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RESEARCH

Inclusion of carotenoids and Eicosapentaenoic Acid (EPA) in sea urchin (*Paracentrotus lividus*) broodstock diets improves gonad development and oocyte quality

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Abstract

In aquaculture, broodstock conditioning diets are essential for enhancing reproductive performance, particularly as offspring survival remains a primary bottleneck in sea urchin production. This study investigated the effects of different broodstock diets on gonad development, fatty acid profiles, and oocyte size in the sea urchin *Paracentrotus lividus*. To determine if maternal investment could be enhanced through specific nutrients, three diets that differed in both ingredient matrix and pigment profile were tested over a two-month feeding period: 1) a formulated pumpkin/algal diet (PA) enriched with β-carotene via pumpkin and *Nannochloropsis* sp.; 2) a formulated pea/corn diet (PC) with a low-pigment profile, and 3) a natural kelp diet based on *Saccorhiza polyschides,* representing one of the natural foods for *P. lividus* in its local habitat. This Kelp diet provided a mixed-pigment reference with relatively high total carotenoids. Following the feeding trial, several key parameters were assessed, including gonadosomatic index (GSI), reproductive stage, fatty acid composition, oocyte size, and fertilization success. Results indicated that females fed the PA diet produced oocytes that were 18% larger and achieved a 7% higher fertilization rate than those on the PC diet. This enhancement is likely linked to the significantly higher deposition of carotenoids and omega-3 polyunsaturated fatty acids (n-3 PUFA) in the gonads. In contrast, while the PC diet promoted a high GSI, it resulted in smaller oocytes and lower fertilization rates, due to a deficiency in n-3 PUFA in their gonads. Females fed the Kelp diet presented the slowest gonadal development and failed to spawn, suggesting that seaweed is insufficient as a sole food source for short-term broodstock conditioning to support reproduction. This research provides valuable insights into developing broodstock conditioning diets by exploring alternative nutrient sources, such as pumpkin and microalgae, to enhance reproductive traits, particularly for emerging species like sea urchins.

Keywords Echinoderm · Conditioning · Reproduction · Carotenoids · Fatty acids

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Introduction

Sea urchins such as *Paracentrotus lividus* (Lamarck, 1816) are exploited across the Mediterranean and Northeastern Atlantic coastal areas for their edible gonads, known as roe or uni. These are considered a delicacy when large, firm, bright orange-yellow in color with a sweet umami taste (Rocha et al. 2019; Boudouresque and Verlaque 2020) yielding high market values, ranging from €60/kg to €221/kg (Monfort 2002; Sun and Chiang 2015; Lourenço et al. 2019). Demand for uni is increasing across Europe, the United States, and Northern Asia (Prato et al. 2018; Boudouresque and Verlaque 2020; Nhan et al. 2020; Plee and Suckling 2024) but is unmet due to declining natural stocks by overfishing, inadequate management, habitat destruction, and climate change (Bertocci et al. 2014; Yeruham et al. 2015). It has been shown that sea urchins can be cultured (propagated) in a hatchery and then grown in open water using a range of gear types (e.g., suspended nets and bottom cages; Chang et al. 2004; Zhanhui et al. 2014), showing an economic opportunity for producing sea urchins through aquaculture (Jong-Westman et al. 1995; Grosjean et al. 1998; Phillips et al. 2010; Carboni et al. 2013; Onomu et al. 2020). Consequently, there has been increasing interest and research efforts on this emerging sector of sea urchin production (Plee and Suckling 2024).

Hatcheries are specifically seeking to optimize diets for gonad enhancement (e.g., somatic growth, gonad yield, gamete yield and quality) and ensure consistent market quality (Fernandez and Pergent 1998; Shpigel et al. 2005; Cyrus et al. 2015; Zhao et al. 2016; Prato et al. 2018; Volpe et al. 2018; Lourenço et al. 2021; Araújo et al. 2023; Tourón et al. 2023). Traditionally, hatchery-reared sea urchins are fed locally sourced wild macroalgae due to their accessibility and low cost (Prato et al. 2018). These macroalgae, including *Ulva* sp., *Padina pavonica*, *Undaria pinnatifida*, *Laminaria* sp., and *Saccorhiza polyschides*, constitute the natural diet of temperate species like *P. lividus* (Tuya et al. 2011; Lawrence et al. 2013; Cardoso et al. 2018; De La Uz et al. 2019). However, a significant limitation of using macroalgae as a dietary resource in hatcheries is their variable nutritional composition and bioavailability (Øverland et al. 2019). In temperate regions, for instance, photosynthetic pigment concentrations (chlorophyll *a,* chlorophyll *b*, and carotenoids) decrease by up to 80% between spring and summer (Altamirano et al. 2000), and saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) concentrations peak during this period, declining by up to 21% the rest of the year (Pereira et al. 2021). These changes occur across the period of time when broodstock ideally need consistent nutritional enhancement in a hatchery setting, resulting in variable gonadosomatic growth rates and gamete quality (Zuo et al. 2022; Ciriminna et al. 2024). In addition, macroalgal diets are often energetically limiting, which can further restrict gonad growth and gamete production. Because successful larval rearing is a primary bottleneck in sea urchin production, hatchery diets must move beyond simple gonad enhancement to prioritizing oocyte quality and viability through maternal investment (Carboni et al. 2015; Brink-Hull et al. 2022a,b; Ciriminna et al. 2024). While formulated diets are often designed to improve gonad yield and color, broodstock diets for hatchery production must focus on the allocation of high-quality nutrient reserves. Optimizing the transfer of these nutrients during oogenesis is critical to ensuring successful embryogenesis and subsequent larval survival (Carboni et al. 2015; Brink-Hull et al. 2022a,b). In a hatchery context, producing high numbers of high-quality eggs is required for profitable aquaculture production. Because maternal nutritional history dictates the efficiency of this allocation (Byrne et al. 2008; Wong et al. 2019), the development of specialized, nutritionally formulated feeds is needed for profitability.

To maximize reproductive success, these diets must optimize ratios of digestible protein and energy, essential fatty acids (e.g., PUFAs), and pigments to enhance gonad development and reproductive success (Carboni et al. 2015; Prato et al. 2018; Zupo et al. 2019; Lourenço et al. 2019, 2021; Raposo et al. 2019; Ciriminna et al. 2024). Although, accessible, low-cost and highly effective formulated feeds that improve reproductive performance are still lacking (Carboni et al. 2013; Walker et al. 2015; Zupo et al. 2019; Suckling et al. 2022).

The incorporation of vegetable-based ingredients (e.g., pea, corn, maize, spinach, lettuce) and agricultural discards (e.g., endive leaves) as sustainable, low-cost components of formulated diets has produced positive outcomes for sea urchin gonadosomatic growth and gonad quality. These benefits are largely attributed to their favorable profiles of proteins, lipids, fatty acids, and β-carotene, which improved gonad quality in *P. lividus* (McLaughlin and Kelly 2001; Fabbrocini et al. 2015; Sartori et al. 2016; Vizzini et al. 2018; Raposo et al. 2019; Vizzini et al. 2019; Zupo et al. 2019; Ciriminna et al. 2020, 2024, 2025). Carotenoid accumulation in sea urchin gonads plays a critical role in egg production, offspring quality, and immune function (McBride et al. 2004; Shpigel et al. 2005; Suckling et al. 2011, 2020; Tan et al. 2019; Leiva et al. 2022; Brink-Hull et al. 2022a). Because sea urchins cannot synthesize carotenoids de novo, dietary provision of these pigments is essential (Pearce et al. 2003; Shpigel et al. 2006).

While much of the existing literature has focused on gonad development, a major knowledge gap remains regarding how formulated diets can be optimized to meet specific nutritional requirements for maternal investment and reproductive output. Evidence from *Pseudocentrotus depressus* indicates that carotenoids including: fucoxanthin, β-carotene, and β-echinenone positively affect egg production (Tsushlma et al. 1997; Kawakami et al. 1998). Similarly, in *Strongylocentrotus droebachiensis*, β-carotene enrichment enhanced gonad growth (15–50%), egg energy content (2–3%), increased the fertilization rate (up to 90%), and metamorphosis rate (up to 86%) compared to diets lacking carotenoids (Jong-Westman et al. 1995). In this context, pumpkin represents a promising ingredient for sea urchin diets aimed at enhancing reproductive performance. It is naturally high in β-carotene and provitamin A (Ninčević Grassino et al. 2023), and its processing by-products, including seeds (3.1–4.4%), peels (2.6–16%), and pulp (72–76%), contain high levels of antioxidants, polyphenols, and carotenoids (Gavril et al. 2024). Incorporating by-products aligns with the circular economy principles and offers a sustainable alternative for aquaculture feed production (De la Caba et al. 2019; Gavril et al. 2024). Support for this approach is evident in other taxa, where pumpkin inclusion in fish diets has enhanced pigmentation in ornamental carp (*Cyprinus carpio*) (Ayi et al. 2018) and improved growth, antioxidant capacity, and immune responses in Nile tilapia (*Oreochromis niloticus*) (Mounes et al. 2024).

While terrestrial ingredients such as lettuce and pumpkin are valuable sources of essential nutritional components, combining vegetable and marine ingredients may be a more effective strategy for supporting reproduction by also supplying key fatty acids. Both the concentration and composition of fatty acids are critical determinants of spawning success and larval performance (Zuo et al. 2022). In particular, omega-3 polyunsaturated fatty acids (n-3 PUFA) play an important role in maintaining membrane integrity, and the n-3/n-6 PUFA ratio is considered an indicator of egg and larval quality (Yanes-Roca et al. 2009; Faleiro and Narciso 2010; Pettersen et al. 2010). Specific n-3 PUFAs, including eicosapentaenoic acid (20:5n-3; EPA), have been shown to strongly influence reproductive performance, egg quality, and hatching success and larval survival in sea urchins (Liu et al. 2007; Carboni et al. 2012; Brink-Hull et al. 2022a,b; Gomes et al. 2022). Consequently,

increasing attention has been directed toward incorporating marine-derived ingredients that supply these fatty acids into formulated feeds across the industry.

Microalgae are emerging as a promising marine ingredient in this context (Sultana et al. 2022; Gharbi et al. 2023). For example, *Nannochloropsis* sp. is a good source of chlorophyll, and carotenoids including β-carotene, zeaxanthin, canthaxanthin, and astaxanthin (Gbadamosi and Lupatsch 2018; Zanella and Vianello 2020; Sultana et al. 2022) and is also a valuable source of EPA and other n-3 PUFAs (Zanella and Vianello 2020). It functional benefits have been shown across taxa, with the inclusion of *Nannochloropsis* sp at 15%, increased fecundity in guppies (*Poecilia reticulata*) (Sultana et al. 2022), and its' partial replacement of fish meal (7.8%) improved the antioxidant capacity of juvenile turbot (*Scophthalmus maximus* L.) (Qiao et al. 2019).

This study aims to optimize nutritional supplies to *P. lividus* by investigating whether dietary supplementation with *Nannochloropsis* sp. and pumpkin (*Cucurbita* sp.) as sources of carotenoid and n-3 PUFA, particularly EPA, will improve gonad development and oocyte quality. Sea urchins were fed a range of diets focusing on differing carotenoid and n-3 PUFA concentrations and profiles, using these dietary resources, and responses measured included gonad development, gonad fatty acid composition, and oocyte size.

Materials and methods

Urchin collection and experimental design

Sea urchins (*P. lividus,* n = 147) with a test diameter (TD) of 38.34 ± 0.35 mm (excluding spines) were collected by hand from the intertidal zone of Peniche, Portugal (39°19′N; 9°21′W) in December 2022. The urchins were randomly allocated to three recirculating aquaculture systems (RAS) at MARE-Polytechnic of Leiria aquaculture facilities (Peniche, Portugal). Each RAS system consisted of three 40 L replicate tanks, each containing 13 sea urchins, connected to a 250 L sump equipped with a protein skimmer, mechanical and biological filters. All individuals were measured for test diameter (TD; 0.1 mm) and whole wet weight (WW, 0.01 g), which were similar across tanks (TD: $F = 1.97$, $df = 2$, $p = 0.142$; WW: $F = 0.79$, $df = 2$, $p = 0.456$). Sea urchins were starved for one month (T0) to standardize their nutritional condition (Carboni et al. 2015; Prato et al. 2018). Tanks were cleaned and water exchanged as needed (see Seawater parameters).

Feeding trial

The urchins were then fed one of three experimental diets that differed in both ingredient matrix and pigment profile: 1) a formulated pumpkin/algal diet (PA) enriched with β-carotene via pumpkin (*Cucurbita* sp.) and *Nannochloropsis* sp.; 2) a formulated pea/corn diet (PC) with a low-pigment profile, and 3) a control diet containing *Saccorhiza polyschides*, which provided a mixed-pigment natural reference with relatively high total carotenoids (Kelp) (Table 1). The diets included other cost-effective and common ingredients for aquafeed, such as pea protein and corn bran, supplying the energy needed for growth and gonad development (Montero-Torreiro and Garcia-Martinez 2003; Marsh et al. 2013; Heflin et al. 2016). Both diets, PA and PC, were carefully balanced to maintain the same protein (isoproteic) and carbohydrate content (Table 1, based on percentage of dry matter-DM). This was achieved by adjusting the amounts of each ingredient, particularly by using pumpkin,

Table 1 Formulation (per 100 g) and proximate composition of the jellified diets (Pumpkin/algal diet—PA, Pea/corn diet—PC, and natural kelp diet—Kelp). Values are presented as mean ± standard deviation, SD (n = 3). Different superscript letters indicate statistically significant differences between diets ($p < 0.05$). n.d. means not detected

per 100 g	PA	PC	Kelp
Pea protein[1]	18.00	30.00	0
Corn bran[2]	0	50.00	0
Kelp [3]	8.50	8.50	93.50
Pumpkin [4]	40.00	0	0
Colza oil [5]	5.00	5.00	0
Nannochloropsis sp.[6]	22.00	0	0
Antioxidant [7]	0.50	0.50	0.50
Agar [8]	6.00	6.00	6.00
Proximate composition			
Dry matter (% DM)	17.94 ± 0.31	17.24 ± 0.65	17.41 ± 0.75
Protein (% DM)	37.34 ± 0.16	37.37 ± 1.36	7.43 ± 0.02
Carbohydrates (% DM)	23.79 ± 1.08	22.53 ± 1.17	9.73 ± 0.48
Lipids (% DM)	9.60 ± 0.17	21.21 ± 0.82	4.41 ± 0.26
Total Carotenoids (mg 100 g^{-1})	21.46 ± 0.91	1.63 ± 0.04	24.31 ± 0.09
β-carotene (mg 100 g^{-1})	11.78 ± 2.33	0.76 ± 0.13	9.06 ± 0.09
Ash (% DM)	1.40 ± 0.05	1.03 ± 0.03	7.86 ± 0.11
Fatty acid profile (% Total FA)			
SFA			
C11:0	0.14 ± 0.02	n.d	n.d
C12:0	0.05 ± 0.01	n.d	n.d
C13:0	0.42 ± 0.03^{a}	n.d	0.24 ± 0.16^{b}
C14:0	1.35 ± 0.05^{b}	0.22 ± 0.02^{c}	5.07 ± 0.37^{a}
C15:0	0.22 ± 0.02^{b}	0.12 ± 0.01^{c}	0.36 ± 0.05^{a}
C16:0	11.23 ± 0.12^{b}	9.61 ± 0.09^{c}	32.13 ± 0.87^{a}
C17:0	0.25 ± 0.01	0.25 ± 0.02	0.30 ± 0.06
C18:0	2.19 ± 0.09^{b}	3.02 ± 0.17^{a}	1.12 ± 0.09^{c}
C20:0	0.34 ± 0.04	0.57 ± 0.49	0.74 ± 0.02
C21:0	0.13 ± 0.01	0.27 ± 0.18	0.21 ± 0.18
C22:0	1.72 ± 0.06^{b}	0.29 ± 0.02^{c}	15.00 ± 0.92^{a}
C23:0	0.04 ± 0.01	0.02 ± 0.02	n.d
C24:0	0.16 ± 0.01^{ab}	0.18 ± 0.03^{a}	0.11 ± 0.01^{b}
∑ SFA	18.23 ± 0.12^{b}	14.56 ± 0.81^{c}	55.29 ± 1.39^{a}
MUFA			
C14:1n-5	0.19 ± 0.04	n.d	n.d
C15:1n-5	0.05 ± 0.03	n.d	n.d
C16:1n-7	5.44 ± 0.17^{a}	0.20 ± 0.02^{c}	2.27 ± 0.05^{b}
C17:1n-7	0.17 ± 0.01^{a}	0.07 ± 0.00	0.07 ± 0.06
trans C18:1n-9	0.07 ± 0.04	0.11 ± 0.03	0.10 ± 0.10
C18:1n-9	43.09 ± 0.76^{b}	49.99 ± 0.39^{a}	15.03 ± 0.23^{c}
C18:1n-7	2.03 ± 0.03^{a}	1.76 ± 0.07	1.83 ± 0.07
C18:1n-11	n.d	n.d	n.d
C20:1n-9	0.78 ± 0.07	0.85 ± 0.02	7.37 ± 0.56^{a}

Table 1 (continued)

per 100 g	PA	PC	Kelp
C22:1n-9	0.07 ± 0.02	0.03 ± 0.01	0.60 ± 0.07[a]
C24:1n-9	0.04 ± 0.01	0.05 ± 0.01	n.d
∑ MUFA	51.95 ± 0.59	53.06 ± 0.39	27.28 ± 0.65[a]
n-6—PUFA			
trans C18:2n-6	0.02 ± 0.00	0.01 ± 0.00	0.06 ± 0.06
C18:2n-6 (LA)	16.64 ± 0.07[b]	25.48 ± 0.68[a]	4.38 ± 0.21[c]
C18:3n-6	0.03 ± 0.00	0.03 ± 0.00	0.45 ± 0.02[a]
C20:2n-6	0.07 ± 0.01	0.03 ± 0.02	0.04 ± 0.06
C20:3n-6	0.10 ± 0.00[b]	0.01 ± 0.01[c]	0.69 ± 0.19[a]
C20:4n-6 (ARA)	0.26 ± 0.03	0.27 ± 0.04	n.d
C22:2n-6	n.d	0.03 ± 0.02	n.d
∑ n-6	17.14 ± 0.08[b]	25.85 ± 0.66[a]	5.62 ± 0.22[c]
n-3 – PUFA			
C18:3n-3 (ALA)	4.79 ± 0.10	6.29 ± 0.39[a]	4.82 ± 0.45
C20:5n-3 (EPA)	7.49 ± 0.44[a]	0.13 ± 0.03[c]	6.14 ± 0.40[b]
C22:5n-3	0.03 ± 0.01	0.05 ± 0.03	0.01 ± 0.02
C22:6n-3 (DHA)	n.d	n.d	n.d
∑ n-3	12.31 ± 0.54[a]	6.47 ± 0.45[c]	10.98 ± 0.87[b]
Other PUFA (%Total FA)			
C16:2n-4	0.08 ± 0.00[b]	0.02 ± 0.01[c]	0.69 ± 0.02[a]
C16:3n-4	0.03 ± 0.01[b]	n.d	0.16 ± 0.45[a]
C16:4n-1	0.26 ± 0.01[a]	0.04 ± 0.00[b]	n.d
∑ Other PUFA	0.11 ± 0.01[b]	0.06 ± 0.01[c]	0.85 ± 0.47[a]
∑ PUFA	29.93 ± 0.46[b]	32.44 ± 1.08[a]	18.28 ± 0.66[c]
n-3/n-6	0.72 ± 0.03[b]	0.25 ± 0.01[c]	1.96 ± 0.19[a]
DHA/EPA	-	-	-
EPA/ARA	29.42 ± 4.72[a]	0.51 ± 0.19[b]	-

[1]Pea protein 80 g/100 g CP, 9.5 g/100 g CF, 0.9 g/100 g CH Alma & Valor, Lda; [2]Corn bran 7.5 g/100 g CP, 3 g/100 g CF, 74 g/100 g CH Diet Import S.A; [3]*Saccorhiza polyschides* powder 14 g/100 g CP, 2 g/100 g CF, 44 g/100 g CH ((Pacheco et al. 2021); [4]Dried Pumpkin 9 g/100 g CP, 6 g/100 g CF, 50 g/100 g CH; [5]Colza oil 100 g/100 g CF Diet import S.A; [6]*Nannochloropsis* sp. powder 45–55 g/100 g CP, 15–20 g/100 g CF, 15–20 g/100 g CH Allmicroalgae; [7]Antioxidant (Vitamin E) Nekton Produkte; [8]Agar- agar (extracted from *Gelidium* spp) 0.4 g/100 g CP, 0.2 g/100 g CF, 79.8 g/100 g CH, Algamar

which, in addition to its carotenoid content, has a high carbohydrate content that helped to balance the carbohydrates provided by the corn bran. Colza oil, a vegetable oil, was selected as a lipid source because it is a viable alternative to fish oil (FO) due to its relatively stable supply and rich content of polyunsaturated fatty acids, particularly Linoleic acid (C18:2n6, LA) and alpha-linolenic acid (C18:3n3, ALA), which are vital for successful reproduction (Zuo et al. 2022). To maintain carotenoid consistency, the macroalgae *S. polyschides* were included as a standardized basal ingredient at an identical concentration (8.50 g per 100 g) in both the PA and PC formulations. Including the same amount of *S. polyschides* across the formulated diets (PA and PC) ensured a constant basal carotenoid contribution, isolating the experimental ingredients as the sole variable affecting gonad carotenoid deposition. The macroalgae were locally collected near the intertidal zone of Peniche, Portugal (39°19′N;

9°21′W) and rinsed with filtered and UV-treated seawater (FSW) to ensure all epifauna was removed before use. The macroalgae were dried in a ventilated oven (Memmert IF110, Wuppertal, Germany) at 40 °C for 48 h. Then, dried biomass was ground to 200 µm using a food processor (Breville VBL120X Personal Active Pro, Cheadle, UK) and a specialized grinder (SilverCrest SKMS 180 A1, Neckarsulm, Germany) to ensure a consistent nutrient profile for incorporation into the experimental diets (Ferreira et al. 2025). All ingredients were blended with agar since it is a promising binder for animal feed, creating firm, water-stable pellets that significantly minimize waste and nutrient loss in rearing environments (Fabbrocini et al. 2012; Volpe et al. 2018). A 6% agar concentration was utilized to achieve the desired pellets firmness and water stability (Klinger 1982). The diets were freshly prepared into 1 cm^3 blocks and stored at −20 °C to maintain their nutritional profile and prevent bacterial growth. Before being fed to the urchins, the frozen blocks were defrosted. The sea urchins were fed every two days, for two months, at 5% of their mean body mass (Gibbs et al. 2015). Food was weighed before feeding, and any uneaten portions were collected before the next feeding and re-weighed. Diets were readily and equally accepted by the sea urchins throughout the trial with consistent consumption across all treatments (PA: 1.36 ± 0.01 g $day^{-1}ind.^{-1}$; PC: 1.34 ± 0.02 g day^{-1} $ind.^{-1}$; Kelp: 1.37 ± 0.01 g day^{-1} $ind.^{-1}$, $F = 20.47$; $df = 2$, $p = 0.365$). Throughout the trial, the experimental diets were observed to have structural integrity without significant rehydration or fragmentation, ensuring consistent availability to the urchins.

The sea urchins were maintained on each experimental diet for two months, which aligns with the minimum duration required to observe a significant effect of nutrition on gonad development and the production of viable gametes (Suckling et al. 2014; Sepúlveda et al. 2021). To ensure that observed effects were exclusively due to the experimental diets, temperature and photoperiod (important environmental drivers of the reproductive cycle) were kept constant at 18 ± 0.2 ºC and a 12 h light: 12 h dark photoperiod (Sepúlveda et al. 2021) using fluorescent light sources at an intensity of 11.1 ± 0.12 µE m^{-2} s^{-1}. The feeding trial was conducted from January to March to capture the crucial transition from the early to mid-stages of gametogenesis when the impact of diet on the gonadosomatic index (GSI) is maximized. This experimental period is also aligned with the reproductive season of *P.lividus* along the Portuguese Atlantic coast, which typically begins in March (Rocha et al. 2019; Raposo et al. 2023).

Sea urchin sampling

At the experimental start ($n = 30$) and end ($n = 5$ per replicate, $n = 15$ per treatment), the sea urchins were measured for TD and WW and dissected for the gonad wet weight (GWW), used to achieve the gonadosomatic index (GSI), calculated as GWW divided by WW and expressed as a percentage. One gonad per individual was fixed in 4% formaldehyde for histological analysis (described in Histology analysis). Samples of four gonads per individual were freeze-dried and stored frozen (−80ºC) until biochemical analysis.

Histology analysis

Gonads were processed in an automated Leica TP1020 tissue processor (Leica Microsystems GmbH, Wetzlar, Germany) by sequential immersion in increasing graded ethanol solutions, followed by xylene for clearing. Subsequently, they were embedded in 100% (v/v) paraffin (Leica® EG 1120 Paraffin Dispenser with integrated Hot Plate; Leica

Microsystems GmbH, Wetzlar, Germany) into blocks at 60 ºC. Then, sectioned at 5 µm thickness transverse to the longitudinal axis using an Accu-Cut SRMTM 200 rotary microtome (Sakura Finetek Europe BV, Netherlands). Sections were stained with Harris' hematoxylin and eosin and observed under a Leica DM 2000 LED light-optical microscope (Leica, Wetzlar, Germany) equipped with a Leica MC170 5MP HD microscope camera (Leica) and combined LAS V4.4.0 (Leica Application Suite) image analysis software (Leica). Gametogenic stages were classified into six stages according to Byrne (1990): recovery (stage I), growing (stage II), premature (stage III), mature (stage IV), partly spawned (stage V), and spent (stage VI).

Biochemical analysis

Biochemical analyses were conducted to characterize the nutritional profile of the experimental diets and to quantify the incorporation of dietary components into sea urchin gonad tissue. The samples analysed included freeze-dried diets (three independent 4 g replicates per diet) and pooled triplicates of freeze-dried gonads per treatment. Only female gonads were processed to specifically assess maternal provisioning effects on gonad development and oocyte quality. The analyses comprised the quantification of total protein, lipid, and carotenoid contents, as well as the characterization of fatty acid profiles. β-Carotene content was determined only in the diets. Due to limited biomass, only total protein, lipid, and carotenoid contents were assessed in T0 gonads.

Protein content in diets (% DM) was estimated from total nitrogen, quantified by the Kjeldahl method using 0.5 g samples and a conversion factor of 6.25, following AOAC (940.25, 2016), as described by Neves et al. 2021. Protein content in sea urchin gonads was determined using the method of Lowry et al. (1951), adapted to microscale according to Lourenço et al. (2021). Briefly, samples (30 mg) were extracted by sonication in 0.1 M NaOH (10 mL), incubated overnight at 4 °C, and centrifuged, after which the supernatant was collected and appropriately diluted. Samples and bovine serum albumin (BSA) standards (33.3–233 µg mL^{-1} in 0.1 M NaOH) were subjected to the Lowry color reaction, and absorbance was measured in triplicate at 750 nm in 96-well flat-bottom microplates using a Synergy H1 Hybrid Reader. Results were expressed as % DM.

Carbohydrate content in both diets and gonads was determined using the colorimetric method of Dubois et al. (1956), with modifications described by Lourenço et al. (2021). In summary, samples (30 mg) were hydrolyzed in 1 M H_2SO_4 at 90 °C for 60 min, and the volume was then adjusted to 10 mL. Hydrolysates and glucose standards (0.04–0.8 mg mL^{-1}) were reacted with 97% H_2SO_4 and phenol, and absorbance at 490 nm was recorded in triplicate. Carbohydrate content was expressed as glucose (% DM).

Total lipid content in diets was quantified using the gravimetric method of Folch, as modified by Neves et al. (2021), and expressed as % DM. Total lipid content in gonads was quantified using the spectrophotometric method of De Coen and Janssen (1997), with adaptations described by Lourenço et al. (2021). Briefly, samples (30 mg) were extracted with a mixture (1.5 mL) of chloroform:methanol: water (1:1:1). The recovered lipid phase was reacted with sulfuric acid for colour development, and absorbance was measured in triplicate at 375 nm. Tripalmitin solutions (0–2.6 mg mL^{-1} in chloroform) were used to construct the calibration curve. Lipid content was expressed as % DM.

For characterization of fatty acid (FA) profiles of both diet and urchin gonad, FA methyl esters (FAME) were obtained by acid transmethylation of samples (50 mg),

recovered in hexane, and then analyzed by gas chromatography using a flame ionization detector (GC-FID) in the same conditions described by Lourenço et al. (2021).

Fatty acid (FA) profiles of both diets and gonads were analysed by gas chromatography with flame ionization detection (GC-FID). Fatty acid methyl esters (FAME) of samples (50 mg) were obtained by acid transmethylation, extracted in hexane (2 mL) and analyzed under the chromatographic conditions described by Lourenço et al. (2021). FAME identification was performed by comparing retention times with those of certified standards (SUPELCO 37, PUFA No. 1 and PUFA No. 3, SUPEPCO, Bellefonte, PA, USA). Fatty acids were expressed as a percentage of total fatty acids (% total FA).

To quantify total carotenoids (TC) concentration, samples of diets (100 mg) and gonads (30 mg) were extracted with 80% acetone under stirring (3 min), and the supernatant was collected after centrifugation at 10,000 rpm for 5 min at 4 °C. Extractions were repeated until the supernatant became colorless. The combined supernatants were brought to a final volume of 10 mL. For diets extracts, due to the mixture of different pigments, absorbance was measured at 663.2, 646.8, and 470 nm. The concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), and TC were calculated using the equations of Lichtenthaler and Buschmann (2001). Pigment contents were expressed as mg100 g^{-1} DM.

$$Chla\left(\mu g\ mL^{-1}\right) = 12.25 * A_{663.2} - 2.79 * A_{646.8} \tag{1}$$

$$Chlb\left(\mu g\ mL^{-1}\right) = 21.50 * A_{646.8} - 5.10 * A_{663.2} \tag{2}$$

$$TC\left(\mu g\ mL^{-1}\right) = \left(1000 * A_{470} - 1.82 * Ca - 85.02 * Cb\right)/198 \tag{3}$$

For gonads extracts, since their pigment content is exclusively carotenoids, the absorbance was measured at 450 nm and total carotenoids were determined in acetone using an extinction coefficient of 2500 L mol cm^{-1} (Tsushima et al. 1993). The concentration of TC was calculated using the equation provided by Pocock et al. (2004) and expressed as µg g^{-1}.

$$TC\left(\mu g\ g^{-1}\right) = (E \times V \times 1000)/[100 \times M \times (E1\%1cm)] \tag{4}$$

where: E is the absorbance of a known volume of carotenoid extract read at 450 nm; V is the sample volume; E1%1 cm is the extinction coefficient of 1% (w:v) solution in a 1 cm cuvette at 450–451 nm; M is the dried sample weight (g).

β-Carotene quantification in diets was performed by extracting 100–200 mg samples with 10 mL of an ethyl acetate: ethanol: water (45%:45%:10%) solution. The extracts were analyzed using a High-Performance Liquid Chromatography (HPLC) system comprising an Alliance 2695 separation module (Waters, Milford, USA) and a Waters 996 photodiode array detector. Chromatographic separation was achieved using a reverse-phase column (YMC Carotenoid, 250 × 4.6 mm I.D., S-5 µm, Japan) at room temperature, with gradient elution consisting of solvent A (methanol: water, 75:25) and solvent B (ethyl acetate). The gradient started at 70% A (0 min), followed by a linear transition to 10% A (20 min), and was returned to the initial condition by 40 min. The flow rate was 0.80 mL min^{-1}, and detection was performed at 450 nm. Quantification was based on an external standard curve of increasing β-carotene concentrations (0–20 mg mL^{-1} in an ethyl acetate: ethanol: water mixture). Results were expressed as mg 100 g^{-1} DM.

Proximate composition and fatty acid dietary profiles are provided in Tables 1, and for females, gonad tissue is presented within the results section (Table 4; Table S1, see supplementary material).

Sea urchin spawning

At the end of the experimental period, a subset of 15 individuals per treatment (n = 5 per replicate) was randomly selected and chemically induced to spawn following the methods described by Gomes et al. (2021). In summary, urchins were injected with 0.5 M potassium chloride in the proportion of 40 µl g^{-1} WW, into the coelom through the peristomial membrane, until a ratio of five females and three males was achieved. Oocytes from each female of each feeding treatment were collected in 200 mL beakers containing FSW for the determination of oocyte average diameter (µm) and preserved in 2% formalin in 0.01 M phosphate-buffered saline at pH 7.4 (Wong et al. 2019; Chamorro et al. 2023). The eggs were then photographed at 200 × magnification using a compound microscope Axio Lab.A1 (Carl Zeiss, Germany) equipped with a Zeiss AxioCam MRc3 camera (Carl Zeiss) and oocyte size (n = 60 oocytes per female; total n = 300 per treatment) was measured using Zen 2.6 Lite software (Carl Zeiss). Sperm was collected dry, directly from the surface of the individuals, by pipetting into 1.5 mL microcentrifuge tubes and stored at 4 °C (Morroni et al. 2016). Before fertilization, gamete quality was visually inspected under a microscope, assessing sperm (e.g., movement, density, color) and oocyte (e.g., shape, color) quality. These parameters are established indicators of high-quality gametes for achieving high fertilization rates (Minuti et al. 2022). To ensure a balanced maternal representation within the pool while maintaining the speed necessary for high oocyte viability, a standardized volumetric approach was used. Specifically, 200 mL of concentrated oocyte suspension from each female was pooled immediately following release. Gametes were then fertilized by the broodstock source to ensure conspecific crosses (e.g., PA oocytes crossed with PA sperm). This process took place in 5L beaker cups filled with FSW and gently aerated. Oocyte concentration was determined using a Sedgewick Rafter counting chamber on a microscope at 200 × magnification. Sperm concentration was determined using a Neubauer counting chamber at 400 × magnification. Based on these concentrations, sperm were added to ensure a ratio of 50 spermatozoa: 1 oocyte (Morroni et al. 2016). Fertilization success was assessed for each batch 2 h after fertilization started, by determining the percentage of oocytes that had developed a visible fertilization membrane. This was quantified by counting the fertilized oocytes within a 1 mL sample volume (in triplicate) using a Sedgewick-Rafter counting chamber.

Seawater parameters

Tank water parameters were measured daily using a YSI Professional Plus (Pro Plus) handheld multiparameter meter (YSI Incorporated, Yellow Springs, USA). Seawater parameters did not differ across all treatments (Table 2). Nitrogenous products (e.g., ammonia, nitrites, nitrate) were measured daily with API test kits before and after a 10% water change, and levels were maintained at 0 mg L^{-1} across all treatments.

Table 2 Mean (±SD) seawater temperature, salinity, pH, and dissolved oxygen levels for sea urchins (*Paracentrotus lividus*) fed with three jellified diets (Pumpkin/algal diet—PA, Pea/corn diet—PC, and natural kelp diet – Kelp) for two months

Diet treatment	Temperature (°C)	Salinity (ppt)	pH (mg L^{-1})	Oxygen (mg L^{-1})
PA	18.32 ± 0.22	33.41 ± 1.21	8.11 ± 0.14	8.02 ± 0.22
PC	18.16 ± 0.39	33.45 ± 1.20	8.13 ± 0.12	8.01 ± 0.25
C	18.28 ± 0.25	33.43 ± 1.22	8.15 ± 0.12	8.04 ± 0.22
Statistical results	F = 17.93, df = 2, P = 0.405	F = 11.51, df = 2, P = 0.978	F = 14.40, df = 2, P = 0.673	F = 19.18, df = 2, P = 0.510

Statistical analysis

Data analysis was performed in the R environment (R version 4.3.2) (R Core Team 2023). All results are expressed as mean ± standard deviation (SD). The effect of broodstock diets on sea urchin growth (TD, WW, and GSI) and biochemical profile (protein, lipids, carbohydrates, pigments, and FA) were tested using a one-way analysis of variance (ANOVA). Data was first tested for normal distribution assumptions using the Shapiro–Wilk test and homogeneity of variances using Levene's test. If the assumptions were not met, the non-parametric Kruskal–Wallis test was applied. Whenever statistically significant differences were found, multiple pairwise comparisons between groups were performed using the post-hoc Tukey's honestly significant difference (HSD) test or the non-parametric Games-Howell test when assumptions of normal distribution and homogeneity of variances were not fulfilled (Zar 2010). The effect of the broodstock diets on fertilization rate and oocyte diameter was tested by an independent *t*-test. Whenever the assumptions of normality and homoscedasticity of the data were not met, the Wilcoxon rank-sum test or the Welch test was applied.

To assess the effect of the broodstock diets on gonad development was assessed by Pearson's chi-square test (Zar 2010). The FA content with a mean concentration higher than 1% Total FA was normalized by log (x + 1) transformation. The normalized FA matrix was then used to evaluate the main effect of the experimental diets through MANOVA. Principal component analysis (PCA) was then performed on the FA correlation matrix to evaluate which fatty acids were more influential in female gonads. A significance level of $P < 0.05$ was used for all analyses.

Results

Sea urchin responses

There was full survival of urchins across all dietary treatments. No significant differences were found across treatments for test diameter (TD; F = 0.63, df = 3, P = 0.596; Fig. 1A), or individual wet weight (WW; H = 5.81, df = 3, P = 0.121; Fig. 1B). For the GSI, the groups fed PA and PC diets showed significant increases from the baseline (T0) by two-fold and three-fold, respectively (H = 53.43, df = 3, P < 0.001; Fig. 1C).

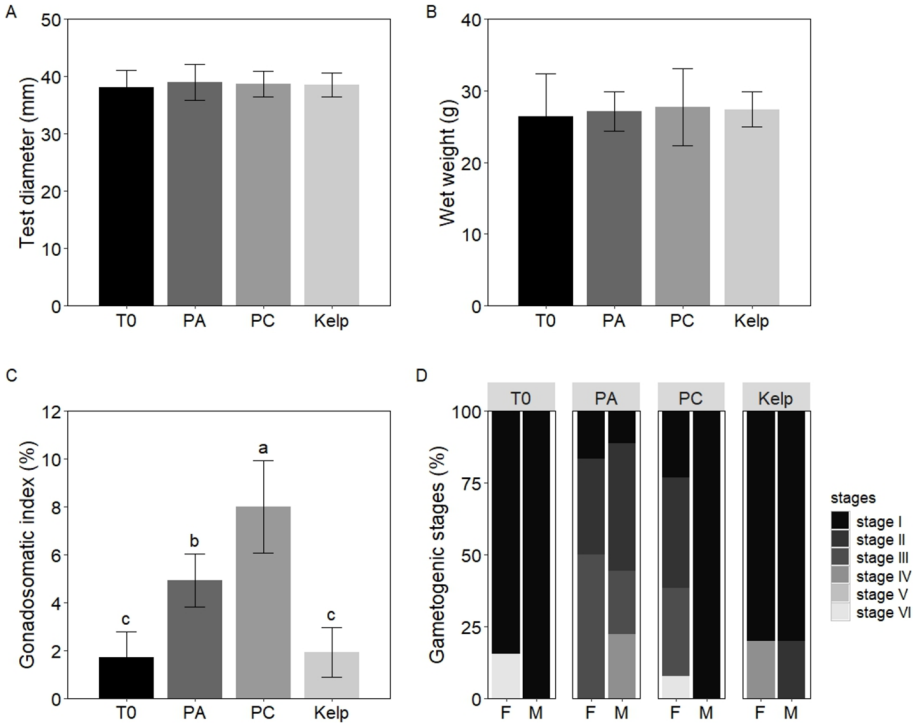

Fig. 1 Mean (±SD) test diameter (**A**), wet weight (**B**), gonadosomatic index (**C**), and proportional distribution of gonad gametogenic stages (**D**) of *Paracentrotus lividus* broodstock after two months of feeding with differing diets. T0 = Baseline, PA = Pumpkin/algal diet, PC = Pea/corn diet, Kelp = natural kelp diet. F represents the females and M the males. Different superscripts indicate statistically significant differences between experimental treatments ($P < 0.05$)

A significant association was observed between gametogenic stage and diet ($\chi 2 = 18.69$, df = 8, $P = 0.01$; Fig. 1D), with sea urchins fed PA and PC diets showing more advanced gametogenic stages than the Kelp diet. For example, 50% of the females fed with PA were at stage III, and 22% of males were at stage III, and another 22% males were at stage V. Approximately a third of females fed the PC diet were at stage III and stage II, and 23% at stage I, but all males remained at stage I. The Kelp diet yielded urchins mostly at stage I, with only 20% of females at stage IV and 20% of males at stage II.

When the urchins were induced to spawn, only those fed the PA and PC diets successfully released gametes. The individuals from the Kelp diet did not spawn, so only PA and PC oocyte sizes and fertilization success data were analyzed. The sea urchins fed the PA diet yielded oocytes that were 18% larger and exhibited a 6.62% higher fertilization rate compared to sea urchins fed the PC diet (Table 3).

Urchin proximate composition and FA profiles

The proximate composition of the gonads from female sea urchins was affected by the nutritional content of the experimental diets (Table 4). Females fed the PA diet exhibited the highest protein levels, showing a 2–5% increase compared to other dietary treatments

Table 3 Mean (±SD) oocyte diameter (µm) and fertilization success (%) of *Paracentrotus lividus* broodstock fed with Pumpkin/algal diet (PA) and Pea/corn diet (PC) for two months. * Indicates statistically significant differences between diets ($p < 0.05$)

	PA	PC	*t*-student
Oocyte diameter (µm)	110.30 ± 8.83 *	93.12 ± 0.61	t = 16.70; df = 185.7, P < 0.001
Fertilization rate (%)	98.61 ± 0.51 *	91.99 ± 5.93	t = 11.30; df = 8, P < 0.001

and a 12% increase relative to the T0 group. Similarly, lipid levels in the PA diet were 1 to 4% higher in relation to the other dietary treatments. Females fed with the PC diet exhibited the lowest lipid content but had the highest carbohydrate content, which was up to 23% higher than the PA diet. The highest carotenoid content (80.58 ± 4.37 µg g^{-1}) was found in the gonads of females from T0, followed by the Kelp diet.

The fatty acid (FA) profiles of female gonads varied significantly among the dietary treatments (Fig. 2A, B; Table S1, see supplementary material). Females fed the PA diet were strongly correlated with palmitoleic acid C16:1n-7 (5.64%), eicosadienoic acid (C20:2n-6, 2.12%) and eicosapentaenoic acid (EPA, 12.59%). Higher levels of myristoleic acid (C14:1n-5, 1.21%) and γ-Linolenic acid (C20:3n-6, 1.13%), along with the lowest EPA (3.68%), characterized females fed the PC diet. In addition, females fed the Kelp diet had higher levels of stearic acid (C18:0, 3.63%), arachidic acid (C20:0, 1.26%), vaccenic acid (C18:1n-7, 3.29%), 9-eicosenoic acid (C20:1n-9, 4.42%), erucic acid (C22:1n-9, 5.10%) and arachidonic acid (ARA,13.10%) (Fig. 2A). The principal component biplot (Fig. 2B) revealed that total PUFA (40.27%) and the EPA/ARA ratio (1.62) were with higher abundance in females fed the PA diet, while high n-6 PUFA (21.23%) was correlated with females fed the PC diet. Furthermore, total SFA (38.30%) and other PUFA (3.06%) were correlated with higher abundance in females fed the Kelp diet. Sea urchins fed PA and Kelp diets had a higher n-3/n-6 ratio, indicating a greater contribution of n-3 PUFA to their fatty acid profile.

Discussion

The availability and quality of food are primary drivers of sea urchin gonad development and the size/quality of oocytes, both of which are crucial for successful reproduction (Pearse and Cameron 1991; Jong-Westman et al. 1995; Bertram and Strathmann 1998; George et al. 2001; Byrne et al. 2008). The primary objective of this study was to evaluate the efficacy of sustainable formulated diets, specifically incorporating pumpkin and *Nannochloropsis* sp., as drivers of reproductive success and maternal investment in *P. lividus*. Our results demonstrate that PA and PC experimental diets significantly increased the GSI and advanced gametogenic stages, leading to successful oocyte production compared to the Kelp diet. This suggests that the higher protein (37%) and carbohydrate (23%) content of these formulated diets allowed more efficient energy allocation toward reproductive tissues than what is achievable with macroalgae alone (Pearce et al. 2002; Eddy et al. 2012). These findings align with previous research indicating that replacing macroalgae with pea-based products (e.g., pea flour, pea meal) and microalgae enhances GSI (McLaughlin and Kelly 2001; Fabbrocini et al. 2015; Zupo et al. 2019). However, while both formulated diets (PA and PC) increased GSI, they produced markedly different outcomes regarding gonad nutritional profile, oocyte size, and fertilization success.

Table 4 Proximate composition of *Paracentrotus lividus* female gonads determined at the beginning of the experiment (T0) and after the feeding trial with three jellified diets (Pumpkin/algal diet—PA, Pea/corn diet—PC, and natural kelp diet – Kelp). Values are presented as mean ± standard deviation, SD (n = 3). Different superscript letters indicate statistically significant differences between diets ($p < 0.05$)

	Experimental diets				
	T0	PA	PC	Kelp	Statistical results
Protein (% DM[1])	23.89 ± 0.48^{d}	35.64 ± 0.30^{a}	30.78 ± 0.30^{c}	33.84 ± 1.02^{b}	$H = 6.17$; $df = 3$, $p < 0.001$
Lipids (% DM[1])	6.58 ± 0.51^{d}	10.70 ± 0.28^{a}	7.14 ± 0.40^{c}	9.01 ± 0.23^{b}	$F = 24.44$; $df = 3$, $p < 0.001$
Carbohydrates (% DM[1])	-	15.38 ± 0.79^{b}	38.44 ± 1.13^{a}	-	$t = 28.90$; $df = 4$, $p < 0.001$
Total Carotenoids (μg g^{-1} DM [1])	80.58 ± 4.37^{a}	30.94 ± 0.41^{c}	16.13 ± 0.48^{d}	34.44 ± 0.77^{b}	$H = 25.18$; $df = 3$, $p < 0.001$

[1] DM stands for dry matter

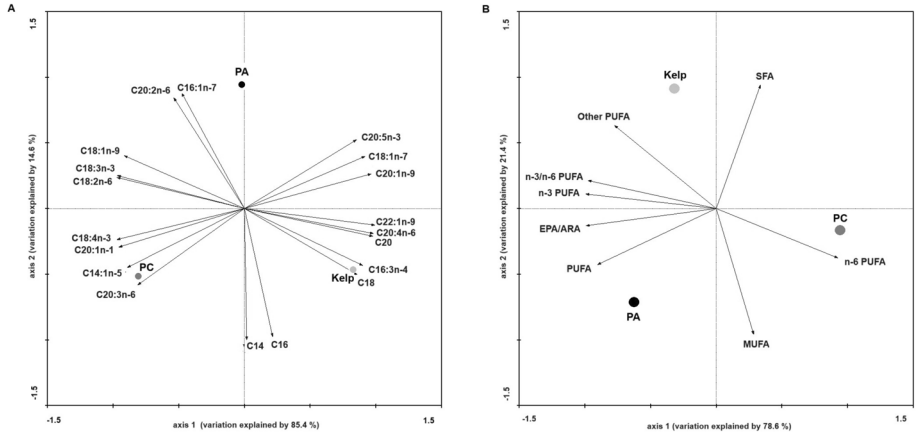

Fig. 2 (**A**) Biplots of Principal Component Analysis of Correlation (PCA) based on the fatty acid profile and (**B**) Biplots of Principal Component Analysis of Correlation (PCA) based on total saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids and EPA/ARA and n-3/n-6 ratios of female gonads fed with three jellified diets (Pumpkin/algal diet—PA, Pea/corn diet—PC, and natural kelp diet – Kelp)

Specifically, females fed with the PA diet showed a significant accumulation of proteins (up to 36%) and lipids (10%) within their gonads, characterized by particularly high concentrations of PUFA and EPA. This is probably due to the influence of the microalga *Nannochloropsis* sp., which is a key component of this diet and is known to be rich in proteins (30% of proteins DW) (Scaglioni et al. 2018) and lipids, particularly EPA (approximately 39% DW) (Kent et al. 2015). This nutrient accumulation in the gonads directly influenced oocyte production, with urchins fed with this diet showing the largest oocytes (91–110 µm) and high fertilization rates, exceeding previous reports for wild *P. lividus* (80–90 µm). Larger eggs are associated with higher metabolic energy reserves and faster larval development (Strathmann et al. 1992; McAlister and Moran 2012). Furthermore, these results align with theoretical models that suggest larger oocytes increase the target area for sperm, thus increasing the fertilization success (Strathmann et al. 1992; Levitan 2006; McAlister and Moran 2012). This PUFA accumulation is particularly important for species like *P. lividus*, which have a specific dietary need for high levels of n-3 PUFA, including EPA, for gamete formation and to fuel the critical early stages of development (Izquierdo et al. 2001; Sui et al. 2009; Liu et al. 2007; Arafa et al. 2012; Carboni et al. 2012; Swanson et al. 2012; Li et al. 2021; Zuo et al. 2022). The gonads from PA treatment also showed high n-3/n-6 (1.15) and EPA/ARA (1.62) ratios, which are correlated with improved oocyte size and higher fertilization rate. This finding aligns with those of Jaya-Ram et al. (2008), who reported that higher n-3/n-6 ratios were associated with improved egg quality and fertilization rates in zebrafish (*Danio rerio*). The efficacy of the PA diet, which provided 21.46 ± 0.92 mg 100 g^{-1} of total carotenoids and 11.78 mg 100 g^{-1} of β-carotene, was further corroborated by the significant accumulation of carotenoids (30.94 µg g^{-1}) in the female gonads. This concentration is likely derived from the synergistic inclusion of pumpkin and *Nannochloropsis* sp., both of which are rich sources of carotenoids (Ambati et al. 2019; Buzigi et al. 2022; Ninčević Grassino et al. 2023). As previously mentioned, carotenoids, including pigments such as β-carotene, are well-documented to enhance oocyte production and fertilization success in sea urchins. Carboni et al. (2015) showed that

β-carotene supplementation in *P. lividus* resulted in a 10% improvement in fecundity, highlighting the crucial role these compounds play in reproductive success. While the present study did not quantify gonad β-carotene content due to limited gonad biomass, its inclusion in future analyses will further clarify the direct relationship between dietary intake and reproductive performance.

Despite females fed the PC diet having a significantly enhanced GSI, this was likely driven by high lipid content (21%) and the influence of corn (Sartori et al. 2016; Wang et al. 2025). However, this increased energy allocation toward GSI did not translate into successful reproduction. These females produced significantly smaller oocytes with lower fertilization rates, which can be attributed to the lack of carotenoids and the low concentration of n-3 PUFA in their gonads. These results were also observed by White et al. (2016) in a 78-day nutritional trial with the sea urchin *Heliocidaris erythrogramma*, where low dietary n-3 PUFAs led to smaller oocytes in females and non-viable sperm in males. This is because n-3 PUFAs are essential for membrane integrity and provide the necessary energy for embryonic development. (Zuo et al. 2022).

The Kelp diet resulted in the lowest GSI (2%), the slowest gonadal development among the dietary treatments, and failure to produce oocytes and sperm. This aligns with previous findings that a diet solely composed of kelp often fails to provide the comprehensive nutrition, specifically an optimal protein-to-energy ratio, required for rapid gonadal development (Pearce et al. 2002, 2004; Phillips et al. 2010; Heflin et al. 2012; Lawrence et al. 2013; Zuo et al. 2017; Prato et al. 2018; Lourenço et al. 2021; Ning et al. 2022). While macroalgae are a natural food source, their lower nutrient density compared to formulated feeds do not support a fast and energy-intensive process of oogenesis. From a commercial hatchery perspective, the low protein-to-energy ratio of macroalgae slows the gametogenic cycle, which often conflicts with the need for rapid and high-yield reproductive output. Although *P. lividus* reproduces successfully on macroalgae in the wild, the slow rate of gonad development is insufficient to meet the economic objectives of hatchery production.

Furthermore, it is important to emphasize that the nutritional profile of the kelp (*S. polyschides*) used in this study reflects the seasonal environmental fluctuations of the temperate region where the broodstock was collected. While these results reflect the natural variability of wild-harvested biomass, land-based aquaculture systems offer the capacity to control key environmental variables (e.g., photoperiod, temperature), thereby mitigating the seasonal declines in pigments and PUFAs often observed in the field (Sebök et al. 2017; Schmitz and Kraft 2022). Consequently, feeding sea urchins with wild-harvested macroalgae appears insufficient for short-term conditioning, where maximizing reproductive output and advancing the gametogenic cycle are primary objectives.

Overall, a common FA pattern was observed in female gonads from all treatment groups, with a greater abundance of PUFAs (36–40%), followed by SFA (31–38%) and MUFAs (22–28%), aligning with previous studies (Cruz-García et al. 2000; Prato et al. 2018). Palmitic acid (C16:0) was the primary SFA, while oleic acid (C18:1n-9) and eicosenoic acid (C20:1n-11) represented the majority of MUFAs. Linoleic acid (LA), arachidonic acid (ARA), and EPA were the predominant PUFAs, confirming earlier observations in *P. lividus* gonads (Liyana-Pathirana et al. 2002; Volpe et al. 2018). The presence of ARA in gonads, despite its absence in the Kelp diet, suggests that sea urchins are capable of endogenous PUFA synthesis. Furthermore, the elevated levels of both ARA and EPA in *P. lividus* female gonads indicate active biosynthesis from LA and ALA via the Δ8 pathway (Carboni et al. 2013; Kabeya et al. 2017; Li et al. 2021; Zuo et al. 2022). Regarding carotenoid concentration, it was significantly higher in the gonads of the initial samples compared to those that were fed the experimental diets. This elevated concentration is likely a

function of the low GSI observed in the initial group. In sea urchins, total carotenoid levels typically peak immediately following spawning when gonad biomass is at its minimum. At this stage, pigments are highly concentrated within a small tissue volume (Symonds et al. 2007). As the gonads transition into mature stages, the rapid accumulation of nutritive phagocytes and gametes leads to a decrease in the concentration of carotenoids as total biomass increases. This relationship between gonad size and carotenoid concentration is consistent with previous observations in *P. lividus* (Symonds et al. 2007) and across several species of the genus *Strongylocentrotus* (Lamare and Hoffman 2004).

The results of this study suggest that broodstock nutrition can strongly enhance reproductive performance beyond natural baselines (Su et al. 2017; Campos 2024) and feeding *P. lividus* with a PA diet positively impacted gonad development and oocyte production. These results highlight the clear advantages of incorporating *Nannochloropsis* sp. powder into sea urchin diets. As a rich source of essential proteins, lipids, and carotenoids, this microalga likely enhanced maternal investment, directly fueling gonad development and oocyte production during the conditioning period. It is worth mentioning that, despite pumpkin being a cost-effective and sustainable feed alternative to synthetic carotenoids, alone it does not provide all the other necessary nutrients for sea urchins. Unlike *Nannochloropsis* sp., pumpkin lacks the high-quality protein and essential FA required to fuel the high metabolic demands of gametogenesis. This nutritional gap explains why the GSI in PA diet was low in relation to the GSI obtained for the PC diet. A similar trend was observed with the kelp diet; although it resulted in gonads with high carotenoid concentrations, the lack of an optimal protein-to-energy ratio slowed gonad development. Consequently, while pumpkin and kelp are excellent natural pigment sources, diets must be supplemented with high-quality protein and lipid sources to fulfill all the nutritional requirements.

Future research should focus on the development of broodstock diets, testing a variety of other cost-effective ingredients to improve reproductive performance. Optimizing maternal feeding strategies is critical for enhancing the gonad nutritional content, which in turn significantly influences oocyte production, embryo, and larval development. While n-3 PUFAs and carotenoids have been identified as key nutrients, future studies must investigate the specific biochemical pathways that enhance reproductive success. Specifically, research should explore how these dietary components modulate gene expression related to development and contribute to proper larval growth and survival. Additionally, while this study focused on maternal investment, evaluating paternal contributions, specifically sperm quality and motility, under these formulated diets, is the necessary next step to fully understand their impact on reproductive success.

Conclusion

Findings from the present study confirm that dietary nutrient availability and quality are fundamental drivers of reproductive success in *P. lividus*. The PA diet, enriched with *Nannochloropsis* sp. and pumpkin, proved to be the best broodstock formulation, yielding the largest oocytes and the highest fertilization rates. This success is attributed to a synergistic nutritional profile – comprising proteins, energy, n-3 PUFAs, and carotenoids—which provide the essential metabolic reserves required for maternal investment. From a hatchery perspective, these improvements in oocyte size and fertilization success represent a significant advancement for profitable sea urchin aquaculture, as consistent output relies entirely on the production of high-quality fertilized eggs. By implementing

the PA diet, hatcheries can transition from relying on seasonal, wild-collected macroalgae to a controlled, year-round production cycle. This transition ensures a supply of high-quality oocytes for larval rearing, significantly enhancing the economic viability.

In contrast, while the PC diet increased the GSI, which can be beneficial for market purposes, it failed to support successful reproduction due to a deficiency in n-3 PUFAs and carotenoids. Similarly, the Kelp diet was inadequate for broodstock conditioning, as it lacked the necessary protein-to-energy ratio to drive rapid gonadal development. Ultimately, these results highlight that a diet that increases GSI is not necessarily optimized for reproduction, and a diet balanced with microalgae and carotenoid-rich vegetables provides the specific nutrients required for successful gametogenesis.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflicts of interest The author HC is employed by the company Allmicroalgae-Natural Products SA. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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