradation of 49–66% was estimated in *Dunaliella* supplemented diets after extrusion. Nevertheless, D2 and D2P β-carotene content varied between 100 and 120 μg g^{-1} DM, which is approximately twice the levels detected in D1 and D1P, i.e. 49–77 μg g^{-1} DM.

The amino acids (AA) content of the experimental diets (Table 1) indicated that glycine supplementation was effective in increasing the glycine content of the GLY diet, with an increase of 32% when compared to the CTRL diet, whereas most diets had slightly lower levels than the CTRL diet, except for D2 with a glycine content increased by 12%. The replacement of a commercial macroalgae mix by *Porphyra* sp., rich in aspartic acid (26.5 mg g^{-1} DM), glutamic acid (25.7 mg g^{-1} DM) and alanine (24.1 mg g^{-1} DM), failed to increase these AA levels in D1P or D2P diets.

Growth performance and gonad yield

All tested diets were well accepted by the sea urchins, but particularly those diets in which a commercial macroalgae mix was replaced by *Porphyra* have significantly increased voluntary feed intake (Table 2). As a result, the groups fed the D1P and D2P displayed increased feed conversion ratio when compared to D2 and a decreased protein efficiency ratio when to the other groups. However, diets containing *Porphyra* tended to be less stable in water, degrading more quickly and potentially leading to an overestimation of voluntary feed intake and feed conversion ratios.

Weight gain was not affected by dietary treatments. Final body weight varied between 52 and 56 g, and diameter between 5.5 and 5.7 cm, with no significant differences being detected between groups (Table 2).

Table 2 Growth performance of sea urchins, during the experimental trial

	Diets						
	CTRL	D1	D2	D1P	D2P	GLY	One-way ANOVA p -value
Initial body weight (g)	51.59 ± 10.88	52.17 ± 11.02	52.64 ± 14.31	51.85 ± 12.82	51.66 ± 14.18	52.01 ± 12.31	0.997
Final body weight (g)	53.37 ± 9.77	55.33 ± 11.01	56.17 ± 14.36	53.59 ± 12.32	52.03 ± 12.02	54.05 ± 11.53	0.458
Initial diameter (cm)	4.98 ± 0.42	4.99 ± 0.42	4.97 ± 0.48	4.96 ± 0.46	4.96 ± 0.46	4.98 ± 0.43	0.998
Final diameter (cm)	5.54 ± 0.43	5.63 ± 0.44	5.67 ± 0.54	5.47 ± 0.44	5.57 ± 0.51	5.58 ± 0.42	0.173
SGR(% day^{-1})	0.09 ± 0.01	0.09 ± 0.02	0.10 ± 0.02	0.08 ± 0.01	0.07 ± 0.02	0.08 ± 0.02	0.269
FCR	5.84 ± 1.41^{AB}	5.27 ± 0.69^{AB}	4.54 ± 0.29^{B}	10.02 ± 1.74^{A}	12.91 ± 4.67^{A}	7.06 ± 3.27^{AB}	0.004
PER	0.54 ± 0.12^{A}	0.57 ± 0.09^{A}	0.66 ± 0.06^{A}	0.31 ± 0.06^{BC}	0.26 ± 0.09^{C}	0.49 ± 0.16^{AB}	< 0.001
VFI (%)	0.50 ± 0.03^{B}	0.50 ± 0.07^{B}	0.45 ± 0.08^{B}	0.75 ± 0.05^{A}	0.81 ± 0.02^{A}	0.50 ± 0.04^{B}	< 0.001

Values are presented as mean ± standard deviation ($n = 72$ for body measurements and $n = 4$ for SGR, FCR, PER and VFI). In each line, different superscript letters indicate significant differences ($p < 0.05$) between diets identified through one-way ANOVA analysis followed by a Tukey post-hoc test. SGR – specific growth rate; FCR – feed conversion ratio; PER – protein efficiency ratio; VFI – voluntary feed intake

All diets enhanced GSI, which increased significantly from 6.4 to 15.8% and from 8.9 to 17% in males and females, respectively, corresponding to an average increment of 91%−147%. Furthermore, gut weight and VSI were not affected by sex or diet, while gonad yield was significantly affected by sex ($p < 0.05$), but not by diet (Table 3).

Throughout the trial the gonads maturation status evolved from stage I – recovery (50% of females and 67% of males) and stage II – growing (50% of females and 33% of males) to stages IV—mature (9% of females and 3% of males), V—partially spawned (51% of females and 74% of males) and VI—spent (40% of females and 23% of males).

Proximate composition of gonads

Gonad dry matter was generally higher in females ($p = 0.015$) than in males, and was not affected by diet (Table 3). However, gonad crude protein and energy as a percentage of wet weight were affected by both sex and diet, with ovaries displaying higher energy, but lower protein content than testes (Table 3). Furthermore, sea urchins fed diet D1 displayed the highest gonadal protein content, significantly different from those fed the D1P diet, whilst sea urchins fed diet D2 presented the highest gonadal energy content, differing only from sea urchins fed D1P, regardless of sex (Table 3). Gonads of sea urchins fed D1P had the lowest protein and energy contents.

Carotenoids content in gut and gonads

Gut carotenoids were significantly affected by both diet and sex ($p < 0.05$, Fig. 2), with no interaction between these two factors. Lutein and β-carotene were the most abundant pigments in *P. lividus* gut, which reflects, to some extent, the dietary carotenoids profile (Fig. 2A and D**;** Table 1). The gut of D1P urchins exhibited significantly higher lutein levels than that of sea urchins fed the CTRL, D1 and GLY diets, in both sexes (Fig. 2A). The β-carotene concentration in the gut of D2 urchins was twice that of D1 urchins, but this increase was not observed in D2P urchins, which held β-carotene levels similar to those of D1P urchins (Fig. 2D). Moreover, D2, D1P and D2P diets led to similar concentrations of echinenone, α-and β-carotene and total carotenoids in sea-urchin gut, all significantly higher than those of CTRL, D1 and GLY urchins (Fig. 2C-F). By the same token, both gut β-carotene (0.77–0.87, $P < 0.01$, Fig. 3A) and echinenone (0.92–0.94, $P < 0.01$, Fig. 3B) were strongly correlated with dietary β-carotene, regardless the sex. Moreover, there was a significant linear correlation between gut echinenone and gut β-carotene (0.96–0.90, $P < 0.01$, Fig. 3C**;** Table 4). Males' gut had higher concentrations of echinenone, α- and β-carotene, but lower concentrations of lutein, zeaxanthin and total carotenoids than females' (Fig. 2).

In general, the carotenoid content in the gonads was much lower than in the gut (Fig. 2), with the exception of echinenone. In males, gonad echinenone levels were 1.4 to 3.8-fold higher than those observed in the gut (Fig. 2C). Gut total carotenoids were higher in females (77%) than in males (63%), yet gonads total carotenoids were lower in females (15%) than in males (18%) (Fig. 2F).

Unlike what was observed for the gut, gonad carotenoids were not affected by diet, but were affected by sex ($p < 0.05$) (Fig. 2). In ovaries the most abundant pigments were lutein (3.7 – 5.3 μg g^{-1} WW) and echinenone (3.3 – 5.9 μg g^{-1} WW), followed by zeaxanthin (1.9–3.2 μg g^{-1} WW). In testes the most abundant pigments were echinenone (12.4 – 17.6 μg g^{-1} WW), followed by β- and α-carotene (2.7 – 4.7 and 0.4 – 1.1 μg g^{-1} WW, respectively), with lutein and zeaxanthin visually undetected (Fig. 2). Gonad β-carotene and echinenone contents were positively correlated (Table 4**;** 0.515, $P < 0.01$), more strongly in females (Table S1; 0.666, $P < 0.01$; Fig. 3D) than in males (Table S1; 0.488, $P < 0.01$; Fig. 3D). There was also a significant correlation between α-carotene and echinenone (Table 4).

The PCA plot shown in Fig. 4, provides an overview of the similarities and dissimilarities between the different diets and sexes. This biplot represents loadings for carotenoid content in the gonads and gut and explains 72.8% of the data intrinsic variability. Female sea urchins were projected in the third quadrant and associated with lutein and zeaxanthin both in gonads and gut, as well as total carotenoids in the gut. Moreover, females fed diets containing *D. salina* were highly associated with lutein and zeaxanthin in the gut and gonads. On the other hand, male sea urchins were projected along the first quadrant and associated with echinenone, α- and β-carotene and total carotenoids in gonads. In addition, males fed with diets D2, D1P and D2P were highly associated with echinenone, and α- and β-carotene in both gonads and gut. There was also a high proximity between males fed with diets CTRL, D1 and GLY, and a tendency for these to be projected on the opposite side of lutein and zeaxanthin in both tissues. Diets D1P, D2 and D2P were also closely associated with each other, for each sex.

Color and texture of gonads

Gonad lightness and hue angle were negatively correlated with lutein and zeaxanthin contents and positively correlated with echinenone and α-carotene contents. On the other hand, redness a^*, yellowness b^* and color saturation C^* were positively correlated with lutein and zeaxanthin content and negatively correlated with echinenone and α-carotene (Table 4).

Generally, gonad color was significantly affected by sex and diet ($p < 0.05$), except for b* and Chroma (C*) that were

Table 3 Gonad and gut yield, final gonad composition (%WW), carotenoids (μg.g^{-1} WW), color and texture of female and male *P. lividus* fed different diets

	Females						Males						*Two-way ANOVA p-value*		
	CTRL	D1	D2	D1P	D2P	GLY	CTRL	D1	D2	D1P	D2P	GLY	*Sex*	*Diet*	*S x D*
Yield															
Gonad weight (g)	10.9 ± 3.6	9.9 ± 2.3	10.8 ± 3.5	9.3 ± 3.8	10.1 ± 3.2	12.1 ± 4.5	10.0 ± 3.3	9.6 ± 3.3	9.4 ± 3.6	8.9 ± 3.1	9.3 ± 3.5	9.5 ± 2.7	*0.032*	*0.407*	*0.783*
GSI (%)	18.0 ± 4.2	16.5 ± 3.6	16.2 ± 3.1	15.7 ± 3.9	16.7 ± 3.2	18.6 ± 3.9	16.3 ± 4.6	16.4 ± 3.9	15.9 ± 4.5	14.3 ± 3.3	15.3 ± 4.1	16.4 ± 3.6	*0.036*	*0.137*	*0.882*
Gut weight (g)	1.5 ± 0.5	1.5 ± 0.5	1.7 ± 0.7	1.4 ± 0.4	1.3 ± 0.4	1.5 ± 0.5	1.5 ± 0.5	1.3 ± 0.3	1.4 ± 0.4	1.3 ± 0.4	1.2 ± 0.5	1.5 ± 0.5	*0.136*	*0.106*	*0.838*
VSI (%)	2.5 ± 0.7	2.5 ± 1.0	2.6 ± 1.0	2.4 ± 0.6	2.2 ± 0.6	2.4 ± 0.6	2.3 ± 0.6	2.2 ± 0.4	2.5 ± 0.6	2.2 ± 0.5	2.1 ± 0.8	2.5 ± 0.6	*0.225*	*0.250*	*0.896*
Final gonad composition (%WW)															
Dry matter	21.8 ± 1.3	22.0 ± 2.3	21.9 ± 0.6	19.5 ± 1.2	20.4 ± 1.2	22.1 ± 0.4	20.2 ± 1.5	20.8 ± 1.9	21.2 ± 0.6	20.0 ± 1.8	19.9 ± 1.0	20.0 ± 1.3	*0.015*	*0.051*	*0.468*
Crude protein	12.9 ± 0.6^{AB}	13.6 ± 1.5^{A}	13.4 ± 0.4^{AB}	12.2 ± 0.8^{B}	12.6 ± 0.7^{AB}	13.5 ± 0.1^{AB}	14.4 ± 0.9^{AB}	15.7 ± 1.2^{A}	15.5 ± 0.8^{AB}	13.8 ± 2.3^{B}	14.3 ± 0.2^{AB}	14.0 ± 0.9^{AB}	*< 0.001*	*0.022*	*0.717*
Gross energy (kJ.g^{-1})	4.7 ± 0.3^{AB}	4.6 ± 0.7^{AB}	5.0 ± 0.1^{A}	4.1 ± 0.2^{B}	4.3 ± 0.2^{AB}	5.0 ± 0.1^{AB}	3.9 ± 0.2^{AB}	4.1 ± 0.5^{AB}	4.2 ± 0.2^{A}	3.8 ± 0.6^{B}	3.9 ± 0.2^{AB}	4.0 ± 0.3^{AB}	*< 0.001*	*0.017*	*0.281*
Color and texture															
*L**	44.9 ± 2.4^{A}	41.4 ± 3.4^{BC}	41.6 ± 3.0^{BC}	43.1 ± 4.6^{BC}	39.9 ± 3.1^{C}	45.2 ± 3.7^{AB}	57.6 ± 4.8^{A}	53.3 ± 6.5^{BC}	56.1 ± 5.2^{BC}	51.4 ± 4.9^{BC}	52.8 ± 6.3^{C}	55.2 ± 4.9^{AB}	*< 0.001*	*< 0.001*	*0.197*
*a**	23.2 ± 1.7^{B}	26.2 ± 1.6^{A}	26.9 ± 2.7^{A}	26.2 ± 3.3^{A}	26.7 ± 2.8^{A}	24.3 ± 2.4^{A}	10.6 ± 3.0^{B}	14.3 ± 4.3^{A}	12.5 ± 2.5^{A}	13.3 ± 3.6^{A}	14.9 ± 3.9^{A}	13.8 ± 3.1^{A}	*< 0.001*	*< 0.001*	*0.374*
*b**	36.9 ± 2.5	36.7 ± 5.2	37.4 ± 4.5	34.5 ± 5.8	35.6 ± 2.9	37.9 ± 2.6	21.6 ± 7.7	26.0 ± 6.0	24.1 ± 2.7	24.3 ± 5.5	25.8 ± 6.2	25.8 ± 5.3	*< 0.001*	*0.402*	*0.351*
*C**	43.6 ± 2.6	45.2 ± 4.5	46.1 ± 4.8	43.4 ± 6.4	44.5 ± 3.6	45.0 ± 3.1	24.2 ± 8.1	29.7 ± 7.1	27.3 ± 3.3	27.8 ± 6.3	29.8 ± 7.2	29.3 ± 6.0	*< 0.001*	*0.164*	*0.541*
*h**	57.8 ± 2.1^{A}	54.2 ± 3.8^{BC}	54.2 ± 2.8^{BC}	52.6 ± 2.8^{C}	53.1 ± 2.4^{C}	57.3 ± 2.2^{AB}	63.1 ± 4.6^{A}	61.3 ± 3.8^{BC}	62.7 ± 3.2^{BC}	61.4 ± 3.8^{C}	60.1 ± 2.7^{C}	61.8 ± 2.4^{AB}	*< 0.001*	*< 0.001*	*0.063*
Resilience (%)	84.8 ± 3.4	86.8 ± 8.4	83.6 ± 2.4	83.7 ± 5.8	82.8 ± 2.9	83.0 ± 3.4	83.6 ± 4.7	85.4 ± 8.9	87.8 ± 6.1	87.5 ± 3.9	83.6 ± 4.0	83.0 ± 6.1	*0.135*	*0.941*	*0.749*
Firmness (g)	15.6 ± 2.8^{AB}	15.1 ± 2.1^{AB}	16.8 ± 3.0^{A}	13.0 ± 3.5^{B}	14.6 ± 3.4^{AB}	15.1 ± 3.0^{AB}	17.1 ± 2.9^{AB}	15.8 ± 3.9^{AB}	17.8 ± 5.1^{A}	15.7 ± 4.5^{B}	18.6 ± 4.3^{AB}	17.9 ± 3.9^{AB}	*< 0.001*	*0.049*	*0.503*
Antioxidant activity															
ABTS	50.3 ± 4.0	75.3 ± 9.5	68.0 ± 6.7	59.1 ± 3.6	64.1 ± 8.9	77.4 ± 4.0	47.9 ± 4.4	43.5 ± 0.6	47.1 ± 2.3	46.3 ± 7.4	44.6 ± 3.6	39.2 ± 2.8	*< 0.001*	*0.072*	*0.119*
DPPH	7.4 ± 2.2^{f}	72.8 ± 14.0^{ab}	51.9 ± 19.8^{abcd}	56.1 ± 6.3^{abc}	46.2 ± 11.3^{bcd}	78.4 ± 2.5^{a}	24.1 ± 7.1^{def}	21.7 ± 5.5^{ef}	22.6 ± 2.9^{ef}	32.3 ± 5.2^{cdef}	24.2 ± 2.8^{def}	21.8 ± 6.8^{ef}	< 0.001	*< 0.001*	*< 0.001*

Initial gonad weight: 5.5 ± 3.1 g for females and 3.2 ± 1.6 g for males; Initial GSI: 8.9 ± 3.9% for females and 6.4 ± 2.6% for males. GSI – Gonadosomatic index; VSI – Viscerosomatic index

Values are presented as mean ± standard deviation (n = 15–18 for yield parameters; n = 4–5 for proximate composition; n = 12–16 for color parameters; n = 13–17 for texture parameters; n = 4 for antioxidant activity parameters). In each line, different superscript letters indicate significant differences ($p < 0.05$) identified through two-way ANOVA analysis followed by Tukey post-hoc tests. Lowercase superscript letters indicate differences between diets and sexes. Uppercase superscript letters indicate differences between dietary treatments within each sex

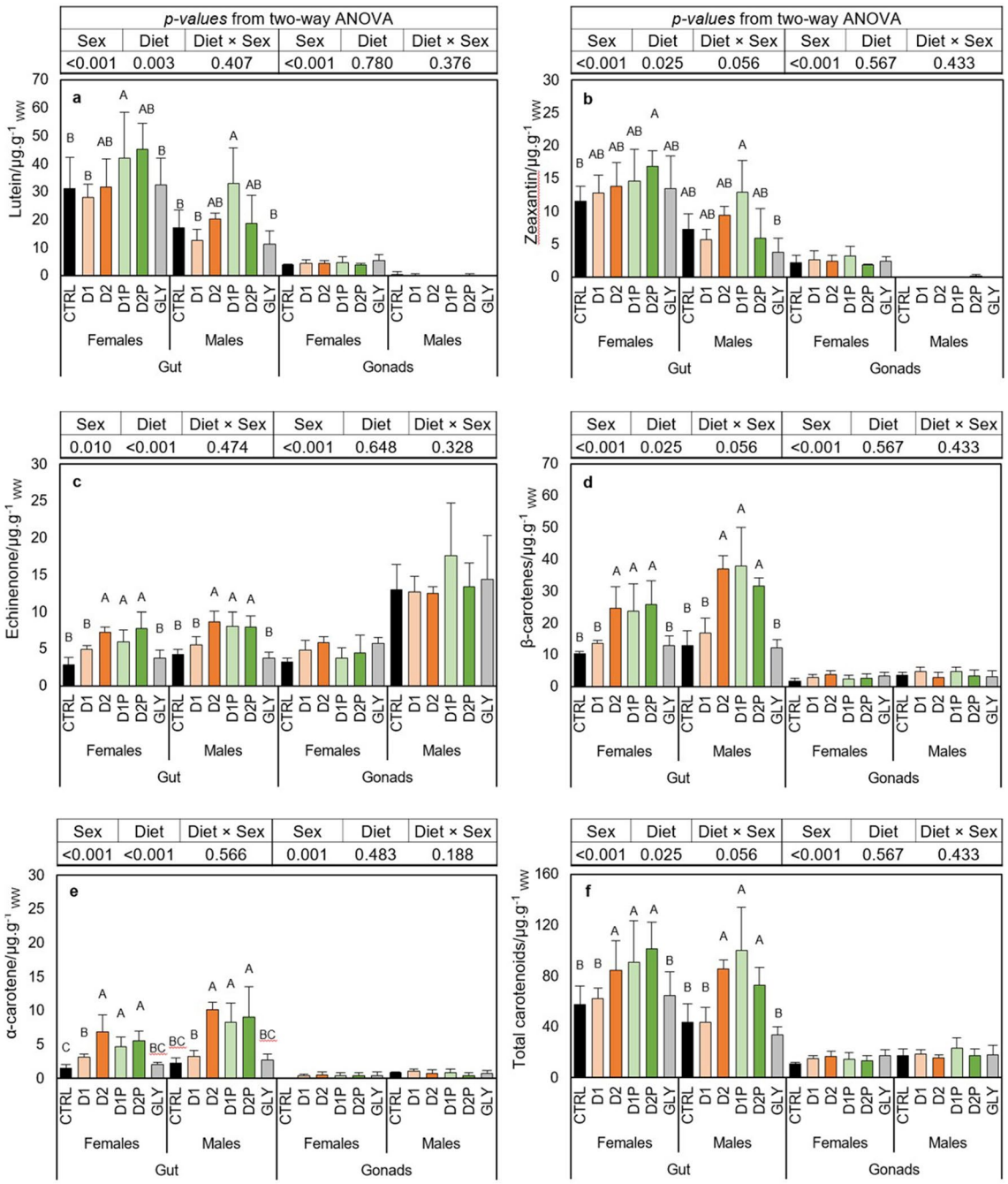

Fig. 2 Gut and gonads carotenoids of female and male *P. lividus* fed different diets: **(a)** – lutein; **(b)** – zeaxanthin; **(c)** – gut echinone; **(d)** β-carotenes; **(e)** α-carotenes; **(f)** Total carotenoids. Values are presented as mean ± standard deviation ($n = 4$–5). Different superscript letters indicate significant differences ($p < 0.05$) between dietary treatments within each sex

only affected by sex, see Table 3**.** Female gonads were less luminous (lower L^*), redder (higher a^*), yellower (higher b^*) and with a more saturated color (higher C^*) than males. Furthermore, sea urchins fed *D. salina*-supplemented diets also produced darker (lower L^*) and more reddish gonads (higher a^* and lower h^*) than those fed the CTRL diet (Table 3**;** Fig. 5).

The PCA plot (Fig. 6) shows the similarities and dissimilarities for the different diets and sexes. The biplot of the data from gonad color and carotenoid content from the gonads accounts for 82.7% of data variation. Four distinct groups along F1 are visible, as well as distancing between females and males. Females were positively projected on F1, and associated with a^*, b^*, C^*, lutein, and

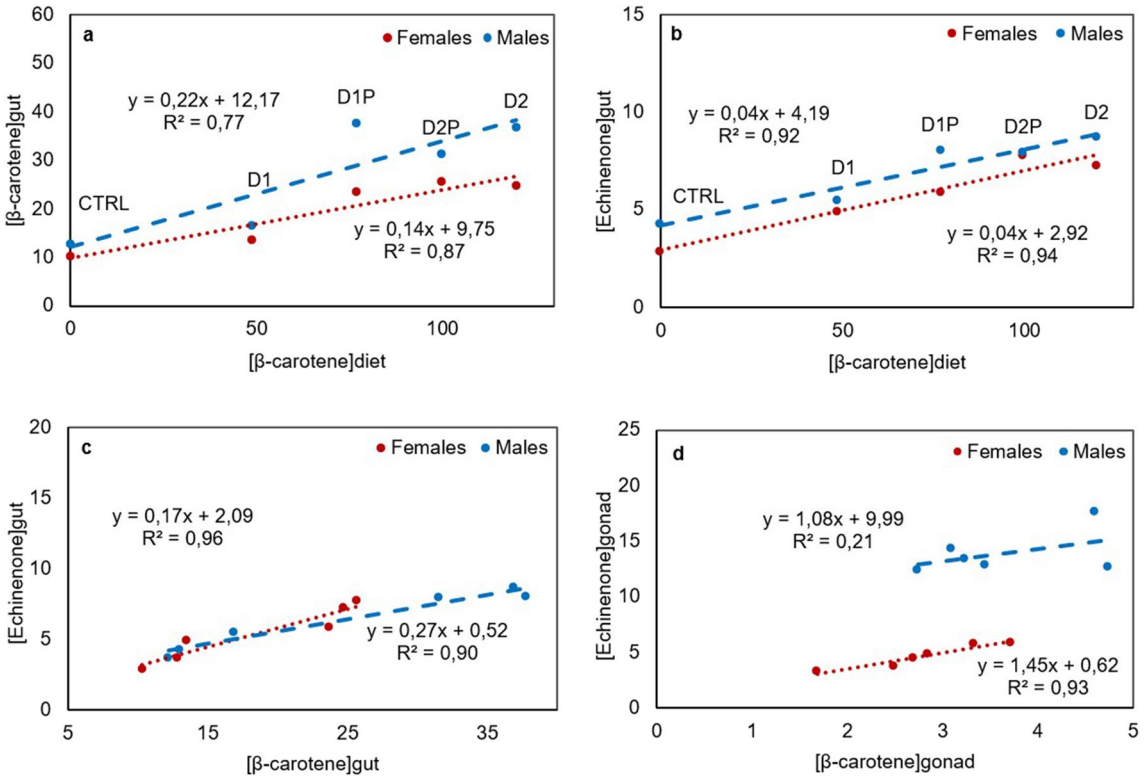

Fig. 3 Linear regression between pigment concentration in diets and/or tissues in *P. lividus*. **(a)** – dietary β-carotene vs. gut β-carotene. **(b)** – dietary β-carotene vs. gut echinenone. **(c)** – gut β-carotene vs. gut echinenone. **(d)** – gonad β-carotene vs. gonad echinenone

zeaxanthin, specifically females fed *Dunaliella*-diets and GLY. Females fed with CTRL diet appear apart from the remaining diets. In contrast, males were negatively projected along F1 and associated with h^*, L^*, echinenone, α- and β-carotene and total carotenoids. Furthermore, males fed with the lowest *D. salina* concentrations were highly associated with echinenone, α- and β-carotene. Males fed with the highest concentration of *D. salina*, as well as CTRL and GLY diets were associated with h^* and L^*.

The color results herein obtained were compared with those of reference sea-urchins with an acceptability level of 8 in a scale from 1 to 9, proposed by Baião et al. (2021b), hereafter labeled as ACC8 (Fig. 7). In general, D2 gonads were the closest to those of ACC8 sea urchins, and displayed similar a^*, C^* and h^* values (Fig. 7).

Regarding the texture of roes, resilience, defined as the ability of a sample to recover its original height after being compressed, was neither affected by sex nor diet (Table 3). However, firmness, defined as the force required to compress the sample by 25% of its initial height, was affected mostly by sex ($P < 0.001$), but also by diet to a lesser extent ($P = 0.049$). Male gonads were generally firmer than their female counterparts (Table 3). Regardless of sex, sea urchins fed diet D2 had firmer gonads than those fed diet D1P, but did not differ from the CTRL group. Firmness was generally positively correlated with protein (0.53, $p < 0.01$, Table 5), being also positively correlated with energy and dry matter in ovaries (Table S2).

Volatile compounds in gonads

Sixteen volatile compounds were identified in the gonads of the sea urchins: four aldehydes, 3-methylbutanal, cis-4-heptenal, heptanal and phenylacetaldehyde; ethylbenzene and benzene derivatives p-xylene, o-xylene, p-ethyltoluene, m-ethyltoluene, 1,2,4-trimethylbenzene and o-cymene; five terpenes, β-pinene, limonene, γ-terpinene, terpinolene and a sesquiterpene-like compound. The gonads' volatile profile was not affected by diet. However, significant differences ($p < 0.05$) were observed between sexes, with ovaries containing higher concentrations of cis-4-heptenal, heptanal, β-pinene, 1,2,4-trimethylbenzene and terpinolene than testes (Fig. 8).

Free amino acids content in gonads

The most abundant free amino acids in *P. lividus* gonads were arginine, glycine, lysine, proline and glutamine, regardless of sex and diet (Table 6). Moreover, most gonad free amino acids contents were affected by diet ($p < 0.001$,

Table 4 Pearson's correlations coefficients between gonadosomatic index, gut and gonads carotenoids and color parameters

		Gut Carotenoids (μg g^{-1} WW)						Gonads Carotenoids (μg g^{-1} WW)					
		Lutein	Zeaxanthin	Echinenone	α-carotene	β-carotene	Total Carotenoids	Lutein	Zeaxanthin	Echinenone	α-carotene	β-carotene	Total Carotenoids
	GSI (%)	NS	NS	-,287*	NS	-,377**	NS	NS	NS	-,290*	NS	NS	NS
Gut Carotenoids	Lutein	1	0.957**	0.354**	NS	0.389**	0.852**	0.632**	0.558**	−0.386**	NS	NS	NS
	Zeaxanthin	0.957**	1	0.342*	NS	0.359**	0.826**	0.657**	0.589**	−0.423**	NS	NS	NS
	Echinenone	0.354**	0.342*	1	0.861**	0.857**	0.739**	NS	NS	NS	NS	0.285*	0.272*
	α-carotene	NS	NS	0.861**	1	0.877**	0.680**	NS	NS	0.309*	NS	NS	0.295*
	β-carotene	0.389**	0.359**	0.857**	0.877**	1	0.805**	NS	NS	0.327*	NS	NS	0.298*
	Total Carotenoids	0.852**	0.826**	0.739**	0.680**	0.805**	1	0.316*	0.275*	NS	NS	NS	NS
Gonads Carotenoids	Lutein	0.632**	0.657**	NS	NS	NS	NS	1	0.898**	−0.687**	−0.239	−0.145	−0.131
	Zeaxanthin	0.558**	0.589**	NS	NS	NS	0.275*	0.898**	1	−0.695**	−0.310*	−0.197	−0.178
	Echinenone	−0.386**	−0.423**	NS	0.309*	0.327*	NS	−0.687**	−0.695**	1	0.515**	0.511**	0.778**
	α-carotene	NS	NS	NS	NS	NS	NS	NS	−0.310*	0.515**	1	0.741**	0.658**
	β-carotene	NS	NS	0.285*	NS	NS	NS	NS	NS	0.511**	0.741**	1	0.774**
	Total Carotenoids	NS	NS	0.272*	0.295*	0.298*	NS	NS	NS	0.778**	0.658**	0.774**	1
Color parameters	L*	−0.654**	−0.660**	NS	NS	NS	−0.417**	−0.739**	−0.710**	0.631**	0.454**	NS	0.273*
	a*	0.585**	0.594**	NS	NS	NS	0.312*	0.845**	0.822**	−0.728**	−0.352**	NS	−0.288*
	b*	0.474**	0.491**	NS	NS	NS	NS	0.757**	0.718**	−0.695**	−0.335*	NS	−0.322*
	Chroma *C**	0.527**	0.542**	NS	NS	NS	NS	0.805**	0.773**	−0.720**	−0.345*	NS	−0.313*
	Hue *h**	−0.576**	−0.570**	NS	NS	NS	−0.370**	−0.784**	−0.782**	0.605**	0.292*	NS	00.167

$n = 53–54$, $^{**}p < 0.01$ (two-tailed) and $^{*}p < 0.05$ (two-tailed)

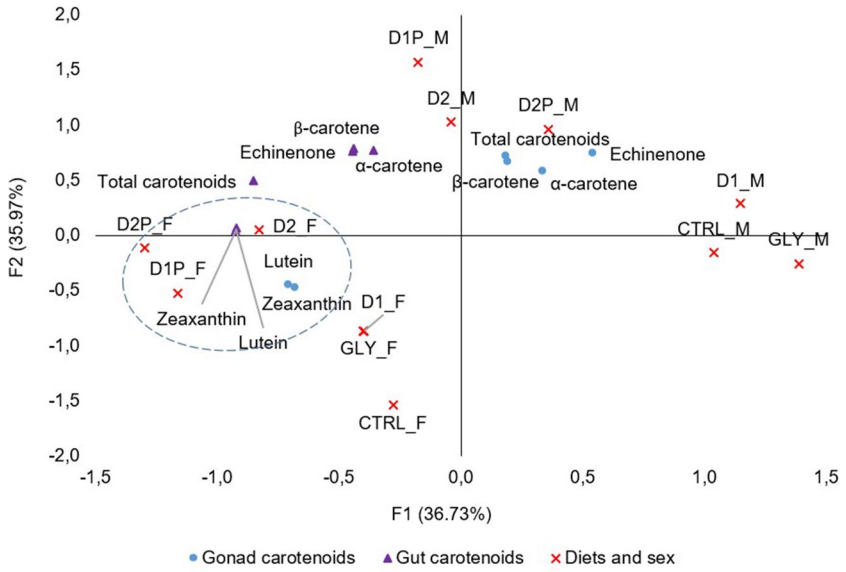

Fig. 4 Principal component analysis of the experimental data of gonads and gut carotenoid concentration of *P. lividus* fed the experimental diets. Dotted lines are a representation of an aggregation of dietary treatment. Abbreviations are: CTRL_F = Females fed with CTRL diet; D1_F = Females fed with D1 diet; D2_F = Females fed with D2 diet; D1P_F = Females fed with D1P diet; D2P_F = Females fed with D2P diet; GLY_F = Females fed with GLY diet; CTRL_M = Males fed with CTRL diet; D1_M = Males fed with D1 diet; D2_M = Males fed with D2 diet; D1P_M = Males fed with D1P diet; D2P_M = Males fed with D2P diet; GLY_M = Males fed with GLY diet

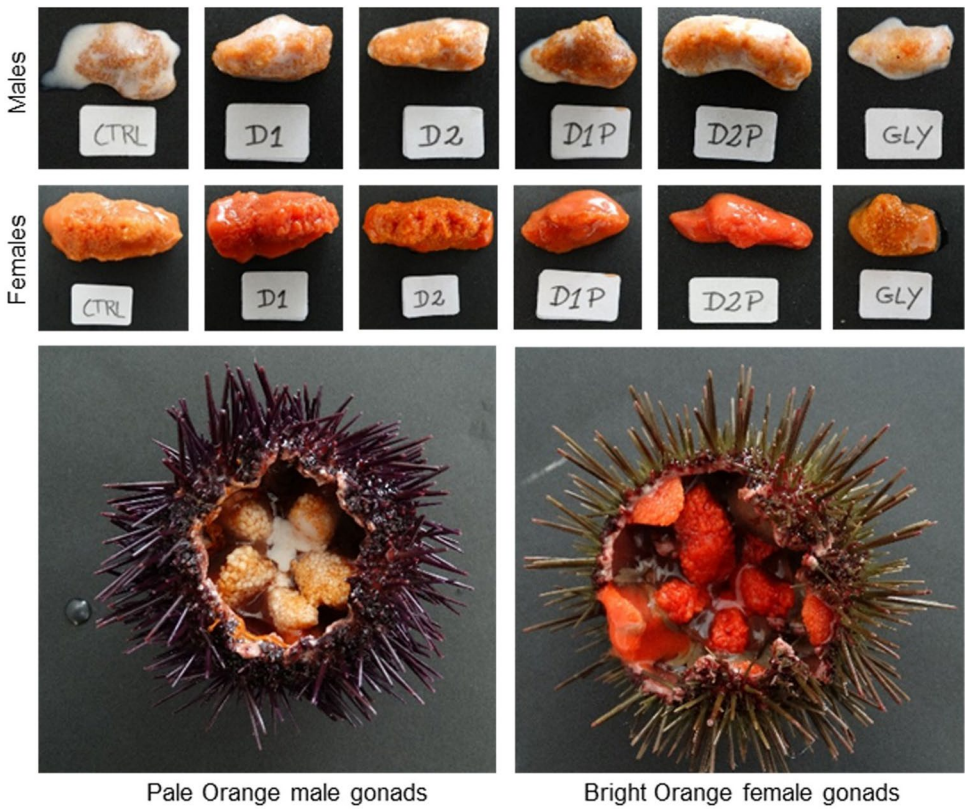

Fig. 5 Gonad of male and female *P.lividus* after being the experimental diets for 8 weeks. The abbreviations for the experimental diets stand for: CTRL—Control diet (not supplemented); D1 – supplemented with 0.75% of a natural source of β-carotene (*D. salina*); D2—supplemented with 1.5% of a natural source of β-carotene (*D. salina*); D1P – supplemented with 0.75% of a natural source of β-carotene (*D. salina*) and substitution of macroalgae mix by *Porphyra*; D2P—supplemented with 1.5% of a natural source of β-carotene (*D. salina*) and substitution of macroalgae mix by *Porphyra*; GLY – supplemented with 1.2% of the crystalline amino acid glycine

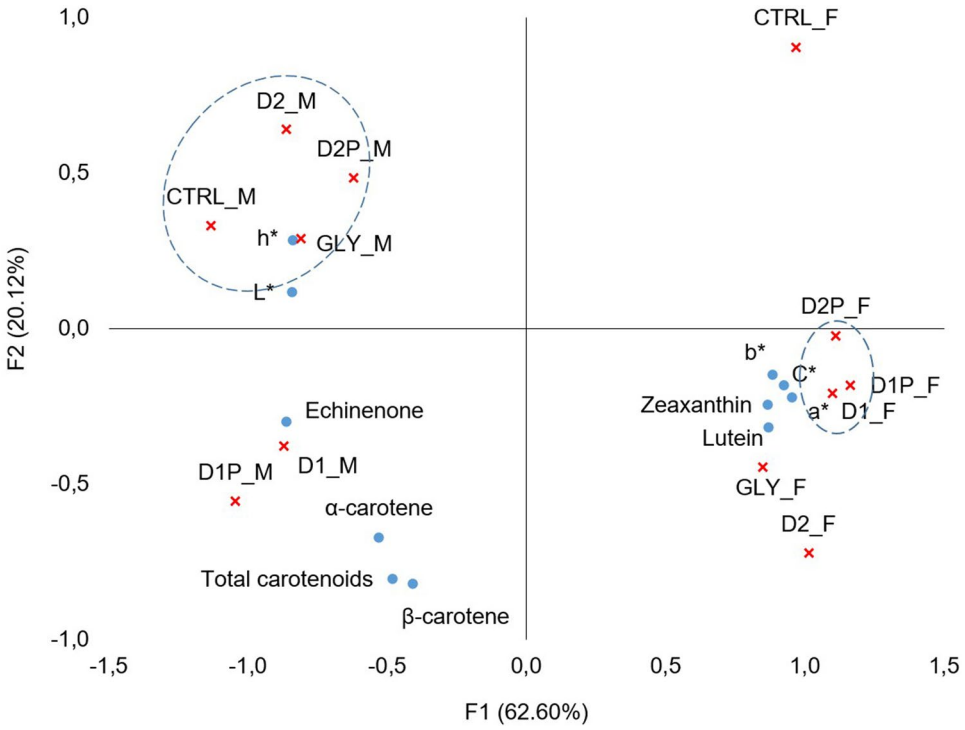

Fig. 6 Principal component analysis of the experimental data of colour parameters and carotenoids concentration in the gonads of *P. lividus* fed the experimental diets. Dotted lines are a representation of an aggregation of dietary treatment. Abbreviations are: CTRL_F = Females fed with CTRL diet; D1_F = Females fed with D1 diet; D2_F = Females fed with D2 diet; D1P_F = Females fed with D1P diet; D2P_F = Females fed with D2P diet; GLY_F = Females fed with GLY diet; CTRL_M = Males fed with CTRL diet; D1_M = Males fed with D1 diet; D2_M = Males fed with D2 diet; D1P_M = Males fed with D1P diet; D2P_M = Males fed with D2P diet; GLY_M = Males fed with GLY diet

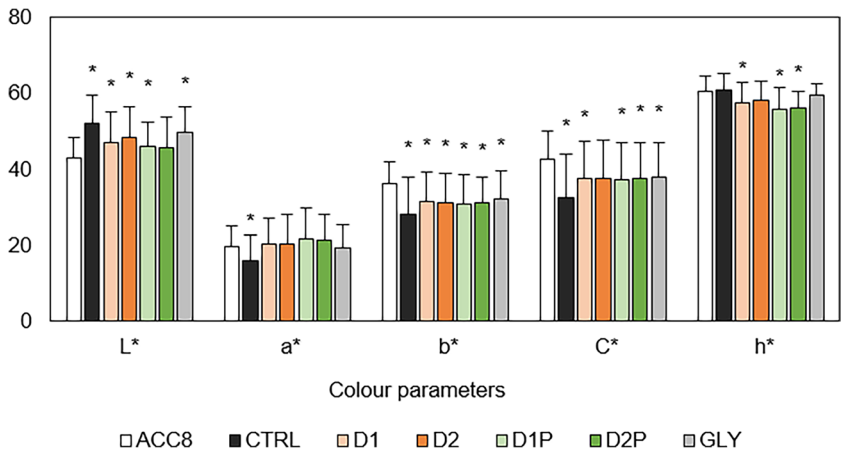

Fig. 7 Colour parameters of female and male sea urchins with a score of 8 (ACC8), in a scale from 1 to 9, and experimental diets. Values are presented as mean and standard deviation ($n = 24–30$). * identify significant differences between the experimental diet and the ACC8, for a Mann–Whitney-U test, considering statistical significance at $p < 0.05$. Data of ACC8 provided by Baião et al. (2021b)

Table 5 Pearson's correlations coefficients between gonad yield, proximate composition and texture parameters

	GSI	Protein	Energy	Dry matter
Firmness	NS	0.530**	NS	NS
Resilience	NS	NS	NS	NS

$n = 51–54$, $^{**}p < 0.01$ (two-tailed) and $^{*}p < 0.05$ (two-tailed)

Table 6), including the abundant arginine, glycine, lysine, and glutamine that were also affected by sex ($p < 0.05$, Table 6). Most amino acids, including arginine and glycine, increased in all groups when compared to the CTRL group, which reflected on total FAA. Conversely, lysine, threonine, aspartic acid, asparagine, serine and ornithine were generally reduced in all groups, when compared to the CTRL (Table 6).

The gonads of sea urchins fed the GLY diet displayed the highest glycine values regardless of sex, when compared to the other groups (Table 6). D2P male gonads also exhibited a high glycine content, similar to that of GLY gonads. Furthermore, D2 and D2P gonads glycine contents were higher than those of D1 (by 5–12%) and D1P (by 27–38%) gonads, respectively, in both males and females. The male gonads of GLY group also displayed the highest proline content. The proline content in D1P ovaries and testes and GLY ovaries were similarly high (Table 6). Alanine was significantly higher in D2P gonads, regardless of sex, by ca. threefold when compared to the CTRL, D2 and GLY groups and by twofold when compared to the D1P group (Table 6).

Regardless of sex, the gonads of sea urchins fed the D2P diet displayed the highest glutamine levels, which increased by fourfold relative to the CTRL group, and by approximately twofold when compared to the other groups, but also the highest glutamic acid levels (Table 6).

The gonads of males fed the D2P diet displayed the highest arginine values and lysine was higher in D2P groups than in D1, D2, D1P and GLY groups (Table 6). D1 gonads contained more methionine, phenylalanine, cysteine and tyrosine than those of all other groups, regardless of sex; while tryptophan was more abundant in the gonads of urchins fed the GLY diet, followed by D2.

Gonad antioxidant activity

Activity against ABTS was affected by sex, but not diet, whereas activity against DPPH was affected by both sex and diet (Table 3). Ovaries generally displayed higher activity against ABTS and DPPH than testes, with the exception of

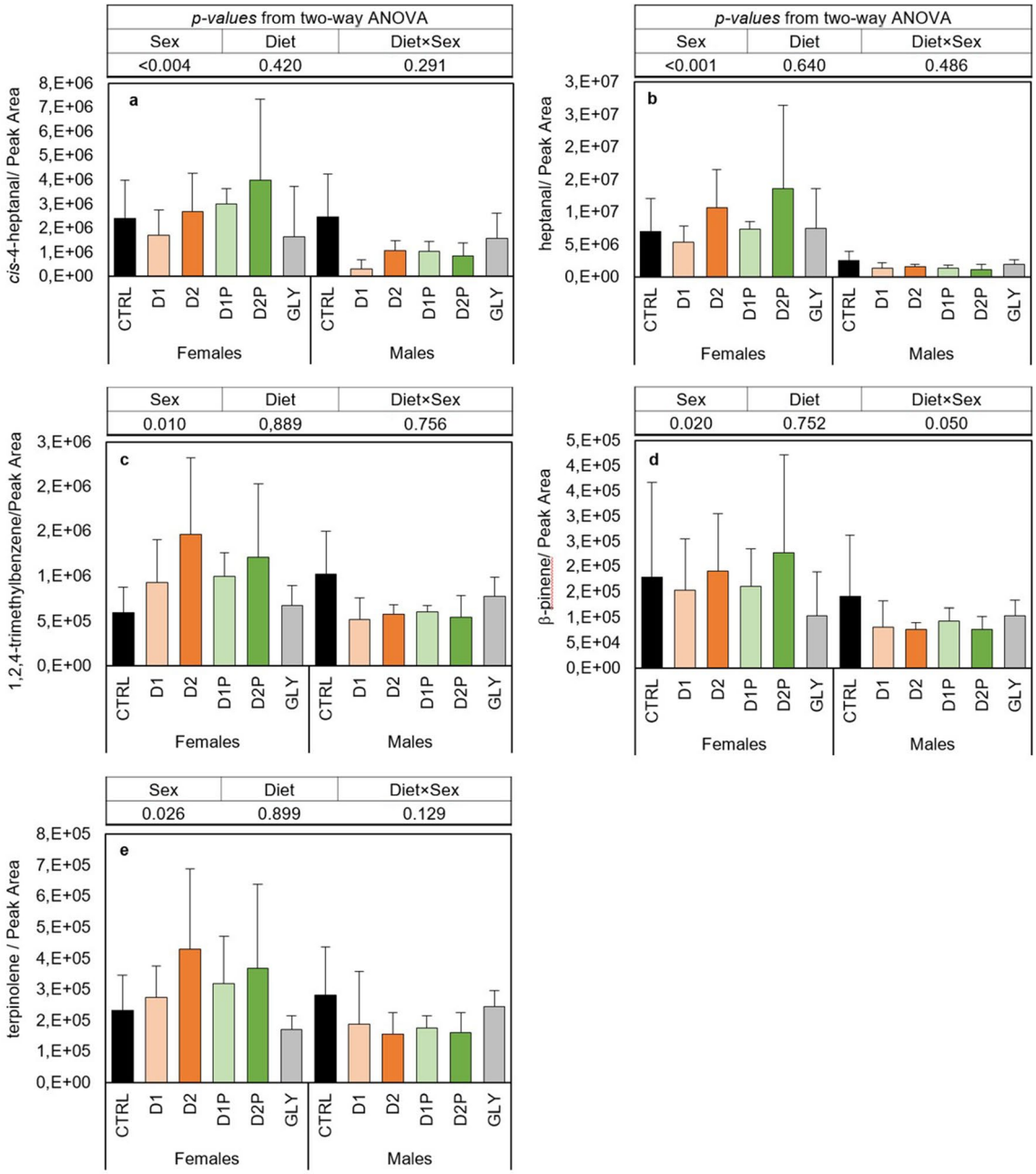

Fig. 8 Gonads volatile compounds affected by sex, considering a significance difference at $p < 0.05$: **(a)** – *cis*-4-heptenal; **(b)** heptanal; **(c)** 1,2,4-trimethylbenzene; **(d)** β-pinene; **(e)** terpinolene. Values are presented as mean ± standard deviation ($n = 4$)

Table 6 Free amino acid content of female and male gonads of *P. lividus* fed the experimental diets

	Females					
	CTRL	D1	D2	D1P	D2P	GLY
Free amino acid concentration mg (100 g)$^{-1}$ DW)						
Arg▼	2011.9 ± 53.8^{e}	2530.4 ± 0.4^{c}	2500.8 ± 11.1^{c}	2232.9 ± 20.8^{d}	2470.9 ± 5.9^{c}	2510.9 ± 8.9^{c}
His▼	17.2 ± 0.2^{D}	74.7 ± 3.5^{A}	23.3 ± 0.2^{C}	30.8 ± 0.1^{B}	27.9 ± 0.9^{B}	29.6 ± 0.7^{B}
Lys▼	225.0 ± 2.2^{A}	107.8 ± 0.1^{E}	142.3 ± 1.9^{D}	190.5 ± 0.4^{C}	215.5 ± 7.2^{B}	109.3 ± 1.0^{E}
Thr▼	37.9 ± 0.7^{C}	49.9 ± 0.1^{A}	31.0 ± 0.6^{D}	32.2 ± 0.2^{D}	43.0 ± 0.8^{B}	31.6 ± 0.2^{D}
Ile▼	8.8 ± 0.1^{f}	30.3 ± 0.6^{a}	20.0 ± 0.2^{d}	17.4 ± 0.1^{e}	24.9 ± 0.5^{b}	20.9 ± 0.2^{c}
Leu▼	15.2 ± 0.6^{f}	75.5 ± 0.3^{a}	29.8 ± 0.1^{e}	35.0 ± 0.1^{d}	63.3 ± 0.2^{b}	30.5 ± 0.5^{e}
Val▼	13.4 ± 0.3^{f}	43.2 ± 0.2^{b}	20.9 ± 0.2^{d}	17.1 ± 0.1^{e}	27.9 ± 0.1^{b,B}	23.0 ± 0.2^{c}
Trp▼	10.4 ± 0.1^{f}	27.9 ± 0.1^{d}	31.1 ± 0.3^{c}	19.1 ± 0.5^{e}	27.5 ± 0.4^{d}	33.1 ± 0.6^{a}
Met▼	7.6 ± 0.3^{E}	28.5 ± 0.1^{A}	20.6 ± 0.1^{B}	19.0 ± 1.1^{C}	20.3 ± 0.4^{B}	17.2 ± 0.1^{D}
Phe▼	11.5 ± 0.1^{F}	39.6 ± 0.1^{A}	33.7 ± 0.3^{C}	27.3 ± 0.2^{E}	31.2 ± 0.5^{D}	35.7 ± 0.2^{B}
Cyst▼	2.3 ± 0.1^{D}	4.4 ± 0.1^{A}	2.5 ± 0.1^{CD}	3.5 ± 0.1^{AB}	2.8 ± 0.1^{BC}	2.4 ± 0.1^{CD}
Tyr▼	12.9 ± 0.3^{f}	143.5 ± 1.1^{a}	56.4 ± 0.6^{e}	66.6 ± 0.1^{c}	121.1 ± 0.3^{b}	60.6 ± 0.9^{d}
Aspartic acid *	24.1 ± 0.5^{B}	25.5 ± 0.3^{A}	17.5 ± 0.3^{DE}	16.2 ± 0.4^{E}	21.0 ± 0.3^{C}	17.9 ± 0.2^{D}
Aspargine *	28.7 ± 0.9^{C}	34.5 ± 0.1^{A}	10.7 ± 0.3^{D}	32.7 ± 0.1^{B}	27.3 ± 0.2^{C}	11.5 ± 0.1^{D}
Glutamic acid *	84.0 ± 0.9^{de}	109.6 ± 2.5^{b}	78.7 ± 1.9^{e}	93.6 ± 0.9^{c}	137.8 ± 2.5^{a}	82.1 ± 0.5^{e}
Glutamine *	126.2 ± 0.8^{e}	243.4 ± 3.0^{d}	234.5 ± 4.4^{d}	244.3 ± 0.2^{d}	528.3 ± 2.5^{a}	269.9 ± 6.7^{c}
Ala +	84.1 ± 2.2^{d}	156.9 ± 0.1^{b}	86.4 ± 0.4^{d}	101.7 ± 0.1^{c}	221.8 ± 3.1^{a}	84.8 ± 0.2^{d}
Gly +	686.5 ± 11.9^{f}	812.1 ± 11.2^{de}	911.8 ± 4.0^{c}	766.2 ± 21.7^{e}	975.7 ± 21.8^{b}	1078.2 ± 20.7^{a}
Pro +	146.5 ± 1.1^{d}	131.5 ± 0.4^{e}	258.9 ± 0.2^{c}	289.3 ± 4.7^{b}	131.4 ± 1.4^{e}	285.8 ± 0.4^{b}
Ser +	54.6 ± 0.9^{c}	92.5 ± 1.2^{b}	50.3 ± 1.9^{d}	43.0 ± 0.1^{e}	50.8 ± 0.3^{cd}	45.1 ± 0.5^{e}
Tau	25.2 ± 0.7^{e}	73.1 ± 0.6^{a}	32.1 ± 0.1^{d}	18.1 ± 0.2^{e}	53.9 ± 0.9^{c}	32.4 ± 0.2^{d}
Ornithine	83.9 ± 1.1^{a}	26.0 ± 0.3^{d}	15.2 ± 0.2^{e}	82.6 ± 3.0^{a}	40.1 ± 1.0^{c}	15.9 ± 0.1^{e}
γ-amino-n-butyric	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
H-Pro	3.2 ± 0.1^{f}	7.5 ± 0.1^{e}	7.9 ± 0.2^{e}	21.2 ± 0.1^{b}	7.7 ± 0.2^{e}	9.8 ± 0.1^{c}
β-alanine	4.3 ± 0.1^{d}	4.8 ± 0.1^{c}	6.0 ± 0.3^{b}	5.3 ± 0.1^{c}	6.8 ± 0.2^{a}	6.8 ± 0.1^{a}
Total FAA	3725.9 ± 77.0^{f}	4873.7 ± 15.0^{c}	4623.1 ± 20.6^{d}	4406.1 ± 35.6^{e}	5279.7 ± 42.5^{b}	4845.6 ± 42.1^{c}

Table 6 (continued)

	Males						*Two-way ANOVA p-value*		
	CTRL	D1	D2	D1P	D2P	GLY	*Sex*	*Diet*	*S x D*
Free amino acid concentration mg (100 g)$^{-1}$ DW)									
Arg▼	2084.7 ± 35.1^{e}	2536.9 ± 26.5^{c}	2652.5 ± 40.1^{b}	2225.7 ± 22.1^{d}	2761.9 ± 21.1^{a}	2547.2 ± 27.7^{bc}	*< 0.001*	*< 0.001*	*< 0.001*
His▼	17.7 ± 0.2^{D}	76.7 ± 0.4^{A}	25.2 ± 0.3^{C}	30.6 ± 0.2^{B}	29.6 ± 0.6^{B}	29.8 ± 0.1^{B}	*0.043*	*< 0.001*	*0.644*
Lys▼	221.6 ± 5.1^{A}	106.7 ± 0.1^{E}	139.0 ± 4.4^{D}	187.7 ± 4.1^{C}	203.6 ± 3.4^{B}	103.5 ± 0.8^{E}	*0.006*	*< 0.001*	*0.349*
Thr▼	38.6 ± 0.6^{C}	49.6 ± 0.1^{A}	30.9 ± 0.2^{D}	31.5 ± 0.5^{D}	44.3 ± 1.2^{B}	31.7 ± 0.3^{D}	*0.422*	*< 0.001*	*0.525*
Ile▼	8.3 ± 0.1^{g}	30.0 ± 0.1^{a}	20.2 ± 0.1^{d}	17.0 ± 0.2^{e}	25.0 ± 0.5^{b}	20.9 ± 0.2^{c}	*0.011*	*< 0.001*	*0.019*
Leu▼	14.7 ± 0.1^{f}	76.4 ± 0.5^{a}	30.2 ± 0.6^{e}	34.8 ± 0.3^{d}	60.5 ± 1.0^{c}	30.8 ± 0.2^{e}	*0.430*	*< 0.001*	*0.004*
Val▼	13.2 ± 0.2^{f}	43.2 ± 0.2^{a}	21.7 ± 0.1^{cd}	16.6 ± 0.1^{e}	28.5 ± 0.4^{b}	23.0 ± 0.5^{c}	*0.183*	*< 0.001*	*0.046*
Trp▼	10.7 ± 0.2^{f}	27.7 ± 0.1^{d}	32.1 ± 0.2^{bc}	19.2 ± 0.3^{e}	28.3 ± 0.9^{d}	33.0 ± 0.2^{ab}	*0.014*	*< 0.001*	*0.034*
Met▼	6.9 ± 0.1^{E}	29.0 ± 0.2^{A}	21.0 ± 0.3^{B}	18.1 ± 0.2^{C}	20.4 ± 0.3^{B}	16.6 ± 0.1^{D}	*0.279*	*< 0.001*	*0.701*
Phe▼	11.1 ± 0.2^{F}	39.5 ± 0.2^{A}	34.9 ± 0.2^{C}	27.3 ± 0.4^{E}	32.0 ± 0.7^{D}	36.5 ± 0.3^{B}	*0.128*	*< 0.001*	*0.114*
Cyst▼	2.1 ± 0.1^{D}	4.2 ± 0.1^{A}	2.5 ± 0.1^{CD}	3.4 ± 0.1^{AB}	2.6 ± 0.1^{BC}	2.5 ± 0.1^{CD}	*< 574*	*< 0.001*	*0.395*
Tyr▼	12.7 ± 0.3^{f}	140.4 ± 1.0^{a}	58.3 ± 0.2^{de}	66.8 ± 1.0^{c}	123.4 ± 1.5^{b}	60.4 ± 1.2^{d}	*0.603*	*< 0.001*	*0.006*
Aspartic acid *	23.5 ± 0.1^{B}	25.5 ± 0.1^{A}	17.5 ± 0.1^{DE}	16.7 ± 0.1^{E}	21.3 ± 0.3^{C}	18.6 ± 0.1^{D}	*0.430*	*< 0.001*	*0.425*
Aspargine *	28.1 ± 0.3^{C}	34.5 ± 0.1^{A}	10.7 ± 0.3^{D}	32.7 ± 0.4^{B}	27.9 ± 0.4^{C}	12.1 ± 0.1^{D}	*1.000*	*< 0.001*	*0.234*
Glutamic acid *	82.6 ± 0.9^{e}	104.4 ± 0.2^{b}	82.1 ± 1.6^{e}	88.6 ± 1.2^{cd}	139.6 ± 1.2^{a}	84.1 ± 0.9^{de}	*0.232*	*< 0.001*	*0.004*
Glutamine *	131.0 ± 2.0^{e}	234.6 ± 1.2^{d}	253.5 ± 6.1^{cd}	238.7 ± 2.8^{d}	542.9 ± 14.2^{a}	294.3 ± 2.7^{b}	*0.003*	*< 0.001*	*0.005*
Ala +	85.3 ± 0.7^{d}	156.3 ± 0.8^{b}	84.9 ± 3.1^{d}	100.0 ± 2.2^{c}	227.6 ± 3.4^{a}	79.8 ± 0.8^{d}	*0.446*	*< 0.001*	*0.020*
Gly +	701.3 ± 10.3^{f}	861.8 ± 0.2^{cd}	905.4 ± 5.5^{c}	760.6 ± 6.9^{e}	1048.2 ± 5.5^{a}	1062.7 ± 20.8^{a}	*0.007*	*< 0.001*	*0.004*
Pro +	142.8 ± 3.2^{d}	130.1 ± 0.8^{e}	257.0 ± 4.4^{c}	285.4 ± 3.6^{b}	136.6 ± 2.3^{de}	301.2 ± 1.0^{a}	*0.178*	*< 0.001*	*0.001*
Ser +	54.6 ± 0.7^{c}	98.7 ± 0.1^{a}	53.6 ± 0.4^{cd}	42.9 ± 1.3^{e}	51.1 ± 0.9^{cd}	45.1 ± 0.7^{e}	*0.001*	*< 0.001*	*0.002*
Tau	26.5 ± 0.3^{e}	70.4 ± 0.5^{a}	32.2 ± 0.2^{d}	18.2 ± 0.4^{e}	58.7 ± 1.4^{b}	34.6 ± 0.6^{d}	*0.010*	*< 0.001*	*< 0.001*
Ornithine	81.9 ± 0.1^{a}	25.7 ± 0.2^{d}	15.4 ± 0.1^{e}	76.5 ± 1.4^{b}	40.1 ± 0.7^{c}	15.7 ± 0.1^{e}	*0.026*	*< 0.001*	*0.019*
γ-amino-n-butyric	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	*0.337*	*0.458*	*0.458*
H-Pro	3.1 ± 0.1^{f}	7.6 ± 0.1^{e}	8.4 ± 0.1^{e}	22.0 ± 0.1^{a}	8.7 ± 0.1^{d}	9.8 ± 0.1^{c}	*< 0.001*	*< 0.001*	*0.006*
β-alanine	3.8 ± 0.1^{d}	4.7 ± 0.1^{c}	7.1 ± 0.1^{a}	5.0 ± 0.1^{c}	7.3 ± 0.2^{a}	6.5 ± 0.1^{ab}	*0.337*	*< 0.001*	*0.006*
Total FAA	3807.3 ± 60.8^{f}	4915.3 ± 32.4^{c}	4796.9 ± 62.6^{cd}	4366.5 ± 35.9^{e}	5670.8 ± 51.4^{a}	4900.9 ± 59.7^{c}	*< 0.001*	*< 0.001*	*< 0.001*

Values are presented as mean ± standard deviation ($n = 2$). In each line, different superscript letters indicate significant differences ($p < 0.05$), identified through two-way ANOVA analysis followed by Tukey post-hoc tests. Lowercase superscript letters indicate differences between diets and sexes. Uppercase superscript letters indicate differences between diets. Amino acids marked + are regarded as sweet tasting, * as Umami tasting, and ▼ as bitter tasting

Table 7 Pearson's correlations coefficients between gut and gonads carotenoids and anti-oxidant activity

	Gut Carotenoids (μg g^{-1} ww)						Gonad Carotenoids (μg.g^{-1} ww)					
	Lutein	Zeaxanthin	Echinenone	α-carotene	β-carotene	Total Carotenoids	Lutein	Zeaxanthin	Echinenone	α-carotene	β-carotene	Total Carotenoids
ABTS	0.409*	0.424**	NS	NS	NS	NS	0.680**	0.603**	−0.454**	NS	NS	NS
DPPH	0.481**	0.512**	NS	NS	NS	NS	0.585**	0.567**	−0.354*	NS	NS	NS

$n = 38$, $^{**}p < 0.01$ (two-tailed) and $^{*}p < 0.05$ (two-tailed)

sea-urchins fed the CTRL diet, which ovaries displayed a significantly weaker DPPH activity than all others.

Anti-oxidant activity appeared to correlate with carotenoids (Table 7), but not with amino acids (results not shown). ABTS and DPPH were positively correlated with both gut and gonads lutein and zeaxantin, and negatively correlated with gonads echinenone (Table 7); no significant correlations were, however, found between gonads ABTS or DPPH and gonads amino acids (data not shown).

Discussion

Experimental diets

This study aimed at developing an extruded diet for *P. lividus* grow-out, able to optimize growth and gonad yield while maximizing the deposition of dietary carotenoids and taste-associated amino acids for eventual enhancement of roe color and flavor. Cold extrusion and soft drying methods were employed to preserve carotenoid properties, thus preventing carotenoid degradation throughout the manufacturing process as compared to classical hot extrusion and hot drying processes. Although the targeted levels for β-carotenes dietary content were still not achieved, the supplementation with *D. salina* as a source of β-carotene in D1, D2, D1P, D2P diets has improved gonad color. The dietary inclusion of *Porphyra* spp., abundant in sweet-tasting and umami amino acids, increased sweet and umami-tasting amino-acids such as proline, alanine and glutamine, whereas the dietary supplementation with glycine was per se quite effective in increasing glycine, but also other sweet-taste amino acids.

Although sea-urchin culture has traditionally relied on the use of raw materials such as macroalgae as dietary sources (Liu and Chang 2015), the expansion of echinoculture calls for the development of extruded diets to ensure a sustainable and safer production of sea urchins. Despite the recent research that proved successful in developing extruded feeds for *P. lividus,* by ensuring growth and good gonad yields (Baião et al. 2019, 2022b; Lourenço et al. 2020), addressing consumer demands for color and taste remains a challenge. Several authors suggested the inclusion of carotenoids sources in diets for sea urchins as an effective way to enhance sea-urchin gonad colour, by resorting to either natural or synthetic sources in extruded moist pellets or gelatin-based feeds (Robinson et al. 2002; McBride 2004; Shpigel et al. 2005, 2006; Suckling et al. 2011; Prato et al. 2018), but none using extruded diets. Furthermore, few of these studies have actually determined the dietary carotenoid content (McBride 2004; Shpigel et al. 2005; Suckling et al. 2011), which should be considered key for a thorough interpretation of experimental results. To the best of our knowledge, only Baião et al. (2022b) and Lourenço et al. (2022) analyzed the impact of the dietary inclusion of β-carotene sources on *P. lividus* gonads quality considering the stability of dietary carotenoids' in extruded diets. Baião et al. (2022b) tested two β-carotene sources, a synthetic and a natural source (paprika), at two incorporation levels (100 and 250 mg β-carotene kg^{-1} feed) and observed a high degradation of dietary β-carotene, which was attributed to the impact of extrusion and drying at high temperatures (70ºC). In this study, the testing diets were manufactured by cold extrusion (< 30 °C) and soft drying (< 45ºC). This manufacturing conditions led to an estimated degradation of 49–66% of dietary β-carotene, thus much lower than the 88–94% reported for paprika β-carotene, and lower than the 69–74% reported for a synthetic β-carotene (Baião et al. 2022b). Using similar manufacturing conditions to those employed in this study, Lourenço et al. (2022) estimated dietary total carotenoids losses at 56–75% in diets supplemented with a synthetic β-carotene at 100 mg kg^{-1} feed. Although the β-carotene losses in this study were below those previously reported, the targeted levels of 150 and 300 μg g^{-1} DM for D1/D1P and D2/D2P diets were far from being achieved. Further research is indeed required to better preserve dietary carotenoids, such as testing the stabilization of *D. salina* by different oils prior to inclusion in an extruded diet.

Growth, macronutrient deposition and gonads texture

The dietary formulations herein tested resulted in similar growth. The replacement of a commercial macroalgae mix by *Porphyra* led to a significant increase in voluntary feed

intake (VFI) that did not reflect on growth, but did reflect on feed conversion ratio (FCR) and protein efficiency ratio (PER). Sea-urchins grown upon D2 exhibited the lowest FCR value. For that reason, D2 may be pointed as the best diet for *P. lividus* grow-out, when considering feeding and growth performance. Baião et al. (2019) found differences in *P. lividus* VFI and FCR in response to dietary protein as previously suggested for other sea-urchins' species (McBride 2004; Heflin et al. 2012; Cuesta-Gomez and Sánchez-Saavedra 2016). However, the diets herein tested had the same protein levels, but used different protein sources that may have affected feed palatability and protein digestibility. On the other hand, a possible miscount of the pellet leftovers and a subsequent impact on FCR and PER estimation cannot be fully discarded, due to sea urchins slow feeding regime and observed differences in the stability of the experimental diets in seawater. *Porphyra* rehydrating and expanding may have contributed to higher feed instability which may partially explain the lower feed conversion ratio observed in groups fed such diets compared to those fed the commercial blend of seaweeds.

All diets increased gonad weight and gonadosomatic index (GSI), rising from 8.9% and 6.4% to 16.9% and 15.8% for females and males, respectively. These values exceed the minimum thresholds (10%) recommended by Stefánsson et al. (2017) for processing and marketing. The GSI values herein obtained were also higher than those reported in previous studies focused on *P. lividus* in 8 weeks trials (Shpigel et al. 2006; Baião et al. 2022b), in a 9 weeks trial (Prato et al. 2018) or a 15 weeks trial (Lourenço et al. 2020), but lower than those obtained for smaller sea-urchins by Baião et al. (2019) in a 15 weeks trial. In the present study, females showed higher gonad weight and GSI than males, in agreement with Baião et al. (2019).

The proximal composition of gonads was affected by both dietary treatments and sex, with ovaries consistently displaying lower protein levels and higher energy content, as well as generally higher dry matter than testes, as previously described by Baião et al. (2019, 2022a). These results suggest sex-related differences in nutrient assimilation or metabolic processes. Regardless of sex, crude protein and energy content were consistently lower in the gonads of D1P urchins, whereas protein was higher in the gonads of D1 urchins and energy content was consistently higher in the gonads of D2 fed urchins, thus suggesting a significant effect of dietary formulation on gonads metabolism.

The texture of sea-urchin gonads was previously claimed to be related to moisture and reproductive stage (McBride 2004). In this study, no differences were found in the reproductive stage of the analyzed urchins and the differences found between sexes in gonads' dry matter did not seem to reflect on texture. Resilience remained unaffected and ovaries showed lower firmness than testes. According to McBride (2004), the opposite would be expected as ovaries displayed higher dry matter than testes. Although overall results do not unfold a correlation between texture and dry matter, ovaries firmness was positively correlated to dry matter, supporting McBride (2004) findings. In the present study, fresh gonad firmness was positively correlated with protein content, supporting previous observations in salted sea-urchins roes (Shimada and Okajima 1989). However, a two-way ANOVA, considering diet and sex as main factors, revealed a weak dietary effect on gonad firmness. Regardless of sex, differences in firmness matched those in energy content rather than protein. In ovaries, there was a positive correlation between firmness and energy, that may be associated to gonadal lipid deposition; however, this cannot be confirmed since lipids were not analyzed in the present study.

Carotenoids deposition

In agreement with Haug et al. (2003), the carotenoid profile of sea-urchins gut in this study reflected in general the dietary carotenoid profile, thus confirming their capacity to assimilate dietary carotenoids. Echinenone was only present in the gut, but not in the diets, which confirms that it is synthesized in the gut wall, as suggested by Symonds et al. (2007). A positive correlation between gut and dietary β-carotene confirms *P. lividus* capacity to retain this carotene. Furthermore, the positive correlations between gut echinenone and gut β-carotene, and between gut echinenone and dietary β-carotene suggest that this carotenoid is synthesized from β-carotene in the gut, making use of dietary β-carotene. A strong correlation between gonads echinenone and gonads β-carotene also points at echinenone synthesis in the gonads. In fact, there has been some controversy about the place where β-carotene is metabolized into echinenone in the sea urchins. It was first proposed to occur only in the gonads (Griffiths and Perrott 1976), but more recently discussion arose on whether it partially occurs in the gonads (Shpigel et al. 2005, 2006) or even only in the gut (Symonds et al. 2007, 2009). In this study, most carotenoids, and β-carotene in particular were more abundant in the gut than in the gonads, but echinenone followed an opposite trend. Gonads' echinenone levels were very close (females) or higher (males) than those in the gut. These results suggest that echinenone can be either synthetized in the gut or the gonads, and if occurring in the gut it may be mobilized into gonads, as previously claimed by Symonds et al. (2007, 2009). Yet further studies are warranted to fully clarify echinenone metabolism.

The impact of sex on the gut and gonad carotenoid profile may reflect different retention or synthesis capacities between females and males. Hagen et al. (2008) reported distinct patterns of carotenoid deposition and depletion in ovaries and testes. If carotenoids are differentially deposited in ovaries and testes, as observed in this study, and gut wall carotenoids are mobilized into gonads, then it is expected to find differences in gut wall carotenoid profiles between males and females. Lutein and zeaxanthin were higher in female gut and gonads, whereas echinenone, α- and β-carotenes were higher in male gut and gonads. The same differences between female and male gonad carotenoid profiles had already been reported in *P. lividus* (Baião et al. 2022b). The matching differences in both gut and gonads, coupled with the positive correlations between gut and gonads lutein, zeaxanthin, and between gut α- and β-carotene and gonads echinenone suggest that carotenoids other than echinenone may also be mobilized from gut into gonads. However, unlike what was observed in the gut, gonads carotenoid profile was not affected by diet. This may be explained by differences in bio-synthesis mechanisms between the gut and the gonads, as put forward for echinenone by Symonds et al. (2007). Other possible explanations are: a) limited metabolism – sea urchins would not be able to metabolize dietary carotenoids at the same rate as they ingest them, thus reaching a saturation point; or b) limited mobilization from the gut to the gonads – possibly due to the saturation of protein-binding transporters, as those involved in echinenone mobilization (Pilbrow et al. 2012).

Impact on gonads colour

The color of sea urchin roes is linked to the deposition of carotenoids. Challenges in enhancing gonad color are often associated with the complex bioconversion of dietary β-carotene into echinenone (Tsushima 2007), with paleness being attributed to a mismatch between gonad growth and echinenone synthesis (Plank et al. 2002). In this study, the gonadosomatic index was negatively correlated with β-carotene and echinenone, which indicates a mismatch between gonad growth and these carotenoids deposition. For being the most abundant carotenoid in sea-urchins roes, echinenone has been singled out as the major carotenoid conferring color to sea-urchins gonad (Shpigel et al. 2005, 2006; Suckling et al. 2011), but there has been some controversy on this topic. Symonds et al. (2007) state that unacceptable gonad coloration can be seen with both very low and high levels of echinenone and total carotenoids. In fact, gonads echinenone and total carotenoids in this study were positively correlated with L* (lightness or paleness). Furthermore, gonads echinenone was negatively correlated with a* (redness), b* (yellowness) and C* (color saturation). On the other hand, the positive correlation between lutein and zeaxanthin and a* (redness), b* (yellowness) and C* (color saturation) point to these carotenoids as determining gonads color, even though lutein and zeaxanthin are actually yellow. Ovaries richer in lutein and zeaxanthin were accordingly darker, redder (a*), yellower (b*) and bearing a higher color saturation (C*) than males. However, other possible factors affecting gonads color cannot be discarded.

Although there was no dietary effect on gonads carotenoids, there was a dietary effect on both ovaries and testes colour, similarly to what was reported by Baião et al. (2022b) when feeding *P. lividus* with extruded diets including different β-carotene sources also for 8 weeks. In this study, sea urchins fed *Dunaliella* supplemented diets produced darker (lower L^*) and more reddish gonads (higher a^* and lower h^*) than those fed the CTRL diet, regardless of sex. Compared to the previous results by Baião et al. (2022b), the ovaries of sea urchins fed *Dunaliella*-supplemented diets displayed better color parameters, thus suggesting *D. salina* as a better promotor of roe color than paprika or a synthetic β-carotene. Shpigel et al. (2006) also reported optimal mango-orange color in the roes of *P. lividus* fed a gelatin-based diet including a *Dunaliella* algal powder, for 8 weeks, surpassing the color of sea-urchins fed other diets including various synthetic carotenoids. Robinson et al. (2002) reported that a *D. salina* powder included in a moist-extruded diet (at a concentration of 250 mg β-carotene kg^{-1}) was the most effective solution for providing good coloration to the roes of *Strongylocentrotus droebachiensis,* when compared to synthetic β-carotene sources.

When comparing the roes obtained in the present study with reference roes classified as gourmet by Baião et al. (2021b), the diets with a higher *Dunaliella* supplementation (D2 and D2P) led to gonads that looked closer to the reference ones. The gonads of D2P urchins were as dark as the reference, and D2 gonads displayed similar color saturation (C*) and tone (h*).

The impact on volatile compounds

Carotenoid metabolism may produce volatile compounds, so-called norisoprenoids. Among the volatile compounds identified in *P. lividus* gonads, some terpenes (β-pinene, limonene, γ-terpinene, terpinolene and sesquiterpene-like compound) can be derived from carotenoids. Likewise gonads carotenoid profile, there were also no significant differences on gonads volatile compounds between sea-urchins fed different diets. However, there was an effect of sex on the concentration of β-pinene and terpinolene. Robinson et al. (2002) proposed sex-specific pathways of carotenoid metabolism and expression in *S. droebachiensis*, males and females likely having distinct metabolism pathways and

deposition rates for the carotenoids metabolites that may include some terpenes. In this study, ovaries displayed higher concentrations of *cis*-4-heptenal and terpinolene (with no reported odour), heptanal (aldehydic, citrus and fatty odour), β-pinene (resinous, woody, pine odour) and 1,2,4-trimethylbenzene (plastic odour) than testes. Baião et al. (2020) has also reported an effect of sex on *P. lividus* gonad volatile profile, namely on β-pinene and 1,2,4-trimethylbenzene concentrations. The observed differences in this study could possibly favour the flavour perception of female gonads.

Free amino acid content in gonads

Regardless of sex and diet, the most abundant free amino acids in *P. lividus* gonads were arginine, glycine, lysine, proline and glutamine, which generally agrees to what was reported by Dincer and Cakli (2007) and Mol et al. (2008) also for *P. lividus.* Several authors have associated the taste of sea urchin gonads with free amino acids (Hirano et al. 1978; Woods et al. 2007; Phillips et al. 2010; Prato et al. 2018; Volpe et al. 2018) and suggested a classification as either enhancing or detracting flavor based on its characteristic taste. Flavor-enhancing amino acids are commonly linked to sweetness, umami or even subtle bitterness, whereas flavor-detracting amino acids are those perceived as strongly bitter or taste. In this study, most flavor-enhancing amino acids including arginine and glycine, were increased in all groups when compared to the CTRL group. The gonads of sea urchins fed the GLY diet exhibited the highest glycine levels irrespective of sex, when compared to other groups; this confirms the dietary supplementation with glycine as an effective way to increase gonads glycine content, possibly increasing their sweetness. Dietary glycine supplementation also led to increased proline (that can be obtained by glycine conversion) in the GLY gonads, especially in testis, which might also contribute to sweetness. Male gonads from the D2P group also showed elevated glycine content, similar to GLY gonads. Additionally, glycine content in D2 and D2P gonads exceeded that of D1 and D1P gonads, respectively, in both sexes; this confirms *D. salina* as a good source of glycine, possibly contributing to the so desired sweetness. The diets including *Porphyra* increased the levels of proline and alanine in D1P and D2P gonads respectively, which confirms *Porphyra* as good source of sweet-taste amino acids other than glycine, as suggested by Hwang et al. (2013). Regarding other marine products, a certain degree of umami coming from glutamic acid, glutamine or aspartic acid is also desired in sea-urchins roes. Glutamic acid and glutamine were generally more abundant than aspartic acid in all gonads. The increased levels of glutamic acid and glutamine contents could have favoured the umami taste of D2P gonads, when compared to others. In addition, D1, D2 and D1P gonads displayed high levels of glutamine, when compared to CTRL and GLY gonads, thus confirming both *Dunaliella* and *Porphyra* as good sources for this amino acid, as suggested by Guedes et al. (2015) and Hwang et al. (2013) or Machado et al. (2020).

A subtle bitterness given by arginine, lysine or leucine is also desirable in sea-urchins gonads. In this study, lysine was more abundant in ovaries, but testes displayed higher arginine levels, which might have favoured a subtle bitterness in testes. Furthermore, all the experimental diets increased gonads arginine content, relative to the CTRL diet. Lysine and, to a lesser extent, other bitter amino acids, with levels significantly affected by diet, may have influenced the flavor of *P. lividus* roes. However, the actual impact of these amino acid variations on the consumers sensorial appreciation and acceptability can only be confirmed through a sensorial evaluation.

As all the experimental diets increased flavor-enhancing amino acids in *P. lividus* gonads when compared to the CTRL diet, they have also increased the flavor-detracting valine. Regardless of sex, D1 gonads displayed the highest content of valine, followed by D2P, GLY, D2 and D1P. Although valine concentration was low and possibly imperceptible, a sensorial perception evaluation would be required to clarify its actual impact.

Gonads antioxidant capacity

Being rich in carotenoids and polyunsaturated fatty acids (Mol et al. 2008; Prato et al. 2018; Baião et al. 2019), sea-urchins roes are susceptible to oxidative reactions that could lead to the development of off-flavours. As a delicacy that is, consumed raw, any off-flavours could have a major impact on this product acceptability. The anti-oxidant activity of sea-urchin gonads was assessed via ABTS and DPPH assays, which respond to the ability of redox molecules to scavenge free radicals. Overall, antioxidant capacity was significantly affected by sex, with ovaries displaying higher activity than testes. Furthermore, ABTS and DPPH were strongly correlated with both gut and gonad carotenoids lutein and zeaxantin, thus making it apparent that these two carotenoids have a considerable effect as antioxidants, when compared with the remaining ones. The fact that females presented higher antioxidant capacity is likely explained by the higher concentration of both lutein and zeaxantin in female gonads, which were practically undetected in males. Lutein has been described as an effective scavenger of superoxide, hydroxyl, nitric oxide, and DPPH and as inhibitor of lipid peroxidation (in vitro) (Sindhu et al. 2010). Furthermore, lutein also inhibited superoxide radical generation in vivo (Sindhu et al. 2010) and was shown to decrease the accumulation rate of hydroperoxide in a study using model membranes (Wisniewska-Becker et al. 2012). Other authors have reported that lutein, α- and β-carotene are key in preventing oxidative damage due to their ability for radical

scavenging (Fernandez-Sevilla et al. 2010; Galasso et al. 2017). In fact, the chemical structure of lutein and zeaxantin suggests strong antioxidant features. Both carotenoids contain alternate double bonds between carbons, that confer a higher stabilization capacity when in the presence of reactive oxygen species (ROS), thus leading to a high antioxidant potential (Bonner and Arbiser 2014). Echinenone, more abundant in testes, was negatively correlated with ABTS and DPPH; this would have contributed for their lower antioxidant capacity. The differences between female and male gonads carotenoids accumulation patterns could result from an evolutionary mechanism that allowed for a higher protection of oocytes against free radicals, as these are highly prone to oxidative stress due to a higher metabolic demand, larger cytoplasmic volume and the involvement of female mitochondrial DNA on embryo development, their oxidative status being highly determinant for offspring survival (Lister et al. 2016, 2017).

The ovaries of sea-urchins fed the supplemented diets exhibited a stronger DPPH activity than those fed the CTRL diet, which confirms the dietary supplementation *Dunaliella* and glycine as an effective way to promote the anti-oxidant capacity of ovaries; this holds especially in ovaries that are richer in lipids, and PUFA that are prone to oxidation (Baião et al. 2019; Rocha et al. 2019b).

Amino acids such as tryptophan, tyrosine, methionine, cysteine, histidine, and phenylalanine, have been acknowledged for their antioxidant capabilities. Although there was a significant effect of diet on the gonads content of all these amino acids and a significant effect of sex for histidine, phenylalanine and cysteine, there was no significant correlation between these amino acids and ABTS or DPPH activities. In fact, these amino acids were less abundant than others, so perhaps their concentration in sea-urchins' gonads was not sufficient to induce a significant effect on anti-oxidant capacity.

Conclusions

This study enabled the production of innovative carotenoid-rich extruded diets, thus setting a useful contribution toward the development of new diets for *P. lividus.* The use of cold extrusion and soft drying techniques in feed manufacturing helped reduce carotenoid losses as compared to hot extrusion and drying processes.

The carotenoid profile of sea-urchin guts reflected in general the dietary carotenoids. However, there was no dietary effect on the carotenoid profile of gonads, possibly due to limited metabolism or limited carotenoid mobilization from gut to gonads. In spite of this, all the supplemented diets improved gonad colour, particularly diet D2, which achieved a gourmet reference standard.

The supplemented diets also increased the gonad content of flavor-enhancing amino-acids. Crystalline glycine and *Porphyra* were effective in increasing the gonads' content of glycine and other sweet-tasting amino-acids, while *D. salina* and *Porphyra* emerged as valuable sources of umami amino acids.

The remarkable differences between males and females for carotenoid deposition in both gut and gonads, gonadal proximal composition, volatile compounds, and anti-oxidant activity suggest sex-related differences in nutrient assimilation and several metabolic processes. This emphasizes the challenges inherent in devising novel diets aimed at enhancing the quality of sea urchin roe.

All in all, the present results highlight diet D2, supplemented with *D. salina* at 1.5%, as the best option for *P. lividus* grow-out, considering not only colour, but also feed conversion ratio as a key criterion in aquaculture.

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Author contributions PC: Investigation; Validation; Roles/Writing – original draft; IG: Formal analysis; Investigation; Methodology; Roles/Writing – original draft; LFB: Investigation; Methodology; Roles/Writing – original draft; TS: Investigation; Methodology; Roles/Writing – original draft; HMA: Investigation; Methodology; Roles/Writing – original draft; ACG: Conceptualization; Funding acquisition; Investigation; Methodology; Resources; Writing – review & editing; TT: Investigation; Methodology IC: Conceptualization; Investigation; FXM: Investigation; Methodology; Writing – review and editing FA: Investigation; Methodology; JP: Investigation; Methodology; PGP: Investigation; Methodology; LMPV: Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Validation; Writing – review & editing. All authors read, revised, and approved the final version of the manuscript.

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Data availability Data are available from the corresponding Author upon reasonable request.

Declarations

Competing interests LV is a member of the Editorial Board for the Journal of Applied Phycology and the peer-review process for this article was independently handled by another member of the journal editorial board. Author LFB is employed by the company Sense Test and the authors ACG and IC are employed by the company Ínclita Seaweed Solutions. The remaining authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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