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Exploitation of food processing discards as main ingredients of sustainable feeds for the aquaculture of *Paracentrotus lividus*

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General content

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ABSTRACT:

Sea urchin gonads are considered a delicacy in many parts of the world, leading to an increase of fishing pressure and resulting in the decline of natural stock of commercially valuable species. Aquaculture could be the best solution to fill the gap between market demand and natural supply, but in a context of Sustainable Development and Blue Growth, aquaculture sector is called to reduce its ecological footprint. Aquaculture feeds are produced using ingredients obtained from natural resources already overexploited by human activities, such as fish meal and fish oil that are considered the best source of lipids, or cereal meals that are included as protein sources. In this context, in order to propose a more sustainable approach, increasing attention has been focused on the formulation of alternative feeds, with low environmental and economic impacts, for the two main field of sea urchin aquaculture: gonad enhancement of wild specimens and full-cycle production, from larval development until the achievement of the commercial size. Here, sustainable diets obtained using food processing discards as main ingredients were produced and tested as feed for the purple sea urchin *Paracentrotus lividus* (Lamarck, 1816), the most important commercial sea urchin in the Mediterranean Sea.

A first short-term feeding experiment was conducted to assess feed stability in seawater, testing two different amounts of binder on formulated feed. The nutritional value of the main ingredients and feed formulations was assessed, and then a bioenergetics trial was conducted to test feed suitability for *P. lividus*. Findings confirmed the goodness of the proposed formulations for sea urchins, due to their satisfactory stability in seawater, palatability and adequate nutritional value.

In the next step, two of the four proposed formulations tested in the previous experiment, were selected to feed adult *P. lividus* for three months, aiming to evaluate gonad development, biomass increase and quality. Results highlighted a progression in sea urchin sexual maturation and a significant rise in gonad biomass coupled with a consistent increase of the gonad somatic index. Finally, sea urchins fed with the experimental feed formulations presented high quality gonads, thanks to a marketable colouration.

A third long-term feeding experiment was conducted on adult females of *P. lividus* reared using five diets: four composed mainly of lettuce discards and one composed of macroalgae, considered their natural food. Diet performance was evaluated employing a new productive protocol, the Raking method, which allows testing the effects of experimental diets on egg production rather than in gonad production, without sacrificing the experimental sea urchins. Thus, ingestion, absorption efficiency, somatic growth, egg production and quality were assessed to evaluate the suitability of the five

experimental diets. Findings showed that the lettuce-based diets resulted more effective than algal diet, presenting an adequate nutritional content, promoting increase in total weight and egg production, and presenting a better egg colour.

Finally, the effect of the five experimental diets on larval development was assessed, using stable isotope and fatty acid analyses to secure dietary nutrient allocation in produced eggs, with the aim to extend the information of nutrient needs for the development of a full cycle production system. Results of SIA and FA confirm diet assimilation, with significant changes in egg composition according with the respective diet. The analysis on larval development showed a lower efficacy of lettuce-based diets than the algal diet, which promoted a faster and more efficient larval development.

The three experiments allowed a comprehensive evaluation of the validity of sustainable diets and feeds for *P. lividus*, presenting encouraging results. Findings suggest that the nutritional content of the diets used allows the production of gonads of adequate size and quality to meet market demands, thus making them suitable for the practice of gonad enhancing. On the other hand, they do not seem to meet the correct composition to produce gametes able to sustain effective and complete larval development.

CHAPTER 1: Introduction

Echinoids (Echinoidea), one of the five classes of the phylum Echinodermata, are heavily exploited due to their high market value. Sea urchins are appreciated for their gonads, from both male and female specimens, which are the eaten part of the organism. Gonads, usually called also roe have been considered a delicacy in Asian countries since ancient times while today they are growing in worldwide popularity as a luxury food and a source of bioactive compounds (Andrew et al., 2002; Rubilar and Cardozo, 2021; Stefánsson et al., 2017). From the nutritional point of view, sea urchin gonads are rich in carotenoids, phospholipids and polyunsaturated fatty acids (PUFAs), mainly arachidonic (ARA) and eicosapentaenoic acids (EPA) (Dincer and Cakli, 2007; González-Durán et al., 2008; Martínez-Pita et al., 2010a), which have a positive effect on human health, in particular for the prevention of hypertension, cardiovascular diseases, inflammation, arrhythmias and cancer (Fetterman and Zdanowicz, 2009). There are about 850 living species of sea urchins distributed in polar, temperate and tropical areas, but just a small part of these are commercially harvested for human consumption (Andrew et al., 2002; Harris and Eddy, 2015). Sea urchin fishery is diffused in Asian and American countries, with the highest level of fishing pressures on *Loxechinus albus* and *Strongylocentrotus* spp. (FAO, 2020). In recent years, Japan, which accounts for around 80% of sea urchin gonad worldwide demand, increased sea urchin import to satisfy its domestic market (Stefánsson et al., 2017). In 2013, Japan imported alive sea urchins, fresh, chilled and frozen roe for a value of about US$300 million to supply about 50 000 metric tonnes of sea urchins (whole weight) yearly consumed. Consequently, several countries (Chile, Russia, Korea, and Mexico) started to harvest their local sea urchin species, to fill the market demand, although gonad produced were qualitatively different from those of Japanese sea urchins. Gonad size, colour, flavour and other sensory attributes that strongly influence gonad market value, greatly vary among sea urchin species (Sun and Fu-Sung, 2015). However, sea urchin market requests rapidly exceeded their natural supplies resulting in a sharper decline in landings starting from around 120000 tons in 1995 to 75000 tons in 2016 (Andrew et al., 2002; Stefánsson et al., 2017). These organisms are characterized by a low growth rate and sedentary lifestyle, characteristics that make sea urchins particularly susceptible to fishery pressure and overexploitation, leading to natural stock depletion (Lawrence, 2013a). In addition, the reduction and fragmentation of sea urchin populations caused by commercial harvesting lead to low fertilisation success and loss of the protection from predators that adults offer to small juveniles (Gascoigne and Lipcius, 2004; Quinn et al., 1993). Finally, the depletion of natural population consistently influenced the equilibrium of benthic communities, due to the key role of sea urchins as herbivorous grazers, able to alter algal community states and to recycle the organic matter (Lawrence, 2013a). To contrast stock overexploitation and collapse, fisheries management were

suggested and adopted introducing specific regulations on harvesting period, numbers and sizes of catches (Botsford et al., 2004; Dewees, 2003; G.U.R.I., 1995; Stotz, 2009). However, the lack of control together with the spread of the illegal catches, supported also by new fishing methods, contributed to the overexploitation of the natural populations of the most important commercial species (Fernández-Boán et al., 2012).

1.2 Sea urchin aquaculture: limits and new challenges facing sustainable development

Sea urchin aquaculture may help to sustain or substitute fisheries, allowing sea urchin production for human consumption, as well as for the extraction of bioactive compounds and for research application, securing sea urchin production throughout all the year rather than only during reproduction seasons (Rubilar and Cardozo, 2021). In addition, aquaculture could support restocking processes with hatchery seed and mass production of small sea urchins for outplanting in the field, as already done for *Strongylocentrotus intermedius, S. nudus* (Sakai et al., 2004) and *Loxechinus albus* (Carcamo, 2004). In Japan, farming of *S. intermedius* was conducted starting from the early life stages to adult production or promoting gonad growth in wild sea urchin collected in the natural environment, but these practices are not widely distributed in the country (Unuma et al., 2015). Similarly, in Chile, sea urchin aquaculture is not developed, despite this country accounts for about half of global roe production with the largest sea urchin fishery in the world based on *L. albus* catches (Moreno et al., 2007; Stotz, 2009; Sun and Fu-Sung, 2015). Sea urchin aquaculture is based on two kinds of practices: the enhancement of gonad size and quality of sea urchin collected from the wild, also known as "bulking", and the development of a full-cycle production starting from larval development to the achievement of commercial sizes (James and Siikavuopio, 2015; Walker et al., 2015). However, due to several bottlenecks sea urchin aquaculture is still considered an uncertain business (Rubilar and Cardozo, 2021). The lack of proper hatchery and breeding techniques able to obtain high survival rate in larval production are the major issues affecting the first stages of sea urchin production (Carboni et al., 2013, Rubilar and Cardozo 2021). In addition, sea urchins are characterized by a low growth rate, and several months are required to achieve the commercial size (Bouduresque and Verlaque, 2020). Similarly, adult sea urchins obtained from laboratory reproduction or collected from the wild, need 3 or 4 months to produce gonad biomass for aquaculture goals (Walker et al., 2015).

Sea urchin gonads have a dual function as reproductive organs, involved in gametes production, and as store tissue, dedicated to nutrient accumulation. This dual function implies that gonad composition is strictly influenced by endogenous factors, related to sea urchin life cycle, and exogenous factors, environmental and nutritional condition, like food composition or feeding frequency (Walker et al.,

2015). Aquaculture farming is usually conducted under controlled environmental conditions, thus feeding regime and feed nutritional quality assume the predominant role in gonad development and gonad quality increase (Lourenço et al., 2019). Gonads contain germinal cells (GC), in which gametes are produced, and somatic cells called nutritive phagocytes (NP), in which extensive nutrient reserves are stored. According to sexual maturity stages, NPs and GCs are present in a different ratio, with gonads characterized by a high content of NPs, before the gametogenesis, while since gametogenesis occurs nutrients were transferred to GCs for gametes production, with a decrease of NPs number and size (Byrne, 1990; Marsh et al., 2013; Walker et al., 2015). Handling gonad sexual maturation is fundamental for aquaculture purposes because high content of NPs positively influences gonad quality (Böttger et al., 2006; Walker et al., 2015). Marketable high-quality gonads are characterized by a large size coupled with a desirable combination of characteristics, such as size, taste, colour, and consistency that are strongly related to diet nutritional content (Stefánsson et al., 2017). In particular, proteins showed a positive effect on gonad production, providing essential amino acids for growth, maintenance and sea urchin reproduction (Cook and Kelly, 2007a; Heflin et al., 2012, 2016; Pearce et al., 2002a). Carbohydrates are an important source of energy (Cuesta-Gomez and Sánchez-Saavedra, 2018; Walker et al., 2001), while lipids have a fundamental role in gonad development since lipids are membrane structural components (Martínez-Pita et al., 2010b). Furthermore, an appropriate amount of lipids, in particular fatty acids, plays a decisive role in reproduction, securing the correct nutritional content of eggs for larval development (Carboni et al., 2013, 2015). Due to the correlation between gonad quality and diet composition, several studies were conducted worldwide to assess a suitable diet to promote gonad production and to improve gonad quality (Lourenço et al., 2019). Macroalgae, considered sea urchin natural food, were tested as diets for gonad enhancement showing positive results on gonad growth. However, the relatively low protein content of macroalgae did not support maximal somatic and gonadal growth of sea urchins, and in addition obtained gonads did not meet commercial quality (Carrier et al., 2016; McBride, 2005; Shpigel et al., 2005). Furthermore, due to the seasonal variability in nutrient content, and the high catch and management cost of macroalgae, their use in large-scale aquaculture is not viable (Cook and Kelly, 2007b; Pearce et al., 2002a). Therefore, alternative feeds were tested, introducing corn or soya meal as protein sources (Pearce et al., 2002a; Robinson et al., 2002; Suckling et al., 2011; Woods et al., 2008), and maintaining just a low amount of macroalgae to secure efficacy in ingestion and assimilation by sea urchin (Dworjanyn et al., 2007). Recently, terrestrial vegetables such as maize (*Zea mays*) and spinach (*Spinacia oleracea*) were tested in artificial diets for sea urchins resulting well-accepted and showing increase in gonad growth (Raposo et al., 2019; Sartori et al., 2014; Sartori and Gaion, 2015). However, most formulated feeds for sea urchin allow an increase in gonad growth but fail to meet

important quality features, such as gonad colour which is considered the most important characteristic as appreciated from first sight (Cuesta-Gomez and Sánchez-Saavedra, 2018). Sea urchin fed artificial diets often produce large but pale yellow or orange gonads (Walker et al., 2015), with consequent low marketability. Roe colour depends on carotenoid concentration in the gonad, synthesised from precursors that are assimilated through the diet (Robinson et al., 2002; Shpigel et al., 2005; Symonds et al., 2009). Carotenoid concentration also protects gonads from photo-oxidative damage, improve gamete development, and enhance the immune system (George et al., 2001; Kawakami et al., 1998). As carotenoid supplements are fundamental for gonad production and are one of the most expensive ingredients in formulated sea urchin feed (Cuesta-Gomez and Sánchez-Saavedra, 2018), terrestrial vegetables such as pumpkin *Cucurbita maxima* were tested as a low-cost alternative of carotenoids, in particular β-carotene and echinenone (Santos et al., 2019). Similarly, it was observed that the addition of animal ingredients, in particular fishmeal (FM) and fish oil (FO), showed a positive effect on sea urchin gonad production when added to vegetal diets, promoting gonad growth and improving gonad quality (Fernandez and Boudouresque, 1998, 2000; Shpigel et al., 2005). FMs and FOs are sources of energy, protein, essential amino acids, minerals and fatty acids, especially of n-3 long-chain polyunsaturated fatty acids, DHA and EPA (Olsen and Hasan, 2012). Today, however, the fast increase of the aquaculture sector imposes new and alternative nutrient sources for feed production. Aquaculture utilizes 20 million tonnes of wild fish, roughly one-fifth of the world's wild-caught fish species, to produce FMs and FOs destined to feed production (FAO, 2018). Both, it was observed, could offer major benefits to animal health, promoting immunity against disease, higher digestibility, higher survival and growth, and reduced incidences of deformities, turning them attractive for widespread use as nutritional ingredients in aquaculture feed (Cho and Kim, 2011). However, the considerable amount of feed used in semi-intensive and intensive farming practices, coupled with the high price achieved by FM and FO in recent years became a key issue for aquaculture development (Salin et al., 2018). Aquaculture is called to guarantee the seafood consumed by a global human population that is estimated to exceed 9 billion by 2050 (Kobayashi et al., 2015), and is expected to play a key role in the achievement of the 17 Sustainable Development Goals (SDGs) (Cavalli et al., 2021). In addition, the development of the Blue Growth, a new strategy with a cohesive approach for environmentally compatible, integrated and socioeconomically sensitive management of aquatic resources, posed more attention to aquaculture utilization of natural resources (Eikeset et al., 2018). Terrestrial vegetal and plant ingredients are considered environmentally sustainable reducing feed dependency on overexploited marine resources (Gatlin et al., 2007). However, substituting macroalgae or fishmeal with plant ingredients would add pressure on the land-based food production

system, affecting the environment, biodiversity and availability and cost of crops (Blanchard et al., 2017; Malcorps et al., 2019; Pelletier et al., 2018; Troell et al., 2014).

1.3 New sustainable feeds

To improve the sustainability and profitability of current aquaculture practices, a step towards the use of alternative nutrient sources, such as food processing discards could secure the aquaculture sector maintenance in the next future also for new emergent species such as sea urchins. Every year one-third of the produced food for human consumption was lost or wasted, accounting for about 1.3 billion tons (Gustavsson et al., 2011) and becoming an economic, social and environmental issue (Mirabella et al., 2014; Wadhwa and Bakshi, 2013). The European Union produce more than 88 million tons of food waste, and it is estimated that this value will continue to grow up to 40% in the following years (Plazzotta et al., 2017; Stenmarck et al., 2016). In addition, a large amount of fruit and vegetable waste is made up of edibles that are discarded during the sale process because they do not fit the food quality standards that retailers and consumers demand (Gustavsson et al., 2011). Vegetal and fruit discards, which account for 80% of total food discards are produced at all the different stages of the food supply chains, starting from the harvesting operation, through the industrial processing stage, to the distribution and sale stages until the final consumer (Gustavsson et al., 2011). The large amount of vegetable and fruit wastes produced by industries affect municipal landfills due to the high biodegradability of their organic matter content, which contribute to greenhouse gas emission (Tedesco et al., 2021). Similarly, fishery discards and waste, described as unwanted catch products and those parts of fish that are not used for human consumption, namely fins, heads, skin and viscera, became a global issue in the last decade accounting for 25% of fishery production (Rustad, 2003). Although food discards represent a significant disposal problem for the industry, they are also a promising source of nutrients that can be productively recycled as ingredients for aquaculture feed production. In particular, vegetal discards are excellent sources of pigments, phenolic compounds, dietary fibres, sugar derivatives, organic acids, and minerals (Esteban et al., 2007, Sagar et al., 2018), while fish discards contain a considerable amount of proteins, lipids, minerals and essential amino acids and fatty acids (Caruso, 2015, Kim and Mendis, 2006; Olsen et al., 2010). Under the assumption that food processing discards have a high potential to be exploited, their use in aquaculture feed production is increasingly taken into account (Bimbo, 2007). First promising attempts to use fish processing discards as valuable resources in aquaculture date from about the year 2000 (Kotzamanis et al., 2001; Turchini et al., 2003). Similarly, increasing attention was focused on the exploitation of vegetal discards as an ingredient for sustainable feeds. Kang et al.(2010) highlighted higher growth rates in the juveniles of the white-leg shrimp *Litopenaeus vannamei* fed with papaya waste. Luo et al. (2014) showed an improvement of gonad flavour in the sea urchin *Strongylocentrotus intermedius*

fed with banana peels. More recently, Mo et al. (2020) recorded weight gain for the grass carp *Ctenopharyngodon idellus* fed with a mix of cereal, meat and fruit waste. These studies confirmed that the introduction of practices of circular economy in feed production may allow turning wastes into resources. This approach could indeed reduce the reliance on high-cost resources, such as fish oil and meal, matching the purpose of Blue Growth, namely the socio-economic growth based on sustainability and biodiversity protection of marine systems and resources (Eikeset et al., 2018).

1.4 Aims and organisation of the thesis

Aligned with these considerations, the aim of this Ph.D. project was the development of eco-friendly feeds looking towards a sustainable approach and development of echinoids aquaculture in the Mediterranean Sea. *Paracentrotus lividus* is the most important sea urchin commercial species, widely distributed throughout the Mediterranean Sea, as well as along the Eastern Atlantic coast, from Scotland to Morocco, (Boudouresque and Verlaque, 2020). *P. lividus* is highly appreciated for their salty and flavoured mango orange gonads (Stefánsson et al., 2017), and despite the adoption of specific laws aiming at natural stock conservation, a reduction in wild population was observed (Guidetti et al., 2004). *P. lividus* is a well-studied species, used also as model species in several scientific fields, whose feeding habits and reproductive processes are known (Boudouresque and Verlaque, 2020; Byrne, 1990; Lozano et al., 1995), but currently there are no facilities for full-cycle aquaculture or adult gonad enhancement. To overcome the main bottleneck of sea urchin aquaculture, experimental sustainable feeds were produced using food-processing discards obtained by local retailers, giving them a new life according to the principles of the circular economy (de la Caba et al., 2019).

Firstly, a preliminary short-term experiment was conducted to evaluate the stability in seawater of four sustainable formulations produced only with vegetal and animal discards from the food retail chain. Then, its effectiveness as feed for sea urchins was evaluated in a fifteen-day bioenergetics trial throughout the assessment of palatability, assimilability and gonad growth (Chapter 2).

Starting from the positive results of the preliminary test, a second long-term experiment (3 months) was carried out testing the effects of the sustainable feeds on gonad development, production and quality. To evaluate the goodness of the proposed feeds the results of the feeding experiment were compared with the pattern observed in wild sea urchins (Chapter 3).

A third experiment was conducted in collaboration with the Experimental Ecology and Aquaculture Laboratory of the Rome Tor Vergata University, to test the effects of five different diets on egg production in females of *P.lividus* farmed in a recirculating aquaculture system (RAS). Four diets made of 90% of *Lactuca sativa* (common lettuce) discards from the food industry and just 10% of

seafood ingredients, while the fifth diet was based on macroalgae, considered the natural sea urchin diet. The effects of the five diets were evaluated by somatic growth and egg production applying the innovative and sustainable Racking method (Chapter 4).

Finally, to evaluate the experimental diets also for sea urchin reproduction, the effect on larval development was measured. Diet assimilation was assessed using stable isotopes and fatty acids as biochemical tracers of organic matter transfers, while to evaluate the effect on larval development the new Integrated Toxicity Index was applied (Chapter 5).

The thesis is structured in stand-alone chapters, two of which (Chapter 2 and Chapter 3) already published in peer-reviewed journals (pdf were attached at the end of the thesis).

CHAPTER 2: Formulation of a new sustainable feed from food industry discards for rearing the purple sea urchin *Paracentrotus lividus*.

This chapter has been published in Aquaculutre Nutrition 26, 1046–1057. Ciriminna L., Signa G., Vaccaro A. M., Messina C. M., Mazzola A., Vizzini S., 2020, Formulation of a new sustainable feed from food industry discards for rearing the purple sea urchin *Paracentrotus lividus*. The pdf article is attached at the end of this thesis.

2.1 Introduction

Looking towards a sustainable approach of farming practices able to contrast the recent overexploitation of natural populations of the edible sea urchins, the exploitation of fresh agricultural discards as a diet for *Paracentrotus lividus,* alone (Vizzini et al. 2014, 2018) or mixed with other ingredients (Vizzini et al., 2019) was evaluated, with encouraging results in terms of gonad yield and organoleptic and nutritional features of the produced roe. In this chapter we propose a sustainable feed for sea urchins, mainly based on discards from the food industry. Two feed formulations with different percentages of vegetable and animal discards were tested to assess their feasibility for feeding adult specimens of *P. lividus* in rearing conditions. Feed stability in seawater and both palatability and assimilability of the new sustainable feed were tested. A preliminary assessment of the effect on gonad growth was also carried out by estimating the gonado-somatic index. Finally, the nutritional composition and quality of both ingredients and feed were assessed through the study of the proximate composition and fatty acid profiles. Indeed, a proper provision of dietary proteins, lipids and fatty acids, such as essential and polyunsaturated fatty acids, especially the *omega*-3 class, is crucial to improve the growth of reared organisms, obtaining also roe of good quality (Carboni et al., 2015; Castell et al., 2004; González-Durán et al., 2008).

2.2 Materials and methods

2.2.1 Feed formulation

Outermost leaves of *Cichorium endivia* (endive), obtained from unprocessed agricultural discards, and industry discards of *Engraulis encrasicolus* (European anchovy), composed mainly by viscera, head, skin and bones, were used as the main ingredients for producing a new sustainable feed for echinoculture. Endive and anchovy discards were freeze-dried and then ground to fine powder. Two formulations were prepared differing for the ratio of the main ingredients: endive leaves and anchovy discards contributed on a rougly 60:40 ratio (60/40 formulation) and 80:20 (80/20 formulation) to the

two feed formulations (Table 2.1). Agar (Agar-Agar fine powder 100% Food Grade, Intra Laboratories, UK), a non-branched polysaccharide extracted from red algae, was dissolved in boiling MilliQ distilled water (385 g/L) and mixed until a homogeneous jelly-like solution was obtained. Then, it was allowed to cool to about 60 °C and added in different proportion (25 and 50 g/Kg) to both feed formulations, and mixtures were stirred and manually converted into bar-shaped feeds (0.5 cm diameter, 2 cm length, ~1 g wet weight) using a 35 ml syringe. The feed bars were air dried for 24 hours at room temperature (24 °C), and then stored at -20°C until further use and analysis.

Table 2.1. Composition (g/kg) of the two feed formulations, 60/40 and 80/20, with different agar content.

Ingredient	Feed formulation			
	60/40		80/20	
	A25 (g/kg)	A50 (g/kg)	A25 (g/kg)	A5 (g/kg)
Cichorium endivia	58.8	57.5	78.8	77.5
Engraulis encrasiculos	38.8	37.5	18.8	17.5
Agar	2.5	5.0	2.5	5.0

2.2.2 Stability trial

All the formulations (two feed formulations at two different agar content) were tested for stability in seawater, hypothesizing a different stability according to the agar amount. Before the stability trial, six feed bars of each formulation were weighed (WW), oven-dried at 60°C for 48 hr to constant weight, and weighed again (DW) to assess the standard dry weight (DW_S % = DW/WW x 100) of each feed formulation.

Afterwards, other six feed bars of each formulation were weighed (WW_I) and put individually inside PVC cylindrical cages (20 cm height and 12 cm diameter) closed on both sides with a nylon net (mesh size 500 µm) and fixed in pairs under the water surface in 80-L tanks (Fig. 2.1a). Environmental conditions were kept stable throughout the stability trial, in terms of seawater temperature: 20.0 ± 1.0 °C, salinity: 38.0 ± 0.5 g kg^{-1}, photoperiod: 8h light and 16h dark, and continuous water flow in/out: 5 L min^{-1}. At three different times: T1 (24hr), T2 (48hr) and T3 (72hr), two bars of each formulation were randomly collected, oven dried at 60 °C for 48h and weighed to assess the final dry weight (DW_F). Feed stability of each formulation was expressed based on the dry weight loss (DW_L) of the feeds at the end of the stability trial, as follows:

$$DW_L\ (\%) = [(DW_I - DW_F)/\ DW_I] \times 100$$

where DW_I is the dry weight of each feed bar provided, calculated based on the standard dry weight, as follows:

DW_I (mg) = (WW_I x DW_S%)/100).

The results of the stability test showed that the agar amount did not affect significantly the feed stability over time (see the "Results" section), and hence, considering the economic advantages and sustainability of using a lower binder quantity, the feed formulations with the lower amount of agar (25 g/kg) were selected for the further steps.

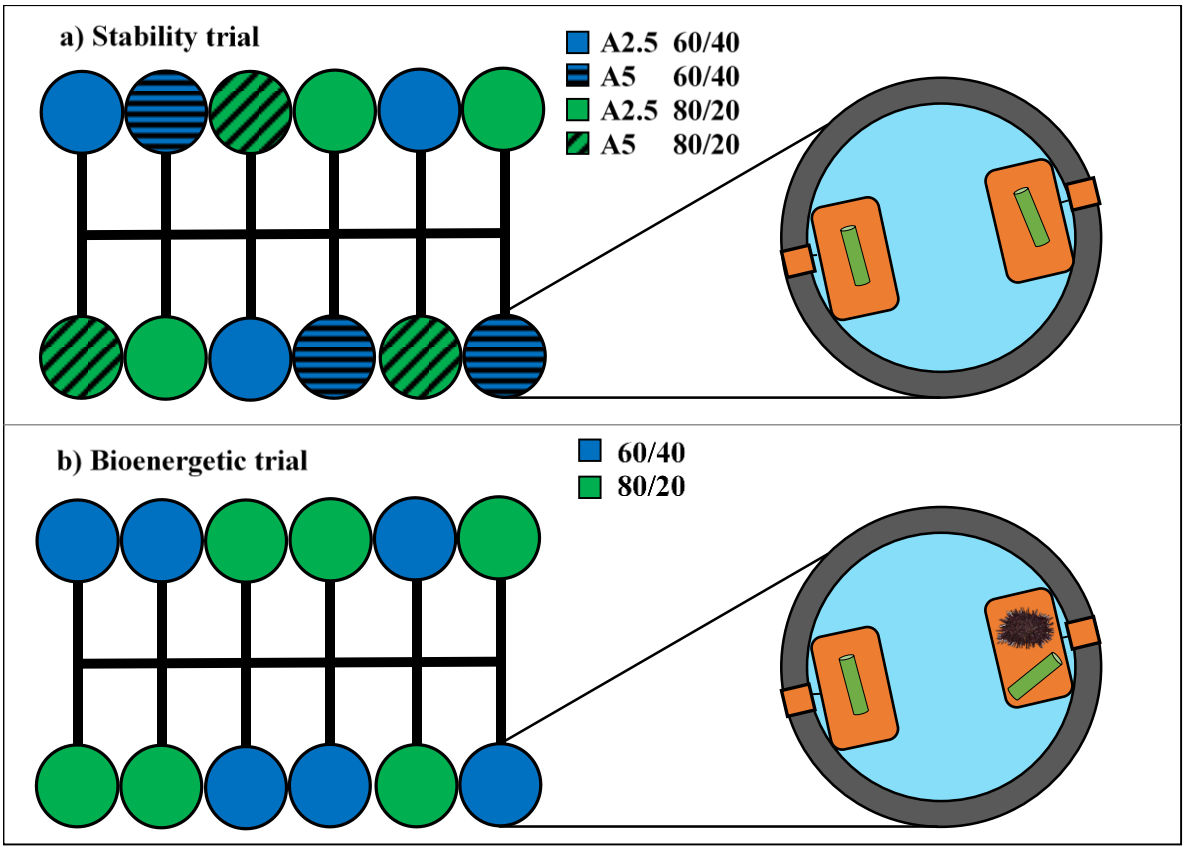


Figure 2.1. Indoor tank system used for the feed stability experiment (a) and the bioenergetic experiment (b). The detail of each tank is showed on the right side of each panel.

2.2.3 Proximate composition and fatty acids analysis

The main ingredients, that is discarded outermost leaves of *Cichorium endivia* (endive) and industry discards of *Engraulis encrasicolus* (European anchovy), and the two selected feed formulations with 25 g/Kg agar, 60/40 and 80/20, were freeze-dried, ground and analysed in triplicate. Ash content was determined by combustion in a muffle furnace at 550 °C for 4 h according to Nielsen (2010), crude

protein content was estimated by the Kjeldahl method, with nitrogen to protein conversion factor of 6.25 (Horowitz and Latimer, 2006). Carbohydrate content was also estimated, according to Baião et al. (2019) as follows:

Carbohydrates = 100 - (lipid + protein + ash)

A modified version of the Bligh and Dyer (1959) method was applied to measure lipids and fatty acids (FA). Lipids were extracted using a MilliQ distilled water : methanol : chloroform mixture (1:2:1 v:v:v) with 0.01% BHT (butylated hydroxyl toluene) to avoid lipid oxidation. Samples were then sonicated to improve lipid extraction and centrifuged twice to separate the lipid phase from the aqueous phase. The lipid extracts were evaporated to dryness under gentle nitrogen stream, weighed, and the lipid content was expressed as mg g^{-1} dw of dry sample and as percentage. Therefore, lipids were resuspended in n-hexane and subjected to acid-catalysed transesterification using methanolic hydrogen chloride to obtain fatty acid methyl esters (FAME). FAME were then analysed by a gas chromatograph (GC-2010, Shimadzu) equipped with a BPX-70 capillary column (30 m length; 0.25 mm ID; 0.25 μm film thickness, SGE Analytical Science) and detected by a flame ionisation detector (FID). Peaks were identified by retention times from mixed commercial standards (37 FAME from Supelco; QUALFISH and BACTERIAL MIX from Larodan). Tridecanoic and tricosanoic acids (C13:0 and C23:0) were used as surrogate standards, while pentacosanoic acid methyl ester (ME C25:0) was used as internal standard for quantification. FA data were expressed as mg g^{-1} of dry sample.

2.2.4 Bioenergetic trial

Twenty-four *Paracentrotus lividus* specimens (Test Diameter: 3.7 ± 0.2 cm, Total Wet Weight 23.4 ± 4.1 g) were collected from natural environment and randomly divided in two 80 L tanks. After a starvation period of two weeks, during which sea urchins were kept fasting, six specimens from each tank were randomly collected, sacrificed, wet weighed, and their gonads were removed and wet-weighed.

The remaining twelve specimens were used for a two-week bioenergetic trial in an indoor tank system made of two groups of six tanks of 80 L, one group per each feed formulation (60/40 and 80/20). In each tank, two PVC cylindrical cages (20 cm height, 12 cm diameter) closed on both sides with a nylon net (mesh size 500 μm) were fixed under the water surface (Fig. 2.1b). The remaining sea urchins were put individually in one of the two PVC cages per tank, while the other cage was left empty as a control treatment, aiming at calculating the feed loss. The same environmental conditions

used in the previous stability trial, were kept during both the starvation period and the bioenergetic trial. At the beginning of the experiment and every 48h (T0-T6), each sea urchin was fed with a known amount of the feed formulations (~1 g WW), and the same amount of feed was put in the correspondent control cage. Before feed provision (T1-T7), all the material contained within both treatment and control cages of each tank was carefully removed, oven dried to constant weight (48hr, 60°C) and reweighed. As far as the treatment cages, the collected material was previously separated in feed particles and sea urchin faeces, under a stereomicroscope.

The daily ingestion rate by sea urchins (IR), expressed as dry weight (mg day^{-1}), was calculated for each specimen at each sampling time (T1-T7), according to Fernandez and Boudouresque (1998) as follows:

IR (mg/day) = (total provided biomass – total uneaten biomass)/2

where the total provided biomass is the dry weight of the feed provided (DW), calculated from the standard dry weight (DW_S %), likewise the previous stability trial. The total uneaten biomass is given by the dry weight of the feed particles collected in the treatment cages, corrected based on the biomass lost from the control cages, 2 are the days between each feed provision.

The absorption efficiency (AE) was calculated for each specimen at each sampling time as follows:

AE (%) = [(total biomass ingested – total faeces biomass)/total biomass ingested] x 100

where total biomass ingested is equal to: total provided biomass – total uneaten biomass. At the end of the trial, sea urchins were sacrificed, weighed, and the gonads extracted and wet-weighed. The gonado-somatic index (GSI) was calculated before the onset (T0), and at the end of the feeding treatment (T7) as follows:

GSI (%) = [gonad wet weight (g)/total wet weight (g)] x 100

2.2.5 Data elaboration and statistical analysis

Univariate permutational analysis of variance was used to test the differences in stability among feed formulations at different percentage of agar (factor Agar fixed with two levels: A25, A50; factor Feed fixed with two levels: 60/40 and 80/20) across time (factor Time fixed and orthogonal, with three levels: T1, T2, T3). The analysis was run on untransformed data resembled using Euclidean distance.

One-way multivariate permutational analysis of variance (PERMANOVA) was carried out to test the differences in fatty acid (FA) profiles between the two selected feed formulations with the lower agar content (factor Feed fixed with two levels: 60/40, 80/20). PERMANOVA was carried out on FA data resembled using Euclidean distance after square root transformation. Principal Coordinates Analysis (PCO) was also run on the FA profiles of the feed formulations, in order to graphically highlight the differences found by PERMANOVA. The nutritional quality of the ingredients and the formulated feed was assessed through a semi-quantitative fatty acid approach: the patterns of the main classes of FA, together with those considered as important biomarkers of nutritional quality in aquaculture [i.e. arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), the sum of ω-3 and ω-6 polyunsaturated fatty acids (Σω-3-PUFA and Σω-6-PUFA), the ratio ω-3/ω-6, and the sum of ω-3 highly unsaturated fatty acids (Σω-3-HUFA), according to Gago et al. (2009), Sargent et al. (1999) and Vizzini et al. (2019)] were assessed.

Difference in ingestion rate (IR) and absorption efficiency (AE) of the sea urchins fed with the two selected feed formulations across time was also tested using univariate permutational analysis of variance (factor Feed fixed with two levels: 60/40, 80/20, factor Time fixed and orthogonal, with 7 levels: T1 – T7). Difference in gonado-somatic index (GSI) between the onset and the end of the trial was also run using univariate permutational analysis of variance with both factors, Feed and Time, fixed and orthogonal, and both with 2 levels (Feed: 60/40, 80/20; Time: T0, T7). All the analyses were based on untransformed data resembled using Euclidean distance.

All the statistical analyses were performed using the software PRIMER 6 v6.1.10 & PERMANOVA+ β20 (Plymouth, UK). When significant differences were found, pair-wise tests were used as *a posteriori* check of significant effects. The Montecarlo test was also carried out to identify significant patterns when the numbers of permutation were less than 100.

2.3 Results

2.3.1 Stability trial

The stability trial carried out on the two new feed formulations (60/40 and 80/20) manufactured with different percentages of agar (25 and 50 g/kg) revealed that the higher feed loss occurred in the first 24hr of immersion in seawater, and then was overall stable in the following times (48 and 72 hr) (Figure 2.2). The higher agar amount did not contribute to provide a higher stability to both formulations at all times (MS= 92.23, Pseudo-$F_{(1,12)}$= 17.27, p= 0.057); indeed, while the interaction of the factors feed and time was significant (MS= 27.30, Pseudo-$F_{(4,12)}$= 3.97, p= 0.036), pair-wise

tests, carried out to compare the two feed formulations at different agar amount over time, revealed only that the stability of the formulation A50 60/40 was significantly lower at T3 than at T1 ($p < 0.05$).

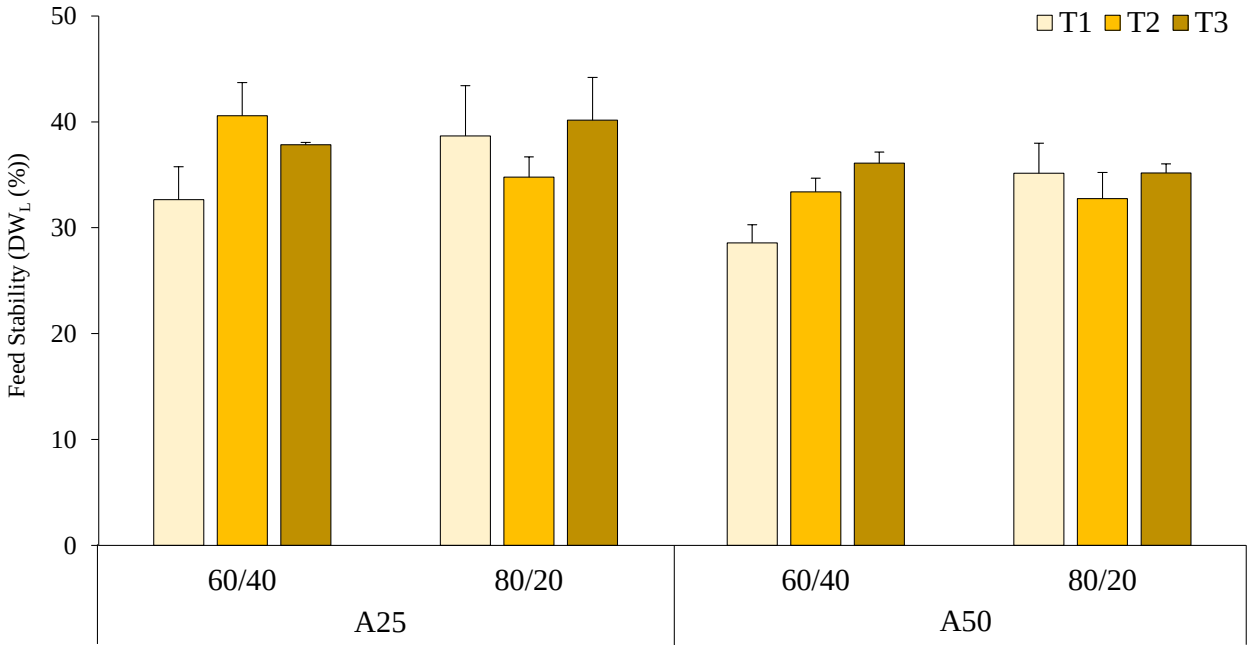


Figure 2.2. Feed stability expressed as dry weight loss (DW_L %, mean ± standard deviation) of the two feed formulations (60/40 and 80/20) prepared with a different agar amount (A25: 25 g/kg, A50: 50 g/kg).

2.3.2 Proximate composition and fatty acid analysis

Proximate composition and fatty acid (FA) profiles of the main ingredients, *Cichorium endivia* and *Engraulis encrasicolus* discards, and the two selected feed formulations, 60/40 and 80/20 are showed respectively in Table 2.2 and Table 3.3 Fish industry discards showed higher lipid, protein and ash content than discarded endive leaves, while endive was richer in carbohydrates than fish discards. These differences were mirrored in the feed formulations: lipids, proteins and ash were more abundant in the formulation with the higher relative content of fish discards (60/40), and carbohydrates were more abundant in the formulation with the higher relative content of endive leaves (80/20) (Table 2.2).

Table 2.2. Proximate compositon (g/kg dry matter, mean ± standard deviation) of the two main ingredients (*Cichorium endivia* and *Engraulis encrasicolus* discards) and the two selected feed formulations.

	Ingredient		**Feed formulation**	
	C. endivia	***E. encrasicolus***	**60/40**	**80/20**
Lipid %	3.80 ± 0.17	14.01 ± 1.90	7.08 ± 0.74	5.55 ± 0.20
Protein %	19.14 ± 0.67	40.58 ± 0.38	29.36 ± 0.28	23.86 ± 0.29
Carbohydrate %	64.42 ± 0.96	4.34 ± 2.27	38.89 ± 0.79	50.69 ± 0.84
Ash %	12.63 ± 0.15	41.07 ± 0.01	24.67 ± 0.39	19.89 ± 0.39

As regards FAs, the two main ingredients showed very different profiles, being the outermost leaves of endive almost exclusively constituted by 18:3 n3 (α-Linolenic acid, ALA), 18:2 n6 (Linoleic acid, LA) and 16:0 (Palmitic acid), and anchovy discards by a high abundance of essential fatty acids (EFA), namely arachidonic (ARA), eicosapentaenoic (EPA), and docosahexaenoic acid (DHA) (Table 2.3). As regards the feed formulations, a higher amount of all the three FA classes, saturated, mono- and polyunsaturated FA characterised the formulation with a higher amount of animal ingredients (∑ SFA, ∑ MUFA, ∑ PUFA: 60/40 > 80/20). Looking through the biomarkers of nutritional quality, the sum of EFA and of ω-3 highly unsaturated FAs (∑ω-3-HUFA) were about twice in the 60/40 formulation, compared with the 80/20. Individual EFAs (i.e. ARA, EPA and DHA) were also higher in the 60/40 formulation, while ALA (18:3n3) and LA (18:2n6), both precursors of EFA (Baião et al., 2019; Castell et al., 2004), showed an opposite trend with a higher amount in the 80/20 formulation than in the 60/40. As a result, the sum of ω-3 and ω-6 PUFA were respectively higher in the 60/40 and the 80/20 feed formulation, and their ratio ω-3/ω-6 was also higher in the former, compared with the latter.

Table 2.3. Fatty acid profiles and lipid content (mg/g dw, mean ± standard deviation) of the two main ingredients (*Cichorium endivia* and *Engraulis encrasicolus* discards) and the two selected feed formulations.

	Main ingredient		**Feed formulation**	
FAs (mg g^{-1} dw)	***C. endivia***	***E. encrasicolus***	**60/40**	**80/20**
8:0	0.04 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
10:0	-	0.11 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
11:0	-	0.03 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
12:0	0.01 ± 0.00	0.24 ± 0.01	0.02 ± 0.00	0.00 ± 0.00
14:0	0.07 ± 0.01	7.57 ± 0.5	1.86 ± 0.04	0.86 ± 0.06
15:0	0.03 ± 0.00	1.43 ± 0.09	0.37 ± 0.00	0.20 ± 0.01
16:0	2.07 ± 0.01	28.7 ± 1.51	10.49 ± 0.12	6.34 ± 0.21
17:0	0.03 ± 0.00	1.32 ± 0.08	0.61 ± 0.00	0.28 ± 0.02
18:0	0.20 ± 0.01	5.98 ± 0.31	1.82 ± 0.08	1.04 ± 0.02
19:0	-	0.31 ± 0.02	0.10 ± 0.00	0.05 ± 0.01
20:0	0.12 ± 0.00	0.39 ± 0.01	0.12 ± 0.00	0.09 ± 0.00
21:0	0.09 ± 0.01	0.07 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
22:0	0.14 ± 0.01	0.27 ± 0.01	0.13 ± 0.00	0.13 ± 0.00
Σ LCFA (>22:0)	0.26 ± 0.01	0.39 ± 0.08	0.48 ± 0.01	0.47 ± 0.02
Σ SFA	**2.98 ± 0.02**	**47.16 ± 2.42**	**16.06 ± 0.21**	**9.52 ± 0.34**
14:1	-	0.06 ± 0.02	0.01 ± 0.00	0.00 ± 0.00
15:1	0.03 ± 0.00	-	0.00 ± 0.00	0.00 ± 0.00
16:1 n7	-	4.15 ± 0.25	1.06 ± 0.01	0.52 ± 0.02
18:1 n7	0.09 ± 0.00	3.01 ± 0.12	0.89 ± 0.01	0.44 ± 0.01
18:1 n9t	-	0.09 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
18:1 n9c	0.19 ± 0.03	14.13 ± 0.68	3.87 ± 0.08	1.92 ± 0.06
20:1 n9	0.03 ± 0.00	0.69 ± 0.03	0.16 ± 0.02	0.08 ± 0.02
20:1 n11	-	0.04 ± 0.01	0.05 ± 0.01	0.02 ± 0.00
22:1 n9	-	0.19 ± 0.01	0.04 ± 0.00	0.01 ± 0.00
Σ MUFA	**0.35 ± 0.04**	**23.35 ± 1.1**	**6.08 ± 0.12**	**2.99 ± 0.09**
18:2 n6c - LA	3.72 ± 0.31	2.25 ± 0.12	4.12 ± 0.13	5.12 ± 0.14
18:2 n6t	-	0.05 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
18:3 n3 - ALA	9.31 ± 0.63	1.51 ± 0.09	6.20 ± 0.56	8.26 ± 0.23
18:3 n6	0.09 ± 0.00	0.19 ± 0.03	0.12 ± 0.00	0.16 ± 0.00
18:4 n3	0.03 ± 0.00	2.41 ± 0.16	0.58 ± 0.01	0.32 ± 0.01
20:2 n6	0.07 ± 0.00	0.43 ± 0.02	0.08 ± 0.01	0.04 ± 0.01
20:3 n3	0.03 ± 0.00	0.15 ± 0.01	0.04 ± 0.00	0.02 ± 0.01
20:3 n6	-	0.16 ± 0.06	0.01 ± 0.00	0.02 ± 0.00
20:4 n3	-	0.59 ± 0.04	0.17 ± 0.00	0.10 ± 0.01
20:4 n6 - ARA	-	1.54 ± 0.07	0.28 ± 0.00	0.10 ± 0.01
20:5 n3 - EPA	-	9.74 ± 0.62	2.50 ± 0.02	1.08 ± 0.04
22:2 n6	-	0.14 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
22:4 n6	-	0.05 ± 0.00	0.02 ± 0.00	0.01 ± 0.01
22:5 n3	-	0.92 ± 0.04	0.95 ± 0.01	0.44 ± 0.01
22:6 n3 - DHA	-	24.88 ± 1.51	7.45 ± 0.05	3.28 ± 0.11
Σ PUFA	**13.26 ± 0.50**	**44.96 ± 2.59**	**22.52 ± 0.70**	**18.94 ± 0.55**
branched	-	1.27 ± 0.11	0.20 ± 0.02	0.08 ± 0.00
-OH	0.30 ± 0.02	0.78 ± 0.03	0.19 ± 0.00	0.17 ± 0.01
-Δ	0.09 ± 0.03	0.53 ± 0.07	0.13 ± 0.01	0.06 ± 0.01
Σ EFA	**-**	**36.16 ± 2.20**	**10.23 ± 0.07**	**4.46 ± 0.15**
Σ ω3-PUFA	**9.38 ± 0.63**	**40.20 ± 2.46**	**17.89 ± 0.58**	**13.49 ± 0.39**
Σ ω6-PUFA	**3.88 ± 0.32**	**4.76 ± 0.13**	**4.63 ± 0.12**	**5.45 ± 0.16**
ω3/ω6	**2.44 ± 0.33**	**8.44 ± 0.28**	**3.86 ± 0.00**	**2.47 ± 0.00**

Σ ω3- HUFA	**0.03 ± 0.00**	**38.54 ± 2.36**	**11.65 ± 0.07**	**5.22 ± 0.16**
Σ FA	**16.98 ± 0.61**	**118.06 ± 6.32**	**45.12 ± 0.00**	**31.72 ± 0.00**
Lipid content (mg g^{-1} dw)	**38.02 ± 1.66**	**140.10 ± 18.97**	**70.82 ± 7.36**	**55.49 ± 1.97**

Note: Main FA classes (SFA: saturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA) and main biomarkers of nutritional quality are indicated in bold. LCFA: long-chain FA; LA: linoleic acid, ALA: α-linolenic acid; ARA: arachidonic acid, EPA: eicosapentaenoic acid, DHA:docosahexaenoic acid, Branched: branched-chain saturated FA, -OH: hydroxyl FA, -Δ: cyclopropyl FA.

PERMANOVA revealed that the FA profiles of the two feed formulations were significantly different (MS= 4.62; Pseudo-$F_{(1,5)}$= 291.15; $p \leq 0.001$) Principal Coordinates Analysis (PCO) of the FA profiles of 60/40 and 80/20 formulations confirmed this result, showing a clear separation along the horizontal axis based on the feed formulations with almost the totality of the explained variance (Figure 2.3). The formulation 60/40 was grouped on the right side of the graph, characterized by a higher abundance of all the FA classes (the sum of SFA, MUFA and PUFA), total and individual EFA, the sum of ω-3 PUFA and HUFA, and the ratio ω-3/ω-6. In contrast, the formulation 80/20 was distributed in the left area of the graph, because of the higher abundance of the sum of ω-6 PUFA and the two dominant fatty acids in the PUFA class, ALA and LA, suggesting that their abundances were an important driver for the distinction between the two formulations.

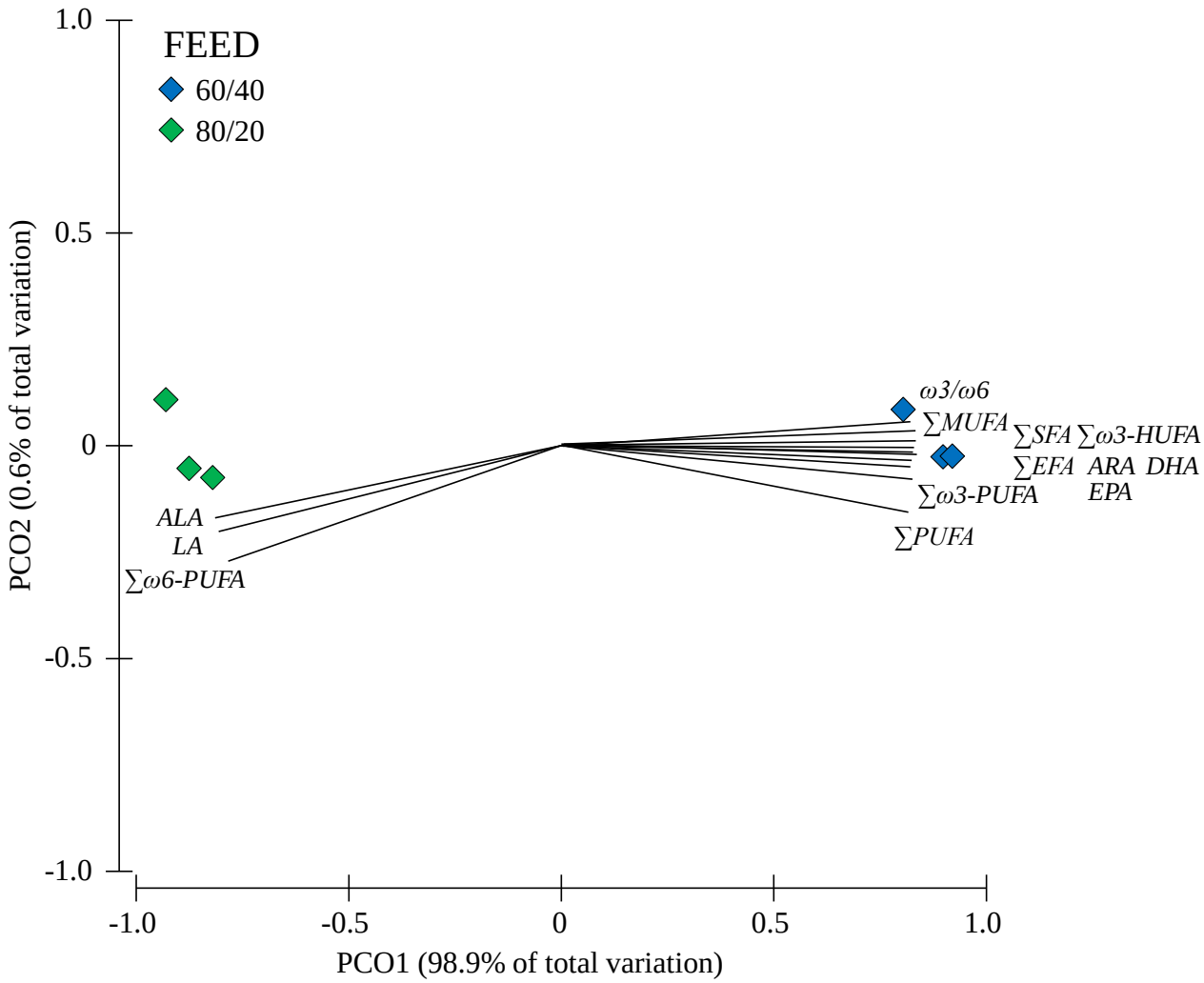


Figure 2.3. Principal Coordinates Analysis (PCO) of the fatty acid profiles of the two feed formulations (60/40, 80/20). The main fatty acid classes and the main indicators of nutritional quality selected in this study are superimposed to the graph. The meaning of the acronyms is the same as in Table 2.3.

2.3.3 Bioenergetic trial

The daily ingestion rate (IR) recorded in *Paracentrotus lividus* fed with the two different feed formulations across the seven sampling periods, showed a fluctuating pattern. The mean value of the daily IR calculated for the entire duration of the trial was rather similar for the two feed formulations: 104.0 ± 25.5 mg DW d^{-1} and 111.9 ± 25.1 mg DW d^{-1} respectively for 60/40 and 80/20. A mean IR decrease was evident in the early stages of the experiment (T1-T3), followed by a slight increase (T4-T5) and a further reduction (T6-T7) (Figure 2.4). This ambiguous temporal trend, coupled with a high

individual variability, resulted in a lack of significant differences between feed formulations, times and their interaction (Table 2.4).

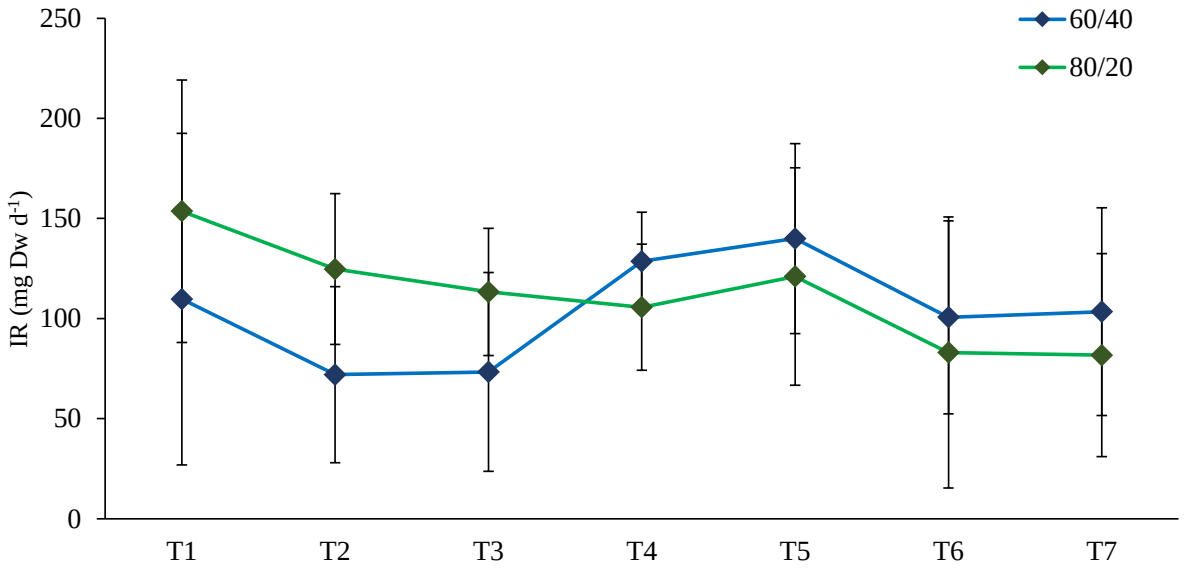


Figure 2.4. Ingestion rate IR (mg dw/day, mean ± standard deviation) of *Paracentrotus lividus* for the two feed formulations across time.

The absorption efficiency (AE) recorded in the sea urchins fed with the two feed formulations showed a fluctuating pattern, similarly to that observed for IR. After the early stages of the trial, where the AE values were similar in the sea urchins fed with the 80/20, and tended to decrease in those fed with the 60/40 formulation, higher AE values were recorded in the sea urchins fed with the formulation with the higher fish content (i.e. 60/40) (Figure 2.5). The average AE calculated for the whole trial was higher, indeed, for the 60/40 formulation than for the 80/20 (63.6 ± 6.4% *vs*. 55.1 ± 10.3% respectively) (Table 2.4), while differences among times and for the interaction of the two factors were not detected.

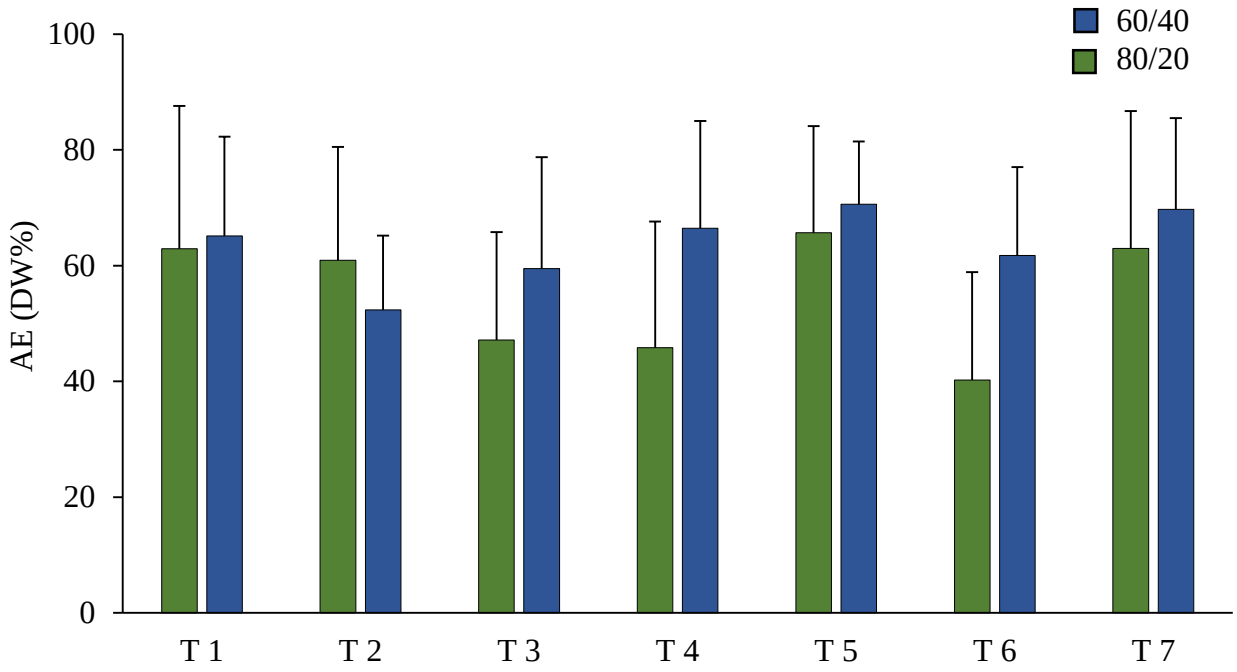


Figure 2.5. Percentage absorption efficiency AE (DW%, mean ± standard deviation) of *Paracentrotus lividus* for the two new feed formulations across time.

Despite the short duration of the trial (2 weeks), the gonado-somatic index (GSI) showed a clear increase in sea urchins fed with both formulations (from 0.8 ± 0.7% to 2.8 ± 0.6% and from 0.9 ± 0.7% to 2.7 ± 1.4% in the sea urchins fed with the 60/40 and 80/20 formulations respectively). Univariate permutational analysis of variance showed significant differences between times (MS= 21.55; Pseudo-$F_{(1, 22)}$= 23.73; p< 0.01), but not between feeds (MS= 0.002 and Pseudo-$F_{(1,22)}$= 0.002, p>0.05) or the interaction of the two factors (Pseudo-$F_{(1 ,22)}$= 0.13, p> 0.05).

Table 2.4. Univariate permutational analysis of variance results testing the effects of the feed formulations across time on the ingestion rate IR (a) and absorption efficiency AE (b) of *Paracentrotus lividus*. Significant p values are highlighted in bold.

PERANOVA Main test		**a) IR**			**b) AE**		
Source of variation	**df**	**MS**	**Pseudo-F**	**P(perm)**	**MS**	**Pseudo-F**	**P(perm)**
Feed	1	1321.7	0.50	0.48	1529.3	4.43	**0.04**
Time	6	3914.9	1.48	0.18	544.3	1.58	0.18
Feed x Time	6	3766.7	1.43	0.24	340.3	0.99	0.45
Residual	70	2636.8			345.4		

2.4 Discussion

To move towards a higher sustainability of echinoculture, this study proposes a new sustainable feed through the reuse of discards from the food industry. Two experimental formulations were prepared

using discarded endive (*Cichorium endivia*) leaves and anchovy (*Engraulis encrasicolus*) industry discards in different proportions, and were tested for stability in seawater. Nutritional composition and quality of the main ingredients and the formulations were evaluated through the analysis of proximate composition and fatty acid profiles and biomarkers. Finally, both formulations were tested for palatability, absorption efficiency and effect on gonad growth of the purple sea urchin *Paracentrotus lividus*.

The stability trial showed a comparable pattern between the feed formulations at different agar amount: the greater feed loss occurred in the first 24 hours of immersion in seawater, and then remained fairly stable in the subsequent times, ranging overall between 30 and 40%. These patterns clearly indicates that the different amount of agar in the feed formulation affected only marginally the feed stability over time, consistent with previous studies (Argüello-Guevara and Molina-Poveda, 2013; Fabbrocini et al., 2012). The macromolecular structure of the gel formed by agar is deemed, indeed, as a strong binder as it confers a high feed stability at ambient temperature by limiting nutrient loss through leaching (Fabbrocini et al., 2012; Leclercq et al., 2015) and water absorption (Paolucci et al., 2015). Moreover, as *P. lividus* takes at least 2-3 days to eat the feed offered in confined conditions (Fabbrocini et al., 2015), the very limited feed loss observed between 24 and 72 hours makes both feed formulations enough stable over time and then resulting a suitable choice in the production of sustainable feeds for sea urchins. Additionally, the present findings revealed that the use of a commercially affordable product (i.e. agar powder for home baking) rather than a laboratory product, for the production of aquaculture feeds ensured good results coupled with a substantial cost reduction. In contrast, although other binders, such as pork gelatine, may result in a higher feed stability in water (Pearce et al., 2002b), the higher cost and quantity needed to produce gelatine-based pellets make them economically unsustainable. Furthermore, agar-based feeds may have a positive effect on growth rate, as previously observed in reared crustaceans (Palma et al., 2008) and on both gamete production and gonad growth of *P. lividus* (Fabbrocini et al., 2012). For all these reasons, chiefly the comparable stability over time coupled with the greater sustainability of using lesser amount of binders in the context of industrial-scale feed production, the further steps were conducted using only the two formulations with the lower agar amount.

Following the stability trial, the two selected feed formulations and their main ingredients were characterised in terms of nutritional composition and quality. Both formulations appeared nutritionally balanced, with carbohydrates as the most representative macronutrient, followed by proteins and lipids. As expected, the differences found between the formulations were essentially driven by the different nutritional contribution of the main ingredients. Indeed, endive discarded leaves and anchovy industry discards showed major differences in both lipid and fatty acid content,

the two ingredients being respectively of plant and animal origin and hence characterized by a different nutritional profile (Rana et al., 2009). Being constituted mainly of fish skin, bones, heads and internal organs, the protein and lipid content of anchovy discards was much higher than that of endive leaves (Ghaly et al., 2013). This was mirrored in the two feed formulations, where proteins and lipids decreased proportionally with the ratio of vegetal *vs*. animal ingredients, consistent with the literature (Fernandez and Boudouresque, 2000). On the other hands, discarded endive leaves and the formulation 80/20 were characterized by the highest content of carbohydrates.

A proper nutritional composition of the feeds is crucial in echinoculture. Previous studies showed that carbohydrate and protein levels similar to those found in this study (~ 40 and 20%) provide the proper amount of energy and essential amino acids needed to foster growth and reproduction (Cuesta-Gomez and Sánchez-Saavedra, 2018; Hammer et al., 2012). Also the source of proteins is important, as revealed by Fernandez and Boudouresque (2000) who found the highest values of gonado-somatic index in the sea urchins fed with diets with intermediate levels of animal ingredients. Also dietary lipids have a key role as structural components, source of energy and precursors of bioactive molecules (Carboni et al., 2013), and additionally they influence the FA composition and organoleptic attributes of the roe (Martínez-Pita et al., 2010a; Siliani et al., 2016; Vizzini et al., 2019). Consequently, a high lipid content of the diet may favour gonad development and contribute to the restoration of energy supplies following the starvation, during which sea urchins tend to consume the nutrients present in their tissues (Guillou and Lumingas, 1998).

Turning to the FA profiles, the high concentration of SFA and MUFA found in the formulation characterised by a higher content of anchovy discards (60/40) is mainly attributable to a higher content of 16:0 and 18:1n9 in fish discards than in endive leaves, consistently with the high typical abundance of SFA and MUFA in the common anchovy (Öksüz and Özyilmaz, 2010; Zlatanos and Laskaridis, 2007). In contrast, the high concentration of PUFA observed in both formulations, is mainly due to the high content of linoleic (LA) and α-linolenic (ALA) acids, being both very abundant in the endive leaves, but not in the fish discards. Endive is a 18:3 metabolism plant and its PUFA profile is composed almost exclusively by 18:3n3 and 18:2n6 (Le Guedard et al., 2008; Vizzini et al., 2019), which, in contrast, are fatty acids generally not abundant in the common anchovy (Öksüz and Özyilmaz, 2010).

The higher abundance of essential FA (EFA) in the 60/40 formulation than in the other (80/20) is also consistent with the high EFA concentration in *E. encrasicolus* discards. In turn, the EFA content in fish discards is consistent with what is reported in the literature for anchovy tissues [about 1, 10, and 15% of the total FA content for arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids respectively, Öksüz and Özyilmaz (2010)], due to the high EFA assimilation and storage

ability of fish (Bendiksen et al., 2011). EFA are deemed suitable indicators of high nutritional quality in aquaculture feeds as they play a key role in many physiological functions, and then represent an added value in the market of sea urchins. The abundance in the proposed formulation may also boost gamete production and gonad growth (Watts et al., 2013). The preliminary assessment of gonado-somatic index carried out in this study confirms this, even if any difference was detected between the two feed formulations, but longer-time experiments are needed for further consideration.

The two selected formulations were provided to sea urchins to assess both palatability and absorption efficiency of *P. lividus.* The bioenergetics trial highlighted a similar fluctuating pattern for both ingestion rate (IR) and absorption efficiency (AE), characterised by high initial values followed by an overall decrease during the first phases of the experiment, and then increased again. The early pattern may have been influenced by the previous period of starvation. Under food limitation, sea urchins rely on internal stores of nutrients to meet their energy requirements for maintenance (Guillou et al., 2000; Lares and Pomory, 1998), while, once food become available, the level of hunger may lead sea urchins to increase the consumption of food regardless of its nutritional content (Castilla-Gavilán et al., 2019). After that, the reduction of food ingestion may be an effect of the stomach fullness (Lawrence et al., 2003). Moreover, the fluctuating IR pattern observed in *P. lividus* may be also due to an intrinsic periodicity of food ingestion resulting in high peaks spaced out by a few fasting days (Nédélec et al., 1983). Comparisons with sea urchins fed with natural food (i.e. macroalgae and seagrasses) revealed contrasting results depending on the species used. Mean IR of the two formulations was higher than that observed for *Corallina elongata, Flabellia petiolata, Halopteris scoparia* and *Ulva lactuca*, comparable to the IR measured for *Dictyota* sp., *Laurencia* sp., *Padina pavonica, U. rigida* and *Posidonia oceanica,* and lower than the IR for *Codium* sp. and *Dictyopteris* sp. (Ruocco et al., 2018; Sartori and Gaion, 2015). Nevertheless, present IR values were overall comparable with those previously measured in *P. lividus* fed with commercial and experimental pellets (Ruocco et al., 2018; Sartori and Gaion, 2015). Although agar was observed to confer a high palatability to manufactured feeds without, however, affecting the digestibility (Barker et al., 1998; Fabbrocini et al., 2012, 2015; Leclercq et al., 2015), the low concentration (2.5%) used here in the preparation of the sustainable feed may have had a negligible influence on the IR values.

Similarly to the IR, the patterns observed for the absorption efficiency (AE) showed that sea urchins responded to the resumption of feed provision with high feed absorption, for meeting their nutritional requirements, regardless of the type of the food provided. After that, there was an evident difference in AE based on the formulation provided, with a higher assimilation of the formulation with a higher content of fish ingredients, than the other. This is consistent with the literature: also Fernandez and Boudouresque (2000) found a different absorption efficiency in *P. lividus* according to the food

provided. In particular, a different AE seems to depend on the assimilation of carbohydrates: vegetables are characterized by a higher amount of insoluble carbohydrates, not digestible by echinoids, that are instead poorly represented in fishmeal (Fernandez and Boudouresque, 2000, present study). This is supported also by the higher biomass of faeces found in the cages where sea urchins were fed with the 80/20 formulation, compared to those where sea urchins were fed with the 60/40 (data not showed), where the ingredients of plant and animal origin are more balanced. Despite the differences found between formulations, the mean absorption efficiency observed for both, was comparable with the AE recorded in the Australian sea urchin, *Heliocidaris erythrogramma*, fed with mixed feed (Senaratna et al., 2005), confirming the suitability of the new sustainable feed.

Finally, as previously mentioned, this preliminary short-time assessment of the effect of the sustainable feed on gonad growth, based on the evaluation of the gonado-somatic index (GSI), revealed a significant increase in GSI for both formulations, regardless of the ratio of vegetal *vs*. animal ingredients. Although GSI is usually estimated over longer time scales, our findings are overall consistent with the literature (e.g. Vizzini et al., 2019; Zupo et al., 2019) and revealed that the sea urchins fed with the new sustainable feed had good feed intake and nutrient conversion even in a very short time (2 weeks).

2.5 Conclusion

A new sustainable feed produced using anchovy and endive food industry discards with the addition of a low amount of agar resulted suitable for feeding *Paracentrotus lividus*. Two formulations at a different ratio of vegetal *vs*. animal ingredients were tested. Both showed a good stability in seawater, and a balanced nutritional composition and fatty acid (FA) profiles, which are basic requirements for feeding sea urchins. Main biomarkers of nutritional quality (PUFA, ω-3 HUFA, EFA and the ratio ω-3/ω-6) were higher in the formulation with the higher content of fish discards. This formulation was also absorbed more efficiently by the sea urchin, resulting as attractive as the other formulation, but more digestible for *P. lividus.* Finally, despite the short experimental period, the gonado-somatic index increased in all the reared sea urchins, regardless of the provided formulation. These encouraging results showed that food industry discards are suitable and promising alternative ingredients for the production of sustainable feeds for sea urchins, by meeting also the requirements of bio- and blue-economy that promote sustainable development. Moreover, on first analysis, the formulation with a more balanced ratio of vegetal *vs*. animal content (60/40) seemed more suitable in echinoculture, but further studies are needed to assess the effect of this new feed on gonad yield, in order to obtain a marketable while sustainable product.

CHAPTER 3: Turning waste into gold: sustainable feed made of discards from the food industries promotes gonad development and colouration in the commercial sea urchin *Paracentrotus lividus* (Lamarck, 1816).

This chapter has been published in Aquacultre Reports, 21: Ciriminna, L., Signa, G., Vaccaro, A.M., Visconti, G., Mazzola, A., Vizzini, S., 2021. Turning waste into gold: Sustainable feed made of discards from the food industries promotes gonad development and colouration in the commercial sea urchin *Paracentrotus lividus* (Lamarck, 1816).
The pdf article is attached at the end of the thesis.

3.1 Introduction

After the preliminary assessment on the suitability of discards from the food retail chain for the production of a sustainable feed for *P. lividus* (Chapter 2), here a further evaluation of the proposed feed was conducted. The two proposed formulations were tested on gonad production, development and colour in adult *P. lividus* reared indoor for 3 months. In more detail, the performance of the two sustainable feed formulations on gonad somatic index, gonad maturity stages and colour of reared sea urchins was tested, and results were compared with those obtained from wild specimens. The main goals were: i) to evaluate the potential of the new sustainable feed as a sea urchin diet, compared to natural patterns observed in wild sea urchins collected in the same sampling area; ii) to assess the best proportion of vegetal and animal ingredients from food industry discards promoting the best production and quality of sea urchin gonads.

3.2 Materials and methods

3.2.1 Feed formulations

A sustainable feed based on food processing discards was produced following the protocol developed by Ciriminna et al. (2020). Briefly, two feed formulations were prepared using outermost leaves of endive *Chicorium endivia* (Linnaeus, 1753) and European anchovy *Engraulis encrasicolus* (Linnaeus, 1758) processing discards (mainly fins, skin, bones and offal) plus a low amount (25 g kg^{-1}) of agar (Agar-Agar fine powder 100% Food Grade, Intra Laboratories, UK) as a binder. The two main ingredients were mixed in different proportions to prepare the two feed formulations: i) the "feed 60/40" was characterised by overall equilibrated proportions of the two ingredients (*i.e.* about 600 and 400 g kg^{-1} dry weight of endive and anchovy discards respectively), and ii) the "feed 80/20" was characterised by a higher relative amount of vegetal ingredients (*i.e.* about 800 and 200 g kg^{-1} of endive and anchovy discards respectively). To prepare the feed, agar was dissolved into boiling MilliQ distilled water (385 g l^{-1}). Then, the solution was allowed to cool to about 60°C and mixed

with the main ingredients. The obtained mixtures were manually transformed into cylindrical pellets (0.5 cm diameter, ~2 cm length, ~1 g wet weight), using 35 ml syringes, air-dried at room temperature (24°C) for 24h and then stored a -20°C until provision.

The proximate composition of the feed formulations was determined according to Ciriminna et al. (2020) and highlighted a high nutritional value of both formulations with the one with the higher amount of vegetable ingredient (feed 80/20) being richer in carbohydrates, and the other (feed 60/40) richer in protein and lipid content (Table 3.1).

Table 3.1. Ingredients and proximate composition of the two feed formulations.

	Feed Formulation	
Ingredients (g kg^{-1} dw)	**Feed 60/40**	**Feed 80/20**
Cichorium endivia	587.5	787.5
Engraulis encrasiculos	387.5	187.5
Agar	25.0	25.0
Proximate Composition (g kg^{-1} dw)		
Lipid	138.0	96.2
Protein	294.8	264.9
Carbohydrates	321.3	423.2
Ash	245.9	215.9

Mean proximate composition of the feed ingredients from Ciriminna et al. (2020): *Cichorium endivia* outermost leaves: lipid: 38.0, protein: 191.4, carbohydrates: 644.2, ash: 126.3 g kg^{-1} dw. *Engraulis encrasicolus* processing discards: lipid: 140.1, protein: 405.8, carbohydrates: 43.4, ash: 410.7 g kg^{-1} dw.

Lipids were measured following a slightly modified version of the Bligh and Dyer (1959) method: a solution of MilliQ distilled water, methanol (CARLO ERBA Reagents, Chaussée du Vexin, France) and chloroform (Panreac Quimica Sau, Barcelona, Spain) ratio1:2:1 (v:v:v) respectively, with 0.01% of butylated hydroxytoluene (Sigma-Aldrich®, St. Louis, United State of America) as an antioxidant, was added to ground sub-samples of freeze-dried feed bars. Samples were then sonicated to improve lipid extraction and centrifuged twice to separate the lipid phase from the aqueous phase. The lipid extracts were evaporated to dryness under a gentle nitrogen stream and weighed. Protein content was estimated by analysing the total nitrogen content in an Elemental Analyzer (FlashEA® 1112, Thermo Fisher Scientific, Monza, Italy), which was subsequently converted in protein content by applying a conversion factor of 6.25 (Horowitz and Latimer, 2006). Ash content was assessed by combustion in a muffle furnace (ASAL ZB/1, Asal s.r.l. Milan, Italy) at 550°C for 4 hr according to Nielsen (2010). Carbohydrates were indirectly determined according to Baião et al. (2019), by applying the following formula: carbohydrates = (100 – (ash + protein + lipid)).

3.2.2 Feeding trial

The feeding trial was conducted on adult sea urchins, as they allocate more energy for reproduction than juveniles (Fabbrocini and Adamo, 2010; Fernandez and Boudouresque, 2000). In the experiment, sea urchins fed on the two new formulations were compared with the natural development of wild sea urchins. Natural diets (*i.e.* macroalgae) were not included in the experiment because of *i*) the low performance of algal diets on *Paracentrotus lividus* (Lamarck, 1816) gonad yield and quality, as already found in a previous study conducted under the same experimental conditions (Vizzini et al., 2014); *ii*) the low sustainability of algal diets in aquaculture and hence the low relevance to the objectives of the present study.

In October 2015, about 160 wild specimens of *P. lividus* of similar size (mean test diameter TD ± standard deviation: 41.2 ± 5.3 mm) were collected by SCUBA divers at Cala Rossa, Terrasini (38°8'34.47" N; 13°4'15.99" E; northern Sicily, Italy, Mediterranean Sea) and transported to laboratories within seawater-filled oxygenated containers. The sampling area was overall characterized by a meadow of *Posidonia oceanica* (L.) Delile, 1813, patches of *Cystoseira* (C. Agardh, 1820) spp. and *Dictyota* (J.V.Lamouroux, 1809) spp. macroalgae on a rocky bottom (author's observations). In the laboratory, 20 specimens were randomly collected, weighed and sacrificed to evaluate the initial gonad conditions in terms of gonad biomass, gonad somatic index, maturity stage and colour (T0 - Wild). Other 140 specimens were transferred into a 150 L tank and kept fasting for 15 days, for acclimatising to laboratory conditions and standardising the relative food appetence before the onset of the feeding trial (Pearce et al., 2002a). After the fasting period, other 20 specimens were randomly collected and sacrificed to evaluate the potential effects of fasting (T0 - Fast) on the gonad conditions. The remaining 120 sea urchins were randomly divided into two groups of 5 tanks with 80 L capacity (in total, 10 tanks with 12 specimens per tank) in a recirculating aquaculture system (RAS) (Figure 3.1). Each group corresponded to a feed formulation (feed 60/40 and feed 80/20, respectively). The animals were fed *ad libitum* every 72h, for 12 weeks (~ 3 months, October 2015 - January 2016), which was considered a suitable period for assessing the effects of controlled feeding according to the literature (Carboni et al., 2015; Schlosser et al., 2005; Vizzini et al., 2014). Before each round of feed provision, feed particle leftovers and sea urchin faeces were carefully siphoned from each tank. The RAS was equipped with a common sand filter, bio-filter and protein skimmer to maintain optimal water quality conditions. Moreover, the environmental conditions in the tanks were kept stable throughout both the fasting period and the feeding trial, with seawater temperature: 20.0 ± 1.0 °C, salinity: 38.0 ± 0.5, photoperiod: 8h light and 16h dark and continuous

water flow in/out: 5 L min^{-1}. Ammonia levels (mean ± s.d.: 0.034 ± 0.005 mg l^{-1}), pH (8.07 ± 0.04) and oxygen saturation (always > 90 %) were measured daily in effluent water from each tank.

Every 4 weeks (T1, T2, T3 corresponding respectively to November, December 2015 and January 2016), 4 specimens were randomly collected from each tank, for a total of 20 specimens for each feed formulation. At the same experimental times, wild sea urchins of similar size (43.1 ± 3.7 mm) were also collected (15 - 20 specimens each time depending on availability in nature) from the same coastal site, to allow the comparison over time between reared and wild specimens. After each sampling, wild sea urchins were kept for 24h in an 80 L tank of the same experimental RAS, to empty their gut and avoid biases in the next weight measurements. For the same reason, reared sea urchins were collected and measured before feeding provision. No mortality was recorded throughout the experimental period.

Total weight (TW) measurement was performed from T0 to T3 using an electronic balance (Sartorius BL120S d ± 0.01 mg). Then, the weighted sea urchins were sacrificed to record the gonad wet weight (GW) and calculate the gonad somatic index (GSI) as:

$$GSI = GW/TW \times 100.$$

After TW and GW measurement, one of the five gonads of each sacrificed sea urchin was taken for microscopic (Leica Leitz DMRB, Leica, Wetzlar, Germany) determination of sex. Then, a subsample of 6 female specimens and 5 male specimens for each formulation was randomly selected for histological analysis aiming at confirming the sex and the maturity stage. Selected gonads were dehydrated, embedded in paraffin, dissected in 7 μm thick slices using a microtome (5040 Rotary Microtome, Bright Instuments, Huntingdon, United Kingdom) and stained with the alcian blue-periodic acid Schiff reagent (AB/PAS) method. Gonads (both ovaries and testis) were preliminarily categorized according to morphologically criteria on a scale of gamete maturity phases and nutritive deterioration of gonads (Byrne, 1990). Therefore, the stages of the *P. lividus* gametogenic cycle were classified into six categories: stage I, recovery; stage II, growing; stage III, premature; stage IV, mature; stage V, partly spawned; stage VI, spent (Byrne, 1990).

To assess the colour, gonads were placed in clean Petri dishes and compared with Pantone® colour standards chart (Colour Formula Guide 1000, 1991) under standard artificial daylight (Reer, 4000K) by three expert observers (Symonds et al., 2007; Vizzini et al., 2014, 2019). The observers assigned each specimen to a single colour category among those defined by Shpigel et al. (2005): dark orange (DO), pale yellow (PY), bright orange (BO), yellow-orange (YO) and mango orange (MO), which were then classified in three quality categories (I: inadequate, A: acceptable and E: excellent) following Symonds et al. (2009).

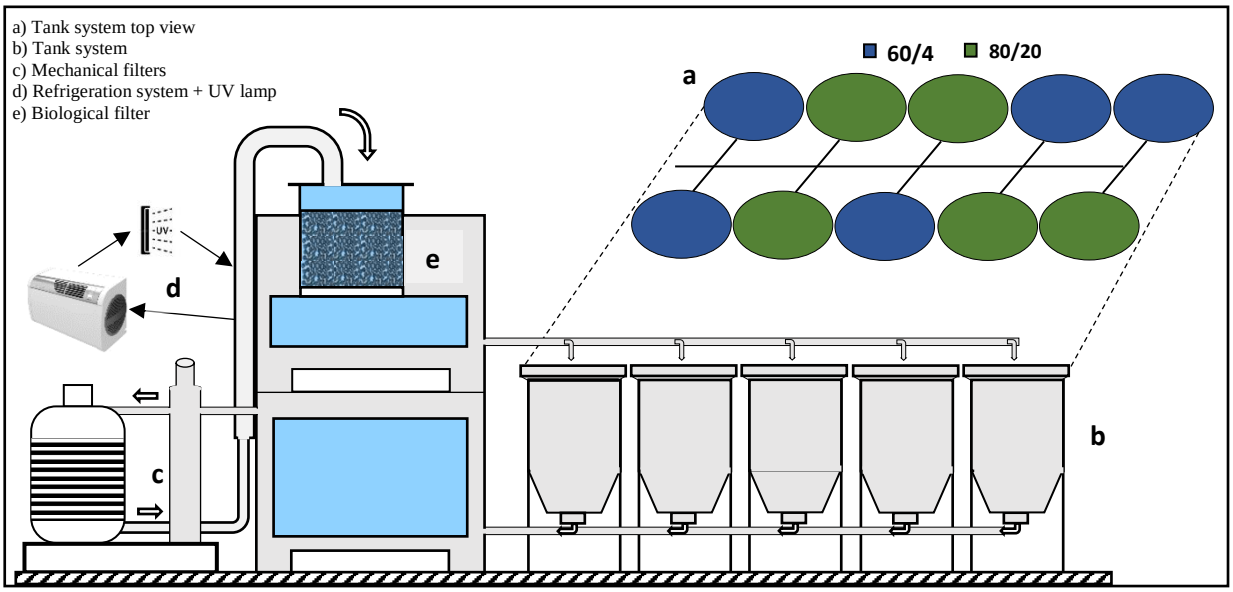


Figure 3.1. Scheme of the indoor recirculating aquaculture system (RAS) used for the feeding trial with *Paracentrotus lividus* Lamarck (1816). Five 80-L tanks were randomly assigned to both experimental feed formulations (feed 60/40 and feed 80/20).

3.2.3 Statistical analysis

Differences in gonad wet weight (GW) and gonad somatic index (GSI) between sea urchins across time were tested using univariate permutational analysis of variance (PERMANOVA, Anderson et al., 2008). The factors Feed and Time were both fixed and orthogonal, the former with 3 levels (Feed: Wild, feed 60/40 and feed 80/20) and the latter with 4 levels (Time: T0, T1, T2, T3). Analyses were based on untransformed data resembled using Euclidean distance and were performed using the software PRIMER 6 v3.1.10 & PERMANOVA+ β20 (Plymouth, UK). When significant differences were found, pair-wise tests were run as a posteriori check for significant effects.

3.3 Results

3.3.1 Gonad growth

Permutational analysis of variance (PERMANOVA) carried out on gonad wet weight (GW) and gonad somatic index (GSI) data showed significant differences for both factors Feed and Time and their interaction (Table 3.2a). In particular, GW showed a significant gradual increase across time only in reared sea urchins, while GW of wild specimens was significantly higher just at T3 than at T0 (Figure 3.2a). On the other hand, while the three treatments did not differ significantly at T0, sea urchins fed with the feed 60/40 presented significantly higher GW than wild ones at all times starting from T1, while those fed with the feed 80/20 from T2. Moreover, the feeding treatment 60/40 showed also the fastest increment of gonad weight over the trial (percentage GW increase from T0 to T3 =

78.1, 329.0 and 488.8% for wild, feed 80/20 and feed 60/40 respectively), as well as the highest GW values at T3.

Similar to GW, wild sea urchins showed significant differences in GSI only between T3 and T0, but also a fluctuating trend over the experimental period (Figure 3.2b). In contrast, all reared sea urchins presented a significant increase in GSI throughout the trial, mirroring the patterns of GW. The comparison between treatments highlighted comparable values at T0, significant differences between wild and reared specimens since T2 and between reared specimens (feed 60/40 > feed 80/20) only at T3.

Table 3.2. Results of permutational analysis of variance (PERMANOVA) testing for differences in gonad weight (GW) and gonad somatic index (GSI) of *Paracentrotus lividus* Lamarck (1816) between feed formulations, times and their interaction.

a) MAIN TEST			**GW**			**GSI**		
Source of variation		**df**	**MS**	**Pseudo-F**	**P(perm)**	**MS**	**Pseudo-F**	**P(perm)**
Feed		2	50.34	13.85	**0.001**	412.26	33.60	**0.001**
Time		3	86.39	23.77	**0.001**	507.63	41.38	**0.001**
Feed x Time		6	11.72	3.22	**0.004**	84.38	6.87	**0.001**
Residual		219	3.6			12.26		
b) PAIR-WISE TESTS			**GW**			**GSI**		
Within time between feeds			**t**	**P(perm)**	**Unique perms**	**t**	**P(perm)**	**Unique perms**
T0	**WILD vs FAST**		1.25	0.215	901	0.58	0.541	995
T1	**WILD vs FEED 60/40**		1.82	**0.029**	963	1.82	0.071	998
	WILD vs FEED 80/20		1.56	0.122	944	0.96	0.360	999
	FEED 60/40 vs FEED 80/20		0.50	0.696	976	0.73	0.480	997
T2	**WILD vs FEED 60/40**		3.19	**0.003**	982	5.72	**0.001**	999
	WILD vs FEED 80/20		2.67	**0.014**	973	4.18	**0.001**	998
	FEED 60/40 vs FEED 80/20		1.16	0.274	957	1.37	0.178	997
T3	**WILD vs FEED 60/40**		4.13	**0.001**	984	6.50	**0.001**	997
	WILD vs FEED 80/20		2.60	**0.018**	985	4.31	**0.001**	996
	FEED 60/40 vs FEED 80/20		2.06	**0.047**	982	2.11	**0.048**	997
Within feed between times								
WILD	**T0 vs T1**		0.25	0.808	913	1.97	0.066	996
	T0 vs T2		0.43	0.646	686	0.97	0.363	996
	T0 vs T3		2.01	**0.043**	975	1.96	**0.032**	995
	T1 vs T2		0.22	0.843	960	0.80	0.440	998
	T1 vs T3		1.80	0.053	962	0.38	0.732	998
	T2 vs T3		1.36	0.193	972	0.99	0.353	995
FEED 60/40	**FAST vs T1**		2.44	**0.001**	960	3.19	**0.003**	997
	FAST vs T2		4.60	**0.001**	956	6.81	**0.001**	998
	FAST vs T3		7.68	**0.001**	975	9.98	**0.001**	994
	T1 vs T2		1.26	0.255	966	3.49	**0.002**	996
	T1 vs T3		3.44	**0.001**	978	6.40	**0.001**	996
	T2 vs T3		2.34	**0.025**	968	2.78	**0.011**	996
FEED 80/20	**FAST vs T1**		2.36	**0.024**	960	2.19	**0.038**	996
	FAST vs T2		4.41	**0.001**	942	5.06	**0.001**	997
	FAST vs T3		6.34	**0.001**	973	7.00	**0.001**	998
	T1 vs T2		1.10	0.264	956	2.60	**0.013**	997
	T1 vs T3		2.81	**0.009**	978	4.43	**0.002**	998
	T2 vs T3		1.95	0.059	965	1.85	0.075	998

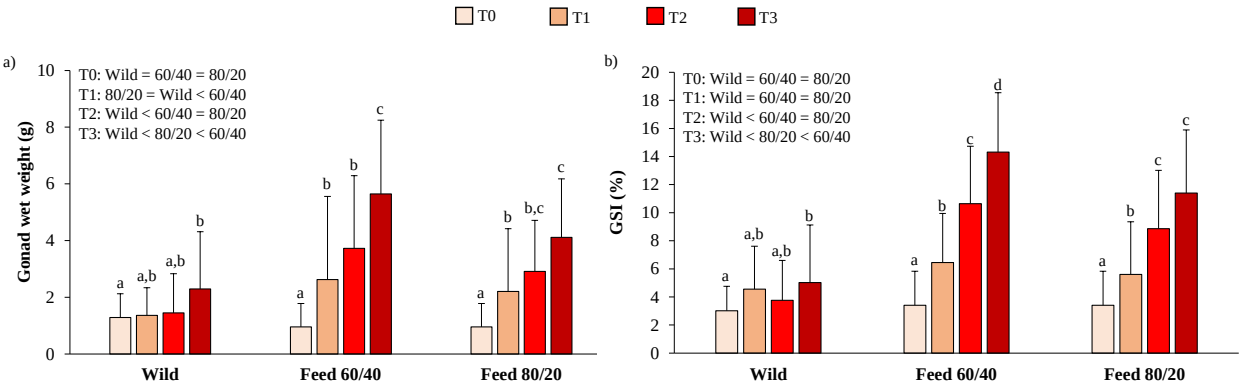


Figure 3.2. a) Gonad wet weight (GW, mean ± standard deviation) and b) gonad somatic index (GSI, mean ± standard deviation) of sea urchins collected from the natural environment (wild), and sea urchins fed with the two experimental feed formulations (feed 60/40, feed 80/20) across experimental times (T0-T3). Lowercase letters on each panel indicate significant differences between times within feeds. The boxes on both panels indicate significant differences between feeds within times.

3.3.2 Gonad development

Histological analysis revealed overall a slower gonad development in wild specimens than reared sea urchins (Figure 3.3, 3.4). Wild females showed a gradual maturation across time, consisting of a decrease of the frequency of the gonads found in the recovery stage (I), up to disappearance at T3, and an increase of those found in the premature stage (III) (Figure 3.3). On the other hand, reared females showed a sharper progression in sexual development, achieving the mature stage (IV) at T2, with a frequency of 50% of all the observed sea urchins, and remained stable up to T3, regardless of the feed formulation provided (Figure 3.3).

Similarly to females, wild males showed a decrease in the recovery stage (I) and an increase in the premature stage (III) from T0 to T3, but the mature stage (IV) was not achieved (Figure 3.4). Moreover, unlike females, reared males showed different patterns according to the formulation provided. In more detail, the specimens fed with the feed 60/40 showed a faster development than the others (feed 80/20), with the achievement of the premature stage (III) just one month after the start of the feeding trial (T1), followed by a further increase up to achieve the 100% of all the observed gonads at the end of the trial (T3). In contrast, the male specimens fed with the feed 80/20 achieved the premature stage at T2, followed by a lower increase at T3, when immature gonads were also found (Figure 3.4).

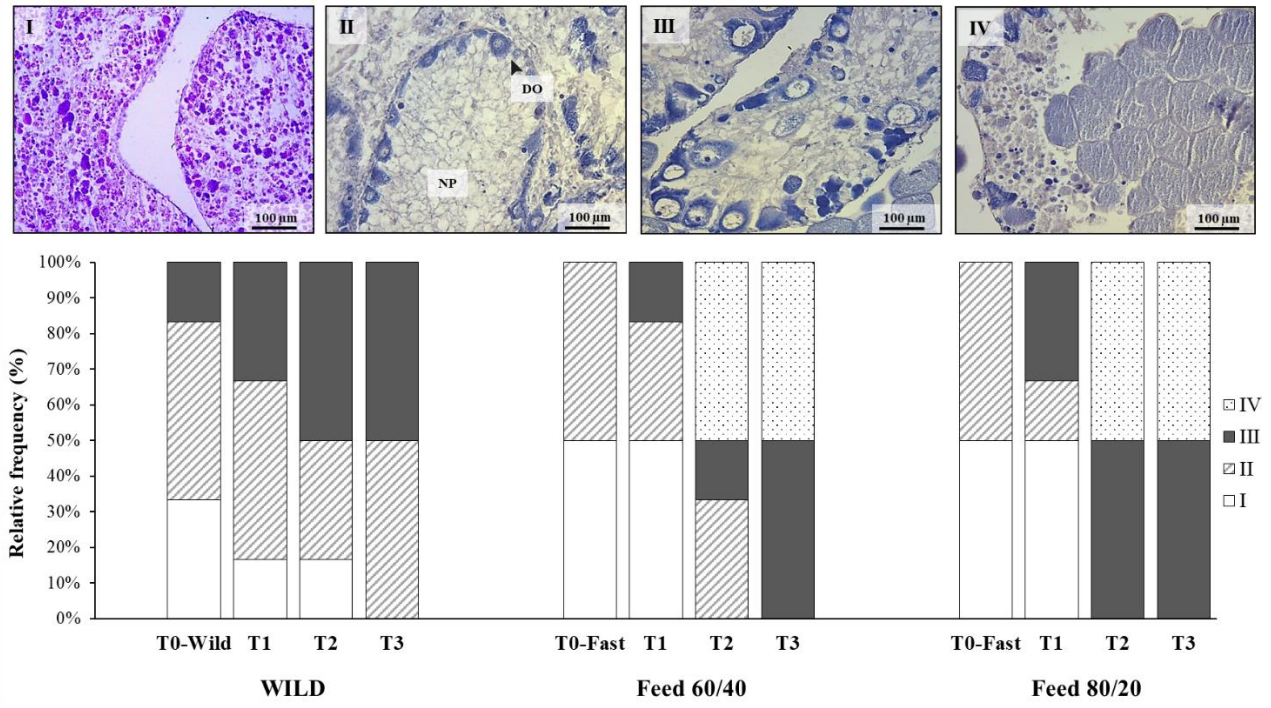


Figure 3.3. Above: histological sections of female gonads. Stage I (recovery): nutritive phagocytes begin forming a meshwork across the ascini. Stage II (growing): nutritive phagocytes (NP) and early vitellogenic oocytes attached to the ascinal wall (DO). Stage III (premature): oocytes at all stages of development. Stage IV (mature): ovary packed with ova. Below: relative frequency (n= 6) of the gametogenic stages of female sea urchins collected from the natural environment (wild), and sea urchins fed with the two experimental formulations (feed 60/40, feed 80/20) across experimental times (T0-T3).

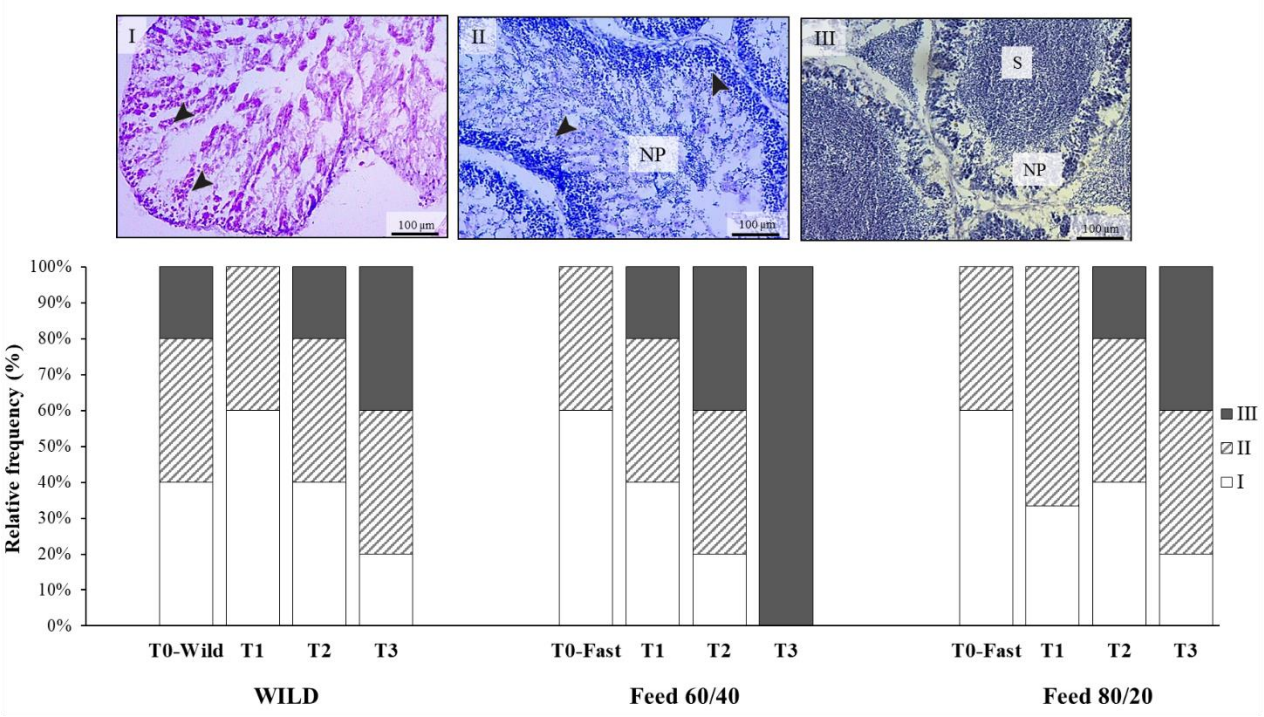


Figure 3.4. Above: histological sections of male gonads. Stage I (recovery): primary spermatocytes along ascinal wall (arrowheads). Stage II (growing): testis with columns of developing spermatocytes (arrowheads) in the meshwork of nutritive phagocytes (NP). Stage III (premature): premature testis with spermatozoa (S) in the ascinal lumen and nutritive phagocytes (NP) around the periphery. Below: relative frequency (n= 5) of the gametogenic stages of male sea urchins collected from the natural environment (wild), and sea urchins fed with the two experimental formulations (feed 60/40, feed 80/20), across experimental times (T0-T3).

3.3.3 Gonad colour

The assessment of gonad colour showed an excellent colouration (E) at the beginning of the trial (T0) in almost 70% of both wild and fasted sea urchins. This value increases up to about 90% if the categories "E: excellent" and "A: acceptable" are summed together (Figure 3.5). Wild specimens showed an overall worsening of the gonad colour over time, consisting of a sharp decrease of the frequency of the gonads with excellent and acceptable colour (E + A) (from 90 to 47 % at T0 and T3 respectively) coupled with a marked increase of those classified as inadequate (I) (from 10 to 53 % at T0 and T3 respectively). In contrast, despite both groups of reared sea urchins showed an initial colour worsening, especially those fed with the feed 80/20 (E + A gonad frequency: from 90 to 55% at T0 and T1 respectively), all the sea urchins fed with the sustainable feed highlighted a subsequent and overall improvement of the gonad colour (A+E gonad frequency: 95% at T3 for both groups of reared sea urchins). However, the frequency of the gonads with excellent colour was always higher in those fed with the feed 60/40 than in those fed with feed 80/20. In particular, at T3, the sea urchins fed with the feed 60/40 showed about 90 + 5% of excellent and adequate gonads, while those fed with

feed 80/20 only about 55 + 40% of excellent and adequate gonads. Less than 10% belonged to the inadequate (I) category in both groups of reared sea urchins (Figure 3.5).

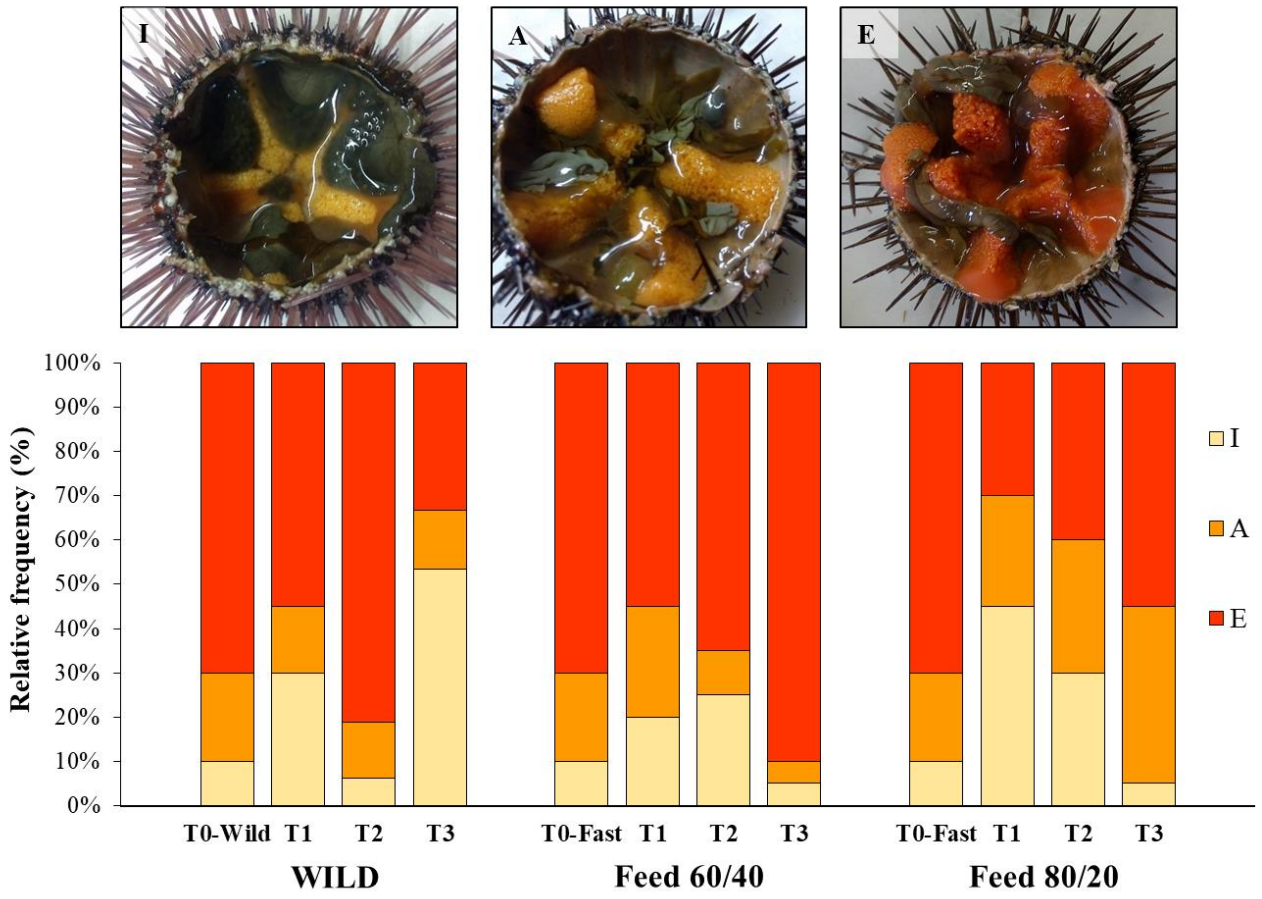


Figure 3.5. Relative frequency of the gonad colour categories of sea urchins collected from the natural environment (wild), and sea urchins fed with the two experimental formulations (feed 60/40, feed 80/20), across experimental times (T0-T3). Values are expressed as the percentage of specimens with gonads within each colour category (E: excellent, A: acceptable, I: inadequate).

3.4 Discussion

A sustainable feed prepared by recycling processing discards of endive and common anchovy from the food retail chains was provided to adult sea urchins *Paracentrotus lividus* (Lamarck, 1816), in controlled conditions, to assess the potential of sustainable feeds based on the circular economy principles for echinoculture. In more detail, sea urchins were fed for 3 months with two feed formulations characterised by a different ratio of vegetal *vs.* animal ingredients to evaluate the most effective formulation promoting gonad production, maturation and quality. The results were compared with those obtained in wild sea urchins, aiming to evaluate differences with natural

patterns. Overall, both formulations led to a greater increase in gonad production in reared *P. lividus* than in wild specimens, in terms of gonad wet weight (GW) and gonad somatic index (GSI). Similarly, reared sea urchins showed faster sexual maturation and a better gonad colouration than wild specimens, confirming the high suitability of the sustainable feed as a diet for sea urchins under farming conditions.

At the beginning of the trial, no significant differences in both GW and GSI were highlighted between fasting and wild sea urchins, indicating that the fasting period did not lead to significant changes in gonad biomass (Guillou et al., 2000; Raposo et al., 2019) and therefore confirming that is suitable for standardizing the initial experimental conditions without affecting gonad production. Afterwards, the significant increase in GW and GSI observed in the sea urchins fed with the two experimental formulations after just one month, suggests a rapid conversion of the provided feed nutrients into gonad biomass, contributing to the higher gonad than somatic growth. Indeed, gonads are the main nutrient storage organ as the nutritive phagocytes of the gonads are responsible for both nutrient accumulation and transfer into the developing gametes (Fabbrocini et al., 2012; Marsh et al., 2013; Walker et al., 2015). For this reason, GSI is considered a good indicator of diet nutritional quality and high-quality feeds are associated with high GSI values (Carboni et al., 2015; Cuesta-Gomez and Sànchez-Saavedra, 2016).

In contrast, the patterns of GW and GSI observed in wild sea urchins did not match perfectly and indicated a much more gradual and lower increase over time, maybe due to a lack of a proper nutritional supply across time (Byrne, 1990; Shpigel et al., 2004; Tenuzzo et al., 2012). Although the growth of sea urchin gonads into the wild may benefit from the low winter temperature and the short daylight period (Byrne, 1990; Shpigel et al., 2004), it is also strongly influenced by food quality and availability, which are typically fluctuant in the natural environment (Cook and Kelly, 2007a) differently from the constant and controlled rearing condition. Although wild *P. lividus* can remove large amounts of vegetal biomass to satisfy their nutritional requirements (Klinger, 1984; Lawrence et al., 2020), their favourite food items (*i.e.* macroalgae) are characterised by low concentrations of macro and micronutrients (Cook et al., 2000; Fernandez and Boudouresque, 2000; Vizzini et al., 2014). Therefore, it is likely that the ingestion of algal biomass (*Cystoseira* spp. and *Dyctiota* spp. are abundant in the collection site) might have led to scarce energy intake and consequently an imbalance between gonad and somatic growth (Schlosser et al., 2005; Shpigel et al., 2005).

In contrast, although the controlled and stable condition of the rearing system used for the feeding trial may have plaid a positive role, the influence of diet on gonad growth is further confirmed by the comparison between the two feeding treatments. Comparing the results from reared sea urchins, it was evident, indeed, that the nutritional differences between the two formulations played a key role

in modulating gonad production. Although *P. lividus* is considered a herbivore (Bouduresque and Verlaque, 2007), the sea urchins fed with the formulation characterised by a more balanced vegetal/animal ratio (*i.e.* feed 60/40) showed higher GW values and a greater gonad increment over time than those fed with the formulation with a higher vegetal content (*i.e.* feed 80/20). GSI was also boosted by the feed 60/40, consistently with Fernandez and Boudouresque (1998; 2000), who also found the highest GSI following the provision of a balanced diet, made of 60% of vegetal ingredient and 40% of fish meal. These findings confirm the key role of animal proteins and lipids as important storage compounds of the nutrients needed for gonad growth, development and maturation (Fernandez and Boudouresque, 2000; González-Durán et al., 2008; Grosso et al., 2021). Furthermore, the common anchovy discards are richer in fatty acids than endive leaves, especially in omega-3-long chain polyunsaturated and essential fatty acids (Ciriminna et al., 2020), which are vital for reproductive fitness and a multitude of physiological functions, including gonad development and enhancement (Glencross, 2009; Liu et al., 2007; White et al., 2016). In contrast, the higher relative amount of vegetal ingredients of the feed 80/20 may have led to slower absorption of nutrients. Vegetal meals have, indeed, a high abundance of insoluble carbohydrates (Bach Knudsen, 1997; Esteban et al., 2007) and fibres (Plazzotta et al., 2017), which are usually poorly digested by sea urchins (Fernandez and Boudouresque, 2000; Powell et al., 2020). However, good performances of artificial diets based on terrestrial vegetables on *P. lividus* GSI have been recently highlighted by several authors. Raposo et al. (2019), Santos et al. (2019) and Sartori and Gaion (2015) recorded high, while different, performances (mean GSI = 9, 9 and 19% respectively) feeding *P. lividus* with maize and spinach, while Vizzini et al. (2014) obtained a high mean GSI (10%) providing lettuce to *P. lividus*. The results obtained in this study are overall comparable to these, but also slightly better than others related to sea urchins fed with macroalgae or mixed diets (Prato et al., 2018; Vizzini et al., 2014; Zupo et al., 2019). This confirms the higher potential of the proposed sustainable feed formulation based on balanced vegetal and animal discards (*i.e.* feed 60/40) in promoting gonad growth, compared to natural or macroalgae-based diet.

Gonad maturation is another important issue in echinoculture, because marketable high-quality gonads, in terms of firmness, colour and taste, are obtained when the ratio between nutritive phagocytes (abundant in the recovery and growth phases) and gametes (abundant in the mature and generation phases) are in favour of the former (Böttger et al., 2006; Walker et al., 2001). Most of the sea urchins collected at the beginning of the trial were in the early reproductive stages, consistent with the annual reproductive cycle of *P. lividus* in the Mediterranean area (Lozano et al., 1995). While the fasting period seems to have induced a partial regression in the reproductive stages, consistent with Guillou et al. (2000) and Raposo et al., (2019), the gonad maturation patterns of reared sea

urchins revealed a subsequent progression in the reproductive stages. In more detail, both formulations promoted similar gonad development in female sea urchins, while the feed 60/40 showed the best performance in males, achieving the totality of specimens in the premature stage at the end of the trial. Different response of females and males may be related to different specific requirements during sexual maturation and gametes production. Indeed, the reproductive effort is greater in females than in males, as the development of embryos and larvae depends on the maternal provisioning of nutrients (Carboni et al., 2015). A suitable advancement in sexual maturation is fundamental for echinoculture purposes. A low progression may result in a limited increase in gonad biomass, due to a slow nutritive phagocytes growth, while a too fast gonad maturation could lead to spawning events with a consistent loss in gonad biomass due to the emission of gametes (Marsh et al., 2013; Walker et al., 2001, 2015). Nevertheless, in this study, the gonad development was faster in reared sea urchins than in wild specimens. These results contrast with those from Fabbrocini et al. (2019), who found a quicker progression in wild specimens than in sea urchins fed with agar-based pellets. These differences, however, may be related to the different environmental conditions (off-shore cages) and the short duration of the trial (one month) conducted by Fabbrocini et al. (2019). Here, both the experimental formulations fostered a suitable gonad development for echinoculture goals, indicating an appropriate energy supply, which is one of the most limiting factors for gonad development in growing and mature stages of the reproductive cycle (Schlosser et al., 2005). Indeed, during these stages, sea urchins need to store great amounts of nutrients for the development of gametes (Walker et al., 2015).

Lastly, the analysis of gonad colour, which is a key factor in determining both the quality of the gonads and its economic value (Stefánsson et al., 2017), revealed also positive effects of the new feed formulations on the sea urchin gonads. The vast majority of reared sea urchins achieved, indeed, a marketable colouration after 12 weeks, differently from wild sea urchins that showed an initial colour improvement, followed by a clear worsening over the experimental period. Colour in echinoid gonads is driven mostly by carotenoid dietary intake, and particularly by echinenone, which sea urchins synthesize from β-carotene (Shpigel et al., 2005). Outermost endive leaves have a greater concentration of carotenoids (lutein and β-carotene), compared with young leaves (de Azevedo-Meleiro and Rodriguez-Amaya, 2005), resulting in well suited dietary sources for obtaining high-quality gonads. The temporal pattern observed for gonad colour, however, suggests that sea urchins need time to absorb carotenoids from the diet, synthesize echinenone and store it in the gonads, consistent with previous studies (Plank et al., 2002; Shpigel et al., 2005). Nevertheless, the overwhelming majority (~ 95%) of the gonads produced by *P. lividus* in 12 weeks presented an excellent and acceptable (E + A) colour, confirming the efficacy of both formulations in producing

marketable gonads. Similarly, Santos et al. (2019) obtained marketable gonad colour from sea urchin fed with diets based on spinach, maize and pumpkin, while Luo et al. (2014) found a better performance in promoting gonad colour in sea urchins fed with kelp-based diet than those fed with pumpkin or banana peel. Moreover, Vizzini et al. (2018, 2019) highlighted the effectiveness of lettuce vegetal discards mixed with animal lipids and proteins (*i.e.*, white eggs and commercial pellet) in improving the gonad colour of *P. lividus* (70-90% of gonads in E + A colour). Here, the present findings suggest that the use of animal discards is also highly functional to improve gonad colour and that vegetal discards may replace added carotenoids, usually considered one of the most expensive complements in feed formulation (Cuesta-Gomez and Sánchez-Saavedra, 2018).

3.5 Conclusion

The present study showed that the outermost leaves of *Cichorium endivia* (Linnaeus, 1753) and *Engraulis encrasicolus* (Linnaeus, 1758) processing discards from the food retail chains have a great potential to be recycled as dietary ingredients in echinoculture. The new sustainable feed based on food processing discards promoted gonad growth and development and contributed to improving the gonad coluor of the purple sea urchins *Paracentrotus lividus* (Lamarck, 1816), the most important commercial sea urchin in the Mediterranean Sea (Stefánsson et al., 2017). In this way, food processing discards are transformed into new valuable biomass that might naturally replace traditional ingredients (e.g., fish oils and meals) in aquaculture feeds. This would reduce the environmental and economic impact of the food discard production and disposal, under the principles of circular economy and sustainability, toward the Blue Growth. Moreover, recycling food processing discards may significantly reduce the pressure exerted on marine organisms (macroalgae and fish) for the production of aquaculture feeds. However, since sea urchin gonads are a high-quality niche product, further research is needed to assess the effect of the new sustainable feed on nutritional value and organoleptic features of gonads, as well as to evaluate their performance in aquaculture, compared with commercial feeds.

CHAPTER 4: *Paracentrotus lividus* rearing under four sustainable diets employing Raking method as a new productive protocol

4.1 Introduction

Today, the increasing demand for sea urchins is not met by the productions currently available through aquaculture techniques, because farming practices are still limited by their low efficacy. The lack of proper hatchery and breeding techniques than can enable high larval survival rate and production (Carboni et al., 2013; Rubilar and Cardozo, 2021), the long period requested by juveniles to achieve the commercial size and to produce gonad biomass for aquaculture goals (Boudouresque and Verlaque, 2020, Walker et al., 2015) are the main bottlenecks of echinoculture development. The aim of this experiment was to test five diets on *Paracentrotus lividus* egg production employing the Raking method, a new generation method developed in 2019 at the Laboratory of Experimental Ecology and Aquaculture of the University of Rome Tor Vergata, which introduces an innovative production approach in echinoculture (Rakaj et al. in press). Unlike the traditional methodology which envisages the entire gonad as the final consumer product, thus imposing the sacrifice of adult sea urchins at the end of the breeding cycle for gonad consumption, the Raking method provides eggs as a final product in form of "sea urchin caviar". The caviar production with this method is obtained by breeding female-only batches, which are induced to spawn with cyclical cadence through a combined wet-dry and thermal shock. The echinoculture thus conceived allows to overcome the main traditional constraints due the long breeding time to reach the market size. With this method, in fact, it is not necessary to sacrifice the sea urchins at the end of the rearing cycle, but the same stock must go through multiple reproductive cycles (Rakaj et al. in press). To test the effect of the four sustainable diets, ingestion rate, absorption efficiency, somatic growth and egg production were evaluated. Finally, a first evaluation on the effect of the experimental diets on gonad quality was conducted by assessment of egg colour. Findings were compared with those obtained from a control group of sea urchin fed with an algal-based diet, considered their natural diet.

4.2 Materials and Methods

4.2.1 Feed formulation

For the feeding experiment, five diets were prepared. Four sustainable diets were obtained using a vegetal base of lettuce *Lactuca sativa* discarded leaves as the main ingredient (86%), and a small amount of animal meal (10%) (Table 4.1). In particular, the four sustainable diets were obtained mixing common lettuce discards with fish meal (Diet-F), krill meal (Diet-K), mussel meal (Diet-M)

and fish processing discards (viscera, heads, bones, skin, Diet-D). The fifth experimental diet, composed by equal proportions of *Ulva* sp. and *Laminaria* sp., was used as control diet (Diet-A). In all experimental formulations, agar (3%) and arabic gum (1%) were used as binders. An extra supplement of *Lithothamnium calcareum* (20% of the total feed) was integrated in each diet to provide an appropriate content of inorganic carbon. Each formulation was prepared with boiling water and mixed to obtain a homogeneous mixture. Pellets were produced using an industrial extruder (8mm diameter), and left to air dry for 24 hours. Finally, the pellets were steamed for 15 minutes in a kitchen steamer and stored at -20°C. Three replicates for each ingredient and diet were taken for evaluation of proximate composition.

4.2.2 Proximate composition

To assess the proximate composition of the five experimental diets three replicates of each diet were freeze-dried, grounded and then analysed. Lipids were measured following a modified version of the Bligh and Dyer (1959) (Chapter 2), protein content was estimated by analysing the total nitrogen content in an Elemental Analyzer (Thermo Fisher Scientific EA 1112), which was subsequently converted in protein content by applying a conversion factor of 6.25 (Horowitz and Latimer, 2006). Ash content was determined by combustion in a muffle furnace at 550°C for 4 hr according to Nielsen (2010), while carbohydrate content was also estimated, according to Baião et al. (2019) as follows:
carbohydrates = (100 – (ash + protein + lipid))

4.2.3 Diet stability

Before starting the feeding experiment, a preliminary evaluation of stability in seawater was conducted in a recirculating aquaculture systems (RAS) with stable environmental condition: seawater temperature, 20.0 ± 1.0 °C, salinity, 35.8 ± 0.3 PSU, pH 8.20 ± 0.20 and natural photoperiod. As first step, tree replicates of each formulation were weighed (WW), oven dried at 60°C until a constant weight was reached (≈ 48h), and then weighed again (DW), in order to assess the standard dry weight ($DW_s\%=DW/WWx100$) of each diet and to be able to calculate the dry weight from the wet weight. Next, three replicates per diet were weighed and left in water for 24h, after which they were oven dried until constant weight and then weighed again to assess the final dry weight. Feed loss was calculated as follow:

DW_{loss} (%) = $[(DW_i-DW_f)/DW_i] \times 100$

where DWi is the dry weight of each feed pellet, calculated on the standard dry weight as follow:

DW_i (g) = $(WW_i \times DW_s\%)/100)$

Table 4.1. Ingredients (%) and proximate composition (mg/g, dry weight) of the five experimental diets, algal diet with *Ulva* and *Laminaria* (Diet-A), diet based on common lettuce discards with *Sardina pilchardus* meal (Diet-F), krill meal (Diet-K), mussel meal (Diet-M), and a meal obtained from *Engraulis encrasicolus* processing discards (Diet-D).

Ingredients (%)	**Diet-A**	**Diet-F**	**Diet-K**	**Diet-M**	**Diet-D**
Laminaria sp.	48	-	-	-	-
Ulva sp.	48	-	-	-	-
Vegetal discards (*Lactuca sativa*)	-	86	86	86	86
Fish meal (*Sardina pilchardus*)	-	10	-	-	-
Krill meal (*Euphausia superba*)	-	-	10	-	-
Mussel meal (*Mytilus galloprovincialis*)	-	-	-	10	-
Fish discards (*Engraulis encrasicolus*)	-	-	-	-	10
Binder	4	4	4	4	4
	100	100	100	100	100
Lithothamnium calcareum (external supplement)	20	20	20	20	20
Proximate composition (dry weight)					
Protein	8.66	25.20	26.41	25.54	27.04
Lipid	1.92	4.95	6.20	6.29	6.85
Ash	34.05	34.50	32.99	34.20	35.51
Carbohydrates	55.35	35.32	34.38	33.96	30.59

4.2.4 Feeding experiment

In March 2020, 220 adult specimens (TD: 47.41 ± 4.18 mm) of *Paracentrotus lividus* were collected by snorkeling (1–5 m depth) at Santa Marinella, Italy (42°3′0″Nord, 11°49′9″Est). Sea urchins were transported to the Laboratory of Experimental Ecology and Aquaculture (L.E.S.A) of the University of Rome "Tor Vergata" inside a 150-Lt tank equipped with aerators and dry ice. Organisms were maintained in a 600 L indoor tank in a closed circulation aquarium, and fed with macroalgae collected in the same site of the sea urchins for one week, in order to adjust to laboratory conditions. Spawning was inducted in all specimens employing the raking protocol (Rakaj et al., in press) and a total of 90 females were randomly selected for the experiment. Fifteen females were randomly selected to evaluate initial (T0) ovosomatic index (OI), while the others 75 were tagged through a Passive Integrated Transponder (PIT) tag (Hagen, 1996), wet weighed (mean total wet weight 48.89 ± 15.07 g) and maintained into the RAS (Figure 4.1), in floating boxes for 4 weeks before the start of the feeding experiment. During the two first weeks, sea urchins were fed with macroalgae collected in the sampling site, while in the lasts two weeks sea urchins were kept fast to standardize initial hungry level (Castell et al., 2004). Then sea urchins were divided in 15 tanks each containing 5 sea urchins.

3 tanks for each diet were randomly assigned with a total of 15 sea urchins for treatment. The feed was provided *ad libitum* six days a week for four months. The experiment was conducted in the same RAS as the stability trial, maintaining the same environmental conditions using a protein skimmer, mechanical, biological and UV filterer and microfiltration in order to remove waste, reduce bacteria load and oxidize nitrogen compounds. Water exchange of 50% of the whole volume was undertaken at least twice a week using 5 μm filtered and UV-sterilized seawater. Temperature, salinity and pH were daily monitored through a multiparameter probe (the sensor EUTECH PCD 650) directly immerged in the aquaria.

The somatic growth of sea urchins was evaluated measuring sea urchin wet weight in three times: before the start of the controlled feeding provision (before the two weeks of fasting period, T0), after 10 weeks (T1), and at the end of the experiment (T2). Sea urchins were always weighed after 48h from the last feed provision in order to empty sea urchin guts. In addition, before weight measurement the specimens were kept for 1 minute outside the aquaria to drip external water, and then weighed with a precision balance (± 0.01 g accuracy). Specific Growth Rate (SGR %) was calculate according to Tolon et al. (2017) as follow:

SGR: $((\ln TWW_f - \ln TWW_i) / t) * 100$

Where "TWW_f" and "TWW_i" are the final and initial wet weights (g) of sea urchin specimen, "t" represents time in days of the experiment.

The ingestion rate (IR) and the absorption efficiency (AE) were assessed daily for three weeks, after one month of the controlled feeding provision, to avoid the influence of the initial fasting period (Castilla-Gavilan et al., 2019). For each aquarium feed provided was wet weighed before distribution, while its dry weight was determined on 3 additional food aliquots (Fernandez and Boudouresque, 2000) after drying for 48h at 60 °C. Before each feed provision faeces and food leftover were carefully siphoned off, separated by a sieve (500 μm), dried in stove (60°C for 48h), and then weighed. For each specimen, the IR was calculated as the difference between the total provided biomass (dry weight) and the total uneaten biomass as follows:

IR (g day^{-1} $individual^{-1}$) = (total provided biomass – total uneaten biomass)/tn

Absorption efficiency was calculated as follows:

AE (%) = [(total biomass ingested – total faeces biomass)/total biomass ingested] x 100

Where total biomass ingested is calculated as follows: total provided biomass – uneaten biomass - biomass loss in seawater.

4.2.5 Spawning induction and Ovosomatic index

Egg release was induced through raking method (Rakaj et al. in press), before the start of the feeding trial (T0) and after four months of controlled feeding (T2). Sea urchins were removed from their tanks and left to dry for approximately three hours. Subsequently, each sea urchin was assigned a separate beaker, in order to isolate the eggs produced by each specimen. Sea urchin were then induced to spawn by thermal shock, filling each beaker with seawater at higher temperature (T=24°C), and left to release eggs for 6h. When the spawning ended, sea urchins were removed from the beakers and returned to the RAS to continue rearing. Eggs were collected and transferred to 50 ml tubes and then centrifuged at 2000 rpm for 10 minutes, to assess the total volume and finally the residual of water was removed and the eggs weighed.

Ovosomatic index (OI %) was calculated from each sea urchin in each condition:

OI: $EWW_g / TWW_g \times 100$

Where EWW_g is the wet weight (g) of the eggs and TWW_g is the total wet weight (g) of the sea urchins.

After EWW measurement, gonads were freeze-dried and stored at -80°C for further analyses.

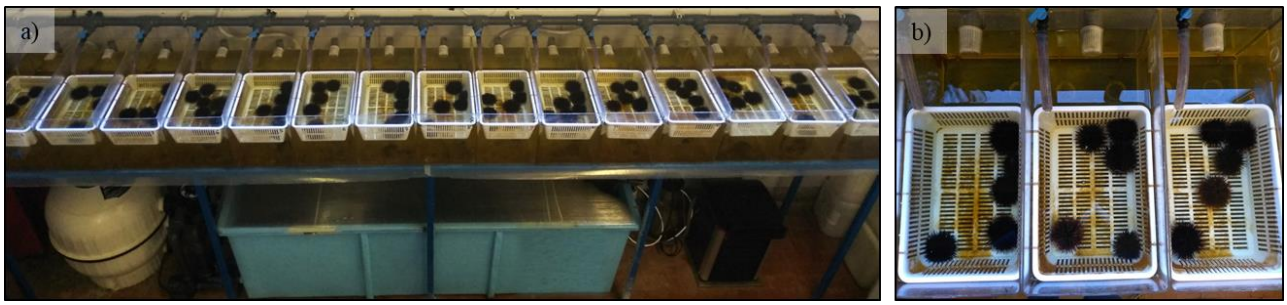


Figure 4.1. Recirculating aquaculture system used for the feeding experiment (a). Detail of the tanks and sea urchins randomly assigned to each experimental diet (b).

4.2.6 Colour

To assess the colour, eggs were compared with Pantone® colour standards chart under standard artificial daylight by an expert observer. Each sample was assigned to a single colour category among those defined by Pearce et al. (2002a):

1 = bright yellow or orange

2 = paler yellow or orange, mustard

3 = yellow-brown, orange-brown, red-brown, cream

4 = any other color (e.g., dark brown, gray)

4.2.7 Statistical analysis

Univariate permutational analysis of variance (PERMANOVA, Anderson et al., 2008) was conducted to find differences in diet stability, ingestion rate, absorption efficiency, somatic growth and egg production. Analysis was conducted on untransformed data resembled with Euclidean distance. Factors considered were "Diet" fixed with five levels (Diet-A, Diet-F, Diet-K, Diet-M, Diet-D), "Time" fixed with three levels (T0, T1, T2) for somatic growth, and with two levels (T0,T2) for egg production.

4.3 Results

Results of the stability trial showed a feed loss of 17.04 ± 5.03%, 28.57 ± 2.21%, 27.90 ± 4.15%, 29.43 ± 0.80% and 30.78 ± 0.89% for Diet-A, Diet-F, Diet-K, Diet-M and Diet-D respectively, without significant differences between the five diets (MS= 61.21, Pseudo-$F_{(4,9)}$= 6.25, P= 0.088).

No mortality or disease were observed across the four months of the feeding trial, and all sea urchins maintained the pit tag without collateral effects.

Analysis of sea urchin ingestion rate (IR) and absorption efficiency (AE) showed significant differences (Table 4.2). Diet-F showed the highest IR, followed by Diet-A, Diet-D and Diet-K, while Diet-M showed the lowest value. AE value peaked for the Diet-A, followed by Diet-K and Diet-F, while Diet-M and Diet-D showed similar and lower values (Figure 4.2).

Table 4.2. Univariate permutational analysis of variance (Main test and Pair-wise tests) of the results of ingestion rate IR (a) and absorption efficiency AE (b) in *Paracentrotus lividus* fed with the five experimental diets (Diet-A, Diet-F, Diet-K, Diet-M and Diet-D. Significant p values are highlighted in bold.

MAIN TEST		**a) IR**			**b) AE**		
Source of variation	**df**	**MS**	**Pseudo-F**	**P(perm)**	**MS**	**Pseudo-F**	**P(perm)**
Diet	4	0.017	9.00	**0.001**	946.010	71.58	**0.001**
Residual	40	0.002			13.215		
PAIR-WISE TESTS		**t**	**P(perm)**	**Unique perms**	**t**	**P(perm)**	**Unique perms**
Diet-A vs Diet-F		2.15	**0.046**	969	7.33	**0.001**	975
Diet-A vs Diet-K		0.83	0.402	980	4.33	**0.003**	977
Diet-A vs Diet-M		3.36	**0.005**	974	12.47	**0.001**	978
Diet-A vs Diet-D		0.43	0.667	982	12.81	**0.001**	975
Diet-F vs Diet-K		3.17	**0.005**	981	2.71	**0.013**	976
Diet-F vs Diet-M		6.11	**0.001**	968	7.26	**0.001**	982
Diet-F vs Diet-D		3.07	**0.009**	976	7.90	**0.001**	977
Diet-K vs Diet-M		2.65	**0.016**	984	8.33	**0.001**	980
Diet-K vs Diet-D		0.54	0.576	985	8.93	**0.001**	980
Diet-M vs Diet-D		3.70	**0.002**	974	1.92	0.079	977

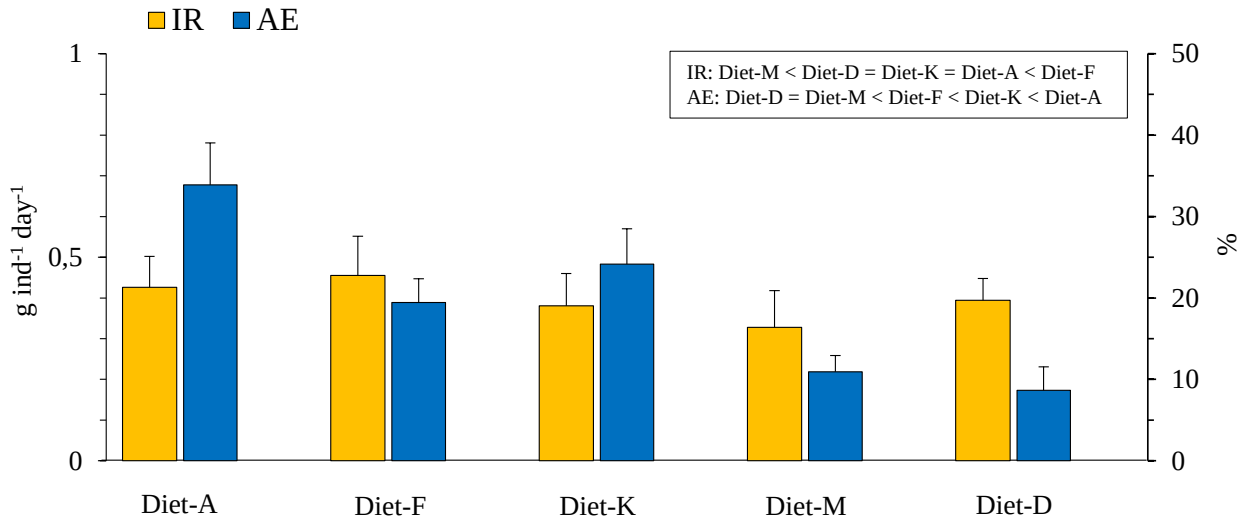


Figure 4.2. Ingestion rate (IR, mean ± standard deviation) and absorption efficiency (AE, mean ± standard deviation) of *Paracentrotus lividus* fed with the five experimental diets. In the box, significant differences between diets are indicated.

Specific growth rate showed similar values for all the experimental diets (MS= 0.009, Pseudo-$F_{(4,79)}$= 0.338, P= 0.858), with Diet-A showing a value of 0.16 ± 0.08 %, followed by Diet-F, Diet-D and

Diet-M with 0.21±0.09, 0.21±0.1 and 0.22±0.09% respectively and then Diet-K 0.24±0.11%. Similarly, total wet weight did not show significant differences among diets but only between times (Table 4.3), with higher values at T2 than T1 and T0, which did not differ each other (Figure 4.3a). Ovosomatic index (OI), on the contrary, showed a significant increase with time and differed among diets. Diet-A led to lower OI (12.82±8.87%) than Diet-M and Diet-K (21.09±10.59%, 20.69±10.34%, respectively), and also lower, but similar, than Diet-F (16.56±11.15%) and Diet-D (19.73±11.04%) (Figure 4.3b).

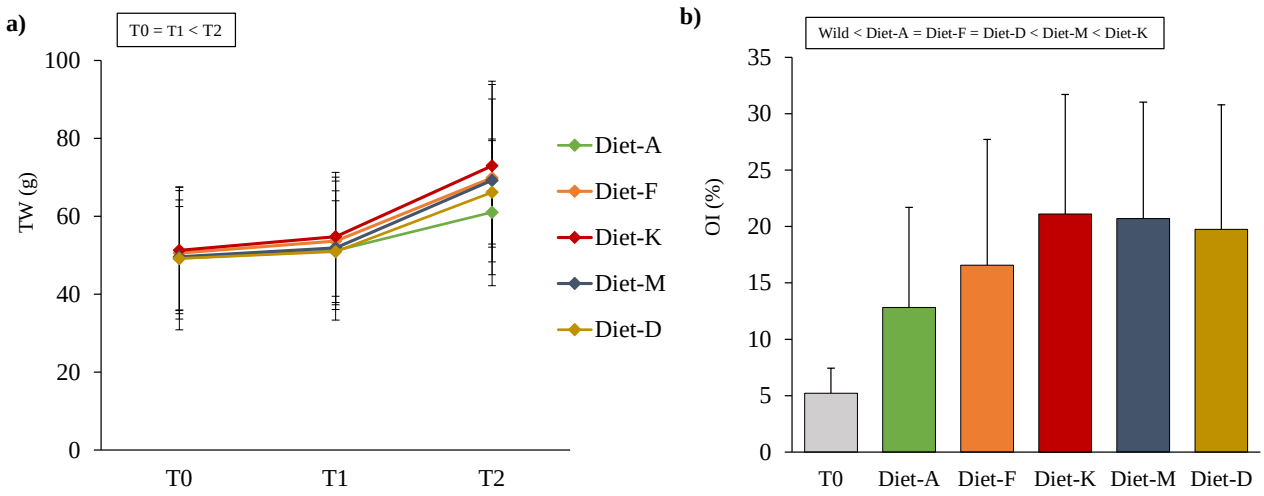


Figure 4.3. a) Total wet weight (TW, mean ± standard deviation) of *Paracentrotus lividus* fed with the five experimental diets across experimental times (T0, T1, T2). Different symbols highlighted significant differences between times. b) Ovosomatic index (OI, mean ± standard deviation) of sea urchin at the start of the experiment (T0) and after the feeding experiment with the five experimental diets (Diet-A, Diet-F, Diet-K, Diet-M and Diet-D). In the box, significant differences between diets are indicated.

Table 4.3. Univariate permutational analysis of variance (Main test and Pair-wise tests) of the total weight (a) measured during the experimental period (T0, T1, T2) and ovosomatic index (b) of *Paracentrotus lividus* collected before the feeding trial (T0) and fed with the five experimental diets (Diet-A, Diet-F, Diet-K, Diet-M and Diet-D) . Significant p values are highlighted in bold.

MAIN TEST			**a) Total weight**	
Source of variation	**df**	**MS**	**Pseudo-F**	**P(perm)**
Time	2	6720.8	22.43	**0.001**
Diet	4	217.31	0.72	0.564
Time x Diet	8	61.91	0.21	0.992
Residual	201	299.52		

PAIR-WISE TESTS	**t**	**P(perm)**	**Unique perms**
T0 vs T1	0.96	0.366	998
T0 vs T2	5.94	**0.001**	998
T1 vs T2	5.10	**0.001**	998

MAIN TEST			**b) Ovosomatic index**	
Source of variation	**df**	**MS**	**Pseudo-F**	**P(perm)**
Diet	5	691.08	7.71	**0.001**
Residual	82	89.61		

PAIR-WISE TESTS	**t**	**P(perm)**	**Unique perms**
T0 vs Diet-A	4.00	**0.001**	996
T0 vs Diet-F	4.53	**0.001**	997
T0 vs Diet-K	6.44	**0.001**	997
T0 vs Diet-M	6.44	**0.001**	997
T0 vs Diet-D	5.71	**0.001**	996
Diet-A vs Diet-F	1.01	0.345	997
Diet-A vs Diet-K	2.25	**0.026**	995
Diet-A vs Diet-M	2.23	**0.028**	997
Diet-A vs Diet-D	1.89	0.072	998
Diet-F vs Diet-K	1.08	0.271	997
Diet-F vs Diet-M	1.03	0.303	998
Diet-F vs Diet-D	0.76	0.456	998
Diet-K vs Diet-M	0.10	0.925	995
Diet-K vs Diet-D	0.33	0.736	995
Diet-M vs Diet-D	0.24	0.808	995

Results of colour assessment showed that Diet-A presented 10% of egg with dark orange (DO) colour, 30% of cream orange (CO) and 60% of paler orange (PO) (Figure 4.4). Eggs produced by sea urchins fed with Diet-F showed the best performance presenting 100% bright orange (BO) colour, followed by eggs produced by Diet-M that presented 90% of BO eggs and 10% of PO eggs. Diet-K and Diet-

D gave the same result with 50, 40 and 10% of eggs characterized by PO, BO and YO colour respectively.

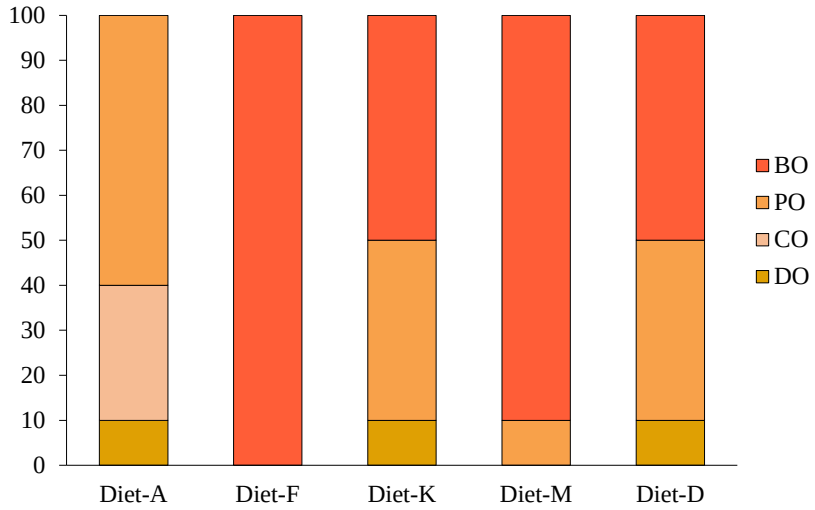


Figure 4.4. Relative frequency of the egg colour categories produced by female *P. lividus* fed with the five experimental diets (Diet-A, Diet-F, Diet-K, Diet-M and Diet-D).

4.4 Discussion

Feasibility of five experimental diets was assessed on purple sea urchin *Paracentrotus lividus* through the recently developed Raking method (Rakaj et al. in press). The racking method resulted a highly sustainable productive approach, which allowed to evaluate the effects of the experimental diets without sacrificing the sea urchin breeding batches. The use of the same pool of sea urchins for subsequent experiments, allows the evaluation of different feeding treatments reducing possible variability associated with changing the experimental pool. In addition, this approach could also reduce the experimental period, limiting the acclimatization, used to allow organism taken from the wild to adapt to laboratory conditions, and the starvation period generally required to standardize the initial condition of the experimental pool (Guillou et al., 2000; Raposo et al., 2019). Finally, the application of this method make it possible to carry out the evaluation of gamete quality for the development of a closed-cycle production system, taking into account the influence of gamete quality on the overall reproductive success (Byrne et al., 2008).

Stability in seawater, i.e. the ability of feed/diet pellets to remain stable enough allowing animal feeding, is a crucial issue in evaluation of experimental formulations, especially for sea urchins that need also 2-3 days to eat the provided feed (Fabbrocini and Adamo, 2010; Pearce et al., 2002b). The result of stability in seawater highlighted a similar behaviour after 24h for all the experimental diets, with the highest feed loss for the diet based on *Engraulis encrasicolus* processing discards (Diet-D about 30% of feed loss). These results were similar with a previous study in which agar was used as

binding agent with terrestrial vegetables (Ciriminna et al., 2020), while were slightly lower than feeds with other binders (Pearce et al., 2002b). For all the experimental diets more than 60% of pellets remaining available between each feed provision, every 24h, confirming pellet efficacy for the feeding trial. Although the results would allow a review of the feeding protocol, distancing feed administrations by 48 hours, daily feeding was maintained to avoid the possible worsening of the environmental condition of the tanks. Sea urchins consumed food with very low rates and thus pellets are expected to remain for long times in the water with consequent nutrient loss, water soaking and low efficiency (Fabbrocini et al., 2015; Pearce et al., 2002b). Although stability has been considered a secondary information respect to feed digestibility and absorption, low stability in prepared pellets can reduce nutritional efficacy (Heflin and Watts, 2016), thus the lack of differences between the five experimental diets suggest that their performance as feed for sea urchins was not influenced by their behaviour in seawater. Finally, the positive results indicate that the arabic gum, a plant-based gum produced from *Acacia arabica* and already widely used in the food industry (Saha et al., 2017), may be a useful alternative to other more expensive binders.

4.4.1 Metabolic rates

The sea urchin growth performance is the resultant of physiological processes (ingestion, digestion, absorption and assimilation), which in turn are strongly influenced by the quality of the foods administered (Lawrence, 2013a). Therefore, metabolic rates and offspring quality and quantity production are excellent measures of the relative quality of a given food source.

Although sea urchins are primarily herbivorous, they are able to consume a great variety of food and production is strongly influenced by both ingestion and absorption of consumed food (Lawrence, 2013a). Ingestion rate (IR) showed low value for all the experimental diets (0.30-0.45 g $ind^{-1}day^{-1}$), also with some differences between diets, suggesting that all the five tested diets were nutritionally adequate for *P. lividus*. In accordance with the compensatory model, food ingestion is strictly related to food availability and quality, and sea urchins usually showed higher ingestion rate for food with low nutrient content, while IR is lower with a high-energy food (Boudouresque and Verlaque, 2020; Fernandez and Boudouresque, 2000; Hammer et al., 2004). Similarly, Ruocco et al. (2018) found higher values of IR for *P. lividus* fed on macroalgae (*Ulva rigida*) or seagrass (*Posidonia oceanica*) than on artificial pellet, while Fernandez and Bouduresque (2000) measured similar values for *P. lividus* fed on a mixed diet composed of vegetal ad animal ingredients. Moreover, in our experiment the differences highlighted among diets suggest that the different origin of the main ingredients did not have a key role in diet palatability. In fact, Diet-A composed by *Laminaria* spp. and *Ulva lactuca* showed similar IR value to Diet-K and Diet-D, composed mainly by *Lactuca sativa* outermost leaves.

Differently to IR, the absorption efficiency results showed a marked difference between the five diets. The algae-based diet emerged as the most efficient, while the lettuce-based diets showed significantly lower values. However, AE is a very sensitive parameter to the different digestibility of the main ingredients of food sources (Cuesta-Gomez and Sánchez-Saavedra, 2018). Absorption efficiency gives important information on the nutritional quality of the proposed diets and could be affected by several factors, including sea urchin age, environmental conditions and feed nutrient composition (Lawrence et al., 2007). Findings of absorption efficiency confirm the efficacy of the diet based on *Laminaria* spp. and *Ulva* sp., in accordance with data observed in other studies (Cyrus et al., 2015; Shpigel et al., 2005). Sea urchins are commonly considered herbivorous, able to transform communities dominated by macroalgae into barren areas due to their grazing activity, thus algae based diets are usually considered an appropriate diet similar to wild food (Lawrence, 2013b). In contrast, the four lettuce-based diets were less effective than macroalgae, despite the appropriate nutritional quality. Proximate composition of the experimental diets showed that the lettuce-based diets were characterized by higher protein (25-27%) and lipid (4-6%) content than Diet-A (about 8% and 2% respectively), which instead was characterized by a higher content of carbohydrates (> 50%). Diet nutritional composition is usually considered the main driver in food consumption for sea urchins, and in particular, protein intake plays a key role (Heflin et al., 2016). However, food efficacy is related not only to food consumption, but also to assimilation, which in turn depends on its digestibility (Bouduresque and Verlaque, 2007; Cuesta-Gomez and Sánchez-Saavedra, 2018), thus the differences highlighted in absorption efficiency among diets may be related to the different source of the main ingredients. Terrestrial vegetables, and in particular of *Lactuca sativa* leaves, are characterized by a high content of fibres and insoluble carbohydrates (Esteban et al., 2007; Kim et al., 2016; Plazzotta et al., 2017), which could be not easily digested by sea urchins (Marsh et al., 2013). In addition, the inclusion of *Lithothamnium calcareum* as a source of inorganic carbon and other minerals (Aslam et al., 2010) may have contributed to the low efficiency of the sustainable diets, in particular of Diet-M and Diet-D. Both diets were prepared with meals containing mussel shells and anchovy bones respectively, increasing the amount of not digestible matter, as suggested also by the higher faeces production by sea urchin fed with these two experimental diets (author's observation).

4.4.2 Somatic growth and egg production

Despite the differences in IR and AE, results of somatic growth showed a similar performance for all the experimental diets. Both specific growth rate (SGR) and total wet weight (TW) did not show significant differences between diets. However, all experimental diets were able to promote significantly higher final TW values than initial ones, confirming the suitability of administrated diets as *P. lividus* feed. SGR ranged between 0.16 and 0.24%$^{-day}$ were in accordance with results obtained

in other studies with adult sea urchins (Grosso et al., 2021). Finally, in adult sea urchins, the best measure of sea urchin's well-being is the offspring production (Vadas, 1977). Our study, through the comparison between ovosomatic index (OI) and gonadsomatic index (GI) values, showed that egg and gonad biomass measurements were comparable, since they are different mainly in composition (different ratio of nutritive:reproductive cells) rather than in biomass (Byrne, 1990; Marsh et al., 2013; Walker et al., 2015). Hence, although in the traditional echinoculture approach the efficacy of administered diets is evaluated in terms of GI, for the raking approach the new parameter OI provides better information. After four months of controlled feeding, all experimental sea urchins showed a marked increase in egg production, in line with the positive results of TW increase but highlighting differences among the five experimental diets. Starting from an OI of about 5%, the lower values were recorded by Diet-A (12%) while the four lettuce-based diets highlighted an higher increase (between 16 and 21%), confirming the efficacy of terrestrial vegetables on *P. lividus* gonad growth (Vizzini et al., 2014, 2019). Of the four lettuce-based diets, Diet-K had the highest OI value (approximately 21%) followed by Diet-M (about 20%), Diet-D (about 19%) and finally Diet-F (about 16%). In particular, results of common anchovy discards confirm findings obtained in a previous study on *P. lividus* gonad enhancement (Ciriminna et al., 2021). Furthermore, a lower but similar increase in gonad production was already observed by Sartori and Gaion (2015) and Santos et al. (2019), which found positive results in GI (about 13% and 9% respectively) feeding *P. lividus* with maize and spinach. Similarly, Luo et al. (2014) tested banana peel in *S. intermedius* with a final GI of about 8%, while Vizzini et al. (2014, 2018) found gonad growth in both juvenile and adult *P. lividus* fed with only outermost leaves of *L. sativa*, but not when fed with *Brassica oleracea* and *Beta vulgaris* discards. Among the four lettuce-based diets, Diet-K presented the higher value of OI (about 21%), in accordance with results obtained from IR and AE measurements, i.e. a low amount of ingested food coupled with the best absorption efficiency between the lettuce-based diets. Similarly, despite the low AE, both Diet-M and Diet-D presented a marked increase in TW e OI, suggesting their feasibility for gonad growth. In particular, results of common anchovy discards confirm findings obtained in a previous study on *P. lividus* gonad enhancement (Ciriminna et al., 2021). Finally, Diet-F, which presented the highest IR coupled with about 20% of AE gave positive results but was the worst between the four sustainable diets.

The nutritional composition of the four lettuce-based diets appeared effective for promoting egg production, as resulted characterized by the adequate protein and lipid content for sea urchin growth, in accordance with results found by Baião et al. (2019) and Lourenço et al. (2020). In addition, better results of mixed diets compared to fully vegetal ones were already observed (Fernandez and Boudouresque, 2000; Grosso et al., 2021; Shpigel et al., 2005), probably thanks to the high nutritional

quality of animal meal. Our results suggested that the low amount of seafood meal promoted nutrient allocation in gonads whit a higher egg production than sea urchin fed with the algae-based diet. Lipids from seafood meal are characterized by a large amount of essential fatty acids (EFAs), which sea urchins usually synthesize through their metabolic pattern (Monroig and Kabeya, 2018). Krill, fish, mussel and fish discards meal (Ciriminna et al., 2020; Ezgeta-Balić et al., 2012; Hagen et al., 2001; Šimat et al., 2020) are all characterized by a high content of EFAs, allowing sea urchin to obtain EFAs from diet without consuming energy for their production. Finally, the introduction of *L. calcareum* in the experimental diets as source of inorganic carbon and other minerals could had a positive effect in sea urchin egg production. Minerals, especially calcium and magnesium, are involved in physiological processes and in particular in test production (Ebert, 2013; Grosjean et al., 1998) and were usually extracted from algae and seawater with energy consumption (Hermans et al., 2010). Despite the effect of dietary mineral content has not been thoroughly investigated, it was observed an increase in somatic growth of juvenile *Stongylocentrotus* when fed with a mineral enriched diet (Kennedy et al., 2007), while Cirino et al. (2017) found positive effect on *P. lividus* reproductive cycle management, adding $CaCO_3$ in the prepared food.

Finally, as regards egg colour, Diet-A promoted the low quality colouration, with the production of pale orange and cream orange eggs, consistent with literature (Pearce et al., 2004), on the contrary all the four lettuce-based diets showed a better performance with Diet-F resulting the most efficient, followed by Diet-M. The colour in gonads is one of the most important features, as visible at a first sight influencing the visual assessment (Cuesta-Gomez and Sánchez-Saavedra, 2018). In sea urchins. gonad colour is related to carotenoid dietary intake and accumulation, in particular the amount of β-carotene plays a fundamental role being an echinone precursor, the most representative carotenoid in sea urchin gonads (Plank et al., 2002; Symonds et al., 2009). As observed by Lopez Garciaà-Alonso et al. (2014) β-carotene is the most representative carotenoid in lettuce leaves, thus the positive performance of the four lettuce-based diets could be influenced by diet carotenoid content, as already found by Vizzini et al. (2014).

4.5 Conclusion

Findings of the present study showed that sustainable diets, composed mainly by *L. sativa* outermost leaves, were efficient as feed for the purple sea urchin *P. lividus*. All the four tested diets resulted characterized by an adequate nutritional content, with a balanced relative amount of proteins, lipids and carbohydrates. Diet assimilation promoted an increase in total weight and egg production, and positively influenced egg colour, important as quality feature. In addition, comparing with results obtained with macroalgae diet, all the four sustainable diets resulted more effective. These results

confirm the suitability of food processing discards as nutrient source to be exploited for aquaculture feed production, as an alternative to more expensive and less sustainable ingredients, in accordance with the principles of the circular economy.

CHAPTER 5: Evaluation of the effect of lettuce-based diets on sea urchin larval development through stable isotope and fatty acid analysis.

5.1 Introduction

Although gonadal and somatic growth remains the most important parameter for assessing the performance of experimental diets for sea urchins (Cyrus et al., 2019), a deeper understanding of the physiological processes of the cultivated species could provide important information to drive diet formulation, also for reseeding and restocking purposes. In sea urchins broodstock diet strongly influences egg composition, thanks to the dual role of their gonads as reproductive organs and storage tissues (Walker et al., 2015). In marine organisms nutritional resources stored in the eggs are fundamental to support a correct embryo development and larval growth (McAlister and Moran, 2013), and in species characterized by larvae able to feed on plankton, such as sea urchins, egg nutrient stores support the attainment of the feeding larval stage (Byrne et al., 2008). Notwithstanding the influence of the broodstock diet on gamete quality, scarce information are today available on the effect of formulated feeds on sea urchin egg composition and larval development (Liu et al., 2007, Gago et al., 2009, Carboni et al., 2013).

Recently, stable isotope analysis (SIA) has been introduced in aquaculture studies to determine the contribution of food sources to organism growth (Cyrus et al., 2019; Gamboa-Delgado, 2021). Consumer isotopic signatures change according to their nutrient sources and, especially in the controlled feeding condition of aquaculture systems, SIA could be an important tool to evaluate the suitability of dietary items (Gamboa-Delgado, 2021). Similarly, fatty acids were introduced as nutritional biomarkers, allowing estimating consumer diets from their fatty acid profiles, also considering the ability of some organisms to transform fatty acids assimilated from the diet (Cook et al., 2000; Kabeya et al., 2017; Yu et al., 2015). In addition, fatty acids are fundamental for sea urchin metabolic maintenance and have a direct effect on larval development and survival (Carboni et al., 2013; Liu et al., 2007). According to these considerations, the aims of this study were: i) to evaluate the assimilation of sustainable diets through stable isotope and fatty acid analysis; ii) to assess the influence of diet and egg fatty acid profiles on larval development. Evaluation of larval development was conducted using the Integrated Toxicity Index (ITI) (Morroni et al., 2016), usually applied in ecotoxicological studies to investigate the presence of contaminants in the environment. Here, the ITI was applied, for the first time, in a farming context to assess diet quality through their ability to generate normoformed larvae, taking into account the delay in development, the gravity and the frequency of observed malformations.

5.2 Materials and Methods

5.2.1 Feeding trial

Details of the experimental diets and feeding trial were already described in chapter 4.

5.2.3 Stable isotope analysis

For stable isotope analysis three samples for each diet were used. Sea urchin eggs were collected at the beginning of the experiment, gathering eggs produced by 5 wild sea urchins (Egg-W), and at the end of the feeding trial, collecting eggs from 5 sea urchins for each experimental formulation (Egg-A, Egg-F, Egg-K, Egg-M, Egg-D). For SIA samples were freeze-dried and ground to a fine powder. Carbon and nitrogen stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$) were analysed using an Isotope Ratio Mass Spectrometer (IRMS; Thermo-Electron Delta Plus XP) coupled to an Elemental Analyser (EA, Thermo-Electron 1112). The isotopic composition was expressed in δ notation following the formula:

$$\delta^{13}C \text{ or } \delta^{15}N\ (‰) = [(R_{sample}/R_{standard}) - 1] \times 10^3$$

where R_{sample} and $R_{standard}$ are the ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ in the sample and the standard, respectively. Isotope ratios were expressed relative to a Vienna Pee Dee Belemnite (VPDB) standard for carbon and atmospheric N_2 in air for nitrogen. The deviation from the standard is denoted by the term δ and the results expressed as parts per thousand (‰). Analytical precision based on the standard deviation of replicates of standards (International Atomic Energy Agency IAEA-NO-3 for $\delta^{15}N$ and IAEA-CH-6 for $\delta^{13}C$) was 0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$.

Lipid content is expected to introduce bias in $\delta^{13}C$ analysis, due to the uneven lipid storage in different tissues/organisms (Post et al., 2007). To normalize $\delta^{13}C$ values (Table 5.2), the linear regression equations suggested by Post et al. (2007) were applied to sea urchin eggs and experimental diets:

Aquatic animals: $\Delta\delta^{13}C = -3.312 + 0.99 \times C{:}N$

Plants: $\Delta\delta^{13}C = -3.02 + 0.09 \times \%C$

Table 5.2. Stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios of the five experimental diets (Diet-A, Diet-F, Diet-K, Diet-M and Diet-D), and of the eggs produced at the start of the experiment (Egg-W) and by sea urchins fed the respective diets (Egg-A, Egg-F, Egg-K, Egg-M, Egg-D).

	$\delta^{15}N$	$\delta^{13}C$	C%	C:N	Corrected $\delta^{13}C$
Diet-A	9.2 ± 0.2	-15.9 ± 0.3	29.2 ± 1.0	21.1 ± 1.8	-16.2 ± 0.2
Diet-F	4.8 ± 0.1	-27.5 ± 0.1	33.2 ± 2.3	8.3 ± 0.6	-27.5 ± 0.1
Diet-K	4.9 ± 0.1	-27.7 ± 0.0	8.1 ± 0.3	8.1 ± 0.1	-27.6 ± 0.1
Diet-M	6.2 ± 0.3	-27.1 ± 0.1	34.4 ± 0.4	8.4 ± 0.0	-27.0 ± 0.1
Diet-D	7.3 ± 0.3	-26.2 ± 0.5	34.4 ± 0.4	7.9 ± 0.2	-26.1 ± 0.4
Egg-W	6.8 ± 0.2	-20.6 ± 1.3	40.0 ± 2.9	4.4 ± 0.1	-19.5 ± 1.4
Egg-A	7.4 ± 0.1	-18.3 ± 0.3	41.1 ± 3.1	5.6 ± 0.5	-16.0 ± 0.2
Egg-F	6.4 ± 0.4	-25.4 ± 0.5	39.5 ± 3.2	4.8 ± 0.3	-23.9 ± 0.6
Egg-K	4.4 ± 0.2	-26.5 ± 0.2	39.1 ± 3.5	4.7 ± 0.2	-25.2 ± 0.4
Egg-M	5.5 ± 0.6	-25.9 ± 1.1	39.5 ± 2.3	5.0 ± 0.3	-24.2 ± 0.8
Egg-D	5.7 ± 0.4	-25.5 ± 0.8	38.1 ± 2.4	4.6 ± 0.5	-24.2 ± 0.5

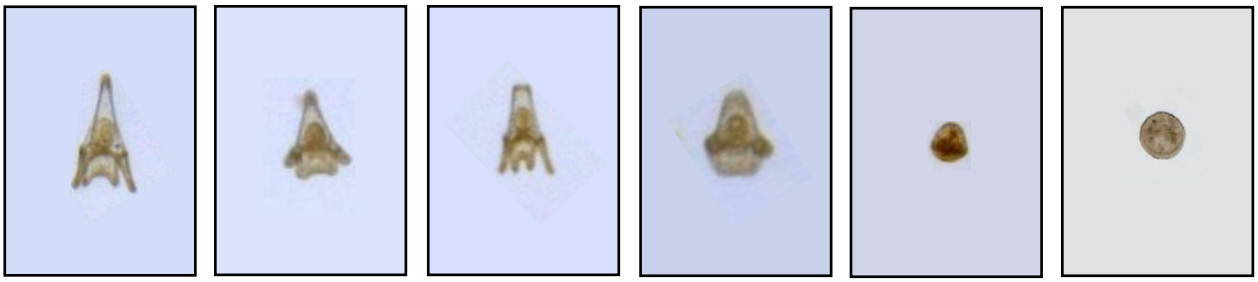
5.2.4 Fatty acids analysis

Fatty acid analysis was conducted on three samples for each experimental diet (Diet-A, Diet-F, Diet-K, Diet-M and Diet-D), and on five sample of eggs produced by 5 wild sea urchins randomly chosen from the initial pool (Egg-W), and at the end of the feeding trial, gathering eggs from 5 sea urchins for each experimental formulation (Egg-A, Egg-F, Egg-K, Egg-M, Egg-D). Fatty acid were extracted following a modified version of the Bligh and Dyer (1959) method (see chapter 2 for more details).

5.2.4 Egg fertilization

To obtain female gametes, the sea urchins involved in the controlled feeding experiment were taken from the tanks and each one was assigned to a beaker. Spawning was induced by thermal and saline shock, following the procedure of the Racking method. After the spawning, eggs for each diet were collected and transferred in multiwells for fertilization. To obtain male gametes, 15 wild sea urchins, collected in the same area of the experimental pool, were inducted to spawn with an injection of 1 M KCl into the coelom via the peristomial membrane (Carboni et al., 2012; Liu et al., 2007). Concentrated sperm of five male specimens was collected "dry", mixed and diluted 10 µl in 10 ml of filtered seawater. An aliquot of 1µl of diluted sperm was added to 10 ml of egg suspension previously collected. Multiwells containing fertilized eggs were stored in a thermostatic chamber at 20°C. After 48h *pluteus* larvae were fixed in by Lugol's iodine and observed under inverted microscope (Axiovert A1) with 15x magnification, to capture 9 images for each diet. For each diet, 300 plutei were analysed using the software ZEN 3.1 (Blue Edition) to assess the embryo-larval development by classifying

each in the flowing category: normal pluteus (P); early pluteus (EP); abnormal pluteus (AP); early abnormal pluteus (AEP); prism (Pr); gastrula (G) (Figure 5.1).



(P) (EP) (AP) (AEP) (Pr) (G)

Figure 5.1. Classification of *Paracentrotus lividus* larvae malformations: normal pluteus (P), early pluteus (EP), abnormal pluteus (AP), early abnormal pluteus (EAP), prism (Pr), gastrula (G).

The Integrate Toxicity Index (ITI) was then calculated according to Morroni et al. (2016), reporting the score assigned to the observed larval abnormalities (Table 5.3) or delay in development:

$ITI = 10 (S_i \times F_i) / 100$

where S_i was the score assigned to larval abnormalities and F_i was the respective frequency.

Table 5.3. Score assigned to observed larvae according to malformation classification. The meaning of the acronyms is the same as in Figure 5.1.

	P	EP	AP	EAP	Pr	G
Score	0	2	4	6	8	10

5.2.4 Statistical Analysis

Multivariate permutational analysis of variance was used to test the differences in $\delta^{13}C$ and $\delta^{15}N$ between experimental diets (Factor diet fixed with 5 levels: Diet-A, Diet-F, Diet-K, Diet-M, Diet-D) and eggs (Factor Tissue fixed with 2 levels: Diet, Egg). Analysis on stable isotopes were conducted on normalized data resembled using Euclidean Distance.

Differences in fatty acid (FA) profiles among the five experimental diets (5 levels: Diet-A, Diet-F, Diet-K, Diet-M, Diet-D) and among eggs (produced through the five diets and from wild specimens; 6 levels: Egg-A, Egg-F, Egg-K, Egg-M, Egg-D and Egg-W) were analysed separately by means of one-way multivariate permutational analysis of variance (PERMANOVA).

PERMANOVA was carried out on FA data resembled using Euclidean Distance after arcsine square root function transformation. Principal coordinates analysis was run on diet and egg FA profiles to graphically highlight the differences found by PERMANOVA. The main classes of FAs and diet biomarkers were superimposed to the graph.

All the statistical analyses were performed using the software PRIMER 6 v6.1.10 & PERMANOVA+ β20 (Plymouth, UK). When significant differences were found, pair-wise tests were used as *a posteriori* check of significant effects. The Montecarlo test was also carried out to identify significant patterns when the numbers of permutations were less than 100.

5.3 Results

5.3.1 Stable Isotope Analysis

Statistical analysis on stable isotope values, $\delta^{13}C$ and $\delta^{15}N$, showed a significant difference between the eggs derived from the tested diets (Table 5.4). In particular, the *pair-wise* test showed that all diets were different except Diet-F and Diet-K. Eggs produced by sea urchin collected before the start of the feeding experiment (Egg-W) resulted significantly different from eggs derived from the rearing conditions. Between the experimental eggs, those produced by sea urchins fed with Diet-A (Egg-A) resulted different from eggs produced by sea urchins fed with the lettuce-based diets. In the group of eggs produced by sea urchins fed with the four sustainable diets, Egg-F and Egg-M and Egg-D resulted similar, while Egg-K were different from all the other eggs. The *pair-wise* test between diets and eggs produced with the respective diets showed significant differences between all the analysed pairs. The average Euclidean Distance resulted smaller for the pair Diet-K:Egg-K, followed by Diet-M:Egg-M, Diet-A:Egg-A, Diet-D:Egg-D and finally Diet-F:Egg-F.

Table 5.4. Multivariate Permutational analysis of variance (PERMANOVA) results, main test (a) and pair-wise test (b), testing the differences in isotopic signatures between the five experimental diets (Diet-A, Diet-F, Diet-K, Diet-M, Diet-D) and the eggs produced by wild sea urchins (Egg-W), and by sea urchins fed with the respective experimental formulation (Egg-A, Egg-F, Egg-K, Egg-M, Egg-D). Significant differences were highlighted in bold.

a) MAIN TEST

	df	**MS**	**Pseudo -F**	**P(perm)**
Diet	5	15.27	135.56	**0.001**
Tissue	1	4.98	44.22	**0.001**
Diet x Tissue	4	2.26	20.07	**0.001**
Residual	37	0.11		

b) PAIR-WISE TESTS

Within tissue between diet

		t	**P(MC) /P(perm)**	**Unique perms**
Diet-A vs	Diet-F	39.07	**0.001**	10
	Diet-K	40.78	**0.001**	10
	Diet-M	20.82	**0.001**	10
	Diet-D	15.71	**0.001**	10
Diet-F vs	Diet-K	0.88	0.452	10
	Diet-M	7.49	**0.002**	10
	Diet-D	12.05	**0.001**	10
Diet-K vs	Diet-M	7.36	**0.005**	10
	Diet-D	12.04	**0.001**	10
Diet-M vs	Diet-D	4.16	**0.017**	10
Egg-W vs	Egg-A	5.31	**0.003**	596
	Egg-F	5.05	**0.002**	611
	Egg-K	11.46	**0.005**	602
	Egg-M	6.01	**0.003**	588
	Egg-D	6.32	**0.002**	603
Egg-A vs	Egg-F	12.07	**0.005**	126
	Egg-K	27.23	**0.007**	126
	Egg-M	10.56	**0.012**	126
	Egg-D	13.1	**0.009**	126
Egg-F vs	Egg-K	7.85	**0.010**	126
	Egg-M	2.49	0.055	126
	Egg-D	2.30	0.060	126
Egg-K vs	Egg-M	3.27	**0.012**	126
	Egg-D	4.76	**0.008**	126
Egg-M vs	Egg-D	0.52	0.677	126

Within diet between tissue

	t	**P(MC) /P(perm)**	**Unique perms**	**Average EA**
Diet-A vs Egg-A	13.27	**0.001**	56	1.35
Diet-F vs Egg-F	6.74	**0.001**	56	1.55
Diet-K vs Egg-K	4.78	**0.001**	56	0.73
Diet-M vs Egg-M	2.82	**0.012**	56	1.04
Diet-D vs Egg-D	5.14	**0.002**	56	1.39

Eggs produced by sea urchins fed with the experimental formulations diverged from $\delta^{13}C$ and $\delta^{15}N$ initial value towards the respective diets (Figure 5.2). In detail, Egg-A were characterized by higher values of both $\delta^{13}C$ and $\delta^{15}N$, according to Diet-A isotopic values. In contrast, eggs produced by sea urchins fed with the four sustainable diets presented a more negative $\delta^{13}C$ than Egg-W, in line with the values found for the lettuce-based diets. Values of $\delta^{15}N$ were also lower respect to those of Egg-W and those of the respective diets, except for Egg-F that presented higher $\delta^{15}N$ than Diet-F.

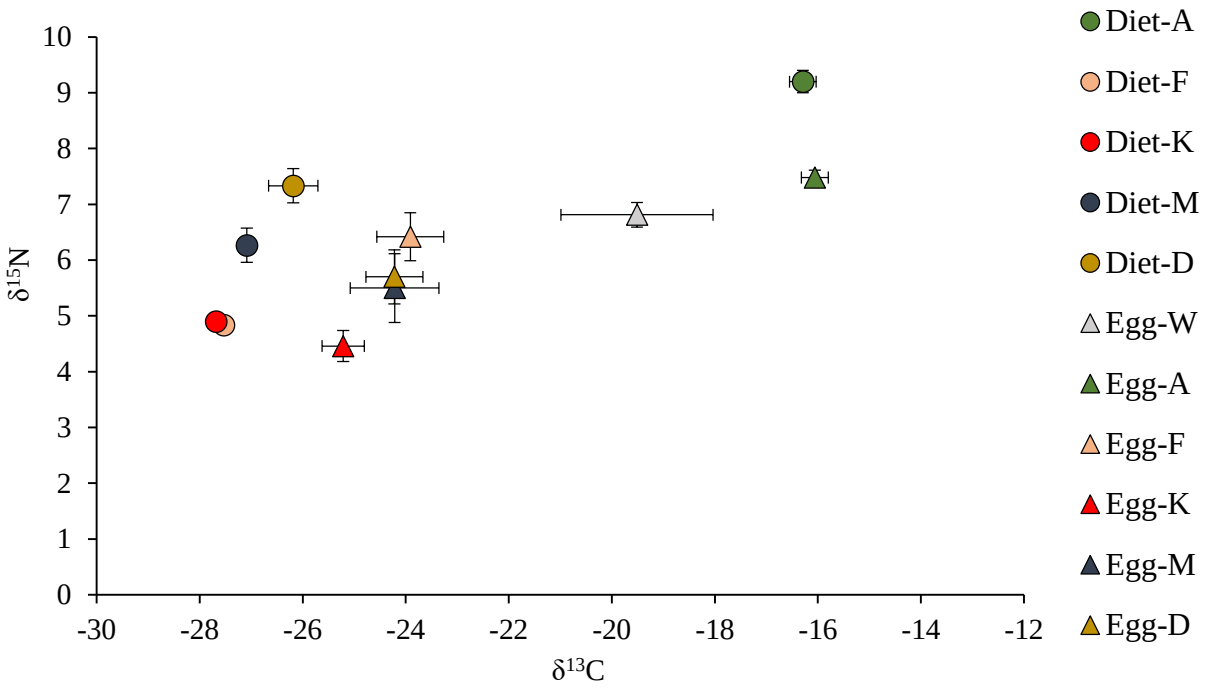


Figure 5.2. Isotopic composition ($\delta^{13}C$ and $\delta^{15}N$, mean ± standard deviation) of the five experimental diets (Diet-A, Diet-F, Diet-K, Diet-M and Diet-D), and of the eggs produced before the start of the feeding experiment (Egg-W) and by sea urchins fed the respective diets (Egg-A, Egg-F, Egg-K, Egg-M, Egg-D).

5.3.2 Fatty acids

Statistical analysis on fatty acid profile of the five experimental diets highlighted significant differences between diets (MS= 0.0030, Pseudo-$F_{(4,14)}$= 26.23, P= 0.001). Diet-A was characterized by the high relative amount, about 32%, of oleic acid (18:1 n9c) that, instead, never reached the 5% in the four sustainable diets (Table 5.5). On the contrary, lettuce-based diets showed a greater relative concentration of linoleic (LA) and α-linolenic acids (ALA) respect Diet-A (about 6% and 7% respectively), in particular, LA was more than twice (13-15%), while ALA was five times higher (32-38%). Arachidonic acid was far higher in Diet-A than the lettuce-based diets, in particular it was not present in Diet-M, while docosahexaenoic acid was absent in Diet-A and showed the highest relative amounts in Diet-K and Diet-D. Finally, eicosapentaenoic acid was similar in Diet-A, Diet-K and Diet-

D and lower in Diet-M and Diet-F. Total essential fatty acids were higher in Diet-A mirroring the patterns of single molecules, with the highest value in Diet-A, followed by Diet-K, Diet-D, and then Diet-F and Diet-M.

Table 5.5. Fatty acid profiles (percentage, mean ± standard deviation) of the five experimental diets (Diet-A, Diet-F, Diet-K, Diet-M and Diet-D). Main FA classes (SFA: Saturated FA; MUFA: Monounsaturated FA; PUFA: Polyunsaturated FA); main biomarkers of nutritional quality are also indicated. LCFA: Long-chain FA; LA: Linoleic acid, ALA: α-Linolenic acid; ARA: Arachidonic acid, EPA: Eicosapentaenoic acid, DHA: Docosahexaenoic acid.

	Diet				
FA	**Diet-A**	**Diet-F**	**Diet-K**	**Diet-M**	**Diet-D**
12:0	0.09 ± 0.03	0.10 ± 0.04	0.04 ± 0.01	0.01 ± 0.00	0.07 ± 0.01
14:0	7.49 ± 0.11	2.26 ± 0.02	2.93 ± 0.21	2.22 ± 0.23	2.36 ± 0.12
15:0	0.18 ± 0.03	0.47 ± 0.01	0.27 ± 0.05	0.36 ± 0.04	0.36 ± 0.04
16:0	11.02 ± 1.08	16.59 ± 0.10	13.92 ± 0.55	13.84 ± 0.33	13.83 ± 0.18
17:0	0.22 ± 0.13	0.64 ± 0.04	0.43 ± 0.26	0.84 ± 0.29	0.85 ± 0.45
18:0	0.69 ± 0.17	2.67 ± 0.15	1.50 ± 0.51	1.53 ± 0.32	1.73 ± 0.14
19:0	0.12 ± 0.14	0.04 ± 0.02	0.00 ± 0.00	0.13 ±0.11	0.30 ± 0.01
20:0	0.25 ± 0.02	0.29 ± 0.19	0.11 ± 0.07	0.28 ± 0.12	0.00 ± 0.00
21:0	0.38 ± 0.66	0.06 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Σ LCFA	0.00 ± 0.00	3.54 ± 0.07	3.04 ± 0.45	3.87 ± 0.50	3.34 ± 0.30
14:1 n5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.34 ± 0.01
15:1 n5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.05
16:1 n9	1.80 ± 0.09	2.64 ± 0.06	3.28 ± 0.13	3.26 ± 0.06	3.91 ± 0.00
16:1 n7	3.90 ± 0.35	3.73 ± 0.02	3.91 ± 0.05	4.16 ± 0.07	4.48 ± 0.06
18:1 n7	3.76 ± 0.53	2.35 ± 0.02	3.07 ± 0.13	2.40 ± 0.09	2.26 ± 0.00
18:1 n9t	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
18:1 n9c	32.15 ± 1.48	3.91 ± 0.13	4.50 ± 0.16	4.79 ± 0.11	3.79 ± 0.07
20:1 n9	0.00 ± 0.00	0.10 ± 0.05	0.14 ± 0.03	1.45 ± 0.11	0.39 ± 0.15
20:1 n11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:1 n9	0.00 ± 0.00	0.00 ± 0.00	0.14 ± 0.04	0.00 ± 0.00	0.08 ± 0.04
18:2 n6c LA	6.07 ± 0.27	15.14 ± 0.43	13.59 ± 0.07	15.33 ± 0.50	13.97 ± 0.17
18:3 n3 ALA	7.38 ± 0.98	37.86 ± 0.57	34.87 ± 0.11	37.15 ± 0.84	32.94 ± 0.51
18:3 n6	0.30 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.11	0.15 ± 0.12
18:4 n3	4.42 ± 0.45	0.00 ± 0.00	0.65 ± 0.02	0.00 ± 0.00	0.85 ± 0.02
20:2 n6	0.85 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:3 n3	0.00 ± 0.00	0.07 ± 0.03	0.05 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
20:3 n6	0.36 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:4 n3	0.46 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.42 ± 0.01
20:4 n6 ARA	8.09 ± 0.21	0.79 ± 0.10	0.56 ± 0.58	0.00 ± 0.00	1.05 ± 0.02
20:5 n3 EPA	5.17 ± 0.17	1.32 ± 0.15	5.95 ± 0.23	1.77 ± 0.20	4.55 ± 0.14
22:2 n6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:4 n6	0.76 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.28
22:5 n3	2.07 ± 0.20	1.91 ± 0.16	1.28 ± 0.14	1.97 ± 0.28	2.15 ± 0.15
22:6 n3 DHA	0.00 ± 0.00	1.28 ± 0.25	3.44 ± 0.17	1.17 ± 0.14	4.07 ± 0.16
branched	0.55 ± 0.63	0.66 ± 0.03	0.82 ± 0.26	1.12 ± 0.16	0.31 ± 0.02
-OH	0.38 ± 0.27	1.13 ± 0.05	0.78 ± 0.28	1.29 ± 0.41	0.84 ± 0.23
-Δ	0.38 ± 0.06	0.25 ± 0.06	0.05 ± 0.00	0.17 ± 0.03	0.05 ± 0.01
Σ SFA	20.89 ± 0.82	26.67 ± 0.48	22.33 ± 0.47	23.11 ± 0.70	22.94 ± 0.34
Σ MUFA	41.79 ± 0.85	12.74 ± 0.18	15.06 ± 0.37	16.17 ± 0.30	15.36 ± 0.17
Σ PUFA	35.99 ± 1.33	58.55 ± 0.66	60.96 ± 0.61	58.14 ± 1.20	60.49 ± 0.30
Σ EFA	13.16 ± 0.25	3.39 ± 0.49	9.95 ± 0.74	3.19 ± 0.23	9.68 ± 0.28
Σ ω3-PUFA	19.57 ± 1.66	42.58 ± 0.53	46.45 ± 0.51	42.21 ± 0.49	44.99 ± 0.26
Σ ω6-PUFA	16.43 ± 0.49	15.97 ± 0.39	14.51 ± 0.28	15.92 ± 0.72	15.50 ± 0.39
Σ ω3-HUFA	12.12 ± 0.72	4.65 ± 0.78	11.53 ± 0.40	5.07 ± 0.36	12.05 ± 0.43
ω3/ω6	1.19 ± 0.13	2.67 ± 0.07	3.20 ± 0.07	2.65 ± 0.09	2.90 ± 0.09

Principal Coordinate Analysis (PCO) of fatty acid profiles of diets highlighted the difference of PERMANOVA analysis with a clear separation between the algal diet and the other four based on lettuce discards (Figure 5.3), due to the different amount of total monounsaturated fatty acids (MUFA), oleic acid (18:1 n9c) and arachidonic acid (ARA). In contrast, the four sustainable diets were divided into two groups with Diet-F and Diet-M characterized by higher SFA concentration, while Diet-K and Diet-D were similar thanks to DHA.

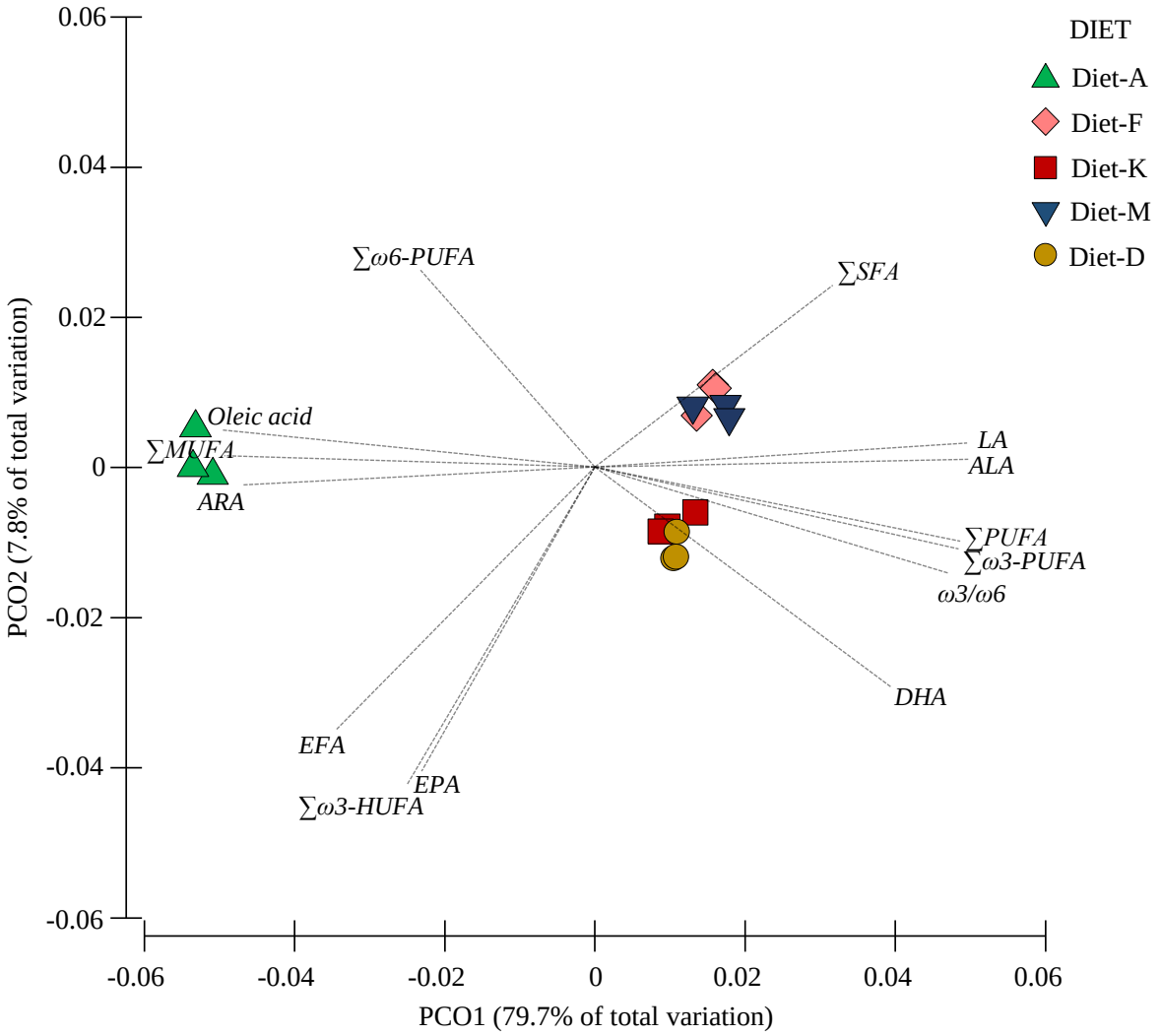


Figure 5.3. Principal Coordinates Analysis (PCO) of the fatty acid profiles of the five experimental diets (Diet-A, Diet-F, Diet-K, Diet-M and Diet-D). The main fatty acid classes and the main indicators of nutritional quality are superimposed to the graph. The meaning of the acronyms is the same as in Table 5.5.

PERMANOVA results of egg fatty acid profiles showed significant differences (MS= 0.0024, Pseudo-$F_{(6,34)}$= 42.61, P= 0.001) between eggs produced by sea urchins collected at the start of the feeding experiment and produced by sea urchins fed with the experimental formulations.

Egg-W collected before the start of the feeding trial were characterized by a high amount of total PUFAs, especially ω3-PUFAs, EFAs and ω3-HUFAs, while ω6-PUFAs showed a low relative amount (Table 5.6). Between single fatty acids, EPA showed the highest percentage, followed by ALA, palmitic acid, ARA and ALA. In Eggs-A PUFAs were still the most representative fatty acid class, but lower than in wild eggs, and characterized by a higher amount of ω6-PUFAs than EFAs, ω3-PUFAs and ω3-HUFAs respectively. ARA was the more abundant fatty acid, followed by gondolic (18:1n9c), EPA and oleic acids (20:1n9). Egg produced by sea urchins fed with lettuce-based diets were characterized by a high content of PUFAs, with a consistent amount of ω3-PUFAs respect to ω6-PUFAs, followed by EFAs and ω3-HUFAs. Low relative concentrations of oleic acid were found, while LA and ALA had the highest relative amount. EPA showed the highest relative amount between EFAs followed by ARA and then DHA. Respect to Eggs-W EFAs were lower, except DHA measured in Eggs-F and Eggs-K. Differently to Eggs-A, EPA and DHA were higher, while ARA was lower.

Table 5.6. Fatty acid profiles (percentage, mean ± standard deviation) of eggs produced by sea urchins collected before the start of the feeding experiment (Egg-W), and fed with the five experimental diets (Egg-A, Egg-F, Egg-K, Egg-M and Egg-D). The meaning of the acronyms is the same as in Table 5.5.

FA	Egg					
	Egg-W	**Egg-A**	**Egg-F**	**Egg-K**	**Egg-M**	**Egg-D**
12:00	0.00 ± 0.00	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.02	0.08 ± 0.02	0.09 ± 0.01
14:00	5.46 ± 1.74	0.83 ± 0.37	5.89 ± 1.63	5.64 ± 0.41	5.51 ± 1.28	4.88 ± 0.66
15:00	1.21 ± 0.18	0.84 ± 0.10	1.13 ± 0.12	0.99 ± 0.06	1.25 ± 0.05	1.51 ± 0.08
16:00	11.62 ± 1.08	11.50 ± 0.28	12.88 ± 1.75	12.29 ± 0.54	11.59 ± 1.11	13.46 ± 0.91
17:00	0.72 ± 0.12	0.42 ± 0.03	0.50 ± 0.05	0. 39 ± 0.04	0.34 ± 0.03	0.51 ± 0.05
18:00	1.91 ± 0.19	2.38 ± 0.22	2.06 ± 0.20	1.88 ± 0.28	1.67 ± 0.18	1,97 ± 0.19
19:00	0.35 ± 0.05	0.12 ± 0.02	0.25 ± 0.08	0.27 ± 0.06	0.24 ± 0.06	0.32 ± 0.03
20:00	0.44 ± 0.10	0.16 ± 0.02	0.41 ± 0.12	0.47 ± 0.08	0.41 ± 0.07	0.52 ± 0.07
21:00	0.06 ± 0.01	0.02 ± 0.01	0.11 ± 0.05	0.09 ± 0.02	0.08 ± 0.02	0.14 ± 0.04
>22	0.18 ± 0.68	0.09 ± 0.01	0.39 ± 0.12	0.40 ± 0.07	0.47 ± 0.07	0. 65 ± 0.14
14:1 n5	0.16 ± 0.11	0.27 ± 0.07	0.31 ± 0.15	0.26 ± 0.09	0.27 ± 0.13	0.24 ± 0.05
15:1 n5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16:1 n9	2.20 ± 0.06	1.73 ± 0.23	3.23 ± 0.29	3.00 ± 0.30	2.60 ± 0.34	2.52 ± 0.19
16:1 n7	2.09 ± 0.09	1.60 ± 0.18	2.81 ± 0.22	2.64 ± 0.25	2.35 ± 0.27	2.25 ± 0.16
18:1 n7	1.67± 0.16	2.09 ± 0.16	1.96 ± 0.14	2.72 ± 0.12	1.81 ± 0.19	1.76 ± 0.14
18:1 n9t	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
18:1 n9c	1.97 ± 0.18	10.93 ± 0.91	1.46 ± 0.08	2.12 ± 0.17	1.65 ± 0.28	1.71 ± 0.13
20:1 n9	2.55 ± 0.56	7.19 ± 0.38	2.57 ± 0.32	2.69 ± 0.22	3.11 ± 0.56	2.48 ± 0.45
20:1 n11	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.02	0.00 ± 0.00	0.22 ± 0.06	0.02 ± 0.03
22:1 n9	1.54 ± 0.31	3.10 ± 0.30	1.24 ± 0.31	1.45 ± 0.26	1.56 ± 0.47	1.33 ± 0.17
18:2 n6c LA	3.49 ± 0.62	4.54 ± 0.69	7.56 ± 1.10	7.66 ± 0.51	9.02 ± 0.89	9.28 ± 0.79
18:3 n3 ALA	13.44 ± 0.45	5.51 ± 0.42	17.12 ± 2.94	17.60 ± 1.20	19.78 ± 3.01	18.90 ± 1.28
18:3 n6	0.37 ± 0.06	0.30 ± 0.03	0.05 ± 0.02	0.05 ± 0.01	0.04 ± 0.02	0.04 ± 0.02
18:4 n3	3.22 ± 0.70	2.68 ± 0.25	0.047 ± 0.08	0.40 ± 0.04	0.34 ± 0.12	0.26 ± 0.09
20:2 n6	3.15 ± 0.41	5.45 ± 0.35	5.73 ± 0.45	6.97 ± 1.11	6.63 ± 0.29	6.65 ± 0.49
20:3 n3	6.85 ± 1.03	2.71 ± 0.46	6.43 ± 0.49	7.10 ± 1.03	6.42 ± 1.12	5.21 ± 0.63
20:3 n6	0.72 ± 0.07	1.14 ± 0.06	1.07 ± 0.13	1.06 ± 0.15	1.13 ± 0.22	1.45 ± 0.37
20:4 n3	0.67 ± 0.15	0.50 ± 0.05	0.58 ± 0.08	0.53 ± 0.16	0.59 ± 0.18	0.65 ± 0.22
20:4 n6 ARA	9.48 ± 0.84	15.37 ± 1.23	7.10 ± 0.84	5.19 ± 0.74	7.45 ± 1.52	8.37 ± 1.65
20:5 n3 EPA	19.89 ± 2.03	9.34 ± 0.79	11.81 ± 0.52	11.62 ± 0.75	10.88 ± 1.02	9.37 ± 0.59
22:2 n6	0.11 ± 0.08	0.02 ± 0.02	0.05 ± 0.03	0.05 ± 0.04	0.08 ± 0.06	0.06 ± 0.02
22:4 n6	0.47 ± 0.13	0.34 ± 0.01	0.14 ± 0.01	0.08 ± 0.01	0.13 ± 0.02	0.15 ± 0.02
22:5 n3	0.88 ± 0.14	1.17 ± 0.09	1.01 ± 0.05	0.54 ± 0.05	0.50 ± 0.07	0.68 ± 0.08
22:6 n3 DHA	2.36 ± 0.73	0.13 ± 0.01	3.11 ± 0.38	3.43 ± 0.49	1.30 ± 0.15	1.64 ± 0.24
branched	0.50 ± 0.06	0.19 ± 0.03	0.33 0.02	0.34 ± 0.03	0.39 ± 0.04	0.43 ± 0.06
-OH	0.11 ± 0.23	0.00 ± 0.00	0.08 0.01	0.00 ± 0.00	0.12 ± 0.04	0.19 ± 0.13
-Δ	0.00 ± 0.00	0.00 ± 0.00	0.00 0.00	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.07
Σ SFA	21.99 ± 1.71	23.63 ± 0.52	23.69 ± 2.75	22.49 ± 1.15	21.63 ± 1.88	23.82 ± 1.07
Σ MUFA	12.21 ± 1.10	26.91 ± 1.01	13.69 ± 1.25	14.90 ± 0.64	13.57 ± 1.92	12. 31 ± 1.04
Σ PUFA	65.17 ± 2.14	49.20 ± 1.18	62.21 ± 3.47	62.27 ± 1.09	64.28 ± 3.56	63.12 ± 0.96
Σ EFA	31.74 ± 3.17	24.84 ± 1.62	22.02 ± 1.06	20.23 ± 1.63	19.63 ± 2.47	19.39 ± 1.94
Σ ω3-PUFA	47.36 ± 2.44	22.04 ± 1.02	40.52 ± 3.02	41.22 ± 1.62	39.81 ± 3.49	37.11 ± 1.41
Σ ω6-PUFA	17.81 ± 1.06	27.17 ± 1.63	21.69 ± 0.98	21.06 ± 1.52	24.47 ± 1.28	26.01 ± 0.99
Σ ω3-HUFA	27.06 ± 2.33	13.81 ± 0.70	16.97 ± 0.45	16.52 ± 1.00	13.61 ± 1.01	12.60 ± 0.90
ω3/ω6	2.66 ± 0.26	0.81 ± 0.08	1.87 ± 0.14	1.97 ± 0.19	1.63 ± 0.18	1.43 ± 0.10

Principal coordinate analysis mirrors the differences highlighted by statistical analysis and fatty acid profiles (Figure 5.4). Eggs produced by wild sea urchins were distributed in the lower part of the graph, while Egg-A were separated from all the other specimens. Eggs produced by sea urchin fed with lettuce-based diets were divided into two main groups on the right, with samples from Diet-M and Diet-D distributed in the upper part of the group, while eggs produced by Diet-K and Diet-F were in the lower.

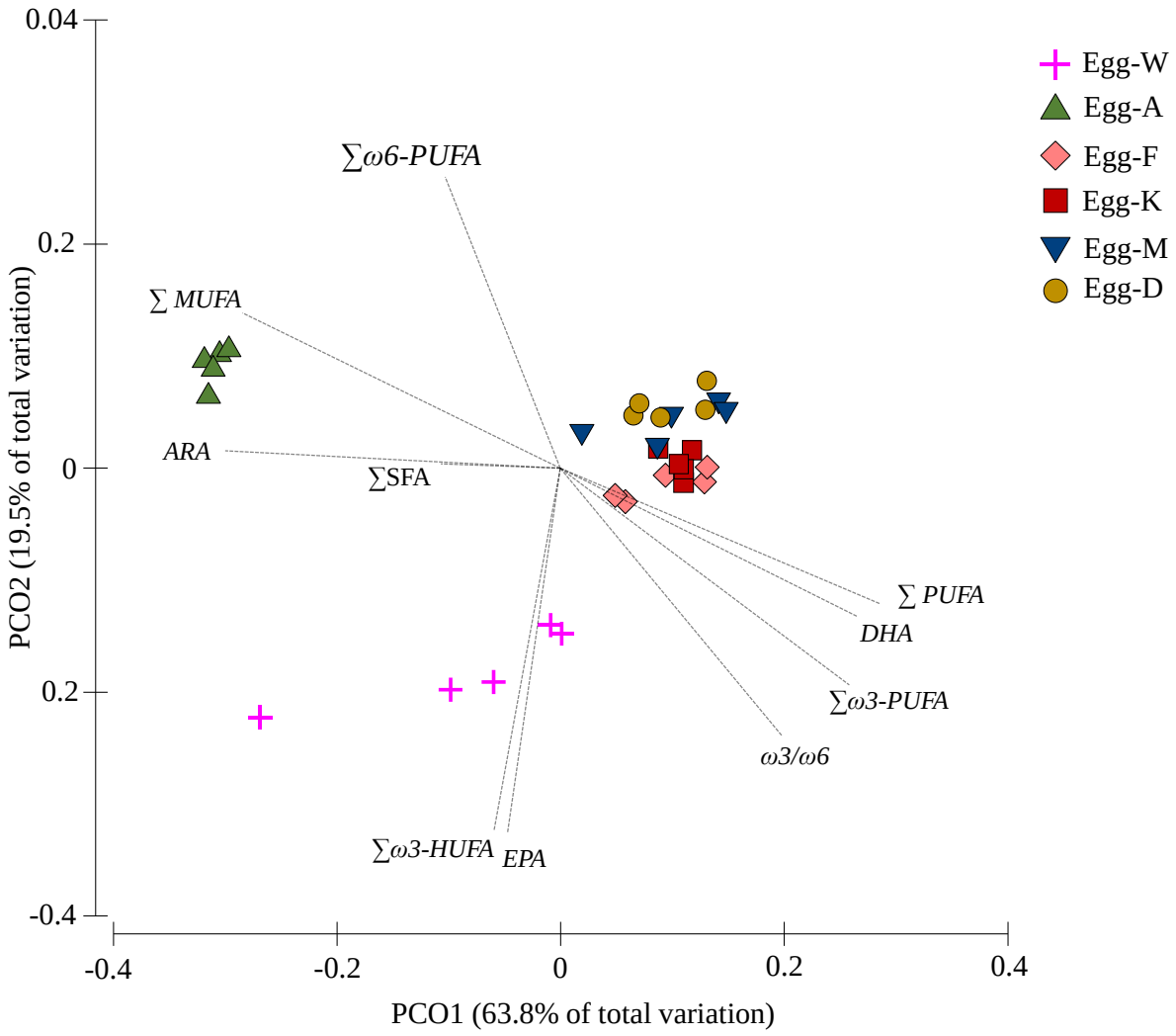
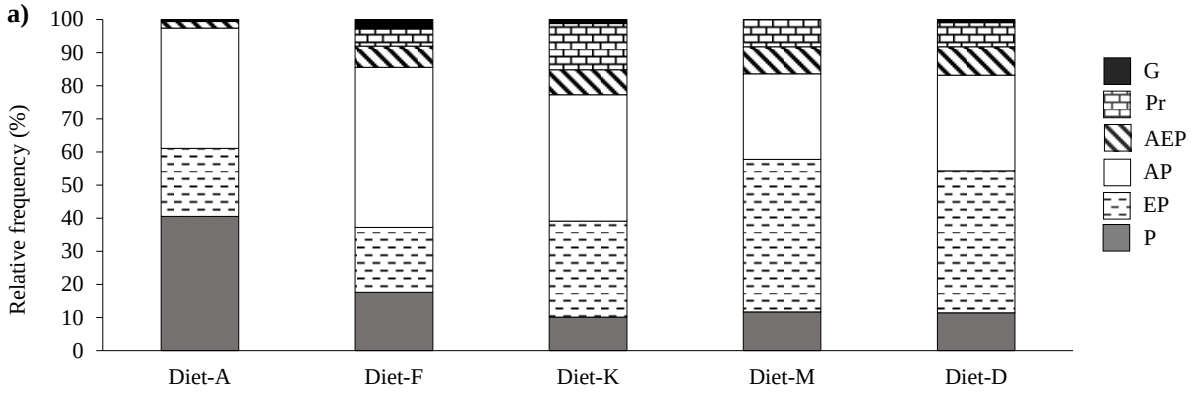


Figure 5.4. Principal Coordinates Analysis (PCO) of the fatty acid profiles of the eggs produced by sea urchin collected from the wild before the start of the feeding experiment (Egg-W) and by sea urchin fed with the five experimental diets (Egg-A, Egg-F, Egg-K, Egg-M and Egg-D). The main fatty acid classes and the main indicators of nutritional quality are superimposed to the graph. The meaning of the acronyms is the same as in Table 5.2.

5.3.3 Larval abnormalities

The five experimental diets showed different performance in larval development (Figure 5.5a), with Diet-A presenting the best performance with the highest amount of normal plutei (40.51%) coupled with the lowest values of abnormal early plutei (1.92%) and prisms (0.32%), resulting in the lowest integrated toxicity index value (Figure 4b). Among the four lettuce-based diet, Diet-M showed the best performance according to ITI value, followed by Diet-D, thanks to the slightly higher amount of normal early plutei (46.05% vs 42.85%), but consistently higher than those produced by Diet-K and Diet-F (29.02 and 19.48 % respectively). Diet-K showed the worst performance due to the low amount of normal plutei (10.55%) and the highest percentage of prisms (13.88%), resulting in the highest ITI value (Figure 5.4b).



b)

ITI	21.13	35.46	40.13	32.80	33.66

Figure 5.5. (a) Relative frequency malformation observed in larvae produced by sea urchin fed with the five experimental diets (Dit-A, Diet-F, Diet-K, Diet-M and Diet-D), and the respective integrate toxicity index (ITI) value. Normal pluteus (P), early pluteus (EP), abnormal pluteus (AP), early abnormal pluteus (EAP), prism (Pr), gastrula (G).

5.4 Discussion

High harvest pressure and the consequent depletion of wild stocks of the most important commercial sea urchin species led to an increased focus on the two areas of echinoculture practices: adult gonad enhancement and full-cycle production. While a huge effort on production and evaluation of formulated diet to promote gonad enhancement was conducted in recent years, scarce information on the effect of formulated diets on *Paracentrotus lividus* larval development is available (Liu et al., 2007, Gago et al., 2009, Carboni et al., 2013). To find new and alternative diets for the implementation of full-cycle echinoculture practices, the evaluation of the effect of four sustainable diets on sea urchin larval development was conducted.

A clear difference emerged between the four lettuce-based diets and the macroalgae diet, mainly related to different $\delta^{13}C$ values. The different sources of carbon, only CO_2 for terrestrial vegetal, CO_2 and HCO_3^- for marine primary producers (Szpak et al., 2013), as well as the different photosynthetic pathways of carbon fixation (Jackson et al., 1993; Raven and Osmond, 1992) lead to the lower $\delta^{13}C$ of lettuce-based diets, according to expected $\delta^{13}C$ value of *Lactuca sativa* leaves and macroalgae (Kroopnick, 1985; Szpak et al., 2013; Wang and Yeh, 2003). Values of $\delta^{15}N$ of the four sustainable diets were also influenced by the low amount (10%) of seafood enrichment, and in particular sardine and krill presented similar $\delta^{15}N$ value, maybe because both organisms can be herbivorous according to seasonal changes (Šimat et al., 2020), resulting in a similar isotopic composition of Diet-F and Diet-K.

The initial isotopic values of the eggs reflect sea urchin natural diet with values suggesting the consumption of brown algae or marine plants (Hanson et al., 2010; Lepoint et al., 2003), such as *Dichotoma dichotoma* and *Posidonia oceanica*, both consumed by *P. lividus* when available (Boudouresque and Verlaque, 2020) and present in the sampling area of the experimental sea urchin pool (Author's observation). Diet effect on produced eggs was clearly highlighted by changes in isotopic values, with Egg-A that showed an increase in both $\delta^{13}C$ and $\delta^{15}N$, according to Diet-A isotopic signature, while egg produced by sea urchin fed the four lettuce-based diets were characterized by a more negative $\delta^{13}C$ and a lower $\delta^{15}N$, resembling isotopic values of the respective diets. As carbon is a conservative tracer, used in food web studies to determine energy sources, the marked difference found in initial and final $\delta^{13}C$ values of sea urchin eggs confirms the assimilation of the consumed organic matter (Post, 2002). Great assimilation promotes growth, tissues regenerations as well as nutrient and energy storage, thus its assessment, in particular for new and alternative diets, is fundamental for aquaculture purposes (Gamboa-Delgado, 2021). These results confirm the goodness of vegetal terrestrial discards as nutritional source for sea urchins, despite the high insoluble carbohydrates content, not digested by echinoids, as well as the presence of anti-nutritional compounds that could negatively affect nutrient assimilation (Fernandez and Boudouresque, 1998; Hardy, 2010; Marsh et al., 2013).

However, consumer isotopic signature did not perfectly resemble those of its diet, as highlighted by the *pair-wise* between diets and the respective eggs. Comparing average Euclidean Distance values, Diet-K resulted better assimilated than the other diets, presenting the lowest mean ED value, followed by diet-M. Diet-A- and Diet-D showed similar ED values while Diet-F and Egg-F was the most different pair. These results are in contrast with those of ingestion rate (IR) and absorption efficiency (AE) measured during the feeding experiment. Although all diets showed similar IR values, the best-

absorbed diet was Diet-F, followed by Diet-A and Diet-K. Diet M and Diet-D showed significantly lower AE, but in terms of egg production, their performance was comparable to or better than those of the other experimental diets. However, although these indices provide important information on the effectiveness of experimental diets, they could be easily affected by technical bias (Lawrence et al., 2007). It is therefore recommended, especially when evaluating diets based on new emerging ingredients, like food discards, to couple indirect index with analytical measurements to obtain an accurate assessment of their suitability.

Fatty acid profile analysis confirmed the clear difference between the five experimental diets and the produced eggs, previously described for SIA. Diet-A was characterized by a higher amount of oleic acid, abundant in both used macroalgae, and arachidonic acid more consistent in *Laminaria* spp (Ortiz et al., 2006). In contrast, values of eicosapentaenoic acid (EPA), essential fatty acids (EFAs) and ω3-highly unsaturated fatty acids (ω3-HUFAs) were comparable with those observed in the lettuce-based diets. In aquaculture feeds these fatty acids were supplied from fish meal or oil due to the low abundance in terrestrial plants, while macroalgae can synthesise some long-chain polyunsaturated fatty acids (PUFAs) (Olsen et al., 2010; Twining et al., 2016). The four diets based on lettuce discards were instead rich in linoleic acid (LA) and α-linolenic acid (ALA), both abundant in terrestrial plants and vegetal discards (Ciriminna et al., 2020; Vizzini et al., 2019). Seafood enrichment characterized the four sustainable diets, with Diet-K and Diet-D showing high content of EPA, DHA, EFA, ω3-HUFA and consequently also of ω3-PUFA and ω3/ω6 rather than Diet-F and Diet-M, according to the fatty acid content of krill (Hagen et al., 2001), anchovy discards (Ciriminna et al., 2020), sardine (Šimat et al., 2020) and mussels (Fernández-Reiriz et al., 2015). As observed by SIA, the value of wild eggs reflects sea urchin natural diets with a high variability among wild samples due to the omnivorous feeding behaviour of *P. lividus*, able to feed on several nutrient sources (Boudouresque and Verlaque, 2020). Differently, eggs produced by experimental sea urchins were distributed in different groups according to fatty acid profile of the respective diets. Egg-A were clearly separated from the other eggs due to the far higher content of oleic acid and ARA, assimilated from Diet-A, and presented in low amount in both Egg-W and eggs produced by sea urchin fed with the four lettuce-based diets, which were in contrast, rich in LA and ALA. In addition, eggs produced by sea urchin fed with the four sustainable diets, showed higher amount of DHA and EPA suggesting the activation of their specific pathways for fatty acid elongations (Castell et al., 2004; Kabeya et al., 2017) and reducing the dependence on the limited EFAs content of diets. However, the low amount of EFAs highlighted in experimental eggs, despite the consistent amount of precursors in the respective diets, confirm that fatty acid production could be inhibited by high intake of dietary precursors (Zuo et al., 2018).

Analysis of larval malformation confirm that broodstock diets could deeply influence reproductive success affecting larval development, and, as males sperm was collected from the same sea urchins for all fertilization, results also confirm that egg nutrient stores play the major role in plutei development (Byrne et al., 2008). The best results were observed for Diet-A, in contrast with findings obtained regards somatic egg production presented in chapter 4. According to literature, formulate diets that promote gonad growth and increase gonad quality for commercial purposes may not satisfy nutritional requirements for a correct larvae development (Carboni et al., 2013). However, differences in performance are highlighted thanks to the application of the Integrated Toxicity Index, which allows a detailed evaluation considering both the gravity and the frequency of the observed malformations (Morroni et al., 2020). The performance of Diet-A, Diet-M and Diet-D would appear the same, if taking into account only the absence or the presence of abnormalities, considering that they presented a similar amount of plutei without malformations (i.e. normal and early normal plutei). However, the higher relative amount of normal plutei in diet A suggests faster development and thus better performance in larval production. A better larval survival rate is related to a shorter larval stage that occurs when females are farmed in favourable condition, especially food availability, and translate this favourable condition into the production of high-quality eggs able to sustain larval body development (Byrne et al., 2008; George, 1995). Present findings are in contrast with De Jong-Westman et al. (1995) that found the worst performance in larval development with sea urchin fed kelp rather than in sea urchin fed formulated feed, due to the low quality of broodstock diet. Protein and lipid, which were scarce in Diet-A, are the most important energy reserves in echinoderms eggs and are assimilated by adult diets (George et al., 1997; Meyer et al., 2007; Unuma et al., 2003). In particular, proteins varied significantly in eggs produced by sea urchins fed high-quality diet rather than in those produced by poorly fed sea urchins (George et al., 1990), while lipids and fatty acids of broodstock diets have been identified as major dietary factors for a successful reproduction and survival offspring (Izquierdo et al., 2001; Liu et al., 2007). Nevertheless, high lipid concentration could result in high production of abnormal plutei, as observed by Zupo et al. (2018) comparing commercial pellets with natural items (*P. oceanica* and *U. rigida*). Lipid content found in the diets with 10% of seafood meal resulted suitable for *P. lividus* gonad growth and development (Baião et al., 2019; Lourenço et al., 2020) but the high production of malformed or delayed larvae, confirmed that lipid quality could affect larval development, especially EFA and HUFA content (Liu et al., 2007; Nevejan et al., 2003; Payne and Rippingale, 2000). During larval development, this long chain–PUFAs can influence gene expression, regulate stress response and modify composition of membrane cells (Carboni et al., 2013; Ganga et al., 2006). According to Gago et al. (2009) prisms and four-armed plutei produced by wild *P. lividus* showed high content of Oleic acid, ARA and EPA, with a

great amount of HUFA, suggesting their importance in larval development, even if analysis on larval abnormalities were not conducted. Although echinoid larvae can also meet EFAs requirements through endogenous synthesis from 18C PUFAs (Liu et al., 2007), differences in dietary fatty acid profiles could explain the different larval developmental performance.

5.5 Conclusion

Five experimental diets were tested on larval development of *Paracentrotus lividus*, the most harvested Mediterranean sea urchin and thus a potential target species for a full cycle aquaculture production (Carboni et al., 2013). According to stable isotopes analysis (SIA), all the presented formulations resulted assimilated by sea urchins, despite terrestrial plants could negatively affect palatability and assimilation (Hardy, 2010). Fatty acid analysis confirms the results highlighted by SIA, allows tracking egg changes according to respective diets. The four sustainable diets were characterized by high concentration of essential fatty acid precursors that sea urchins transformed to meet their requirements of EFAs and HUFAs. Finally, the analysis on larval development showed a lower efficacy of lettuce-based diets in larval development, confirming that diets formulated to boost gonad production for commercial purposes are not necessarily suitable for larvae production (Carboni et al., 2013). The better performance of Diet-A suggests that macroalgae, usually considered low-quality diet due to their scarce nutrient content, contains macro or micronutrients in adequate proportion to promote larval development. Thus, the introduction of an aliquot of macroalgae in a formulated diet for full-cycle production must be taken into account. However, a deep analysis on nutritive requirement for promoting larvae development is needed, as important nutrient as ammino acids and carotenoids are not included in this study.

CHAPTER 6: General conclusion

The overall objective of this research was to propose and evaluate the utilization of food processing discards as ingredients for the production of aquaculture sustainable feeds. In order to develop farming practices for the sea urchin *Paracentrotus lividus*, severely threatened by commercial fishing (Guidetti et al., 2004; Pais et al., 2011), embracing the principles of sustainable development and Blue Growth (Boyd et al., 2020; Eikeset et al., 2018), feeding experiments were conducted using sustainable feeds and diets.

A nutritional assessment of the proposed ingredients and formulations was carried out to ensure the suitability of food processing discards as nutritional sources for the production of *P. lividus* gonads, the final product of echinoculture. *Cihcorium endivia* and *Engraulis encrasicolus* were rich in key micro- and macronutrients, fundamental for supporting growth and development of sea urchin gonads (Chapter 2). In particular, the outermost leaves of endive resulted rich in carbohydrates, while anchovy discards presented high concentrations of protein and lipids. In addition, the analysis of fatty acid profiles revealed high concentrations of essential fatty acids (EFAs), which are necessary in aquaculture feed formulation, as involved in several metabolic processes of the reared organisms, and may increase the quality of the final product, (Mitra, 2021; Olsen and Hasan, 2012). Finally, terrestrial vegetal discards showed a consistent amount of essential fatty acid precursors, such as Linoleic and α-Linolenic acids, important in echinoculture context due to the ability of echinoids to transform them and synthesize *de-novo* EFAs, meeting metabolic requirements (Monroig and Kabeya, 2018). Similarly, the feed formulations produced using both vegetal anchovy discards presented a protein and lipid content suitable for the nutritional requirements of *P. lividus*, as confirmed by gonad (Chapter 3) and egg (Chapter 4) production. Furthermore, the positive results obtained concerning gonad and egg colouration of reared sea urchins suggest an adequate carotenoid content in the leaves of tested vegetables, although specific analyses were not conducted.

Particular attention was paid to the stability of the proposed formulations, as maintaining the integrity of feed pellets in the rearing tanks is essential for efficient performances (Palma et al., 2008; Pearce et al., 2002b). Dissolving in water, pellets release organic matter into the tanks, promoting a worsening of the environmental conditions and, at the same time reducing food availability to organisms (Secci et al., 2021). The binders used to provide stability to the tested feeds, agar and arabic gum, resulted adequate allowing sufficient pellet stability (Chapter 2 and Chapter 4). Both tested binders are low-cost and low impact alternatives, as agar can be produced in a multitrophic aquaculture context using as an example *Gracilaria* spp., able to grow on nutrient excess resulting from aquaculture activities (Sousa et al., 2010), while arabic gum could be extracted by *Acacia*

arabica (Saha et al., 2017). Thus, they can be considered as viable alternatives to more expensive and less sustainable binders of animal origin (Caltagirone et al., 1992).

Palatability and assimilation of the proposed formulation were evaluated in both experiments, as these parameters are fundamental to assess the performance of a new feed. In particular, the assessment of diet assimilation allows evaluating not only food consumption, but also the effective storage of nutrients provided by the diets (Gamboa-Delgado, 2021). The assimilation of the proposed diets was therefore assessed by applying indirect measurements and using the analysis of organic tracers such as stable isotopes and fatty acids (Cook et al., 2000; Cyrus et al., 2019). In both cases, formulations were well assimilated by sea urchins, even though they consisted mostly or completely of discards from the food retail chain, and therefore, rich in inorganic components (fish discards) as well as low digestible compounds (vegetable waste). Some slight differences were found between direct and indirect measurements; therefore, it is recommended to use both analyses when evaluating new and alternative ingredients and diets.

Gonad production, the main objective of sea urchin aquaculture, was evaluated using the gonado somatic index (GSI), considered the standard index to evaluate diet efficacy in promoting gonad growth (Walker et al., 2015), and the application of the new and sustainable Racking method. The GSI allows the assessment of diet suitability by measuring the growth of the gonads in relation to the total weight of the individual, confirming the transfer of nutrients assimilated from the diet into the gonads. In contrast, through the racking method, production is expressed by measuring the amount of gametes released after the controlled feeding period, without sacrificing the farmed organisms. This approach, which allows the use of the same pool of sea urchins for subsequent experiments, allows the evaluation of different feeding treatments while eliminating variability due to individual-specific responses. The use of the Racking method can also reduce the rearing period. In fact, sea urchins subjected to gamete release will start from similar sexual maturity conditions, stage I (Byrne et al., 1990), which can be further standardised by only one week of starvation instead of the suggested 2 to 4 weeks (Guillou et al., 2000; Raposo et al., 2019). In addition, the individuals subjected to Racking will already be adapted to laboratory environmental conditions, eliminating the acclimatization period generally required before starting the experiments. Finally, the application of this method also makes it possible to carry out the evaluation of experimental diets for the larval production of *P. lividus*, one of the main bottlenecks for the development of a closed-cycle production system (Carboni et al., 2013; James and Siikavuopio, 2015). Although most research efforts have focused on the evaluation of experimental diets for the growth and development of planktotrophic

larvae, the importance of the broodstock diet has also been recognised as influencing the nutritional content of the eggs and thus the overall reproductive success (Byrne et al., 2008).

The growth in gonad biomass, and the consequent increase in GSI values (Chapter 3), confirmed the sea urchin ability to ingest and assimilate the proposed formulations, transferring and storing dietary nutrients in their gonads. Furthermore, the effect of the proposed formulations on the sexual maturity of reared urchins was determined by analysing histological sections. The results showed an appropriate progression in sexual maturation that resulted appropriate for aquaculture goals, favouring nutrient accumulation and gonad biomass growth, without presenting pre-spawning or spawning organisms (Walker et al., 2015). In fact, these two stages of sexual maturity are usually associated with reduced size gonads, due to gamete emission, and of poor nutritional quality due to the consumption of the nutritional reserves accumulated in the gonads (Böttger et al., 2006). The advancement of sexual maturity in farmed sea urchins also confirmed the validity of the sustainable diets when feeding a breeding pool. As proved by the second feeding experiment (Chapter 4), by increasing the feeding period from 3 to 4 months, sea urchins reached the mature stage and, when stimulated, released gametes.

Between the two sustainable formulations tested in Chapter 3, the more balanced formulation (60% endive outermost leaves and 40% anchovy discards) presented the best results in terms of gonad development and growth, confirming the importance of a substantial proportion of animal proteins (Fernandez and Boudouresque, 2000, 1998). The formulation characterised by a clear predominance of the vegetable ingredient (80% escarole outermost leaves and 20% anchovy discards) still presented encouraging results, although less effective both in terms of production and sexual development. Looking towards the development of a large-scale aquaculture systems, innovative processes aimed at the reduction of the amount of ingredients of animal origin are crucial to reduce economic and environmental impact. However, the animal ingredient employed in this experiment was produced from fish discards, thus its use in a farming context would both decrease production costs and facilitate the management and disposal of discards produced by the fish industry.

Comparison with patterns observed in wild specimens demonstrated a better performance in farmed urchins, confirming the validity of sustainable feed for feeding *P. lividus*, although certainly influenced by stable environmental conditions. Indeed, sea urchins fed with the sustainable formulations showed a higher GSI than wild specimens, which are highly appreciated by consumers (Stefannson et al., 2019), but also compared to sea urchins reared on other artificial diets (Santos et al., 2019; Sartori et al., 2014).

Similarly, sustainable diets made up of more than 85% lettuce discards were able to stimulate gonad growth and accumulation of nutrients. Comparison with the algal diet, considered the natural diet of *P. lividus*, showed a better performance of the lettuce-based diets in egg production, measured by the ovosomatic index, but not in larval production. Larvae developed from eggs produced on the algae diet showed a higher amount of plutei without malformations, whereas the lettuce-based diets showed a high relative amount of delayed embryos.

The controlled feeding experiments allowed a comprehensive evaluation of the validity of sustainable diets and feeds for *P. lividus*, presenting encouraging results. Findings suggest that the nutritional content of the used diets allows the production of gonads of adequate size and quality to meet market demands, thus making them suitable for the practice of gonad enhancing. On the other hand, they do not seem to meet the correct composition to produce appropriate gametes to sustain effective and complete larval development. It is, therefore, advisable to maintain different formulations according to the purpose for which the diets are prepared, and considering the overall production cycle, a portion of the ingredient of macroalgal origin should be included.

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ORIGINAL ARTICLE

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Formulation of a new sustainable feed from food industry discards for rearing the purple sea urchin *Paracentrotus lividus*

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Abstract

The lack of suitable feeds for echinoculture has led to use natural resources already widely exploited by human activities. To move towards a higher sustainability of echinoculture, this study proposes a sustainable feed for *Paracentrotus lividus*. Two experimental formulations were obtained using discarded endive (*Cichorium endivia*) leaves and anchovy (*Engraulis encrasicolus*) industry discards in different proportions, and agar as a binder. The evaluation of the feed stability showed that the feed was stable for 72 hr, allowing a suitable feeding for sea urchins. Both formulations showed a proper nutritional value and fatty acid profile, corresponding to the features of the main ingredients and resulting suitable for echinoculture. A bioenergetic trial was carried out to measure daily ingestion rate, absorption efficiency and gonado-somatic index in sea urchins. They resulted also palatable and well absorbed by sea urchins, especially that one with higher fish content. At the end of the experiment, an increase in gonado-somatic index was also recorded. Despite further analysis is needed to assess the performance of the feed in terms of gonad yield and quality, these encouraging results indicate that food industry discards may be suitable alternative ingredients for the production of sustainable feeds for sea urchin aquaculture.

KEYWORDS

aquaculture, blue economy, echinoculture, fatty acids, feedstuff, sustainability

1 | INTRODUCTION

The edible sea urchin *Paracentrotus lividus* is the most commercially exploited echinoid in Europe (Baião et al., 2019). It is a widespread species along the North Atlantic Ocean and the Mediterranean Sea (Boudouresque & Verlaque, 2007), and the gonads, commonly called roe, are considered a delicacy in many countries worldwide. In the last decades, the increment of its demand has resulted in the overexploitation of natural populations and the consequent collapse of stocks (Gianguzza et al., 2006; Pais, Serra, Meloni, Saba, & Ceccherelli, 2012). Aquaculture is recognized as a possible solution to mitigate harvesting pressure on wild sea urchins. Therefore, many studies have dealt with feeding strategies and diet formulation for optimizing gonad yield and quality (e.g. Cook & Kelly, 2007b; Gibbs, Watts, Lawrence, & Lawrence, 2009; Pearce, Daggett, & Robinson, 2002) and to fill the gap between the growing market request and the natural supply (Carboni, Hughes, Atack, Tocher, & Migaud, 2015). However, so far, one of the main bottlenecks of echinoculture is the lack of an effective and sustainable diet, able to increase gonad production while keeping good nutritional and organoleptic features.

The use of macroalgae in the diet of reared sea urchins has been widely explored (Carrier, Eddy, & Redmond, 2017), as sea urchins are predominantly herbivores and grazers on macroalgae (Boudouresque & Verlaque, 2007). Nevertheless, the use of macroalgae is unlikely to be commercially viable for large-scale culture, since their availability varies throughout the year and transport

and storage costs are very high. In addition, their nutritional value and edibility are strongly influenced by the season and sampling site (Cook & Kelly, 2007a; Vadas, Beal, Dowling, & Fegley, 2000). Other ingredients, such as wheat, soybean meals or microalgae, have been added as a partial replacement of macroalgae, and the effect on somatic and gonadic growth (Pearce, Daggett, & Robinson, 2002a, 2004; Pearce et al., 2002b; Woods, James, Moss, Wright, & Siikavuopio, 2008), organoleptic characteristics (Robinson, Castell, & Kennedy, 2002; Suckling, Symonds, Kelly, & Young, 2011) and biochemical composition (Carboni, Hughes, Atack, Tocher, & Migaud, 2013; Liyana-Pathirana, Shahidi, & Whittick, 2002) of the roe was evaluated. Thanks to the promising results of some of these studies, the exploitation of constantly available land-based vegetables is nowadays a better option for formulating aquaculture feeds, due to the reduction in the use of natural marine resources generally included in sea urchin diets. Nevertheless, if the use of proteins and lipids derived from terrestrial plants is widespread in fish aquaculture (Gatlin et al., 2007; Torstensen et al., 2008), that is not the case in sea urchin aquaculture. Sartori and Gaion (2015) evaluated the effect of a diet composed of a mixture of *Maize kernel* and *Spinacia oleracea* on reared *P. lividus*, highlighting good feed ingestion rates and significant increases in the gonado-somatic index. Other studies evaluated the exploitation of fresh agricultural discards as a diet for *P. lividus*, alone [*Beta vulgaris*, *Brassica oleracea*, and *Lactuca sativa* in Vizzini, Miccichè, Vaccaro, and Mazzola (2015) and Vizzini, Visconti, Vaccaro, and Mazzola (2017)] or mixed with egg white and a little amount of commercial fish feed (Vizzini, Visconti, Signa, Romano, & Mazzola, 2019), and reported encouraging results in terms of gonad yield and organoleptic and nutritional features of the roe. More recently, also Raposo et al. (2019), by studying both gonad growth and fatty acid profile of sea urchins fed with terrestrial vegetables, encouraged the use of vegetables instead of cropped macroalgae or commercial feeds.

In this context, this study proposes a sustainable feed for sea urchins, mainly based on discards from the food industry. These discards, which are commonly treated as waste to be disposed, with management costs and environmental impact, could have instead the potential to be recycled as raw materials for the production of formulated feeds, in accordance with the principles of the circular economy. Two feed formulations with different percentages of vegetable and animal discards were tested to assess their feasibility for feeding *P. lividus* in rearing conditions. Feed stability in seawater and both palatability and assimilability of the new sustainable feed for *P. lividus* were tested. A preliminary assessment of the effect of the new feed on gonad growth was also carried out by estimating the gonado-somatic index. The nutritional composition and quality of both ingredients and feed were also assessed through the study of the proximate composition and fatty acid profiles. Indeed, a proper provision of dietary proteins, lipids and fatty acids, such as essential and polyunsaturated fatty acids, especially the *omega*-3 class, is crucial to improve the growth of reared organisms, obtaining also roe of good quality (Carboni et al., 2015; Castell et al., 2004; González-Durán, Castell, Robinson, & Blair, 2008).

2 | MATERIALS AND METHODS

2.1 | Feed formulation

Outermost leaves of *Cichorium endivia* (endive), obtained from unprocessed agricultural discards, and industry discards of *Engraulis encrasicolus* (European anchovy), composed mainly by viscera, head, skin and bones, were used as the main ingredients for producing a new sustainable feed for echinoculture. Endive and anchovy discards were freeze-dried and then ground to fine powder. Two formulations were prepared differing for the ratio of the main ingredients: endive leaves and anchovy discards contributed on a roughly 60:40 ratio (60/40 formulation) and 80:20 ratio (80/20 formulation) to the two feed formulations (Table 1). Agar (Agar-Agar fine powder 100% Food Grade, Intra Laboratories, UK), a non-branched polysaccharide extracted from red algae, was dissolved in boiling Milli-Q distilled water (385 g/L) and mixed until a homogeneous jelly-like solution was obtained. Then, it was allowed to cool to about 60°C and added in different proportions (25 and 50 g/kg) to both feed formulations, and mixtures were stirred and manually converted into bar-shaped feeds (0.5 cm diameter, 2 cm length, ~1 g wet weight) using a 35-ml syringe. The feed bars were air-dried for 24 hr at room temperature (24°C) and then stored at −20°C until further use and analysis.

2.2 | Stability trial

All the formulations (two feed formulations at two different agar content) were tested for stability in seawater, hypothesizing a different stability according to the agar amount. Before the stability trial, six feed bars of each formulation were weighed (WW), oven-dried at 60°C for 48 hr to constant weight and weighed again (DW) to assess the standard dry weight (DW_S % = DW/WW × 100) of each feed formulation.

Afterwards, other six feed bars of each formulation were weighed (WW_I) and put individually inside PVC cylindrical cages (20 cm height and 12 cm diameter) closed on both sides with a nylon net (mesh size 500 µm) and fixed in pairs under the water surface in 80-L tanks (Figure 1a). Environmental conditions were kept stable throughout the stability trial, in terms of seawater temperature: 20.0 ± 1.0°C, salinity: 38.0 ± 0.5 g/kg, photoperiod: 8-hr

TABLE 1 Composition (g/kg) of the two feed formulations, 60/40 and 80/20, with different agar content

	Feed formulation			
	60/40		80/20	
Ingredients	A25 (g/kg)	A50 (g/kg)	A25 (g/kg)	A50 (g/kg)
Cichorium endivia	588	575	788	775
Engraulis encrasiculos	387	375	187	175
Agar	25	50	25	50

light and 16-hr dark, and continuous water flow in/out: 5 L/min. At three different times: T1 (24 hr), T2 (48 hr) and T3 (72 hr), two bars of each formulation were randomly collected, oven-dried at 60°C for 48 hr and weighed to assess the final dry weight (DW_F). Feed stability of each formulation was expressed based on the dry weight loss (DW_L) of the feeds at the end of the stability trial, as follows:

$$DW_L\,(\%) = \left[\left(DW_I - DW_F\right)/DW_I\right] \times 100.$$

where DW_I is the dry weight of each feed bar provided, calculated based on the standard dry weight, as follows: DW_I (mg) = ($WW_I \times DW_S$%)/100).

The results of the stability test showed that the agar amount did not affect significantly the feed stability over time (see Section 3), and hence, considering the economic advantages and sustainability of using a lower binder quantity, the feed formulations with the lower amount of agar (A25) were selected for the further steps.

2.3 | Proximate composition and fatty acids analysis

The main ingredients, that is discarded outermost leaves of *C. endivia* (endive) and industry discards of *E. encrasicolus* (European anchovy), and the two selected feed formulations with 25 g/kg of agar, 60/40 and 80/20, were freeze-dried, ground and analysed in triplicate. Ash content was determined by combustion in a muffle furnace at 550°C for 4 hr according to Nielsen (2010), and crude protein content was estimated by the Kjeldahl method, with nitrogen to protein conversion factor of 6.25 (Horowitz & Latimer, 2006). Carbohydrate content was also estimated, according to Baião et al. (2019) as follows:

$$\text{Carbohydrates} = 100 - (\text{lipid} + \text{protein} + \text{ash}).$$

A modified version of the Bligh and Dyer (1959) method was applied to measure lipids and fatty acids (FA). Lipids were extracted using a Milli-Q distilled water: methanol: chloroform mixture (1:2:1 v:v:v) with 0.01g/kg of BHT (butylated hydroxyl toluene) to avoid lipid oxidation. Samples were then sonicated to improve lipid extraction and centrifuged twice to separate the lipid phase from the aqueous phase. The lipid extracts were evaporated to dryness under gentle nitrogen stream and weighed, and the lipid content was expressed as mg/g dw of dry sample and as percentage. Therefore, lipids were resuspended in n-hexane and subjected to acid-catalysed transesterification using methanolic hydrogen chloride to obtain fatty acid methyl esters (FAME). FAME were then analysed by a gas chromatograph (GC-2010, Shimadzu) equipped with a BPX-70 capillary column (30 m length; 0.25 mm ID; 0.25 μm film thickness, SGE Analytical Science) and detected by a flame ionization detector (FID). Peaks were identified by retention times from mixed commercial standards (37 FAME from Supelco; QUALFISH and BACTERIAL MIX from Larodan). Tridecanoic and tricosanoic acids (C13:0 and C23:0) were used as surrogate standards, while pentacosanoic acid methyl ester (ME C25:0) was used as internal standard for quantification. FA data were expressed as mg/g of dry sample.

2.4 | Bioenergetic trial

Twenty-four *P. lividus* specimens (Test Diameter: 3.7 ± 0.2 cm, Total Wet Weight 23.4 ± 4.1 g) were collected from natural environment and randomly divided into two 80 L tanks. After a starvation period of two weeks, during which sea urchins were kept fasting, six specimens from each tank were randomly collected, sacrificed and wet-weighed, and their gonads were removed and wet-weighed.

The remaining twelve specimens were used for a two-week bioenergetic trial in an indoor tank system made of two groups

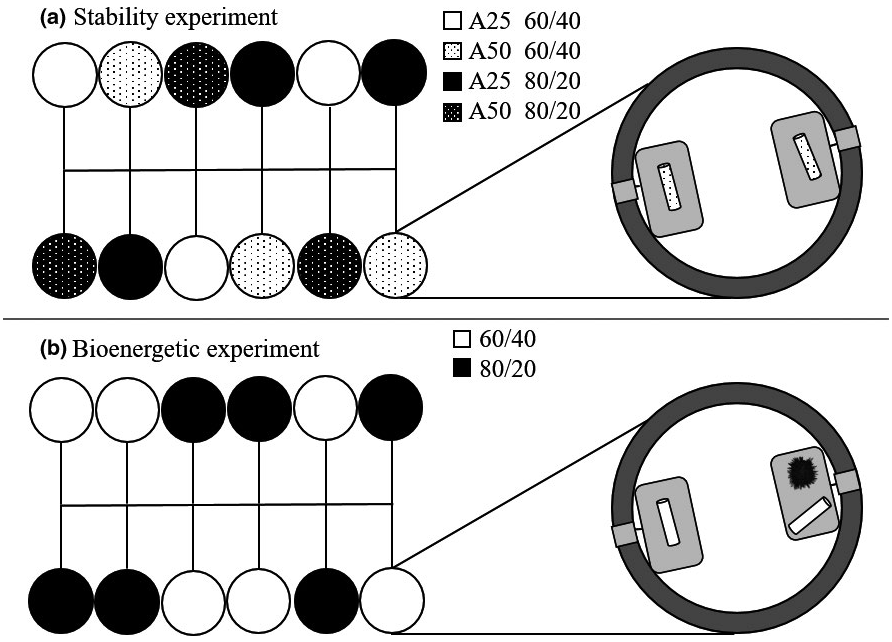

FIGURE 1 Indoor tank system used for the feed stability experiment (a) and the bioenergetic experiment (b). The detail of each tank is showed on the right side of each panel

of six tanks of 80 L, one group per each feed formulation (60/40 and 80/20). In each tank, two PVC cylindrical cages (20 cm height, 12 cm diameter) closed on both sides with a nylon net (mesh size 500 μm) were fixed under the water surface (Figure 1b). The remaining sea urchins were put individually in one of the two PVC cages per tank, while the other cage was left empty as a control treatment, aiming at calculating the feed loss. The same environmental conditions used in the previous stability trial were kept during both the starvation period and the bioenergetic trial. At the beginning of the experiment and every 48 hr (T0-T6), each sea urchin was fed with a known amount of the feed formulations (~1 g WW), and the same amount of feed was put in the correspondent control cage. Before feed provision (T1-T7), all the material contained within both treatment and control cages of each tank was carefully removed, oven-dried to constant weight (48 hr, 60°C) and reweighed. As far as the treatment cages, the collected material was previously separated in feed particles and sea urchin faeces, under a stereomicroscope.

The daily ingestion rate by sea urchins (IR), expressed as dry weight (mg/day), was calculated for each specimen at each sampling time (T1-T7), according to Fernandez and Boudouresque (1998) as follows:

$$\text{IR (mg/day)} = (\text{total provided biomass} - \text{total uneaten biomass})/2$$

where the total provided biomass is the dry weight of the feed provided (DW) and calculated from the standard dry weight (DW_S %), likewise the previous stability trial. The total uneaten biomass is given by the dry weight of the feed particles collected in the treatment cages and corrected based on the biomass lost from the control cages, and two are the days between each feed provision.

The absorption efficiency (AE) was calculated for each specimen at each sampling time as follows:

$$\text{AE (\%)} = \left[\frac{(\text{total biomass ingested} - \text{total faeces biomass})}{[\text{total biomass ingested}]}\right] \times 100$$

where total biomass ingested is equal to the following: total provided biomass – total uneaten biomass. At the end of the trial, sea urchins were sacrificed and weighed, and the gonads were extracted and wet-weighed. The gonado-somatic index (GSI) was calculated before the onset (T0), and at the end of the feeding treatment (T7) as follows:

$$\text{GSI (\%)} = [\text{gonad wet weight (g)}/\text{total wet weight (g)}] \times 100.$$

2.5 | Data elaboration and statistical analysis

Univariate permutational analysis of variance was used to test the differences in stability among feed formulations at different agar content (factor Agar fixed with two levels: A25, A50; factor Feed fixed with two levels: 60/40 and 80/20) across time (factor Time fixed and orthogonal, with three levels: T1, T2, T3). The analysis was run on untransformed data resembled using Euclidean distance.

One-way multivariate permutational analysis of variance (PERMANOVA) was carried out to test the differences in fatty acid (FA) profiles between the two selected feed formulations with the lower agar content (factor Feed fixed with two levels: 60/40, 80/20). PERMANOVA was carried out on FA data resembled using Euclidean distance after square root transformation. Principal coordinates analysis (PCO) was also run on the FA profiles of the feed formulations, in order to graphically highlight the differences found by PERMANOVA. The nutritional quality of the ingredients and the formulated feed was assessed through a semi-quantitative fatty acid approach: the patterns of the main classes of FA, together with those considered as important biomarkers of nutritional quality in aquaculture [i.e. arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), the sum of ω-3 and ω-6 polyunsaturated fatty acids (Σω-3-PUFA and Σω-6-PUFA), the ratio ω-3/ω-6, and the sum of ω-3 highly unsaturated fatty acids (Σω-3-HUFA), according to Gago, Luis, and Repolho (2009), Sargent, Bell, McEvoy, Tocher, and Estevez (1999) and Vizzini et al. (2019)] were assessed.

Difference in ingestion rate (IR) and absorption efficiency (AE) of the sea urchins fed with the two selected feed formulations across time was also tested using univariate permutational analysis of variance (factor feed fixed with two levels: 60/40, 80/20, factor time fixed and orthogonal, with seven levels: T1–T7). Difference in gonado-somatic index (GSI) between the onset and the end of the trial was also run using univariate permutational analysis of variance with both factors, feed and time, fixed and orthogonal, and both with two levels (Feed: 60/40, 80/20; Time: T0, T7). All the analyses were based on untransformed data resembled using Euclidean distance.

All the statistical analyses were performed using the software PRIMER 6 v6.1.10 & PERMANOVA + β20 (Plymouth, UK). When significant differences were found, pairwise tests were used as a posteriori check of significant effects. The Montecarlo test was also carried out to identify significant patterns when the numbers of permutation were <100.

3 | RESULTS

3.1 | Stability trial

The stability trial carried out on the two new feed formulations (60/40 and 80/20) manufactured with different agar content (25 and 50 g/kg) revealed that the higher feed loss occurred in the first 24 hr of immersion in seawater and then was overall stable in the following times (48 and 72 hr) (Figure 2). The higher agar amount did not contribute to provide a higher stability to both formulations at all times (MS = 92.23, Pseudo-$F_{(1,12)}$ = 17.27, p = .057); indeed, while the interaction of the factors feed and time was significant (MS = 27.30, Pseudo-$F_{(4,12)}$ = 3.97, p = .036), pairwise tests, carried out to compare the two feed formulations at different agar amount over time, revealed only that the stability of the formulation A50 60/40 was significantly lower at T3 than at T1 ($p \leq .05$).

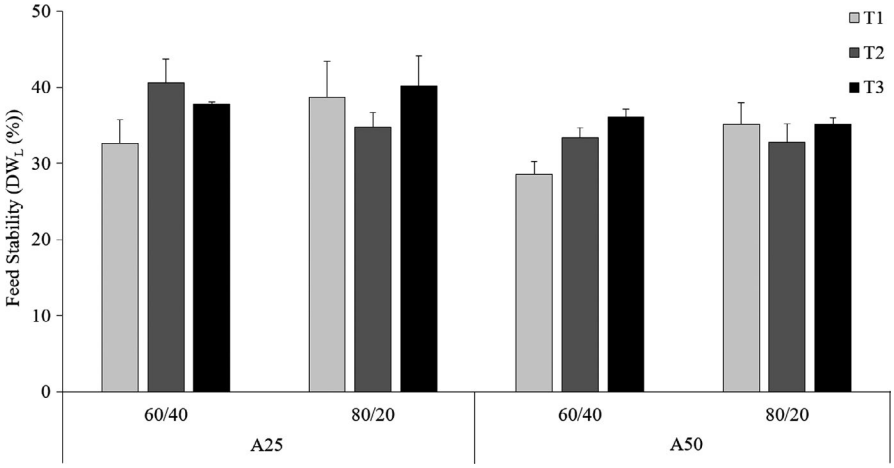

FIGURE 2 Feed stability expressed as dry weight loss (DW_L %, mean ± standard deviation) of the two feed formulations (60/40 and 80/20) prepared with a different agar amount (A25: 25 g/kg, A50: 50 g/kg)

3.2 | Proximate composition and fatty acid analysis

Proximate composition and fatty acid (FA) profiles of the main ingredients, *C. endivia* and *E. encrasicolus* discard, and the two selected feed formulations, 60/40 and 80/20, are shown respectively in Tables 2 and 3. Fish industry discards showed higher lipid, protein and ash content than discarded endive leaves, while endive was richer in carbohydrates than fish discards. These differences were mirrored in the feed formulations: lipids, proteins and ash were more abundant in the formulation with the higher relative content of fish discards (60/40), and carbohydrates were more abundant in the formulation with the higher relative content of endive leaves (80/20) (Table 2).

As regards FAs, the two main ingredients showed very different profiles, being the outermost leaves of endive almost exclusively constituted by 18:3 n3 (α-linolenic acid, ALA), 18:2 n6 (linoleic acid, LA) and 16:0 (palmitic acid), and anchovy discards by a high abundance of essential fatty acids (EFA), namely arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) (Table 3). As regards the feed formulations, a higher amount of all the three FA classes, saturated, mono- and polyunsaturated FA, characterized the formulation with a higher amount of animal ingredients (∑ SFA, ∑ MUFA, ∑ PUFA: 60/40 > 80/20). Looking through the biomarkers of nutritional quality, the sum of EFA and of ω-3 highly unsaturated FAs (∑ω-3-HUFA) were about twice in the 60/40 formulation, compared with the 80/20. Individual EFAs (i.e. ARA, EPA and DHA) were also higher in the 60/40 formulation, while ALA (18:3n3) and LA (18:2n6), both precursors of EFA (Baião et al., 2019; Castell et al., 2004), showed an opposite trend with a higher amount in the 80/20 formulation than in the 60/40. As a result, the sum of ω-3 and ω-6 PUFA were, respectively, higher in the 60/40 and the 80/20 feed formulation, and their ratio ω-3/ω-6 was also higher in the former, compared with the latter.

PERMANOVA revealed that the FA profiles of the two feed formulations were significantly different (MS = 4.62; Pseudo-$F_{(1,5)}$ = 291.15; $p \leq .001$). Principal coordinates analysis (PCO) of the FA profiles of 60/40 and 80/20 formulations confirmed this result, showing a clear separation along the horizontal axis based on the feed formulations with almost the totality of the explained variance (Figure 3). The formulation 60/40 was grouped on the right side of the graph, characterized by a higher abundance of all the FA classes (the sum of SFA, MUFA and PUFA), total and individual EFA, the sum of ω-3 PUFA and HUFA, and the ratio ω-3/ω-6. In contrast, the formulation 80/20 was distributed in the left area of the graph, because of the higher abundance of the sum of ω-6 PUFA and the two dominant fatty acids in the PUFA class, ALA and LA, suggesting that their abundances were an important driver for the distinction between the two formulations.

3.3 | Bioenergetic trial

The daily ingestion rate (IR) recorded in *P. lividus* fed with the two different feed formulations across the seven sampling periods, showed a fluctuating pattern. The mean value of the daily IR calculated for the entire duration of the trial was rather similar for the two feed

TABLE 2 Proximate composition (g/kg dry matter, mean ± standard deviation) of the two main ingredients (*Cichorium endivia* and *Engraulis encrasicolus* discards) and the two selected feed formulations

	Main ingredient		Feed formulation	
	C. endivia	*E. encrasiculos*	60/40	80/20
Lipid g/kg	38.0 ± 1.7	140.1 ± 19.0	70.8 ± 7.4	55.5 ± 2.0
Protein g/kg	191.4 ± 6.7	405.8 ± 3.8	293.6 ± 2.8	238.6 ± 2.9
Carbohydrate g/kg	644.2 ± 9.6	43.4 ± 22.7	388.9 ± 7.9	506.9 ± 8.4
Ash g/kg	126.3 ± 1.5	410.7 ± 0.1	246.7 ± 3.9	198.9 ± 3.9

TABLE 3 Fatty acid profiles and lipid content (mg/g dw, mean ± standard deviation) of the two main ingredients (*Cichorium endivia* and *Engraulis encrasicolus* discards) and the two selected feed formulations

	Main ingredient		Feed formulation	
FAs (mg/g dw)	*C. endivia*	*E. encrasicolus*	60/40	80/20
8:0	0.04 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
10:0	–	0.11 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
11:0	–	0.03 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
12:0	0.01 ± 0.00	0.24 ± 0.01	0.02 ± 0.00	0.00 ± 0.00
14:0	0.07 ± 0.01	7.57 ± 0.5	1.86 ± 0.04	0.86 ± 0.06
15:0	0.03 ± 0.00	1.43 ± 0.09	0.37 ± 0.00	0.20 ± 0.01
16:0	2.07 ± 0.01	28.7 ± 1.51	10.49 ± 0.12	6.34 ± 0.21
17:0	0.03 ± 0.00	1.32 ± 0.08	0.61 ± 0.00	0.28 ± 0.02
18:0	0.20 ± 0.01	5.98 ± 0.31	1.82 ± 0.08	1.04 ± 0.02
19:0	–	0.31 ± 0.02	0.10 ± 0.00	0.05 ± 0.01
20:0	0.12 ± 0.00	0.39 ± 0.01	0.12 ± 0.00	0.09 ± 0.00
21:0	0.09 ± 0.01	0.07 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
22:0	0.14 ± 0.01	0.27 ± 0.01	0.13 ± 0.00	0.13 ± 0.00
Σ LCFA (>22:0)	0.26 ± 0.01	0.39 ± 0.08	0.48 ± 0.01	0.47 ± 0.02
Σ SFA	**2.98 ± 0.02**	**47.16 ± 2.42**	**16.06 ± 0.21**	**9.52 ± 0.34**
14:1	–	0.06 ± 0.02	0.01 ± 0.00	0.00 ± 0.00
15:1	0.03 ± 0.00	–	0.00 ± 0.00	0.00 ± 0.00
16:1 n7	–	4.15 ± 0.25	1.06 ± 0.01	0.52 ± 0.02
18:1 n7	0.09 ± 0.00	3.01 ± 0.12	0.89 ± 0.01	0.44 ± 0.01
18:1 n9t	–	0.09 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
18:1 n9c	0.19 ± 0.03	14.13 ± 0.68	3.87 ± 0.08	1.92 ± 0.06
20:1 n9	0.03 ± 0.00	0.69 ± 0.03	0.16 ± 0.02	0.08 ± 0.02
20:1 n11	–	0.04 ± 0.01	0.05 ± 0.01	0.02 ± 0.00
22:1 n9	–	0.19 ± 0.01	0.04 ± 0.00	0.01 ± 0.00
Σ MUFA	**0.35 ± 0.04**	**23.35 ± 1.1**	**6.08 ± 0.12**	**2.99 ± 0.09**
18:2 n6c - LA	3.72 ± 0.31	2.25 ± 0.12	4.12 ± 0.13	5.12 ± 0.14
18:2 n6t	–	0.05 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
18:3 n3 - ALA	9.31 ± 0.63	1.51 ± 0.09	6.20 ± 0.56	8.26 ± 0.23
18:3 n6	0.09 ± 0.00	0.19 ± 0.03	0.12 ± 0.00	0.16 ± 0.00
18:4 n3	0.03 ± 0.00	2.41 ± 0.16	0.58 ± 0.01	0.32 ± 0.01
20:2 n6	0.07 ± 0.00	0.43 ± 0.02	0.08 ± 0.01	0.04 ± 0.01
20:3 n3	0.03 ± 0.00	0.15 ± 0.01	0.04 ± 0.00	0.02 ± 0.01
20:3 n6	–	0.16 ± 0.06	0.01 ± 0.00	0.02 ± 0.00
20:4 n3	–	0.59 ± 0.04	0.17 ± 0.00	0.10 ± 0.01
20:4 n6 - ARA	–	1.54 ± 0.07	0.28 ± 0.00	0.10 ± 0.01
20:5 n3 - EPA	–	9.74 ± 0.62	2.50 ± 0.02	1.08 ± 0.04
22:2 n6	–	0.14 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
22:4 n6	–	0.05 ± 0.00	0.02 ± 0.00	0.01 ± 0.01
22:5 n3	–	0.92 ± 0.04	0.95 ± 0.01	0.44 ± 0.01
22:6 n3 - DHA	–	24.88 ± 1.51	7.45 ± 0.05	3.28 ± 0.11
Σ PUFA	**13.26 ± 0.50**	**44.96 ± 2.59**	**22.52 ± 0.70**	**18.94 ± 0.55**
Branched	–	1.27 ± 0.11	0.20 ± 0.02	0.08 ± 0.00
-OH	0.30 ± 0.02	0.78 ± 0.03	0.19 ± 0.00	0.17 ± 0.01

(Continues)

TABLE 3 (Continued)

	Main ingredient		Feed formulation	
FAs (mg/g dw)	*C. endivia*	*E. encrasicolus*	60/40	80/20
-Δ	0.09 ± 0.03	0.53 ± 0.07	0.13 ± 0.01	0.06 ± 0.01
Σ EFA	–	**36.16 ± 2.20**	**10.23 ± 0.07**	**4.46 ± 0.15**
Σ ω3-PUFA	**9.38 ± 0.63**	**40.20 ± 2.46**	**17.89 ± 0.58**	**13.49 ± 0.39**
Σ ω6-PUFA	**3.88 ± 0.32**	**4.76 ± 0.13**	**4.63 ± 0.12**	**5.45 ± 0.16**
ω3/ω6	**2.44 ± 0.33**	**8.44 ± 0.28**	**3.86 ± 0.00**	**2.47 ± 0.00**
Σ ω3- HUFA	**0.03 ± 0.00**	**38.54 ± 2.36**	**11.65 ± 0.07**	**5.22 ± 0.16**
Σ FA	**16.98 ± 0.61**	**118.06 ± 6.32**	**45.12 ± 0.00**	**31.72 ± 0.00**
Lipid content (mg/g dw)	**38.02 ± 1.66**	**140.10 ± 18.97**	**70.82 ± 7.36**	**55.49 ± 1.97**

Note: Main FA classes (SFA: saturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA) and main biomarkers of nutritional quality are indicated in bold. LCFA: long-chain FA; LA: linoleic acid, ALA: α-linolenic acid; ARA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, Branched: branched-chain saturated FA, -OH: hydroxyl FA, -Δ: cyclopropyl FA.

formulations: 104.0 ± 25.5 mg DW per day and 111.9 ± 25.1 mg DW per day, respectively, for 60/40 and 80/20. A mean IR decrease was evident in the early stages of the experiment (T1-T3), followed by a slight increase (T4-T5) and a further reduction (T6-T7) (Figure 4). This ambiguous temporal trend, coupled with a high individual variability, resulted in a lack of significant differences between feed formulations, times and their interaction (Table 4).

The absorption efficiency (AE) recorded in the sea urchins fed with the two feed formulations showed a fluctuating pattern, similarly to that observed for IR. After the early stages of the trial, where the AE values were similar in the sea urchins fed with the 80/20, and tended to decrease in those fed with the 60/40 formulation, higher AE values were recorded in the sea urchins fed with the formulation with the higher fish content (i.e. 60/40) (Figure 5). The average AE calculated for the whole trial was higher, indeed, for the 60/40 formulation than for the 80/20 (63.6 ± 6.4% vs. 55.1 ± 10.3%, respectively) (Table 4), while differences among times and for the interaction of the two factors were not detected.

Despite the short duration of the trial (2 weeks), the gonado-somatic index (GSI) showed a clear increase in sea urchins fed with both formulations (from 0.8 ± 0.7% to 2.8 ± 0.6% and from 0.9 ± 0.7% to 2.7 ± 1.4% in the sea urchins fed with the 60/40 and 80/20 formulations, respectively). Univariate permutational analysis of variance showed significant differences between times (MS = 21.55;

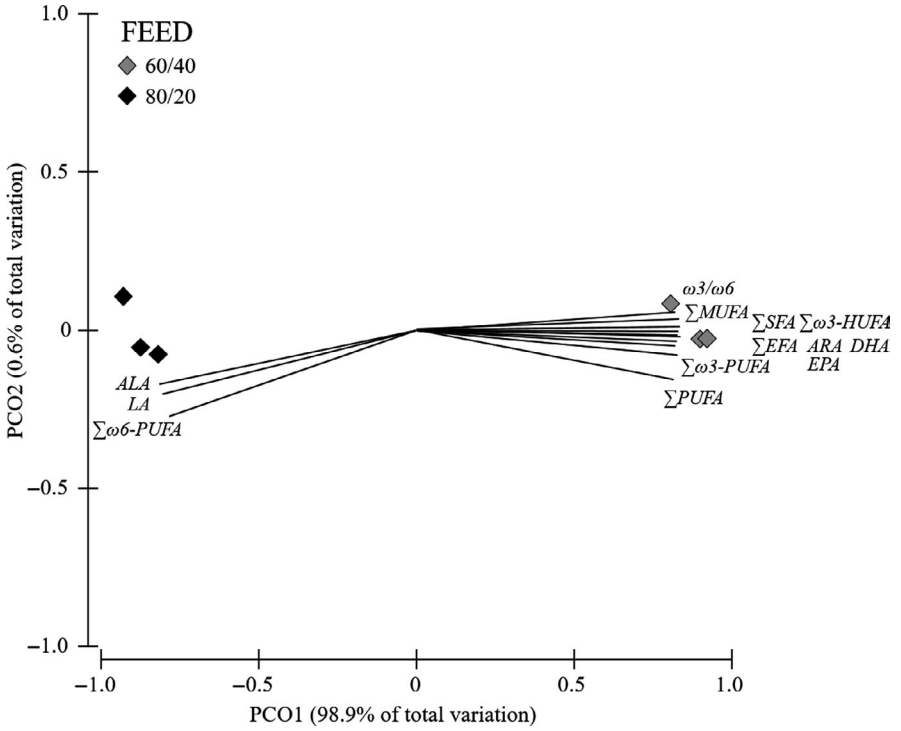

FIGURE 3 Principal coordinates analysis (PCO) of the fatty acid profiles of the two feed formulations (60/40, 80/20). The main fatty acid classes and the main indicators of nutritional quality selected in this study are superimposed to the graph. The meaning of the acronyms is the same as in Table 3

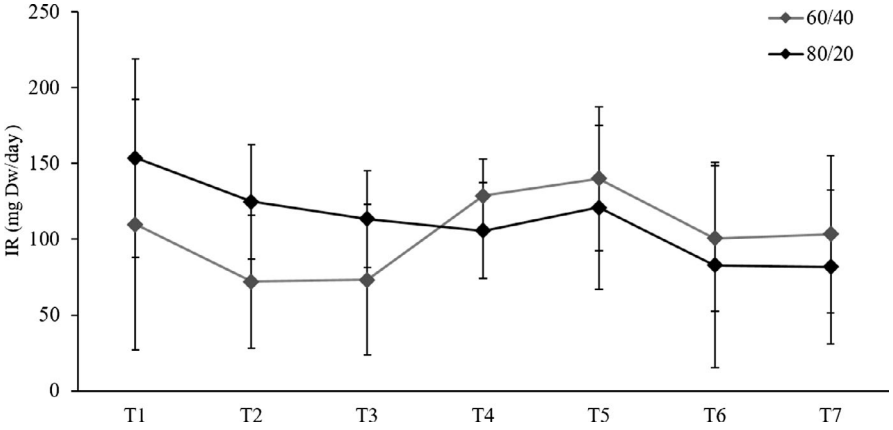

FIGURE 4 Ingestion rate IR (mg dw/day, mean ± standard deviation) of *Paracentrotus lividus* for the two feed formulations across time

Pseudo-$F_{(1,22)}$ = 23.73; $p \leq .01$), but not between feeds (MS = 0.002 and Pseudo-$F_{(1,22)}$ = 0.002, $p > .05$) or the interaction of the two factors (Pseudo-$F_{(1,22)}$ = 0.13, $p > .05$).

4 | DISCUSSION

To move towards a higher sustainability of echinoculture, this study proposes a new sustainable feed through the reuse of discards from the food industry. Two experimental formulations were prepared using discarded endive (*C. endivia*) leaves and anchovy (*E. encrasicolus*) industry discards in different proportions and were tested for stability in seawater. Nutritional composition and quality of the main ingredients and the formulations were evaluated through the analysis of proximate composition and fatty acid profiles and biomarkers. Finally, both formulations were tested for palatability, absorption efficiency and effect on gonad growth of the purple sea urchin *P. lividus*.

The stability trial showed a comparable pattern between the feed formulations at different agar amount: the greater feed loss occurred in the first 24 hr of immersion in seawater and then remained fairly stable in the subsequent times, ranging overall between 30% and 40%. These patterns clearly indicate that the different amount of agar in the feed formulation affected only marginally the feed stability over time, consistent with previous studies (Argüello-Guevara & Molina-Poveda, 2013; Fabbrocini et al., 2012). The macromolecular structure of the gel formed by agar is deemed, indeed, as a strong binder as it confers a high feed stability at ambient temperature by limiting nutrient loss through leaching (Fabbrocini et al., 2012; Leclercq, Graham, & Migaud, 2015) and water absorption (Paolucci, Fasulo, & Volpe, 2015). Moreover, as *P. lividus* takes at least 2–3 days to eat the feed offered in confined conditions (Fabbrocini, Volpe, Coccia, D'Adamo, & Paolucci, 2015), the very limited feed loss observed between 24 and 72 hr makes both feed formulations enough stable over time and then resulting a suitable choice in the production of sustainable feeds for sea urchins. Additionally, the present findings revealed that the use of a commercially affordable product (i.e. agar powder for home baking) rather than a laboratory product, for the production of aquaculture feeds ensured good results coupled with a substantial cost reduction. In contrast, although other binders, such as pork gelatine, may result in a higher feed stability in water (Pearce, Daggett, & Robinson, 2002a), the higher cost and quantity needed to produce gelatine-based pellets make them economically unsustainable. Furthermore, agar-based feeds may have a positive effect on growth rate, as previously observed in reared crustaceans (Palma, Bureau, & Andrade, 2008) and on both gamete production and gonad growth of *P. lividus* (Fabbrocini et al., 2012). For all these reasons, chiefly the comparable stability over time coupled with the greater sustainability of using lesser amount of binders in the context of industrial-scale feed production, the further steps were conducted using only the two formulations with the lower agar amount.

Following the stability trial, the two selected feed formulations and their main ingredients were characterized in terms of nutritional composition and quality. Both formulations appeared nutritionally balanced, with carbohydrates as the most representative macronutrient, followed by proteins and lipids. As expected, the differences found between the formulations were essentially driven by the different nutritional contributions of the main ingredients. Indeed, endive discarded leaves and anchovy industry discards showed major differences in both lipid and fatty acid content, the two ingredients being respectively of plant and animal origin and hence characterized by a different nutritional profile (Rana, Siriwardena, & Hasan, 2009). Being constituted mainly of fish skin, bones, heads and internal organs, the protein and lipid content of anchovy discards was much higher than that of endive leaves (Ghaly, Ramakrishnan, Brooks, Budge, & Dave, 2013). This was mirrored in the two feed formulations, where proteins and lipids decreased proportionally with the ratio of vegetal versus animal ingredients, consistent with the literature (Fernandez & Boudouresque, 2000). On the other hand, discarded endive leaves and the formulation 80/20 were characterized by the highest content of carbohydrates.

A proper nutritional composition of the feeds is crucial in echinoculture. Previous studies showed that carbohydrate and protein levels similar to those found in this study (~40% and 20%) provide the proper amount of energy and essential amino acids needed to foster growth and reproduction (Cuesta-Gomez & Sánchez-Saavedra, 2018; Hammer et al., 2012). Also, the source of proteins is important, as revealed by Fernandez and Boudouresque (2000) who found the

TABLE 4 Univariate permutational analysis of variance results testing the effects of the feed formulations across time on the ingestion rate IR (a) and absorption efficiency AE (b) of *Paracentrotus lividus*

Main test		a) IR			b) AE		
Source of variation	*df*	MS	Pseudo-*F*	*p (perm)*	MS	Pseudo-*F*	*p (perm)*
Feed	1	1,321.7	0.50	.48	1,529.3	4.43	**.04**
Time	6	3,914.9	1.48	.18	544.3	1.58	.18
Feed × Time	6	3,766.7	1.43	.24	340.3	0.99	.45
Residual	70	2,636.8			345.4		

Note: Significant *p* values are highlighted in bold.

highest values of gonado-somatic index in the sea urchins fed with diets with intermediate levels of animal ingredients. Also, dietary lipids have a key role as structural components, source of energy and precursors of bioactive molecules (Carboni et al., 2013), and additionally they influence the FA composition and organoleptic attributes of the roe (Martínez-Pita, García, & Pita, 2010; Siliani et al., 2016; Vizzini et al., 2019). Consequently, a high lipid content of the diet may favour gonad development and contribute to the restoration of energy supplies following the starvation, during which sea urchins tend to consume the nutrients present in their tissues (Guillou & Lumingas, 1998).

Turning to the FA profiles, the high concentration of SFA and MUFA found in the formulation characterized by a higher content of anchovy discards (60/40) is mainly attributable to a higher content of 16:0 and 18:1n9 in fish discards than in endive leaves, consistently with the high typical abundance of SFA and MUFA in the common anchovy (Öksüz & Özyilmaz, 2010; Zlatanos & Laskaridis, 2007). In contrast, the high concentration of PUFA observed in both formulations is mainly due to the high content of linoleic (LA) and α-linolenic (ALA) acids, being both very abundant in the endive leaves, but not in the fish discards. Endive is a 18:3 metabolism plant, and its PUFA profile is composed almost exclusively by 18:3n3 and 18:2n6 (Le Guedard, Schraauwers, Larrieu, & Bessoule, 2008; Vizzini et al., 2019), which, in contrast, are fatty acids generally not abundant in the common anchovy (Öksüz & Özyilmaz, 2010).

The higher abundance of essential FA (EFA) in the 60/40 formulation than in the other (80/20) is also consistent with the high EFA concentration in *E. encrasicolus* discards. In turn, the EFA content in fish discards is consistent with what is reported in the literature for anchovy tissues [about 1%, 10% and 15% of the total FA content for arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, respectively, Öksüz and Özyilmaz (2010)], due to the high EFA assimilation and storage ability of fish (Bendiksen, Johnsen, Olsen, & Jobling, 2011). EFA are deemed suitable indicators of high nutritional quality in aquaculture feeds as they play a key role in many physiological functions and then represent an added value in the market of sea urchins. The abundance in the proposed formulation may also boost gamete production and gonad growth (Watts, Lawrence, & Lawrence, 2013). The preliminary assessment of gonado-somatic index carried out in this study confirms this, but longer-time experiments are needed for further consideration.

The two selected formulations were provided to sea urchins to assess both palatability and absorption efficiency of *P. lividus*. The bioenergetics trial highlighted a similar fluctuating pattern for both ingestion rate (IR) and absorption efficiency (AE), characterized by high initial values followed by an overall decrease during the first phases of the experiment and then increased again. The early pattern may have been influenced by the previous period of starvation. Under food limitation, sea urchins rely on internal stores of nutrients to meet their energy requirements for maintenance (Guillou, Lumingas, & Michel, 2000; Lares & Pomory, 1998), while, once food become available, the level of hunger may lead sea urchins to increase the consumption of food regardless of its nutritional content (Castilla-Gavilán, Cognie, Ragueneau, Turpin, & Decottignies, 2019). After that, the reduction of food ingestion may be an effect of the

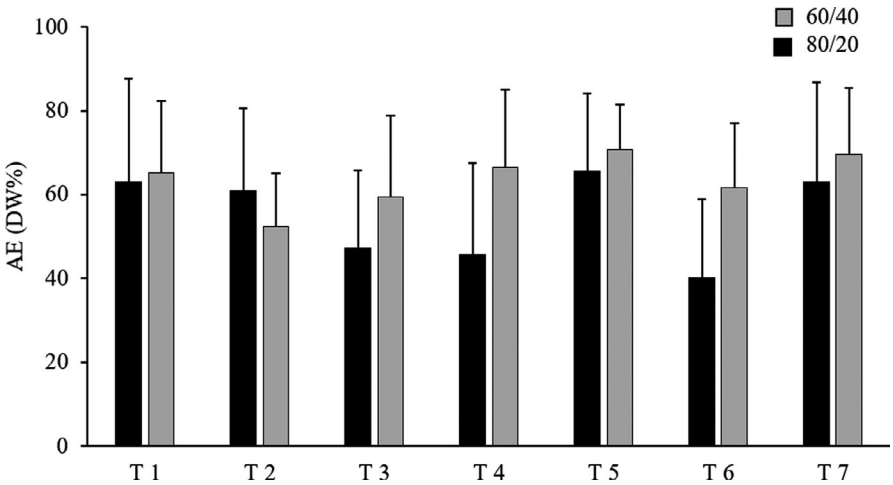

FIGURE 5 Percentage absorption efficiency AE (DW%, mean ± standard deviation) of *Paracentrotus lividus* for the two new feed formulations across time

stomach fullness (Lawrence, Plank, & Lawrence, 2003). Moreover, the fluctuating IR pattern observed in *P. lividus* may be also due to an intrinsic periodicity of food ingestion resulting in high peaks spaced out by a few fasting days (Nédélec, Verlaque, & Dallot, 1983). Comparisons with sea urchins fed with natural food (i.e. macroalgae and seagrasses) revealed contrasting results depending on the species used. Mean IR of the two formulations was higher than that observed for *Corallina elongata*, *Flabellia petiolata*, *Halopteris scoparia* and *Ulva lactuca*, comparable to the IR measured for *Dictyota* sp., *Laurencia* sp., *Padina pavonica*, *U. rigida* and *Posidonia oceanica*, and lower than the IR for *Codium* sp. and *Dictyopteris* sp. (Ruocco et al., 2018; Sartori & Gaion, 2015). Nevertheless, present IR values were overall comparable with those previously measured in *P. lividus* fed with commercial and experimental pellets (Ruocco et al., 2018; Sartori & Gaion, 2015). Although agar was observed to confer a high palatability to manufactured feeds without, however, affecting the digestibility (Barker, Keogh, Lawrence, & Lawrence, 1998; Fabbrocini et al., 2012, 2015; Leclercq et al., 2015), the low concentration (2.5%) used here in the preparation of the sustainable feed may have had a negligible influence on the IR values.

Similarly to the IR, the patterns observed for the absorption efficiency (AE) showed that sea urchins responded to the resumption of feed provision with high feed absorption, for meeting their nutritional requirements, regardless of the type of the food provided. After that, there was an evident difference in AE based on the formulation provided, with a higher assimilation of the formulation with a higher content of fish ingredients, than the other. This is consistent with the literature: also Fernandez and Boudouresque (2000) found a different absorption efficiency in *P. lividus* according to the food provided. In particular, a different AE seems to depend on the assimilation of carbohydrates: vegetables are characterized by a higher amount of insoluble carbohydrates, not digestible by echinoids, that are instead poorly represented in fishmeal (Fernandez & Boudouresque, 2000, present study). This is supported also by the higher biomass of faeces found in the cages where sea urchins were fed with the 80/20 formulation, compared with those where sea urchins were fed with the 60/40 (data not showed), where the ingredients of plant and animal origin are more balanced. Despite the differences found between formulations, the mean absorption efficiency observed for both was comparable with the AE recorded in the Australian sea urchin, *Heliocidaris erythrogramma*, fed with mixed feed (Senaratna, Evans, Southam, & Tsvetnenko, 2005), confirming the suitability of the new sustainable feed.

Finally, as previously mentioned, this preliminary short-time assessment of the effect of the sustainable feed on gonad growth, based on the evaluation of the gonado-somatic index (GSI), revealed a significant increase in GSI for both formulations, regardless of the ratio of vegetal versus animal ingredients. Although GSI is usually estimated over longer-time scales, our findings are overall consistent with the literature (e.g. Vizzini et al., 2019; Zupo et al., 2019) and revealed that the sea urchins fed with the new sustainable feed had good feed intake and nutrient conversion even in a very short time (2 weeks).

5 | CONCLUSION

A new sustainable feed, produced using anchovy and endive food industry discards with the addition of a low amount of agar, resulted suitable for feeding *P. lividus*. Two formulations at a different ratio of vegetal versus animal ingredients were tested. Both showed a good stability in seawater, and a balanced nutritional composition and fatty acid (FA) profiles, which are basic requirements for feeding sea urchins. Main biomarkers of nutritional quality (PUFA, ω-3 HUFA, EFA and the ratio ω-3/ω-6) were higher in the formulation with the higher content of fish discards. This formulation was also absorbed more efficiently by the sea urchin, resulting as attractive as the other formulation, but more digestible for *P. lividus*. Finally, despite the short experimental period, the gonado-somatic index increased in all the reared sea urchins, regardless of the provided formulation. These encouraging results showed that food industry discards are suitable and promising alternative ingredients for the production of sustainable feeds for sea urchins, by meeting also the requirements of bio- and blue economy that promote sustainable development. Moreover, on first analysis, the formulation with a more balanced ratio of vegetal versus animal content (60/40) seemed more suitable in echinoculture, but further studies are needed to assess the effect of this new feed on gonad yield, in order to obtain a marketable while sustainable product.

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DATA AVAILABILITY STATEMENT

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

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Turning waste into gold: Sustainable feed made of discards from the food industries promotes gonad development and colouration in the commercial sea urchin *Paracentrotus lividus* (Lamarck, 1816)

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ABSTRACT

Development of sustainable aquaculture practices is a suitable solution to reduce the pressure on overexploited stocks of the Mediterranean sea urchin, *Paracentrotus lividus*, and to respond to the increasing market demand. To move forward the Blue Growth and following the principles of circular economy, a three-month feeding experiment was conducted to test a sustainable feed based on food processing discards on sea urchins. Two feed formulations differing on the proportions of the two main ingredients (endive outermost leaves and European anchovy discards in a ratio of 60:40 and 80:20 respectively) were prepared and tested on *P. lividus* gonad yield, development and quality. The results were compared with those of wild sea urchins to assess the differences with natural patterns. Both feed formulations promoted gonad growth resulting in a significantly higher percentage increase in gonad biomass compared with wild specimens (490%, 330% and 78% increase in gonad weight in the feed 60/40, 80/20 and wild sea urchins respectively). Similarly, GSI of reared sea urchins varied by about 3–12% and 14% for sea urchins fed with feeds 80/20 and 60/40 respectively, while that found in wild sea urchins varied by about 3–5%. Gonad development was also boosted by the provision of the sustainable feed, as sexual maturation was faster in reared specimens than in wild ones. At the end of the trial, reared sea urchins showed also a very high (> 90%) frequency of marketable gonad colour. Lastly, the formulation with a more balanced vegetal/animal ratio (feed 60/40) gave the best results overall, combining the highest GSI and the best gonad colouration. Outcomes of this study confirm the suitability of food processing discards as ingredients for sea urchin feeds, although further research is needed to evaluate the effects on nutritional quality and organoleptic features of sea urchin gonads.

1. Introduction

Sea urchin gonads, also known as roe, are a prized seafood product worldwide. Consequently, a dramatic reduction of natural stocks of sea urchin commercial species, due to uncontrolled over-exploitation, has been recently observed (Lawrence, 2013; Pais et al., 2011; Yeruham et al., 2019). In many countries of the Mediterranean area, *Paracentrotus lividus* (Lamarck, 1816) is the most exploited sea urchin for commercial purposes, with substantial detrimental impacts on local populations and the whole ecosystems (Guidetti et al., 2004; Pais et al., 2007). In particular, France has one of the oldest artisanal sea urchin fisheries, but also in Spain and Italy purple sea urchins are widely harvested as gonads are traditionally considered a delicacy (Stefánsson et al., 2017). The introduction in the last century of new fishing methods, together with the spread of illegal and unlicensed fishing, contributed to the over-exploitation of *P. lividus* natural populations (Addis et al., 2012; Carboni et al., 2014; Fernández-Boán et al., 2012), despite the existence of local laws for managing and regulating its catches (Fernández-Boán et al., 2012; G.U.R.I, 1995).

The development of sustainable and effective aquaculture practices could be a valuable solution to reduce the pressure on natural sea urchin stocks and satisfy its market demand (Carboni et al., 2013). However, today echinoculture is still based on harvesting sea urchins, due to the lack of proper sea urchin hatcheries able to provide juveniles (Rubilar

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and Cardozo, 2021) and the low growth rates of juveniles to reach marketable size (Boudouresque and Verlaque, 2007). Moreover, another critical bottleneck for echinoculture, and for aquaculture in general, is the exploitation of natural resources as ingredients for feed production (e.g., kelp, seaweeds, fish oils and meals). While the consumption of natural resources is critical to obtain high-quality gonads, their use in the feed industry is not sustainable for several ecological, economic and management reasons (Pearce et al., 2002). Size, colour, taste, and firmness of the gonads are strictly related to the proper nutritional quality of the diet provided and, at the same time, they strongly influence the gonad market value (Baião et al., 2019; Cuesta-Gomez and Sánchez-Saavedra, 2018; Stefánsson et al., 2017). At the same time, large-scale harvesting of natural resources is very expensive, may compromise their population dynamics, viability and ecosystem functioning, and may promote conflicts with other marine users (Pearce et al., 2002). Therefore, the reuse of by-products and wastes, by reducing the exploitation of natural resources, is to be considered as a valuable solution to move towards a sustainable approach in echinoculture, in agreement with the circular economy principles (de la Caba et al., 2019).

Food loss and waste have been steadily increasing worldwide, reaching about 1.3 billion tons per year, with one-third of food for human consumption being lost or wasted throughout the food retail chain, with strong socio-economic and environmental implications (Gustavsson et al., 2011). A large amount of food loss consists of fruits and vegetables, whose inedible parts are discarded during collection, handling, transportation, and processing steps (Gustavsson et al., 2011). This high amount of vegetal biomass with a high fibre, protein and mineral content might instead be recycled and returned to the food chain as raw material (Laufenberg et al., 2003). Similarly, fish processing discards, such as organs, skin, bones, cut-offs, and damaged or spoiled fish from fisheries and aquaculture industries, are a global issue nowadays, as they amount to about a third of the fish mass processed (Kim and Mendis, 2006). Despite the low economic value, these by-products have a high nutritional content, due to a high amount of proteins, minerals and lipids, as well as being an extremely valuable source of bioactive molecules (Esteban et al., 2007; Kim and Mendis, 2006; Olsen et al., 2010). In particular, fish processing discards are natural sources of essential fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are required by fish for growth, reproduction and disease resistance (Glencross, 2009). Under the assumption that food processing discards have a high potential to be exploited, their use in aquaculture feed production is increasingly taken into account (Bimbo, 2007). First promising attempts to use fish processing discards as valuable resources in aquaculture date from about the year 2000 (Kotzamanis et al., 2001; Turchini et al., 2003). Similarly, increasing attention was focused on the exploitation of vegetal discards as an ingredient for sustainable feeds. Kang et al. (2010) highlighted higher growth rates in the juveniles of the white-leg shrimp *Litopenaeus vannamei* (Bonne, 1931) fed with papaya wastes. Luo et al. (2014) showed an improvement of gonads flavour in the sea urchin *Strongylocentrotus intermedius* (Agassiz, 1863) fed with banana peels. More recently, Mo et al. (2020) recorded weight gain for the grass carp *Ctenopharyngodon idellus* (Valenciennes, 1844) fed with a mix of cereal, meat and fruit wastes. These studies confirmed that the introduction of practices of circular economy in feed production may allow turning wastes into resources. This approach could indeed reduce the reliance on high-cost and qualitatively scarce resources, such as fish oil and meal, matching the purpose of Blue Growth, namely the socio-economic growth based on sustainability and biodiversity protection of marine systems and resources (Eikeset et al., 2018). In this context, Ciriminna et al. (2020) assessed the suitability of food processing discards as ingredients for *P. lividus* diet. Outermost leaves of endive *Cichorium endivia* (Linnaeus, 1753), usually discarded before the sale, and processing discards of the European anchovy *Engraulis encrasicolus* (Linnaeus, 1758), mainly fins, skin, bones and offal, were used as the main ingredients based on the high content in carbohydrates (especially from endive leaves: ~ 65%), proteins and lipids (especially from anchovy discards: ~ 40% and 15% respectively) (Ciriminna et al., 2020). A new sustainable feed was produced and tested as a sea urchin diet, showing very promising results in nutritional content and quality (e.g. ratio ω3/ω6 fatty acids from 2.5 to 4), good stability in seawater (mean feed loss < 40% after 72 h), palatability (mean ingestion rate: 107 mg day^{-1}) and absorption efficiency (31%), confirming its suitability for feeding sea urchins (Ciriminna et al., 2020). Here, the suitability of the sustainable feed for rearing sea urchins was further evaluated by testing its effects on gonad production, development and colour in adult *P. lividus* reared indoor for 3 months. In more detail, the performance of two formulations of the new sustainable feed on gonad somatic index, gonad maturity stages and colour of reared sea urchins was tested, and results were compared with those obtained from wild specimens. The main goals were: i) to evaluate the potential of the new sustainable feed as a sea urchin diet, compared to natural patterns observed in wild sea urchins collected in the same sampling area; ii) to assess the best proportion of vegetal and animal ingredients from food industry discards promoting the best production and quality of sea urchin gonads.

2. Materials and methods

2.1. Feed formulations

A sustainable feed based on food processing discards was produced following the protocol developed by Ciriminna et al. (2020). Briefly, two feed formulations were prepared using outermost leaves of endive *Chicorium endivia* (Linnaeus, 1753) and European anchovy *Engraulis encrasicolus* (Linnaeus, 1758) processing discards (mainly fins, skin, bones and offal) plus a low amount (2.5 %) of agar (Agar-Agar fine powder 100% Food Grade, Intra Laboratories, UK) as a binder. The two main ingredients were mixed in different proportions to prepare the two feed formulations: (i) the "feed 60/40" was characterised by overall equilibrated proportions of the two ingredients (i.e. about 600 and 400 g kg^{-1} dry weight of endive and anchovy discards respectively), and (ii) the "feed 80/20" was characterised by a higher relative amount of vegetal ingredients (i.e. about 800 and 200 g kg^{-1} of endive and anchovy discards respectively). To prepare the feed, agar was dissolved into boiling MilliQ distilled water (385 g l^{-1}). Then, the solution was allowed to cool to about 60 °C and mixed with the main ingredients. The obtained mixtures were manually transformed into cylindrical pellets (0.5 cm diameter, ~2 cm length, ~1 g wet weight), using 35 ml syringes, air-dried at room temperature (24 °C) for 24 h and then stored a −20 °C until provision.

The proximate composition of the feed formulations was determined according to Ciriminna et al. (2020) and highlighted a high nutritional value of both formulations with the one with the higher amount of

Table 1
Ingredients and proximate composition of the two feed formulations.

	Feed Formulation	
Ingredients (g kg^{-1} dw)	**Feed 60/40**	**Feed 80/20**
Cichorium endivia	587.5	787.5
Engraulis encrasiculos	387.5	187.5
Agar	25.0	25.0
Proximate Composition (g kg^{-1} dw)		
Lipid	138.0	96.2
Protein	294.8	264.9
Carbohydrates	321.3	423.2
Ash	245.9	215.9

Mean proximate composition of the feed ingredients from Ciriminna et al. (2020): *Cichorium endivia* outermost leaves: lipid: 38.0, protein: 191.4, carbohydrates: 644.2, ash: 126.3 g kg^{-1} dw. *Engraulis encrasicolus* processing discards: lipid: 140.1, protein: 405.8, carbohydrates: 43.4, ash: 410.7 g kg^{-1} dw.

vegetable ingredient (feed 80/20) being richer in carbohydrates, and the other (feed 60/40) richer in protein and lipid content (Table 1).

Lipids were measured following a slightly modified version of the Bligh and Dyer (1959) method: a solution of MilliQ distilled water, methanol (CARLO ERBA Reagents, Chaussée du Vexin, France) and chloroform (Panreac Quimica Sau, Barcelona, Spain) ratio1:2:1 (v:v:v) respectively, with 0.01% of butylated hydroxytoluene (Sigma-Aldrich®, St. Louis, United State of America) as an antioxidant, was added to ground sub-samples of freeze-dried feed bars. Samples were then sonicated to improve lipid extraction and centrifuged twice to separate the lipid phase from the aqueous phase. The lipid extracts were evaporated to dryness under a gentle nitrogen stream and weighed. Protein content was estimated by analysing the total nitrogen content in an Elemental Analyzer (FlashEA® 1112, Thermo Fisher Scientific, Monza, Italy), which was subsequently converted in protein content by applying a conversion factor of 6.25 (Horowitz and Latimer, 2006). Ash content was assessed by combustion in a muffle furnace (ZB/1, Asal s.r.l. Milan, Italy) at 550 °C for 4 hr according to Nielsen (2010). Carbohydrates were indirectly determined according to Baião et al. (2019), by applying the following formula: carbohydrates = (100 – (ash + protein + lipid)).

2.2. Feeding trial

The feeding trial was conducted on adult sea urchins, as they allocate more energy for reproduction than juveniles (Fabbrocini and Adamo, 2010; Fernandez and Boudouresque, 2000). In the experiment, sea urchins fed on the two new formulations were compared with the natural development of wild sea urchins. Natural diets (i.e. macroalgae) were not included in the experiment because of (i) the low performance of algal diets on *Paracentrotus lividus* (Lamarck, 1816) gonad yield and quality, as already found in a previous study conducted under the same experimental conditions (Vizzini et al., 2014); (ii) the low sustainability of algal diets in aquaculture and hence the low relevance to the objectives of the present study.

In October 2015, about 160 wild specimens of *P. lividus* of similar size (mean test diameter TD ± standard deviation: 41.2 ± 5.3 mm) were collected by SCUBA divers at Cala Rossa, Terrasini (38°8'34.47" N; 13°4'15.99" E; northern Sicily, Italy, Mediterranean Sea) and transported to laboratories within seawater-filled oxygenated containers. The sampling area was overall characterised by a meadow of *Posidonia oceanica* (L.) Delile, 1813, patches of the macroalgae *Cystoseira* (C. Agardh, 1820) spp. and *Dictyota* (J.V. Lamouroux, 1809) spp. growing on a rocky bottom (author's observations). In the laboratory, 20 specimens were randomly collected, weighed and sacrificed to evaluate the initial gonad conditions in terms of biomass, gonad somatic index, maturity stage and colour (T0 - Wild). Other 140 specimens were transferred into a 150 L tank and kept fasting for 15 days, for acclimatising to laboratory conditions and standardising the relative food appetence before the onset of the feeding trial without affecting gonad production (Pearce et al., 2002). After the fasting period, other 20 specimens were randomly collected and sacrificed to evaluate the potential effects of fasting (T0 - Fast) on the gonad conditions. The remaining 120 sea urchins were randomly divided into two groups of 5 tanks with 80 L capacity (in total, 10 tanks with 12 specimens per tank) in a recirculating aquaculture system (RAS) (Fig. 1). Each group corresponded to a feed formulation (feed 60/40 and feed 80/20, respectively). The animals were fed *ad libitum* every 72 h, for 12 weeks (~ 3 months, October 2015 - January 2016), which was considered a suitable period for assessing the effects of controlled feeding according to the literature (Carboni et al., 2015; Schlosser et al., 2005; Vizzini et al., 2014). Before each round of feed provision, feed particle leftovers and sea urchin faeces were carefully siphoned from each tank. The RAS was equipped with common sand filter, bio-filter and protein skimmer to maintain optimal water quality conditions. Moreover, the environmental conditions in the tanks were kept stable throughout both the fasting period and the feeding trial, with seawater temperature: 20.0 ± 1.0 °C, salinity: 38.0 ± 0.5, photoperiod: 8 h light and 16 h dark and continuous water flow in/out: 5 L min^{-1}. Ammonia levels (mean ± s.d.: 0.034 ± 0.005 mg l^{-1}), pH (8.07 ± 0.04) and oxygen saturation (always > 90%) were measured daily in effluent water from each tank.

Every 4 weeks (T1, T2, T3 corresponding respectively to November, December 2015 and January 2016), 4 specimens were randomly collected from each tank, for a total of 20 specimens for each feed formulation. At the same experimental times, wild sea urchins of similar size (43.1 ± 3.7 mm) were also collected (15–20 specimens each time depending on availability in nature) from the same coastal site, to allow the comparison over time between reared and wild specimens. After each sampling, wild sea urchins were kept for 24 h in an 80 L tank of the same experimental RAS, to empty their gut and avoid biases in the next weight measurements. For the same reason, reared sea urchins were collected and measured before feeding provision. No mortality was recorded throughout the experimental period.

Total weight (TW) measurement was performed from T0 to T3 using an electronic balance (Sartorius BL120S d ±0.01 mg). Then, the weighted sea urchins were sacrificed to record the gonad wet weight (GW) and calculate the gonad somatic index (GSI) as:

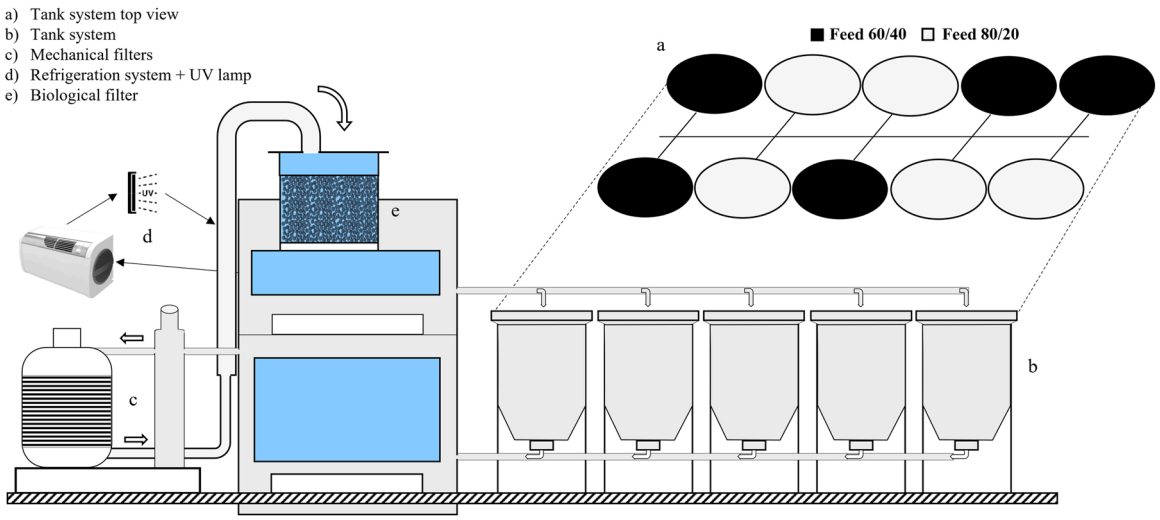

Fig. 1. Scheme of the indoor recirculating aquaculture system (RAS) used for the feeding trial with *Paracentrotus lividus* Lamarck (1816). Five 80 L tanks were randomly assigned to both experimental feed formulations (feed 60/40 and feed 80/20).

$$GSI = GW/TW \times 100.$$

After TW and GW measurement, one of the five gonads of each sacrificed sea urchin was taken for microscopic (Leitz DMRB, Leica, Wetzlar, Germany) determination of sex. Then, a subsample of 6 female specimens and 5 male specimens for each formulation was randomly selected for histological analysis aiming at confirming the sex and the maturity stage. Selected gonads were dehydrated, embedded in paraffin, dissected in 7 μm thick slices using a microtome (5040 Rotary Microtome, Bright Instuments, Huntingdon, United Kingdom) and stained with the alcian blue-periodic acid Schiff reagent (AB/PAS) method. Gonads (both ovaries and testis) were preliminarily categorised according to morphologically criteria on a scale of gamete maturity phases and nutritive deterioration of gonads (Byrne, 1990). Therefore, the stages of the *P. lividus* gametogenic cycle were classified into six categories: stage I, recovery; stage II, growing; stage III, premature; stage IV, mature; stage V, partly spawned; stage VI, spent (Byrne, 1990).

To assess the colour, gonads were placed in clean Petri dishes and compared with Pantone® colour standards chart (Colour Formula Guide 1000, 1991) under standard artificial daylight (Reer, 4000 K) by three expert observers, according to the literature (Symonds et al., 2007; Vizzini et al., 2014, 2019). The observers assigned each specimen to a single colour category among those defined by Shpigel et al. (2005): dark orange (DO), pale yellow (PY), bright orange (BO), yellow-orange (YO) and mango orange (MO), which were then classified in three quality categories (I: inadequate, A: acceptable and E: excellent) following Symonds et al. (2009).

2.3. Statistical analysis

Differences in gonad wet weight (GW) and gonad somatic index (GSI) between sea urchins across time were tested using univariate permutational analysis of variance (PERMANOVA, Anderson et al., 2008). The factors Feed and Time were both fixed and orthogonal, the former with 3 levels (Feed: Wild, feed 60/40 and feed 80/20) and the latter with 4 levels (Time: T0, T1, T2, T3). Analyses were based on untransformed data resembled using Euclidean distance using the software PRIMER 6 v3.1.10 & PERMANOVA+β20 (Plymouth, UK; Anderson et al., 2008). When significant differences were found, pair-wise tests were run as a posteriori check for significant effects.

3. Results

3.1. Gonad growth

Permutational analysis of variance (PERMANOVA) carried out on gonad wet weight (GW) and gonad somatic index (GSI) data showed significant differences for both factors Feed and Time and their interaction (Table 2a). In particular, GW showed a significant gradual increase across time only in reared sea urchins, while GW of wild specimens was significantly higher just at T3 than at T0 (Fig. 2a). On the other hand, while the three treatments did not differ significantly at T0, sea urchins fed with the feed 60/40 presented significantly higher GW than wild ones at all times starting from T1, while those fed with the feed 80/20 from T2. Moreover, the feeding treatment 60/40 showed also the fastest increment of gonad weight over the trial (percentage GW increase from T0 to T3 = 78.1%, 329.0% and 488.8% for wild, feed 80/20 and feed 60/40 respectively), as well as the highest GW values at T3.

Similar to GW, wild sea urchins showed significant differences in GSI only between T3 and T0, but also a fluctuating trend over the experimental period (Fig. 2b). In contrast, all reared sea urchins presented a

Table 2
Results of permutational analysis of variance (PERMANOVA) testing for differences in gonad weight (GW) and gonad somatic index (GSI) of *Paracentrotus lividus* Lamarck (1816) between feed formulations, times and their interaction.

a) MAIN TEST	**GW**				**GSI**		
Source of variation	**df**	**MS**	**Pseudo-F**	**P(perm)**	**MS**	**Pseudo-F**	**P(perm)**
Feed	2	50.34	13.85	**0.001**	412.26	33.60	**0.001**
Time	3	86.39	23.77	**0.001**	507.63	41.38	**0.001**
Feed x Time	6	11.72	3.22	**0.004**	84.38	6.87	**0.001**
Residual	219	3.6			12.26		
b) PAIR-WISE TESTS		**GW**			**GSI**		
Within time between feeds		**t**	**P(perm)**	**Unique perms**	**t**	**P(perm)**	**Unique perms**
T0	**WILD vs FAST**	1.25	0.215	901	0.58	0.541	995
T1	**WILD vs FEED 60/40**	1.82	**0.029**	963	1.82	0.071	998
	WILD vs FEED 80/20	1.56	0.122	944	0.96	0.360	999
	FEED 60/40 vs FEED 80/20	0.50	0.696	976	0.73	0.480	997
T2	**WILD vs FEED 60/40**	3.19	**0.003**	982	5.72	**0.001**	999
	WILD vs FEED 80/20	2.67	**0.014**	973	4.18	**0.001**	998
	FEED 60/40 vs FEED 80/20	1.16	0.274	957	1.37	0.178	997
T3	**WILD vs FEED 60/40**	4.13	**0.001**	984	6.50	**0.001**	997
	WILD vs FEED 80/20	2.60	**0.018**	985	4.31	**0.001**	996
	FEED 60/40 vs FEED 80/20	2.06	**0.047**	982	2.11	**0.048**	997
Within feed between times							
WILD	**T0 vs T1**	0.25	0.808	913	1.97	0.066	996
	T0 vs T2	0.43	0.646	686	0.97	0.363	996
	T0 vs T3	2.01	**0.043**	975	1.96	**0.032**	995
	T1 vs T2	0.22	0.843	960	0.80	0.440	998
	T1 vs T3	1.80	0.053	962	0.38	0.732	998
	T2 vs T3	1.36	0.193	972	0.99	0.353	995
FEED 60/40	**FAST vs T1**	2.44	**0.001**	960	3.19	**0.003**	997
	FAST vs T2	4.60	**0.001**	956	6.81	**0.001**	998
	FAST vs T3	7.68	**0.001**	975	9.98	**0.001**	994
	T1 vs T2	1.26	0.255	966	3.49	**0.002**	996
	T1 vs T3	3.44	**0.001**	978	6.40	**0.001**	996
	T2 vs T3	2.34	**0.025**	968	2.78	**0.011**	996
FEED 80/20	**FAST vs T1**	2.36	**0.024**	960	2.19	**0.038**	996
	FAST vs T2	4.41	**0.001**	942	5.06	**0.001**	997
	FAST vs T3	6.34	**0.001**	973	7.00	**0.001**	998
	T1 vs T2	1.10	0.264	956	2.60	**0.013**	997
	T1 vs T3	2.81	**0.009**	978	4.43	**0.002**	998
	T2 vs T3	1.95	0.059	965	1.85	0.075	998

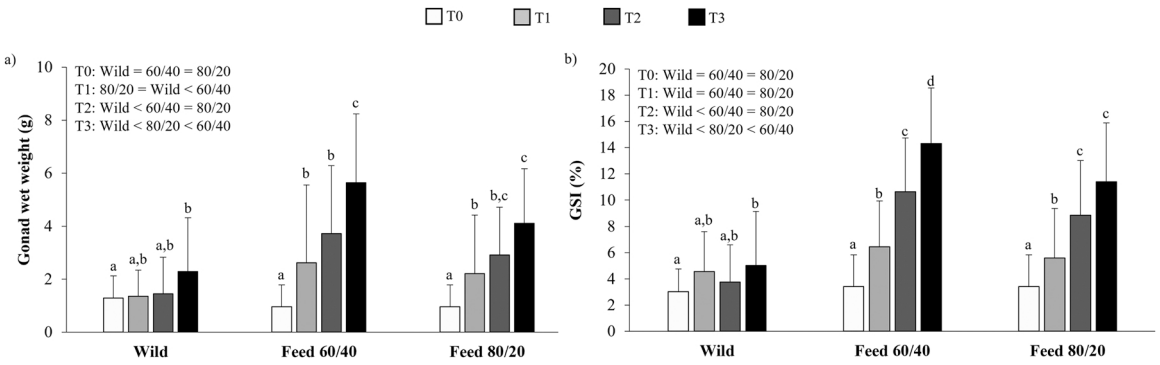

Fig. 2. (a) Gonad wet weight (GW, mean ± standard deviation) and (b) gonad somatic index (GSI, mean ± standard deviation) of sea urchins collected from the natural environment (wild), and sea urchins fed with the two experimental feed formulations (feed 60/40, feed 80/20) across experimental times (T0-T3). Lowercase letters on each panel indicate significant differences between times within feeds. The boxes on both panels indicate significant differences between feeds within times.

significant increase in GSI throughout the trial, mirroring the patterns of GW. The comparison between treatments highlighted comparable values at T0, significant differences between wild and reared specimens since T2 and between reared specimens (feed 60/40 > feed 80/20) only at T3.

3.2. Gonad development

Histological analysis revealed overall a slower gonad development in wild specimens than reared sea urchins (Figs. 3 and 4). Wild females showed a gradual maturation across time, consisting of a decrease of the frequency of the gonads found in the recovery stage (I), up to disappearance at T3, and an increase of those found in the premature stage (III) (Fig. 3). On the other hand, reared females showed a sharper progression in sexual development, achieving the mature stage (IV) at T2, with a frequency of 50% of all the observed sea urchins, and remained stable up to T3, regardless of the feed formulation provided (Fig. 3).

Similarly to females, wild males showed a decrease in the recovery stage (I) and an increase in the premature stage (III) from T0 to T3, but the mature stage (IV) was not achieved (Fig. 4). Moreover, unlike females, reared males showed different patterns according to the formulation provided. In more detail, the specimens fed with the feed 60/40 showed a faster development than the others (feed 80/20), with the achievement of the premature stage (III) just one month after the start of the feeding trial (T1), followed by a further increase up to achieve the

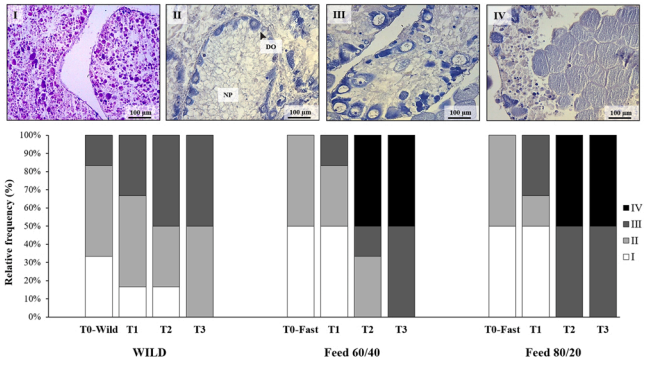

Fig. 3. Above: histological sections of female gonads. Stage I (recovery): nutritive phagocytes begin forming a meshwork across the ascini. Stage II (growing): nutritive phagocytes (NP) and early vitellogenic oocytes attached to the ascinal wall (DO). Stage III (premature): oocytes at all stages of development. Stage IV (mature): ovary packed with ova. Below: relative frequency (n = 6) of the gametogenic stages of female sea urchins collected from the natural environment (wild), and sea urchins fed with the two experimental formulations (feed 60/40, feed 80/20) across experimental times (T0-T3).

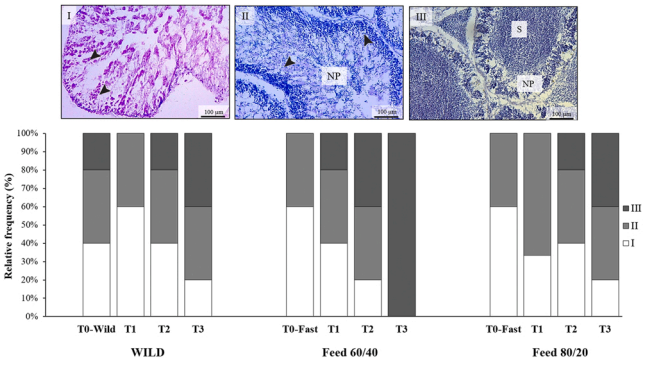

Fig. 4. Above: histological sections of male gonads. Stage I (recovery): primary spermatocytes along ascinal wall (arrowheads). Stage II (growing): testis with columns of developing spermatocytes (arrowheads) in the meshwork of nutritive phagocytes (NP). Stage III (premature): premature testis with spermatozoa (S) in the ascinal lumen and nutritive phagocytes (NP) around the periphery. Below: relative frequency (n = 5) of the gametogenic stages of male sea urchins collected from the natural environment (wild), and sea urchins fed with the two experimental formulations (feed 60/40, feed 80/20), across experimental times (T0-T3).

100% of all the observed gonads at the end of the trial (T3). In contrast, the male specimens fed with the feed 80/20 achieved the premature stage at T2, followed by a lower increase at T3, when immature gonads were also found (Fig. 4).

3.3. Gonad colour

The assessment of gonad colour showed an excellent colouration (E) at the beginning of the trial (T0) in almost 70% of both wild and fasted sea urchins. This value increases up to about 90% if the categories "E: excellent" and "A: acceptable" are summed together (Fig. 5). Wild specimens showed an overall worsening of the gonad colour over time, consisting of a sharp decrease of the frequency of the gonads with excellent and acceptable colour (E + A) (from 90% to 47% at T0 and T3 respectively) coupled with a marked increase of those classified as inadequate (I) (from 10% to 53% at T0 and T3 respectively). In contrast, despite both groups of reared sea urchins showed an initial colour worsening, especially those fed with the feed 80/20 (E + A gonad frequency: from 90% to 55% at T0 and T1 respectively), all the sea urchins fed with the sustainable feed highlighted a subsequent and overall improvement of the gonad colour (A+E gonad frequency: 95% at T3 for both groups of reared sea urchins). However, the frequency of the gonads with excellent colour was always higher in those fed with the feed

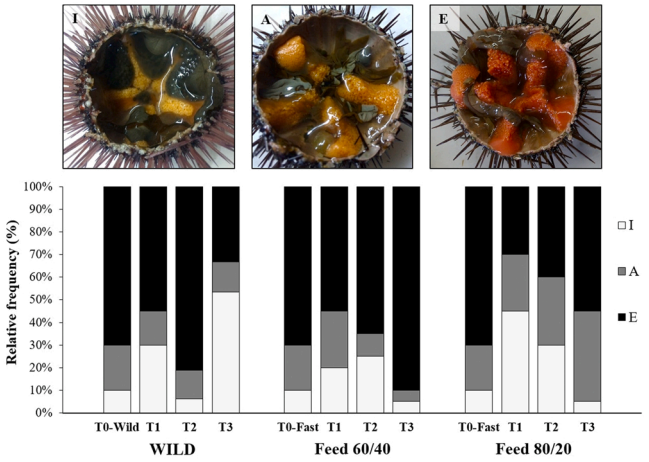

Fig. 5. Relative frequency of the gonad colour categories of sea urchins collected from the natural environment (wild), and sea urchins fed with the two experimental formulations (feed 60/40, feed 80/20), across experimental times (T0-T3). Values are expressed as the percentage of specimens with gonads within each colour category (E: excellent, A: acceptable, I: inadequate).

60/40 than in those fed with feed 80/20. In particular, at T3, the sea urchins fed with the feed 60/40 showed about 90 + 5% of excellent and adequate gonads, while those fed with feed 80/20 only about 55 + 40% of excellent and adequate gonads. Less than 10% belonged to the inadequate (I) category in both groups of reared sea urchins (Fig. 5).

4. Discussion

A sustainable feed prepared by recycling processing discards of endive and common anchovy from the food retail chains was provided to adult sea urchins *Paracentrotus lividus* (Lamarck, 1816), in controlled conditions, to assess the potential of sustainable feeds based on the circular economy principles for echinoculture. In more detail, sea urchins were fed for 3 months with two feed formulations characterised by a different ratio of vegetal vs. animal ingredients to evaluate the most effective formulation promoting gonad production, maturation and quality. The results were compared with those obtained in wild sea urchins, aiming to evaluate differences with natural patterns. Overall, both formulations led to a greater increase in gonad production in reared *P. lividus* than in wild specimens, in terms of gonad wet weight (GW) and gonad somatic index (GSI). Similarly, reared sea urchins showed faster sexual maturation and a better gonad colouration than wild specimens, confirming the high suitability of the sustainable feed as a diet for sea urchins under farming conditions.

At the beginning of the trial, no significant differences in both GW and GSI were highlighted between fast and wild sea urchins, indicating that the fasting period did not lead to significant changes in gonad biomass (Guillou et al., 2000; Raposo et al., 2019) and therefore confirming that is suitable for standardising the initial experimental conditions without affecting gonad production (Pearce et al., 2002). Afterwards, the significant increase in GW and GSI observed in the sea urchins fed with the two experimental formulations after just one month, suggests a rapid conversion of the provided feed nutrients into gonad biomass, contributing to the higher gonad than somatic growth. Indeed, gonads are the main nutrient storage organ as the nutritive phagocytes of the gonads are responsible for both nutrient accumulation and transfer into the developing gametes (Fabbrocini et al., 2012; Marsh et al., 2013; Walker et al., 2015). For this reason, GSI is considered a good indicator of diet nutritional quality, and high-quality feeds are commonly associated with high GSI values (Carboni et al., 2015; Cuesta-Gomez and Sànchez-Saavedra, 2016).

In contrast, the patterns of GW and GSI observed in wild sea urchins did not match perfectly and indicated a much more gradual and lower increase over time, maybe due to a lack of a proper nutritional supply across time (Byrne, 1990; Shpigel et al., 2004; Tenuzzo et al., 2012). Although the growth of sea urchin gonads into the wild may benefit from the low winter temperature and the short daylight period (Byrne, 1990; Shpigel et al., 2004), it is also strongly influenced by food quality and availability, which are typically fluctuant in the natural environment (Cook and Kelly, 2007) differently from the constant and controlled rearing condition. Although wild *P. lividus* can remove large amounts of vegetal biomass to satisfy their nutritional requirements (Klinger, 1984; Lawrence et al., 2020), their favourite food items (i.e. macroalgae) are characterised by low concentrations of macro and micronutrients (Cook et al., 2000; Fernandez and Boudouresque, 2000; Vizzini et al., 2014). Therefore, it is likely that the ingestion of algal biomass (*Cysoseira* spp. and *Dyctiota* spp. are abundant in the collection site) might have led to scarce energy intake and consequently an imbalance between gonad and somatic growth (Schlosser et al., 2005; Shpigel et al., 2005).

In contrast, although the controlled and stable condition of the rearing system used for the feeding trial may have plaid a positive role, the influence of diet on gonad growth is further confirmed by the comparison between the two feeding treatments. Comparing the results from reared sea urchins, it was evident, indeed, that the nutritional differences between the two formulations played a key role in modulating gonad production. Although *P. lividus* is considered an herbivore (Bouduresque and Verlaque, 2007), the sea urchins fed with the formulation characterised by a more balanced vegetal/animal ratio (i.e. feed 60/40) showed higher GW values and a greater gonad increment over time than those fed with the formulation with a higher vegetal content (i.e. feed 80/20). GSI was also boosted by the feed 60/40, consistently with Fernandez and Boudouresque (1998, 2000), who also found the highest GSI following the provision of a balanced diet, made of 60% of vegetal ingredient and 40% of fish meal. These findings confirm the key role of animal proteins and lipids as important storage compounds of the nutrients needed for gonad growth, development and maturation (Fernandez and Boudouresque, 2000; González-Durán et al., 2008; Grosso et al., 2021). Furthermore, the European anchovy discards are richer in fatty acids than endive leaves, especially in omega-3 long chain polyunsaturated and essential fatty acids (Ciriminna et al., 2020), which are vital for reproductive fitness and a multitude of physiological functions, including gonad development and enhancement (Glencross, 2009; Liu et al., 2007; White et al., 2016). In contrast, the higher relative amount of vegetal ingredients of the feed 80/20 may have led to a slower absorption of nutrients. Vegetal meals have, indeed, a high abundance of insoluble carbohydrates (Bach Knudsen, 1997; Esteban et al., 2007) and fibres (Plazzotta et al., 2017), which are usually poorly digested by sea urchins (Fernandez and Boudouresque, 2000; Powell et al., 2020). However, good performances of artificial diets based on terrestrial vegetables on *P. lividus* GSI have been recently highlighted by several authors. Raposo et al. (2019), Santos et al. (2020) and Sartori and Gaion (2015) recorded high, while different, performances (mean GSI = 9%, 9% and 19% respectively) feeding *P. lividus* with maize and spinach, while Vizzini et al. (2014) obtained a high mean GSI (10%) providing lettuce to *P. lividus*. The results obtained in this study are overall comparable to these, but also slightly better than others related to sea urchins fed with macroalgae or mixed diets (Prato et al., 2018; Vizzini et al., 2014; Zupo et al., 2019). This confirms the higher potential of the proposed sustainable feed formulation based on balanced vegetal and animal discards (i.e. feed 60/40) in promoting gonad growth, compared to natural or macroalgae-based diet.

Gonad maturation is another crucial issue in echinoculture, because marketable high-quality gonads, in terms of firmness, colour and taste, are obtained when the ratio between nutritive phagocytes (abundant in the recovery and growth phases) and gametes (abundant in the mature and generation phases) are in favour of the former (Böttger et al., 2006; Walker et al., 2001). Most of the sea urchins collected at the beginning of the trial were in the early reproductive stages, consistent with the

annual reproductive cycle of *P. lividus* in the Mediterranean area (Lozano et al., 1995). While the fasting period seems to have induced a partial regression in the reproductive stages, consistent with Guillou et al. (2000) and Raposo et al. (2019), the gonad maturation patterns of reared sea urchins revealed a subsequent progression in the reproductive stages. In more detail, both formulations promoted similar gonad development in female sea urchins, while the feed 60/40 showed the best performance in males, achieving the totality of specimens in the premature stage at the end of the trial. Different response of females and males may be related to different specific requirements during sexual maturation and gametes production. Indeed, the reproductive effort is greater in females than in males, as the development of embryos and larvae depends on the maternal provisioning of nutrients (Carboni et al., 2015). A suitable advancement in sexual maturation is fundamental for echinoculture purposes. A low progression may result in a limited increase in gonad biomass, due to a slow nutritive phagocytes growth, while a too fast gonad maturation could lead to spawning events with a consistent loss in gonad biomass due to the emission of gametes (Marsh et al., 2013; Walker et al., 2001, 2015). Nevertheless, in this study, the gonad development was faster in reared sea urchins than in wild specimens. These results contrast with those from Fabbrocini et al. (2019), who found a quicker progression in wild specimens than in sea urchins fed with agar-based pellets. These differences, however, may be related to the different environmental conditions (off-shore cages) and the short duration of the trial (one month) conducted by Fabbrocini et al. (2019). Here, both the experimental formulations fostered a suitable gonad development for echinoculture goals, indicating an appropriate energy supply, which is one of the most limiting factors for gonad development in growing and mature stages of the reproductive cycle (Schlosser et al., 2005). Indeed, during these stages, sea urchins need to store great amounts of nutrients for the development of gametes (Walker et al., 2015).

Lastly, the analysis of gonad colour, which is a key factor in determining both the quality of the gonads and its economic value (Stefánsson et al., 2017), revealed also positive effects of the new feed formulations on the sea urchin gonads. The vast majority of reared sea urchins achieved, indeed, a marketable colouration after 12 weeks, differently from wild sea urchins that showed an initial colour improvement, followed by a clear worsening over the experimental period. Colour in echinoid gonads is driven mostly by carotenoid dietary intake, and particularly by echinenone, which sea urchins synthesise from β-carotene (Shpigel et al., 2005). Outermost endive leaves have a greater concentration of carotenoids (lutein and β-carotene), compared with young leaves (de Azevedo-Meleiro and Rodriguez-Amaya, 2005), resulting in well suited dietary sources for obtaining high-quality gonads. The temporal pattern observed for gonad colour, however, suggests that sea urchins need time to absorb carotenoids from the diet, synthesise echinenone and store it in the gonads, consistent with previous studies (Plank et al., 2002; Shpigel et al., 2005). Nevertheless, the overwhelming majority (~ 95%) of the gonads produced by *P. lividus* in 12 weeks presented an excellent and acceptable (E + A) colour, confirming the efficacy of both formulations in producing marketable gonads. Similarly, Santos et al. (2020) obtained marketable gonad colour from sea urchin fed with diets based on spinach, maize and pumpkin, while Luo et al. (2014) found a better performance in promoting gonad colour in sea urchins fed with kelp-based diet than those fed with pumpkin or banana peel. Moreover, Vizzini et al., (2018, 2019) highlighted the effectiveness of lettuce vegetal discards mixed with animal lipids and proteins (i.e., white eggs and commercial pellet) in improving the gonad colour of *P. lividus* (70–90% of gonads in E + A colour). Here, the present findings suggest that the use of animal discards is also highly functional to improve gonad colour and that vegetal discards may replace added carotenoids, usually considered one of the most expensive complements in feed formulation (Cuesta-Gomez and Sánchez-Saavedra, 2018).

5. Conclusion

The present study showed that the outermost leaves of *Cichorium endivia* (Linnaeus, 1753) and *Engraulis encrasicolus* (Linnaeus, 1758) processing discards from the food retail chains have a great potential to be recycled as dietary ingredients in echinoculture. The new sustainable feed based on food processing discards promoted gonad growth and development and contributed to improving the gonad colour of the purple sea urchins *Paracentrotus lividus* (Lamarck, 1816), the most important commercial sea urchin in the Mediterranean Sea (Stefánsson et al., 2017). In this way, food processing discards are transformed into new valuable biomass that might naturally replace traditional ingredients (e.g., fish oils and meals) in aquaculture feeds. This would reduce the environmental and economic impact of the food discard production and disposal, under the principles of circular economy and sustainability, toward the Blue Growth. Moreover, recycling food processing discards may significantly reduce the pressure exerted on marine organisms (macroalgae and fish) for the production of aquaculture feeds. However, since sea urchin gonads are a high-quality niche product, further research is needed to assess the effect of the new sustainable feed on nutritional value and organoleptic features of gonads, as well as to evaluate their performance in aquaculture, compared with commercial feeds.

CRediT authorship contribution statement

Laura Ciriminna: Formal analysis, Writing – original draft, Writing – review & editing. **Geraldina Signa:** Writing – original draft, Writing – review & editing. **Antonino Maurizio Vaccaro:** Conceptualization, Methodology, Validation, Investigation. **Giulia Visconti:** Validation, Investigation. **Antonio Mazzola:** Resources, Funding acquisition, Project administration. **Salvatrice Vizzini:** Resources, Funding acquisition, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data Availability

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

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