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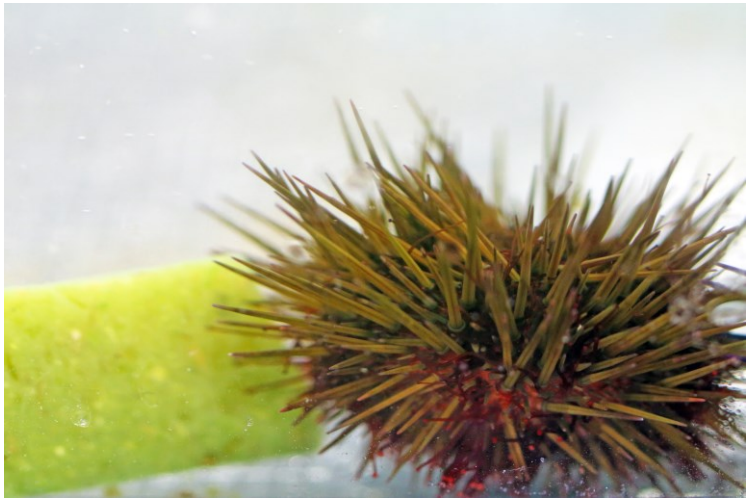

Pedro Castelo Filipe Moreira dos Santos

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Resumo

O ouriço-do-mar *Paracentrotus lividus* (Lamarck, 1816) distribui-se ao longo do Mar Mediterrâneo, Mar Adriático e Costa do Atlântico Nordeste, incluindo a costa de Portugal. As gónadas vermelho-alaranjadas, de alto valor de mercado, são consideradas uma iguaria, especialmente em França, Espanha, Itália e Grécia. Nas últimas décadas, a apanha intensiva tem resultado no colapso de várias populações, sendo agravado pelas suas baixas taxas de crescimento. Logo, a aquacultura comercial desta espécie, aliada à investigação científica, são cruciais para compensar o desequilíbrio entre a procura e oferta, permitindo a recuperação dos *stocks*. Tem sido estudado o uso de rações artificiais para a nutrição dos ouriços-do-mar, de forma a obter gónadas de qualidade, com aceitação de mercado ao longo de todo o ano e de forma rentável. Devido aos constrangimentos ambientais e económicos relativos ao uso de farinha e óleo de peixe em rações, existe a necessidade de encontrar produtos alternativos para o *P. lividus*, enquanto espécie herbívora, nomeadamente produtos ou subprodutos hortícolas. Neste estudo, de forma a determinar o efeito de diferentes dietas no crescimento somático e no crescimento gonadal, cor e estado reprodutivo das gónadas do *P. lividus*, foram desenvolvidas três dietas, usando agar como agente aglutinante: milho e espinafre (A); milho, espinafre e macroalga *Laminaria digitata* (B); milho, espinafre e abóbora *Cucurbita maxima* (C). Os indivíduos foram inicialmente submetidos a um período de jejum de 30 dias e o ensaio de alimentação durou 90 dias, com registo diário das taxas de ingestão. As dietas tiveram boa aceitação, sendo a dieta C a mais consumida (6,21 ± 1,63 g dia^{-1} $indivíduo^{-1}$). Os resultados foram heterogéneos em todos os parâmetros, comparando as três dietas. A dieta B foi a que mais promoveu o crescimento somático, relativamente ao diâmetro da carapaça (1,2 mm $mês^{-1}$) e peso húmido total (79,9 mg ind^{-1} dia^{-1}). Pelo contrário, a dieta C, e particularmente a dieta A, foram as que mais promoveram o crescimento das gónadas. Os indivíduos da dieta A apresentaram um índice gonadossomático médio final de 9,07 ± 2,39%, tendo iniciado com um índice médio estimado de 3,33 ± 0,02% após o jejum. Enquanto as dietas A e C promoveram um desenvolvimento acentuado do ciclo gametogénico (66,7% e 46,7% dos indivíduos com gâmetas maduros, respetivamente), a dieta B resultou numa progressão mais lenta do ciclo reprodutivo, mais apropriado para o consumo. A análise da cor das gónadas confirmou uma diferença significativa entre géneros e também, a eficácia da dieta C e da abóbora *C. maxima* como intensificadores da cor das gónadas.

Palavras-chave: *Paracentrotus lividus*; dietas; aquacultura; ciclo reprodutivo; nutrição; maturação gonadal.

Abstract

The sea urchin *Paracentrotus lividus* (Lamarck, 1816) is distributed throughout the Mediterranean Sea, Adriatic Sea and in the North-Eastern Atlantic coast, including the entire coast of Portugal. The high-value reddish-orange gonads are regarded as a luxury delicacy, having France, Spain, Italy and Greece as the main markets. Intense harvesting has resulted in the collapse of many populations over the last decades, which is further aggravated by the slow growth rates of sea urchins. Therefore, commercial sea urchin aquaculture, associated with scientific research, are crucial to bridge the gap between supply and demand for this product, allowing the stocks to recover. The use of formulated feeds has been studied for sea urchins' nutrition, in order to achieve market acceptable gonads throughout the year, in a cost-effectively manner. Given the environmental and economical issues regarding the use of fishmeal and fish oil for aquafeeds, there is the need to find alternative products for the herbivorous *P. lividus*, such as vegetable products or by-products. In this study, in order to assess the effect of different diets on *P. lividus* somatic and gonadal growth, and gonad colour and reproductive state, there were developed three jellified diets, using agar: maize and spinach (A); maize, spinach and the macroalga *Laminaria digitata* (B); maize, spinach and the pumpkin *Cucurbita maxima* (C). Sea urchins were first subjected to a starvation period of 30 days and the feeding trial lasted for 90 days, with daily measure of the ingestion rates. The diets were well accepted, with diet C as the most consumed (6.21 ± 1.63 g $day^{-1}ind^{-1}$). Results showed heterogeneity in all parameters, between the three diets. Diet B was the most successful in promoting somatic growth, regarding test diameter (1.2 mm $month^{-1}$) and total wet weight (79.9 mg ind^{-1} day^{-1}). On the contrary, diet C, and particularly diet A, were the most successful in promoting gonadal growth. The individuals from diet A presented a final mean gonadosomatic index of 9.07 ± 2.39 %, starting from an estimated 3.33 ± 0.02% from the starvation period. While diets A and C led to a marked advance in the gametogenic cycle (66.7% and 46.7% of the individuals with mature gametes, respectively), diet B resulted in a slower progression in the reproductive cycle, more appropriated for consumption purposes. The gonad colour analysis confirmed a significant difference between gender and also the suitability of diet C and *C. maxima* as gonad colour enhancers for *P. lividus*.

Keywords: *Paracentrotus lividus*; diets; aquaculture; reproductive cycle; nutrition; gonad maturation.

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1. Introduction

1.1 Biology and morphology

Paracentrotus lividus (Lamarck, 1816) belongs to the Echinodermata phylum, Echinoidea class and Echinoida order (Lawrence, 2007). The name assigned to the group, Echinoderms (echinos - spiny; derma - skin), of Ancient Greek origin, refers to the fact that these animals are frequently covered with spines. The genus *Paracentrotus* belongs to the family Echinidae (Saldanha, 1995).

P. lividus has a spherical and slightly flattened dermal skeleton, often referred as test, formed by calcareous plates and covered in spines. Although purple is the predominant colour of the spines, there are several colour variations depending on the proportion of the different chromophores (Mortensen, 1943; Tortonese, 1965; Gamble, 1966). Sea urchins have pentamerous structure, with each sector consisting of two zones, radial (ambulacral) and interradial (interambulacral). Along the ambulacral areas originate the tube feet (aquifer system), which have locomotive, tactile and prehensile functions. On both areas, there are primary tubercles on which are implanted the spines. In the centre of the oral area (facing the substrate) is placed the peristome, covered by a membrane and small plates, where the mouth is positioned. It is formed by an ossicles structure called Aristotle's Lantern. On the opposite side of the oral zone is the aboral area, where the periproct is located, which surrounds the anus (Sartori, 2013). Regarding the reproductive system, the gonads are constituted by five branches, covered by peritoneum and adhering to the aboral side of the test. Branches of the genital coelomic and hemal sinuses interconnect all five gonads. These branches are located in the interambulacral areas and a single gonoduct exits each gonad and emerges through the test, via a pore on each of the five genital plates that surround the periproct. In both sexes, the gonadal wall is composed by an outer and inner sack, each comprised of several characteristic layers. The genital coelomic sinus (GCS) separates the two sacks (Walker, 1979; Walker *et al.*, 2007; Sartori, 2013). With reference to the digestive system, gut structure and histology are very consistent in all regular echinoids. The main regions of the gut consist in the buccal cavity, pharynx, esophagus, stomach (with the siphon along its internal edge), intestine, and rectum (De Ridder & Jangoux, 1982). Mucus cells are found in the pharynx and esophagus in most regular echinoids (Holland & Ghiselin, 1970). Mucus surrounds ingested food to form pellets (De Ridder & Jangoux, 1982) that remain intact through defecation. During the growth period, modifications occur in diameter, mass and shape of the sea urchin’s test, which

involve several processes, such as calcification, expansion or production of soft tissues (Ebert, 2007).

1.2 Biogeography and ecology

Parecentrotus lividus is geographically distributed throughout the rocky intertidal and shallow subtidal zones of the Mediterranean Sea, Adriatic Sea and in the North-Eastern Atlantic coast, from western Scotland and SW Ireland to southern Morocco, including the Canary Islands and the Azores Islands (Fernandez, 1997; Hayward & Ryland, 1990; San Martin, 1995; Boudouresque & Verlaque, 2007). It is present along the entire coast of Portugal (Gago *et al.*, 2003; Bertocci *et al.*, 2012; Jacinto *et al.*, 2013). These geographic areas are characterized by water temperatures that range from 10 to 15 °C in the winter and from 18 to 25 °C in the summer, typical in regions like western Mediterranean, Portugal and Biscayne Bay (Lawrence, 2001; Boudouresque & Verlaque, 2007). *P. lividus* is, in fact, the largest and most abundant echinoid along the Portuguese coast (Hayward & Ryland, 1990; Gago *et al.*, 2003). The species has a broad ecological distribution in the infralittoral area, ranging from shallow subtidal rocky habitats (horizontal and vertical walls) to less stable substrata such as *Posidonia oceanica* and *Zostera marina* meadows (Palacin *et al.*, 1997). It can be found between the mean limit of low tide and 10-20 m depth, and in intertidal rock pools, in which the upper limit is generally determined by desiccation (Régis, 1978; Crook *et al.*, 2000). This sea urchin is also present in areas of high hydrodynamism in the first metres of exposed shores to calmer waters in bays or deeper zones. Echinoid species have the ability to adapt their biological parameters to differing abiotic conditions, such as temperature, food, wave action or predation (Ebert, 1968; Gonor, 1973; Régis, 1979; Himmelman, 1986). The density of *P. lividus* populations usually ranges from a few to a dozen individuals per square metre, however, very high density (> 50-100 individuals per square meter) can occur in particular environments (Pastor, 1971; Crapp & Willis, 1975; Harmelin *et al.*, 1981; Delmas & Régis, 1986; Delmas, 1992). *P. lividus* appears to be relatively insensitive to organic pollution, given that these compounds seem to enhance the growth rate of the individuals (Tortonese, 1965; Allain, 1975; Delmas, 1992). However, laboratory experiments have shown high sensitivity of *P. lividus* to ammonia (Lawrence *et al.*, 2003), at concentrations levels only found in a closed recirculation system rather than in natural environments (Boudouresque & Verlaque, 2007). In addition, *P. lividus* is able to tolerate high concentrations of heavy metals, and even accumulate them, although they can affect the growth rate of the organisms, particularly in early life stages (Walter *et al.*, 1989; Brunetti *et al.*, 1991; Delmas, 1992; Warnau & Pagano, 1994; San Martin, 1995).

1.3 Feeding habits

The analysis of gut contents indicates that *P. lividus* is mainly an herbivore, when the resource is non-limited, consuming algae, seagrass, or fragments of these transported by current flow (Neill & Pastor, 1973; Verlaque, 1987). However, if macroalgae become scarce or unavailable, this species develops a detritivore and browser activity, and may consume animal food (Paine & Vadas, 1969; Fernandez & Caltagirone, 1998; Boudouresque & Verlaque, 2007). Boudouresque & Verlaque (2007) characterize this species as an opportunistic generalist, displaying a wide range of adaptive responses, such as feeding on the resources available and "shifting" from a preferred, but insufficient food source, to another less appreciated, but plentiful (frequency-dependent food-choice) (Régis, 1978; Nédélec, 1982). However, there are several species that are clearly favoured than others, such as *Rissoella verruculosa*, *Cymodocea nodosa*, *Cystoseira amentacea*, *Padina pavonica, Posidonia oceanica*, *Undaria pinnatifida, Corollospora maritima* or *Laminaria* sp. (Cuomo *et al.*, 1982; Spirlet *et al.*, 2001; Lawrence, 2007). Photosynthetic unicellular organisms, sponges, hydroids, copepods, dead fish and mussels, among other organisms can also be found in the gut contents of *P. lividus* (Mortensen, 1943; Tortonese, 1965; Pastor, 1971; Neill & Pastor, 1973; Delmas & Régis, 1986; Fernandez, 1990; Mazzella *et al.*, 1992). Therefore, the designation omnivorous scavengers would be more accurate (Lodeiros & García, 2004).

1.4 Reproduction and life cycle

In *P. lividus*, like in the majority of echinoid species, sexes are separate although some cases of hermaphroditism have been observed (Neefs, 1937; Byrne, 1990). The sex ratio changes throughout the year and from one year to the next (Allain, 1975; Guettaf, 1997). In both sexes, gonads include germinal cells (GC's) and somatic cells, called nutritive phagocytes (NP's) (Ghisaura *et al.*, 2016). Gonads increase in volume during the reproductive cycle in consequence of the increment in size and number of the germinal cells (GC's) during gametogenesis, but also because of the intragonadal nutritive phagocytes (NP's). These cells store extensive nutrient (proteins, lipids and carbohydrates) and energy reserves that are later necessary in gametogenesis (Harrington *et al.*, 2007; Walker *et al.*, 2007; Ghisaura *et al.*, 2016). GC's include the oogonium, oocyte and ovum in females and the spermatogonium, spermatocyte and spermatozoon in males (Walker, 1982; Byrne, 1990). Ova and spermatozoa represent the mature gametes (Byrne, 1990; Spirlet *et al.*, 1998a). The proportion of GC's and NP's varies according to the maturation of the gonads. Before gametogenesis, the gonadal acini are filled with NP's, rich in nutrients.

During the gametogenic process, these nutrients are supplied to the developing GC's, resulting in a decrease in size of the NP's. The fully mature gonads are filled with gametes, although immature gonads (with more NP's than GC's) are preferred for human consumption (Walker *et al.*, 2001). Byrne (1990) identified 6 annual developmental stages: I (recovery), II (growing), III (premature), IV (mature), V (partly-spawned) and VI (spent), applied to both sexes. However, Spirlet *et al.* (1998a) distinguished 8 gonadal stages, introducing two distinct spent stages and a post-spawned stage. The composition of the gonads depends on endogenous factors, primarily related to the life cycle, and exogenous factors, mainly environmental and nutritional, like food composition or feeding frequency (Arafa *et al.*, 2012). The fatty acid profile and abundance in the gonads, in particular, are dependent on the diet and reproductive status (Carboni *et al.*, 2013). In *P. lividus*, according to Pereira *et al.* (2013), using organisms from Peniche (Portugal), found that the main saturated fatty acids present in the coelomic fluid and gonads are tetradecanoic and palmitic acids, while the predominant unsaturated fatty acids are arachidonic, EPA and cis-11,14-eicosadienoic acids. Fatty acids are a relevant source of energy and are essential for gametes maturation and larvae survival, influencing reproductive performance. Particularly, long-chain polyunsaturated fatty acids (PUFA) are major structural and physiological constituents in cell membranes and also are precursors of eicosanoids, lipids involved in reproduction (Cook *et al.*, 2007; Martinez-Pita *et al.*, 2010).

In vitro, sexual maturity is reached in individuals of size ranging between 13 and 20 mm and/or after 5 months (Cellario & Fenaux, 1990), although in natural populations, sexual maturity can be reached in later phases. In general, spawning occurs once or twice in a year, during spring and late summer (Lozano *et al.*, 1995). However, considering all the variables that affect the release of gametes, such as water temperature, photoperiod, habitat and individual variability, independently of the single or double emission, spawning can occur almost year-round, usually at much lower levels. Generally, during the spawning events, males and females of *P. lividus* aggregate and simultaneously release their gametes (Cherbonnier, 1954). The life cycle is characterized by two stages: larval planktonic and adult benthic. The eggs are fertilized and develop into embryos and planktonic echinopluteus larvae, which are capable of swimming and consume phytoplankton. After roughly three weeks, the larvae become "competent" and start approaching the substratum (Gosselin & Jangoux, 1998; Boudouresque & Verlaque, 2007). The newly settled post-larvae acquire the appearance of miniature adults within hours. It then takes a further week for them to reach the true juvenile stage, as they must develop mouths and the rest of the digestive tract first (Cameron & Hinegardner, 1974;

Gosselin & Jangoux, 1998). During the settlement phase, sea urchins are very sensitive to environmental changes (Dupont *et al.*, 2013).

1.5 *P. lividus* consumption and market

There were discovered indications of prehistoric human consumption of sea urchins in several locations, for instance, *P. lividus* occurs with other invertebrates exploited by Cantabrian Mesolithic groups on the Iberian Peninsula (Arias, 1999; Lawrence, 2007). Nowadays, sea urchin roe (gonads) is highly regarded as a luxury food item. Around 100,000 tons of sea urchins are landed annually from the world's fisheries, with a value of over 0.5 billion Euros (Kelly, 2004). In 2010, marine aquaculture produced 384,300 tonnes of echinoderms for consumption world-wide (FAO, 2012), in which an estimated value of 88,000 tonnes corresponds to sea urchins (Carboni *et al.*, 2012b). The most important markets for sea urchin gonads are represented by Japan and the USA. In Europe, France represents the main market for sea urchin gonads, although in a smaller scale (Le Gall, 1990). Sea urchin fisheries first developed on the Atlantic and Mediterranean coasts of Europe, North Asia (Japan and Korea), New Zealand and Chile in the beginning of the 17th century (Andrew *et al.*, 2002). The peak of production was reached in 1995 with a landing of 113,654 tonnes, a value three times higher compared to 1970 (Williams, 2002), a consequence of the expansion of the Chilean, US (Californian and Maine) and Canadian fisheries (Andrew *et al.*, 2002). With regard to *P. lividus*, the most consumed sea urchin in Europe (Carboni *et al.*, 2012a), its reddish-orange gonads are considered a delicacy since ancient Greece. Nowadays, they are marketed fresh, frozen, dried, salted and pasteurized (Hagen, 1996; Sartori, 2013). This species represents an important economic resource in France, Spain, Italy (Guidetti *et al.*, 2004; Boudouresque & Verlaque, 2007) and, to a lesser extent, in Portugal (Jacinto *et al.*, 2013) and is therefore, subject to intense harvesting particularly in the Mediterranean Sea (Ceccherelli *et al.*, 2009, 2011), on Atlantic coasts of France and Iberian peninsula (Barnes & Crook, 2001) and in Ireland (Byrne, 1990). The consumption of *P. lividus* is mainly limited to France and Spain and to a lesser extent to Italy and Greece, although harvesting occurs over a much larger area, including Ireland, Portugal and Croatia, mainly for export (Lawrence, 2007). Regarding the retail price of this product, it is possible to find, in supermarkets, sea urchin gonads packed in small jars (50-70 grams), with an approximate price of around 15 € (Sartori, 2013). According to Unuma (2002), the price varies with the quality of the gonad. In the case of high quality gonads, one sea urchin weighing 100 g with a gonadosomatic index (GSI) of 20% would yield gonads worth US$6.67. Carboni *et al.* (2014) refer that the retail price of unprocessed fresh sea urchin in Europe varies greatly depending on the season, country, and retailer

type and can vary between 0.30 and 3€ per individual, while the price of processed gonads can reach 150€ kg^{-1}. Since the early 70's, the market's demand for sea urchin's gonads has increased globally, particularly in Japan (Williams, 2002), with its annual importation of 20 000 tonnes of sea urchins and a local production of 15 000 tonnes (Carboni *et al.*, 2012b). In Sardinia, 30 million sea urchins (1800 tonnes) are consumed annually, with an associated profit of more than €10 million.

1.6 *P. lividus* stocks

The collapse of many populations and a sharp decline of the world's urchin harvest in the late 90's and first decade of the new millennium are mainly due to overfishing (Andrew *et al.*, 2002; Pearce, 2010). Over the last 15 years, world fishery production of sea urchins has dramatically declined from 115 000 to about 82 000 tonnes. In some of the most important fishery grounds in USA and Japan, there were recorded productivity declines of 75% and 50%, respectively (Carboni, 2012b; Carboni, 2013). In Europe, France represents the most important commercial activity, where the overexploitation of *P. lividus*, between the 60's and the 70's, caused a dramatic depletion in populations, with an estimated annual landing of 1000 tonnes (Sartori *et al.*, 2016). In the 1980's, the stocks of *P. lividus* of France and Ireland were fished to the point of decimation and have never recovered (Barnes & Crook, 2001; Barnes *et al.*, 2002). Today, over half of the world's sea urchin landings come from Chile, harvesting 55 000 tonnes $year^{-1}$, and this fishery is not yet managed in a sustainable way (Cook & Kelly, 2007a). In north Portugal, in particular, populations of *P. lividus* have been commercially harvested in the last decade in order to supply markets of nearby regions, such as Spain, where the strong demand has already caused a situation of overfishing and a drastic reduction of the local stocks (FAO, 2004; Bertocci *et al.*, 2014). Moreover, the situation is further aggravated by the slow growth rates of sea urchins, in which 2 centimetres *P. lividus* are considered to be 2 years old, on average, and 4 centimetres individuals, 4-5 years old (Gago *et al.*, 2003; Grosjean *et al.*, 2003).

1.7 Echinoculture

It is becoming mandatory to adopt specific management strategies that allow the stocks to recover (Sartori *et al.*, 2015). Fisheries management has been the primary response in many parts of the world where overfishing has occurred: Chile (Stotz, 2004), Spain (Catoira, 2004), Japan (Agatsuma *et al.*, 2004), California (Dewees, 2004), and North America (Botsford *et al.*, 2004). Furthermore, several possible solutions have been tested:

reseeding natural habitats with farmed juveniles (Gomez *et al.*, 1995); mariculture (Fernandez, 1996), raising in immerged cages, including polyculture (Robinson *et al.*, 1997; Kelly *et al.*, 1998) and land-based, closed-system aquaculture, allowing the control of each phase of the echinoid biological cycle (Le Gall, 1990; Grosjean *et al.*, 1998). Nowadays, it is becoming more difficult to think of a future without an aquaculture project for any species that have economical interest, including sea urchins. In fact, the worldwide supply of high quality sea urchin gonads will be unable to meet market demand unless commercial sea urchin aquaculture develops to, at least, partially replace the steady decline in natural captures (Grosjean *et al.*, 1998). Therefore, the goal of scientific investigation is to bridge the gap between supply and demand of aquaculture products (Pearce, 2010), so that adult individuals, with excellent quality gonads, could be available for the market throughout the whole year (Carboni, 2013). Furthermore, the taste and texture of the gonads are negatively affected by spawning seasons, with harvest occurring only between November and March. In aquaculture, by artificially controlling abiotic parameters, it is possible to produce good quality gonads for the consumers throughout the year (Carboni, 2013). The aquaculture of echinoderms, including sea urchins and sea cucumbers is known as echinoculture (Hagen, 1996) and over the past 15 to 20 years, its commercial interest has increased in a number of countries, such as Japan, Canada, Chile, Australia, China, New Zealand, Ireland, Norway, Italy and Scotland (Carboni *et al.*, 2012b). According to FAO (2013), the production of *P. lividus* in aquaculture systems corresponds to 10 tonnes year^{-1} in Europe, in contrast to a landing activity of 108 tonnes year^{-1} (Sartori *et al.*, 2016).

1.8 Nutrition

The quantity and quality (biochemical composition) of food have direct influence on the physiology, morphology and nutritional parameters of sea urchins, as well as changes in food supply and the individual's nutritional state may affect several reproductive characteristics (George, 1996; Fernandez & Boudouresque, 2000). Feed formulations are essential to aquaculture, given their constant availability and nutritional composition, water stability and ease of use. Characteristics that are also required for a large-scale aquaculture of *P. lividus*. Moreover, aquafeeds may sometimes represent an upgrade to the natural food, as research conducted with different sea urchin species demonstrates that formulated feed can promote a faster gonadal growth, when compared with macroalgae (Lawrence *et al.*, 2001). During the last two decades, several researchers have studied the use of formulated feeds for sea urchins' nutrition, including Caltagirone *et al.* (1992), Jong-Westman *et al.* (1995), Fernandez & Pergent (1998), Watts *et al.* (1998), Fernandez & Boudouresque (2000), McLaughlin & Kelly (2001), Pearce *et al.* (2002, 2003, 2004),

Robinson *et al.* (2002), George *et al.* (2004), McBride *et al.* (2004), Mortensen *et al.* (2004), Vidal (2004), Senaratna *et al.* (2005), Siikavuopio *et al.* (2006), Dworjanyn *et al.* (2007), Taylor *et al.* (2009), Lawrence *et al.* (2011), Watts *et al.* (2011), Eddy *et al.* (2012), Fabbrocini *et al.* (2012), Hammer *et al.* (2012), Cyrus *et al.* (2013, 2015), Carboni *et al.* (2015), Tomšić *et al.* (2015), Heflin *et al.* (2016a,b), Wei *et al.* (2016) and Zhao *et al.* (2016). However, the aquaculture development brings extensive negative effects on the environment, mainly related to the ingredients source for aquafeeds (White *et al.*, 2016). Fishmeal is the crude flour obtained by milling and drying wild, small oceanic fish or fish by-products, while fish oil is a brown or yellow liquid obtained through the pressing of cooked fish (Miles & Chapman, 2006; FAO, 2016a). Given that these products are still the main protein and lipid source in the aquaculture sector, 46% of the total annual production of fishmeal is directed to this industry (Miles & Chapman, 2006; Tacon & Metian, 2015). However, total wild fish landings show a regular decline since 1996, creating a problem for the supplying of fish meal and fish oil that cannot rely entirely on finite stocks of marine pelagic fish (Turchini *et al.*, 2009; Pauly & Zeller, 2016). As a consequence, the costs of these products are increasingly higher, which forces this industry to reduce its dependence on them and use fish meal and fish oil for particular stages of production, at lower concentrations (Tacon & Metian, 2015; FAO, 2016a). Indeed, feeding represents 50% of the total production cost in aquaculture, which emphasises the need for other food sources (Sartori, 2013).

1.8.1 Alternative feed sources

Against this background mentioned before, modern aquaculture is turning to alternative products, in particular land vegetable products (Turchini *et al.*, 2009), which are constantly available and allow the recycling of unprocessed agricultural discards into biomass of high commercial value (Vizzini *et al.*, 2015). In this context, the rearing of *P. lividus* with alternative formulated feeds brings some advantages. First, the replacement of fish meal and fish oil with land-based vegetables is much more feasible with herbivorous or omnivorous species, when compared to carnivorous ones (Hardy & Tacon, 2002). Although high values of growth rate and gonad size normally result from the presence of animal proteins in the diet, the herbivorous *P. lividus* does not benefit from high dietary protein levels, making the growth not proportional and negatively affect the flavour of the gonads (Pearce *et al.*, 2002; Cook & Kelly, 2007a; Phillips *et al.*, 2009). In fact, Robinson *et al.* (1997) obtained the best gonad growth for the sea urchin *Strongylocentrotus droebachiensis* fed with carrots and cabbage, in comparison with the *Laminaria longicruris* diet. In this context, maize and spinach have emerged as one of the best options for

alternative ingredients to promote gonadal growth and flavour of *P. lividus*, for several reasons.

1.8.2 Maize (*Zea mays* L.)

Regarding the use of maize for sea urchin's nutrition, there is already some related research found in the literature, namely Basuyaux & Blin (1998), Basuyaux & Mathieu (1999), Luís *et al.* (2005), Gago *et al.* (2009), Gago & Luís (2010), Gago *et al.* (2010), Repolho *et al.* (2011), Gago & Luís (2011), Sartori (2013), Sartori *et al.* (2015), Tomšić *et al.* (2015) and Sartori *et al.* (2016). Maize (*Zea mays* Linnaeus, 1753) is originated from Mexico and Central America and is still nowadays, one of the most important cereal crops in the world, with the greatest range of adaptability. Primarily used for the zootechnical sector, as a major component of livestock feed, maize is used for food, feed and industrial uses. For decades, 50% of the dietary protein produced in the world is derived from cereals (Galinat, 1977; Glover & Mertz, 1987; Sartori, 2013; Ranum *et al.*, 2014). The production of maize has been steadily increasing over the last decades, mainly in the United States, the world major producer. For 2016, total world production is estimated at 1 026 million tonnes, more 22.6 million tonnes when compared to 2015 (FAO, 2016b). Moreover, maize carotenoid concentrations are one of the highest found in cereals (Howitt & Pogson, 2006), with a carotene concentration of 1.8 mg kg^{-1} (Luís *et al.*, 2005). The use of this ingredient for animal's feeds can contribute up to 30% protein, 60% energy and 98% starch of the diet, being more economical in comparison to commercial feeds as a source of high energy and protein for *P. lividus* (Dado, 1999; Luís *et al.*, 2005). According to Basuyaux & Blin (1998), maize represents an ingredient for sea urchin's feed with constant quality, it is cheap and easy to store, transport and manage, and offers good resistance when immersed in seawater.

1.8.3 Spinach (*Spinacia oleracea* L.)

Spinacia oleracea (Linnaeus, 1753) is a flowering plant with a high nutritional value, referred as a promising source of nutrients for *P. lividus* by Sartori (2013), Sartori *et al.* (2015), Sartori & Gaion (2015) and Sartori *et al.* (2016). Hur's (1988) work suggested the suitability of vegetables for sea urchin's nutrition in intensive aquaculture, by obtaining a higher gonadosomatic index for *Hemicentrotus pulcherrimus* individuals fed with different vegetables, including spinach, when compared to individuals fed with macroalgae species. Spinach contains large amounts of carotenoids (yellow, orange and red, although hidden by chlorophyll), ascorbic acid (vitamin C), minerals, vitamins, polyphenols (flavonoids),

phylloquinone (vitamin K_1), α-Tocopherol (vitamin E) and folate (vitamin B_9) (Müller, 1997; Davey *et al.*, 2000; Bunea *et al.*, 2008; Cho *et al.*, 2008; Lester *et al.*, 2010). According to Guerra *et al.* (2012), several factors in marine ectotherm organisms, such as aging, somatic growth, maturation and reproduction lead to an increase in oxidative stress and simultaneously, a reduction in cellular maintenance. Cao *et al.* (1996, 1997) using a method for quantifying the oxygen radical absorbance capacity (ORAC) of antioxidants in biological tissues, reported the spinach's high antioxidant activity against hydroxyl radicals, thus protecting biological tissues against oxidative damage (Golden *et al.*, 2002).

1.8.4 Carotenoids

In echinoculture, there is a great need for improving diets in order to maintain or enhance an acceptable commercial colour and brightness of the gonads, and carotenoids must be part of the artificial diets (Britton *et al.*, 2004; Lawrence, 2007). Carotenoids are organic pigments that can be distributed in two categories, carotenes (β-carotene and α-carotene as the main isomers) and xanthophylls, which include β-echinenone, astaxanthin, canthaxanthin, diatoxanthin, alloxanthin, lutein, zeaxanthin and fucoxanthin (Matsuno & Tsushima, 2001; Britton *et al.*, 2004; Tsushima, 2007). The biological properties of carotenoids, particularly in animals, can be divided into several functions and corresponding actions, including energy transfer in photoprotection, pigmentation, improving reproductive capacity (e.g. antioxidant protection of the gametes), positive effects on specific enzymes, antioxidant activity and antitumor activity. Furthermore, β-carotene, β-echinenone and fucoxanthin seem to enhance the phagocytic activity in sea urchins, thus improving the biological defense (Kawakami *et al.*, 1998). With respect to nutrition, carotenoids are very important as provitamin A precursors, having β-carotene as the main source (Matsuno, 2001; Tsushima, 2007). Since animals, in general, do not have the capacity to biosynthesize carotenoids *de novo,* they accumulate these pigments directly from the diet and can structurally modify them through metabolic reactions (Goodwin, 1980; Britton, 1998, 2008). In sea urchins, gonads are the main organ of carotenoid accumulation and the average carotenoid and vitamin concentrations in the fresh gonads of *P. lividus* (mg 100 g^{-1} dry weight) are 11.35 for β-echinenone, 7.86 for α-tocopherol, 3.44 for lutein, 1.89 for β-isocryptoxanthin and 0.79 for β-carotene (de Quirós *et al.*, 2001). Several studies have investigated the relation between carotenoids in sea urchin's artificial diets (mainly β-carotene and β-echinenone) and gonad colour and enhancement (Griffiths & Perrott, 1976; Tsushima & Matsuno, 1990; Goebel & Barker, 1998; Plank, 2000; Matsuno & Tsushima, 2001; McLaughlin & Kelly, 2001; Plank *et al.*, 2002; Pearce *et al.,* 2003; Robinson *et al.,* 2002, 2004; McBride *et al.*, 2004; Tsushima, 2007; Symonds *et al.*,

2009; Suckling *et al.*, 2011; Pilbrow, 2014). Other researchers, such as Shpigel *et al.* (2005), Shpigel *et al.* (2006), Symonds *et al.* (2007), Carboni *et al.* (2015), Sartori & Gaion (2015) and Sartori *et al.* (2016) studied this subject specifically for *P. lividus*. Artificial diets usually produce large gonads, however, pale in colour (Shpigel *et al.*, 2005). Thought maize and spinach have become a promising solution for the aquaculture of sea urchins, there is the need to find other suitable sources of carotenoids to attain a gonad colour equal to wild individuals. On this basis, pumpkins may represent a new potential source of nutrients, mainly carotenoids for *P. lividus* nutrition.

1.8.5 Pumpkin (*Cucurbita maxima* Duchesne)

Pumpkin (genus *Cucurbita*), originated from Central America and Southern Mexico (Whitaker, 1956) and belonging to the family of *Cucurbitacea*, is a squash orange fruit that has been used for human and animal feed (Guiné *et al.*, 2011; Sedigheh *et al.*, 2011). It is a source of water-soluble vitamins, antioxidants, tocopherols, polysaccharides (including pectin) and minerals (iron, manganese, zinc, copper fibres and amino acids) (Pawar *et al.*, 1985; Alibas, 2007; Stevenson *et al.*, 2007; Guiné *et al.*, 2011). Pumpkin seeds, in particular, constitute a good source of zinc, polyunsaturated fatty acids and phytosterols (Glew *et al.*, 2006; Ryan *et al.*, 2007; Kim *et al.*, 2012). *Curcubita pepo* (Linnaeus, 1753), *Cucurbita moschata* (Duchesne ex Lam.) and *Cucurbita maxima* (Duchesne) are three species from these family that are globally cultivated and provide high production yields (Sedigheh *et al.*, 2011; Kim *et al.*, 2012).

According to Kim *et al.* (2012), *C. maxima* has significantly more carbohydrates and protein in the flesh and peel, with the highest aminoacid content when compared to the other two species. *C. maxima* also presents a superior lipid content in the flesh, peel and seed (4.20 g kg^{-1} raw weight, 8.69 g kg^{-1} and 524.34 g kg^{-1}, respectively). Regarding the fatty acid composition, *C. maxima* contains myristic acid, palmitic acid, heptadecanoic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, eicosenoic acid, α-linolenic acid and behenic acid, some of them not detected in the other species, which highlights the high nutritional value of *C. maxima*. Furthermore, according to the same authors, the content in polyunsaturated fatty acids in this species is also significantly higher, as the α-tocopherol concentration in the flesh and peel. With respect to carotenoid concentrations, *C. maxima* presents much higher contents for β-Carotene (123.19 mg kg^{-1} RW in the peel) and β-Cryptoxanthin. *C. maxima* is one of the mostly cultivated species in Portugal and the growing season occurs from April to July (Marreiros & Rosa, 2011; Boschi, 2015). de Escalada Pla *et al.* (2007) concluded that pumpkin-fiber products may be used as food

ingredients and for technological and nutritional purposes. The only scientific article, to date, relating sea urchin's nutrition and pumpkin, as a source of nutrients, is Luo *et al.* (2014), in which *Cucurbita spp.* is used as feed for *Strongylocentrotus intermedius,* as a potential ingredient for improving gonadal growth, colour and flavour. In fact, the use of pumpkin, in several forms, is being investigated as a potential ingredient for aquaculture feed (Dada & Abiodun, 2014; Murray *et al.*, 2014; Lovatto *et al.*, 2017), with the need of extending it to other organisms, such as sea urchins. In addition, given that pumpkin powders and flakes are much utilized in the food industry, the use of food processing by-products, such as peel and skin, and wastes from agriculture, would create accessible ingredients that can be integrated in food products. In this way, the available resources are optimized and therefore, can mitigate waste disposal problems (El-Adawy & Taha, 2001). Moreover, according to Norfezah *et al.* (2011), pumpkin flour can be made from different types of waste and then used for different purposes, namely incorporated with corn grit for extruded feeds.

1.8.6 Kelp (*Laminaria digitata* Huds.)

To attain several organoleptic characteristics of the gonads, some authors refer the need to include natural algae, in order to improve overall quality, colour, firmness, taste, texture (Pearce *et al.*, 2004; Shpigel *et al.*, 2005) and enhance the palatability of artificial feed for sea urchins (Dworjanyn *et al.*, 2007). Traditionally, the aquaculture of sea urchins have depended exclusively on seaweeds found locally (Cipriano-Maack, 2016). The highly productive Kelp forests (Laminariales order) constitute important components of coastal ecosystems (Steneck *et al.*, 2002). *Laminaria digitata* (Laminariaceae) is one of the most preferred natural food for *P. lividus*, serving as feeding stimulant in artificial diets (Dworjanyn *et al.*, 2007; Lawrence, 2007). *Laminaria digitata* (Hudson) J.V. Lamouroux, 1813, belonging to the Laminariales order, is a large brown algae, often reaching 4 metres in length, with a life span of 3-5 years (Werner & Kraan, 2004; Dittert *et al.*, 2014). It is found in the upper part of the sublittoral zone (Rueness, 1977; Nitschke *et al.*, 2011) and is a common kelp on the rocky shores from Norway to the Atlantic coast of Portugal, where high summer temperatures define the southern limit (Hoek, 1982). *L. digitata* is one of the most harvested species in Europe (Valero *et al.*, 2011; Kadam *et al.*, 2015) and in Portugal, large quantities can be found on the beaches, representing a potential resource for different sectors, including cosmetics and the food industry, mainly as an animal food additive (Dittert *et al.*, 2014; Schiener *et al.*, 2015). In 2014, approximately 28.5 million tonnes of seaweed and other algae were harvested (FAO, 2016a). In addition, the aquaculture industry already produces great quantities of *Laminaria* (Kim, 2011). *L. digitata*, like most of seaweeds, is rich in polysaccharides, peptides, omega-3 fatty acids, carotenoids (78-83%

fucoxanthin, 4-6% β,β-carotene and 3-13% violaxanthin), polyphenols, vitamins (mainly A, B3, B6 and C), minerals (notably calcium, potassium, magnesium and sodium) and other bioactive components such as alginic acid and laminarin (Haugan & Liaaen-Jensen, 1994; MacArtain *et al.*, 2007; Kadam *et al.*, 2015). However, the macroalgae use in large scale aquaculture is marked by some advantages. First, suitable macroalgae species are not permanently available throughout the year and some regions restrict their harvest. Besides, it is expensive and impractical to collect and store large amounts of macroalgae, like *L. digitata* (Pearce *et al.*, 2002; Basuyaux & Blin, 1998). Secondly, the seaweed's chemical composition, nutritional value and edibility go through significant variations according to the season, environmental conditions, seaweed's maturity, sampling site or habitat (Vadas *et al.*, 2000; Cook & Kelly, 2007a; Schiener *et al.*, 2015; FAO, 2016). Indeed, the highest concentrations of the different components of macroalgae, rarely coincide at the same season (Black, 1950). Furthermore, kelp forests have become severely deforested over the last three decades, mainly induced by sea urchins (Steneck *et al.*, 2002). All these reasons emphasize the necessity to create an economically viable diet that is able to simultaneously improve gonadosomatic index and organoleptic attributes of sea urchins' gonads (Symonds *et al.*, 2007).

1.8.7 Agar as a binding agent

In aquaculture, for the implementation of a nutrition research project, there is the need to adjust the formulated feed's stability according to the organism. For sea urchins, like *P. lividus*, food must remain intact in water for a significant period, ideally for several days (Caltagirone *et al.*, 1992). In this context, agar, a natural polysaccharide obtained from marine algae (Rhodophyceae), with its binding properties and three-dimensional network, allows the formulation of biocomposites, in which different food sources can be incorporated and nutrients become entrapped (Volpe *et al.*, 2010; Atef *et al.*, 2014). Caltagirone *et al.* (1992) and Fabbrocini *et al.* (2015) concluded that agar gave the best results for the formulation of a diet for *P. lividus*, when compared to other binders. This represents a first step for the creation of research-oriented trial-diets that can later lead to extruded or pelleted industrial feed (Fabbrocini *et al.*, 2015). Among the favourable properties of agar, it can be mentioned its insolubility in cold water, thus avoiding nutrient loss and food waste, its competitive price for large-scale use and its sustainable and biodegradable characteristics (Volpe *et al.*, 2010; Fabbrocini *et al.*, 2012, 2015). Some researchers have referred the use of agar biopolymer for the formulation of *P. lividus*' artificial diets, including Lawrence *et al.* (1989), Caltagirone *et al.* (1992), Lawrence *et al.* (1992), Shpigel *et al.* (2006), Vergés *et al.* (2007), Vergés *et al.* (2011) and notably, Fabbrocini *et al.* (2012, 2015). Other authors

have also used agar-based diets for other sea urchin species: Klinger (1982), Levin & Naidenko (1987), Lares & McClintock (1991), Klinger *et al.* (1994), Hammer *et al.* (2006), Hiratsuka & Uehara (2007), Heflin *et al.* (2016b) and White *et al.* (2016).

1.9 Objectives

In this study, we aimed to evaluate the effect of three diets on the gonads of the sea urchin *P. lividus*. The primary objectives of this study are the following:

- Evaluate the gonads enhancement with three artificial diets for *P. lividus*: maize and spinach (diet A); maize, spinach and kelp (diet B); maize, spinach and pumpkin (diet C);
- Determine the daily ingestion rates with each diet;
- Identify the best diet to enhance the gonadosomatic index and colour characteristics of the gonads and the somatic growth of *P. lividus*;
- Evaluate the reproductive development in sea urchins fed with the three diets.

2. Materials and Methods

2.1 Experimental design

The experimental design was comprised of three recirculating aquaculture systems (RAS), each for one of the tested diets. Each system consisted of three 60 L tanks, used as replicates, and a 70 L sump tank which was divided into three compartments. Each system was equipped with an air-cooled water chiller Frimar F200 (Fernando Ribeiro, Ltd., Barcarena, Portugal), mechanical filtration (wool and sponge filters), biological filtration through the use of plastic bio-balls, a Hailea HX-6530 water pump (Guangdong Hailea Group Co., Ltd., Guangdong, China) and a Bubble Magus C3.5 Needle Wheel Protein Skimmer (Jiyang Aquarium Equipment Co., Ltd., Jiangmen, China). Each tank was equipped with an air stone located at the bottom, for moderate continuous aeration that was adjusted to a similar rate for all of the tanks. During the trial period, including the first month of starvation, temperature, pH, dissolved oxygen and salinity were daily registered in the three rearing systems with the YSI Professional Plus (Pro Plus) handheld multiparameter meter (YSI Inc., Yellow Springs, OH, USA). Water samples from each system were collected every week for the determination of ammonia (± 0.04 mg L^{-1} accuracy), nitrate (± 0.5 mg L^{-1} accuracy), nitrite (± 4 mg L^{-1} accuracy for high range and ± 0.02 mg L^{-1} for low range) and phosphate (± 0.04 mg L^{-1} accuracy) concentrations, by photometric method using a Hanna HI 83203 Multiparameter Bench Photometer for Aquaculture (Hanna Instruments Inc., Rhode Island, USA).

2.2 Sea urchins collection

Wild adult individuals of *P. lividus* were collected from rocks in the intertidal zone of Praia do Abalo in Peniche, Portugal (39°22'12.7" N, 9°23'08.2" W) on the 21st and 22nd of September, 2016. Only individuals with a test diameter ranging from 30 to 45 mm, spines not included, were considered. The sea urchins were manually collected and immediately placed in a cool box, previously filled with seawater from the sampling site, in order to ensure the minimum physiological stress possible during the brief transportation to the Aquaculture laboratory of MARE (Polytechnic Institute of Leiria, Peniche, Portugal). There were selected 153 sea urchins for the trials, excluding the more damaged individuals. The sea urchins were carefully cleaned, in order to remove fragments of algae and small stones from the spines. Before randomly placing the individuals in the tanks, 17 per tank, all of them were weighed with an Adam PGL 3002 (Adam Equipment Co. Ltd, Milton Keynes, UK) precision scale (total wet weight of the individual, ± 0.01 g). Sea urchins were then

acclimatized to the laboratory conditions for approximately 2 weeks, at a temperature of 20 ± 1º C, and fed with macroalgae (*Codium tomentosum* and *L. digitata*) from Praia do Abalo.

2.3 Starving period

By the end of acclimation period, water temperature was decreased by 1º C every 5 days until reaching a temperature of 16º C. Thereafter, sea urchins were subjected to a period of starvation for 30 days, in order to induce the re-absorption of the gonads through the consumption of their content and hence, resetting the reproductive cycle to the spent stage (Spirlet *et al.*, 1998b). The water parameters were daily measured and were the following during this period: 16.17 ± 0.68 ºC water temperature; 35.5 ± 0.33 salinity; 8.1 ± 0.05 pH and 93 ± 1 % dissolved oxygen. In the end of the starvation period of 30 days (T1), 2 individuals (18 in total) were randomly selected from each tank and were weighed (total wet weight of the individual, ± 0.01 g), measured with a caliper (horizontal test diameter, ± 0.1 mm accuracy) and dissected. The respective gonads were carefully removed, weighed (± 0.01 g) and immediately fixed in 10% formalin solution for 24 hours. The gonadosomatic index (GI) was calculated as follows (Sartori & Gaion, 2015; Carboni *et al.*, 2015):

$$GI = \frac{\text{gonads wet weight (g)}}{\text{total wet weight (g)}} \text{x } 100$$

2.4 Feeding trial

Before the feeding trial (T2), all individuals (n=135) were, once again, weighed (total wet weight of the individual, ± 0.01 g) and measured with a caliper (horizontal test diameter, ± 0.1 mm accuracy). Regarding the sea urchins' mean size (test diameter) per tank, there were no significant statistical differences in the test diameter between the 3 systems/diets and between the 9 tanks, as assessed by one-way ANOVA. A value of $p < 0.05$ was chosen as level for statistical significance. Subsequently, the feeding experiment was carried out over a period of 3 months, from 10th November 2016 to 7th February 2017. The stock density was one individual per 4 L (Fabbrocini *et al.*, 2012) and individuals were kept in ambient light. Three jellified diets were tested: maize and spinach (diet A); maize, spinach and the macroalga *L. digitata* (diet B); maize, spinach and the pumpkin *C. maxima* (diet C). The three-tank systems were daily cleaned and partially refilled with fresh seawater.

2.5 Agar-diets formulation and feeding routine

Regarding the formulation and preparation of the feed pellets, also designated as biocomposites (Fabbrocini *et al.*, 2015; Paolucci *et al.*, 2015), it was used agar powder (Próvida, Mem Martins, Portugal) as a binder. The procedure was identical for the three diets: the agar powder was first weighed in order to attain a final concentration of 6%, in relation to the other ingredients (Klinger, 1982; Fabbrocini *et al.*, 2012). Preliminary tests confirmed the suitability of this concentration, given the substantial firmness of the final product. The agar was then dissolved in boiling water for some minutes, by gently stirring, in the proportion of 10 g of agar to 1 L of water, for the subsequent inclusion of the other ingredients in the following proportions:

- Diet A - 47% maize (*Z. mays*), 47% spinach (*S. oleracea*) and 6% agar;
- Diet B - 50% macroalga (*L. digitata*), 22% maize (*Z. mays*), 22% spinach (*S. oleracea*) and 6% agar;
- Diet C - 50% pumpkin (*C. maxima*), 22% maize (*Z. mays*), 22% spinach (*S. oleracea*) and 6% agar.

All ingredients were grinded into particles of few millimetres and blended before adding the agar solution, in order to obtain the most homogeneous biocomposites possible. Thereafter, the hot mixture was immediately poured into ice trays and allowed to cool and solidify at ambient temperature, for the subsequent storage in a refrigerator. Preliminary tests demonstrated the impossibility of freezing the agar-based feed, since it negatively affected the agar structure. Therefore, the three diets were manufactured fresh every week. It was used organic fresh spinach, of which only the leaves were considered for the diet, and canned maize. *L. digitata* was collected in large quantities from the sampling site, without visible evidences of epiphytic growth, and was frozen, given its expected unavailability during the period of the trials. The pumpkin *C. maxima* was originated from organic farming and was used fresh, including the peel, flesh and seeds.

Regarding the feeding routine, the three diets were daily cut into feed pellets of approximately 3 x 3 x 1.5 cm (L x W x H) and manually administered *ad libitum* (Fernandez & Boudouresque, 2000; Heflin *et al.*, 2016a). The ingestion rates were daily measured over the 90-days trial. Thus, at the beginning of each day, a known amount of feed was weighed (± 0.01 g) and then supplied to each of the nine tanks. Nine hours later, the feed not ingested was carefully collected, dried on absorbent paper and weighed (wet weight). The feed intake (wet weight) per tank was daily calculated as the difference between the given feed and the leftovers. With this information, the amount of feed provided to each tank was adapted on

a daily basis, in order to consistently obtain a significant portion of waste (at least 20% of the provided biomass). Posteriorly, feed intake (mean ± SD) was presented as g day^{-1} $individual^{-1}$.

2.6 Water parameters

The water parameters were daily measured during the three-month trial and are represented in Table I.

Table I - Physico-chemical parameters of the rearing systems water during the feeding trial (90 days) with three artificial diets. Data are expressed as mean ± SD

	Temperature (ºC)	Dissolved O_2 (%)	Salinity	pH
Diet A	19.06 ± 0.75	94.6 ± 1.16	35.8 ± 0.87	8.0 ± 0.16
Diet B	19.24 ± 1.10	94.3 ± 1.34	35.7 ± 0.83	8.0 ± 0.16
Diet C	18.98 ± 0.88	94.3 ± 1.31	35.7 ± 0.77	8.0 ± 0.18

The ammonia, nitrite, nitrate and phosphate levels during the three-month trial are represented in Table II.

Table II - Ammonia, nitrite, nitrate and phosphate levels of the rearing systems during the feeding trial (90 days) with three artificial diets. Data are expressed as mean ± SD

	Ammonia (mg L^{-1})	Nitrite (mg L^{-1})	Nitrate (mg L^{-1})	Phosphate (mg L^{-1})
Diet A	0.56 ± 0.32	0.07 ± 0.05	0.65 ± 1.59	0.18 ± 0.15
Diet B	0.56 ± 0.33	0.04 ± 0.00	0.68 ± 1.73	0.24 ± 0.10
Diet C	0.52 ± 0.26	0.07 ± 0.07	0.68 ± 1.68	0.16 ± 0.16

2.7 End of trial

At the end of the trial (T3), after 90 days of rearing period, all individuals (n=135) were measured with a caliper (horizontal test diameter, ± 0.1 mm accuracy) in order to calculate the mean ± SD test diameter. Then, they were briefly drip-dried with absorbent paper and weighed with an Adam PGL 3002 precision scale (total wet weight of the individual, ± 0.01 g) to calculate the mean ± SD total wet weight. Then, the sea urchins were

dissected by cutting around the peristomial membrane and the gonads were carefully removed in order to be wet weighed using an electronic analytical balance Sartorius TE214S (Sartorius AG, Göttingen, Germany) with a sensitivity of 0.0001 g. One of the five gonads was randomly chosen to be immediately photographed, under controlled conditions, for posterior colour analysis. Regarding the histological study of gametogenesis, ten sea urchins from each tank were randomly selected and two gonads from each individual were immediately placed in 10% formalin. With the obtained results, there were used equations for the GI, daily growth rate (DGR), total wet weight gain and linear growth rate (LGR), in order to compare the somatic and gonadal growth of the individuals fed with the three diets.

Daily growth rate (DGR in % day^{-1}) was calculated as follows:

$$DGR = \left[\left(\frac{W_{final}}{W_{initial}}\right)^{1/t} - 1\right] \times 100$$

$W_{initial}$ and W_{final} are the mean initial and final total wet weight (g) and *t* the number of days of the trial (Basuyaux & Blin, 1998).

Total wet weight gain (mg $urchin^{-1}$ day^{-1}) was adapted from Shpigel *et al.* (2004) as follows:

$$\text{Weight gain} = \frac{(W_{final} - W_{initial})}{t}$$

In which W_{final} and $W_{initial}$ represent the final and initial average total wet weight (mg), respectively and *t* represents time in days.

The linear growth rates (LGR) were adapted from Cook & Kelly (2009) and calculated in mm $month^{-1}$ as follows:

$$LGR = \frac{(L_f - L_i)}{t}$$

In which L_f and L_i were the final and initial average test diameters (mm), respectively and *t* represents time in days.

2.8 Photography and colour analysis

In order to capture a digital image of each gonad from all individuals, a DSLR Canon EOS 70D (Canon Inc., Tokyo, Japan), coupled with a Tamron SP AF 90mm F/2.8 Di Macro 1:1 lens (Tamron, Tokyo, Japan) was mounted to a Sachtler Ace M GS tripod (Sachtler GmbH & Co., Eching, Germany) in a dark room, facing towards a table covered with black sponge, where the gonads were placed. Two fluorescent lamps were positioned on the two

sides of this area, below the camera, with a constant light intensity of 1435 lux in the area to be photographed, measured with a Milwaukee SM700 Luxmeter (Milwaukee Inc., Rocky Mount, NC, USA). The camera control settings were kept constant for all of the 135 photographs and were the following: manual (exposure mode); Medium Raw format; 800 ISO; 1/125 seconds (shutter speed); F/4.5 (aperture); 0 EV (exposure compensation) and no flash. The resulting digital images were all 4104 x 2736 pixels, in the Canon's uncompressed file extension CR2.

In order to objectively evaluate the colour of all 135 gonads (45 gonads per diet; 30 gonads per diet when analysing males and females separately), the images were analysed with the Adobe Photoshop CC 2015 image software (Adobe Systems Inc., San Jose, California, USA), allowing an advanced and reliable colour analysis (Yam & Papadakis, 2004), according to Svensson *et al.* (2006) and Hu *et al.* (2015). First, there was the need to convert the CR2 digital images into the PNG image format, given its versatility and lossless compression scheme (Wiggins *et al.*, 2001). Thus, allowing the photographs to be compatible with the Photoshop software. The images were then converted CIE L*****a*****b***** (CIELAB, CIE76), a colour notation system of the CIE-Commission Internationale de l'Eclairage, regarded as the most complete and perceptually uniform colour space and is also device-independent (Li *et al.*, 2017; Schur & Tappert, 2017). Since L***** represents lightness, a***** represents the balance between green and red, and b***** includes the blue–yellow range, a***** value was used to define the "redness" of each gonad, which is also highly related to carotenoid concentration (Hatlen *et al.*, 1998). Indeed, higher a***** values are correlated with a more accentuated red colour (Hunter & Harold, 1987). For each photograph, the gonad was first carefully isolated from the black background with the Photoshop's Quick Selection Tool. Then, using the Histogram tool, the mean value of the a***** parameter was registered.

2.9 Sex ratio and gametogenic stages

Two intact gonads from each sea urchin, in a total of 90 individuals, were placed into a pre-labelled plastic tissue cassette and then put in a plastic container to be fixed in 10% formalin solution (Sartori & Gaion, 2015) for 48h. After this period, the 90 cassettes were transferred to a 70% ethanol solution, for storage (Delorme & Sewell, 2016). Later, the samples were processed by a Leica® TP1020 Automatic Tissue Processor (Leica Microsystems GmbH, Wetzlar, Germany), with sequential submersions in graded ethanol (80%, 96% and 100%) for dehydration, followed by xylene for clarification and finally, impregnation with paraffin wax at 60º C. Subsequently, gonad samples were embedded in

100% (v/v) paraffin (Leica® EG 1120 Paraffin Dispenser with integrated Hot Plate, Leica Microsystems GmbH, Wetzlar, Germany), using Tissue-Tek stainless-steel base molds (Sakura Finetek Europe BV, Netherlands). Solid paraffin blocks were obtained with a Kunz Instruments CP-4 Cooling Plate (Kunz Instruments, Denmark), each block containing the two gonads from one individual with the respective identified plastic cassette. The paraffin blocks were cut, minimum of three replicate tissue sections, with a thickness of 5 µm (Carboni *et al.*, 2013; Paredes & Bellas, 2014), using an Accu-Cut® SRM™ 200 Rotary Microtome (Sakura Finetek Europe BV, Netherlands). The paraffin sections were allowed to stretch in a Sakura 1452 Hot Plate (Sakura Finetek Europe BV, Netherlands) for some minutes and were wet mounted onto glass microscope slides (Normax, Marinha, Grande, Portugal) to be dried overnight at 37 ºC (Sartori, 2013). All sections were stained with Harris' haematoxylin solution (Scharlab S.L., Sentmenat, Barcelona, Spain) and eosin Y (yellowish) (VWR International, Leuven, Belgium), remaining in xylene until coverslips were mounted using Coverquick 2000 mounting Medium (VWR Chemicals, Fontenay-sous-bois, France) and left to dry in open air. Gonad slides were analysed using a Leica® DM 2000 LED light optical microscope equipped with a Leica® MC170 5MP HD Microscope Camera and the combined LAS V4.4.0 software (Leica Application Suite), for monitor display (Leica Microsystems GmbH, Wetzlar, Germany). Gametogenic stages were classified according to Spirlet *et al.* (1998a) into eight stages: stage I (spent with relict), stage II (spent empty), stage III (recovery), stage IV (growing), stage V (premature), stage VI (mature), stage VII (partly-spawned) and stage VIII (post-spawned). The size frequencies of the oocytes were obtained using the LAS software distance line tool, performed at 20X amplification. For each female individual, 50 oocytes/ova sectioned through the nucleolus/nucleus were randomly selected in order to measure the corresponding long diameter to the nearest 0.001 µm. Relict oocytes were not considered (Byrne, 1990; Bronstein *et al.*, 2016).

2.10 Statistical analysis

All statistical tests were performed using IBM SPSS™ Statistics for Windows, version 23 (IBM Corporation, Armonk, New York, U.S.). Results are expressed as mean ± 1 standard deviation (SD) and a value of $p < 0.05$ was chosen as level for significance. All data were tested for normality with the *Shapiro-Wilk* normality test and for homogeneity of variances with Levene's test, in order to meet the assumptions of analyses of variance (ANOVA). Differences in test diameter, total wet weight, gonadosomatic index, gonadal wet weight, feed ingestion and gonad colour (a***** parameter) were tested by one-way ANOVA ($F_{(\text{degrees of freedom between groups, degrees of freedom within groups})}$ = F value; significance *p* value) and independent *t*-test ($t_{(\text{degrees of freedom})}$ = t-value; significance *p*-value). Whenever the ANOVA

assumptions failed, it was used the nonparametric *Kruskal-Wallis (*$H_{\text{(degrees of freedom)}}$ = chi-square value*;* significance *p* value*)*. In case of statically significant differences, multiple pairwise comparisons were performed using the post-hoc parametric *Tukey* test or the non-parametric *Games-Howell* test. To test a possible association between diets and gametogenic stages, it was used a likelihood ratio test (LRT) ($\chi^2_{\text{(degrees of freedom)}}$ = value; significance *p*-value), as a consequence of assumptions violation of the chi-square test.

3. Results

In the starving period of 30 days that was performed with 51 sea urchins per system, in a total of 153 individuals, no individual died during this period. Furthermore, during the feeding trial of 90 days, that initiated with 45 sea urchins per diet, in a total of 135 individuals (T2), all of them survived until the end of the experiment (T3).

3.1 Test diameter

The initial test diameter, with 45 sea urchins per diet, was 35.7 ± 3.1 mm in the individuals fed with diet A (maize & spinach); 35.0 ± 3.1 mm in the individuals fed with diet B (maize, spinach & *L. digitata*) and 35.9 ± 3.1 mm in the individuals fed with diet C (maize, spinach & *C. maxima*), as shown in figure 1. As previously mentioned, it was assured that no statistically significant differences in size were found between the individuals of the three diets at the beginning of the trial (T2), as determined by a one-way ANOVA [$F_{(2, 132)} = 0.927$; $p = 0.398$], and also between the nine replicates [$F_{(8, 126)} = 0.465$; $p = 0.878$].

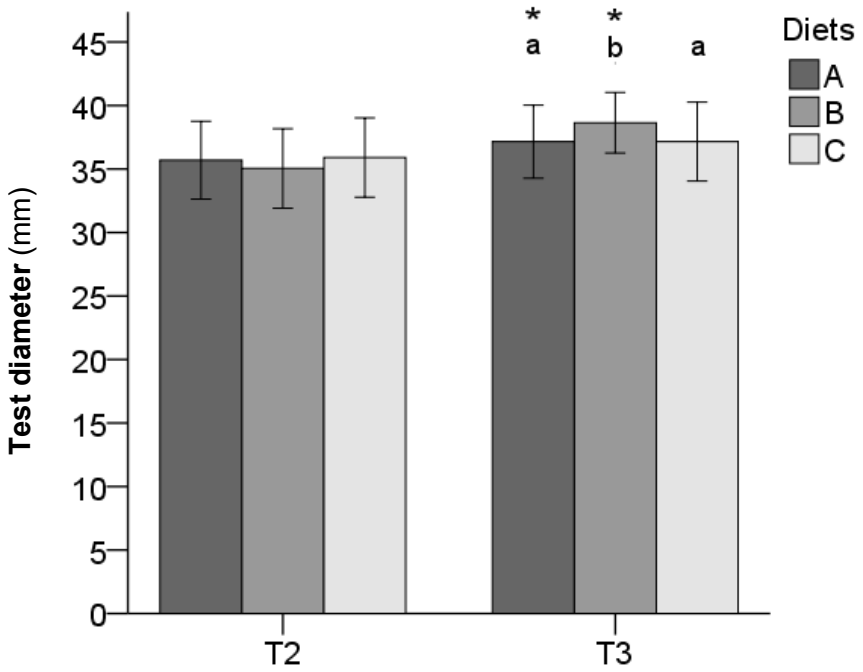

Figure 1 - Test diameter (mm) (mean ± SD) in the beginning of the trial (T2) and after the 90 days of the feeding trial (T3) with three jellified diets. Diet A: maize & spinach. Diet B: maize, spinach & *Laminaria digitata*. Diet C: maize, spinach & *Cucurbita maxima*. Note: * represents statistically significant differences between time periods for each diet; for each time period, bars sharing the same letter are not significantly different according to Tukey's HSD test.

At the end of the experiment, 90 days later, statistically significant size differences were registered between the three diet groups, using one-way ANOVA [$F_{(2, 132)} = 4.22$; $p = 0.017$]. Post hoc comparisons using the Tukey HSD test indicated that the mean test diameter of sea urchins from diet B (38.6 ± 2.4 mm) was significantly higher than the individuals from diet A (37.2 ± 2.9 mm, p= 0.35) and diet C (37.2 ± 3.1 mm, p = 0.35). There was no statistically significant difference between diets A and C (p = 1.0). Therefore, diet B was the most successful in promoting test growth, with a mean monthly growth rate of 1.2 mm, as shown in figure 2. It was followed by diet A (0.5 mm $month^{-1}$) and diet C (0.43 mm $month^{-1}$).

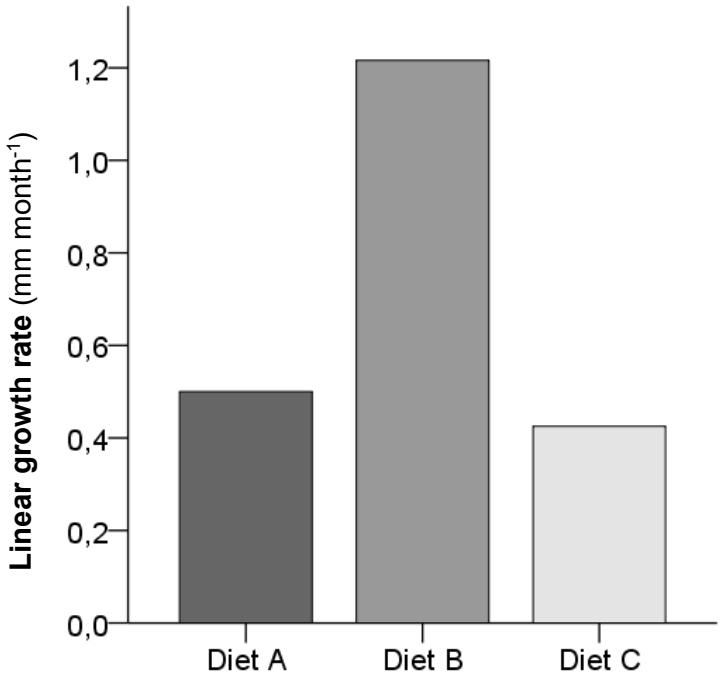

Figure 2 – Linear growth rate (mm $month^{-1}$) of *Paracentrotus lividus* fed with three jellified diets over a period of 90 days. Diet A: maize & spinach. Diet B: maize, spinach & *Laminaria digitata*. Diet C: maize, spinach & *Cucurbita maxima*.

Three independent *t*-tests were conducted to compare differences in size, between the beginning and the end of the trial, for each diet. Sea urchins from diet A were statistically significantly bigger (37.2 ± 2.9 mm) when compared to the beginning of the trial (35.7 ± 3.1 mm), $t_{(88)} = - 2.887$; $p = 0.021$. Similarly for diet B, individuals had also a significantly higher test diameter (38.6 ± 2.4 mm) in comparison to the initial sizes (35.0 ± 3.1 mm), $t_{(88)} = - 6.131$; $p = 0.000$. However, sea urchins fed diet C do not show a

statically significantly higher test diameter (37.2 ± 3.1 mm) in the end, compared to the onset of the trial (35.9 ± 3.1 mm), $t_{(88)}$ = - 1.912; p = 0.059.

3.2 Total wet weight

As shown in figure 3, before the 30 days period of starvation (T0), all individuals (17 per replicate; 51 per system) were wet weighed and no statistically significant differences were found between the individuals cultured in three systems, as assessed by a one-way ANOVA [$F_{(2, 150)}$ = 1.223; p = 0.297]. At the end of the starvation period (T1), after a new weight measurement, there was registered a 2.9% decrease in the mean total wet weight of the individuals in the three systems.

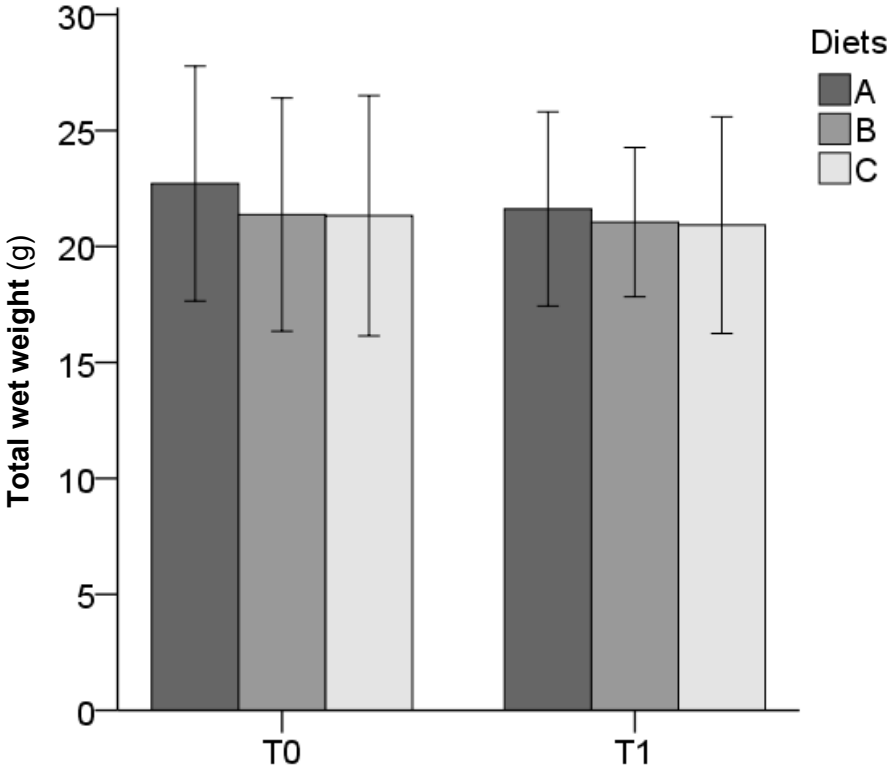

Figure 3 - Total wet weight (g) (mean ± SD) of *Paracentrotus lividus* individuals before the starvation period (T0) and in the end of the starvation period of 30 days (T1).

Before the onset of the feed trials, six individuals were sacrificed per diet. Thus, at this point (T2), the total wet weight of the sea urchins was the following in each system/diet (n=45): 22.46 ± 4.99 g (diet A); 23.00 ± 5.23 g (diet B) and 21.48 ± 4.99 g (diet C), as shown in figure 4. Once again, there were no statistically significant differences regarding the total wet weight, between individuals fed with the three diets in the beginning of the feeding trials (T2), as determined by one-way ANOVA [$F_{(2, 132)}$ = 1.030; p = 0.360].

After the feeding period of 90 days (T3), all the 45 sea urchins per diet were weighed one last time, with the following results (figure 4): 26.10 ± 4.98 g (diet A); 30.19 ± 5.18 g (diet B) and 26.30 ± 5.25 g (diet C). This time, there were statistically significant differences in weight between diets, as showed by a one-way ANOVA [$F_{(2, 132)} = 9.058$; $p = 0.000$]. The Tukey HSD tests showed that the total wet weight of individuals from diet B, similarly to the results in test diameter, was significantly higher than the individuals fed with diet A (p=0.001) and diet C (p=0.001). Therefore, suggesting, once again, a superior efficiency of diet B in promoting somatic growth. There was no statistically significant difference in weight between diets A and C (p = 0.981).

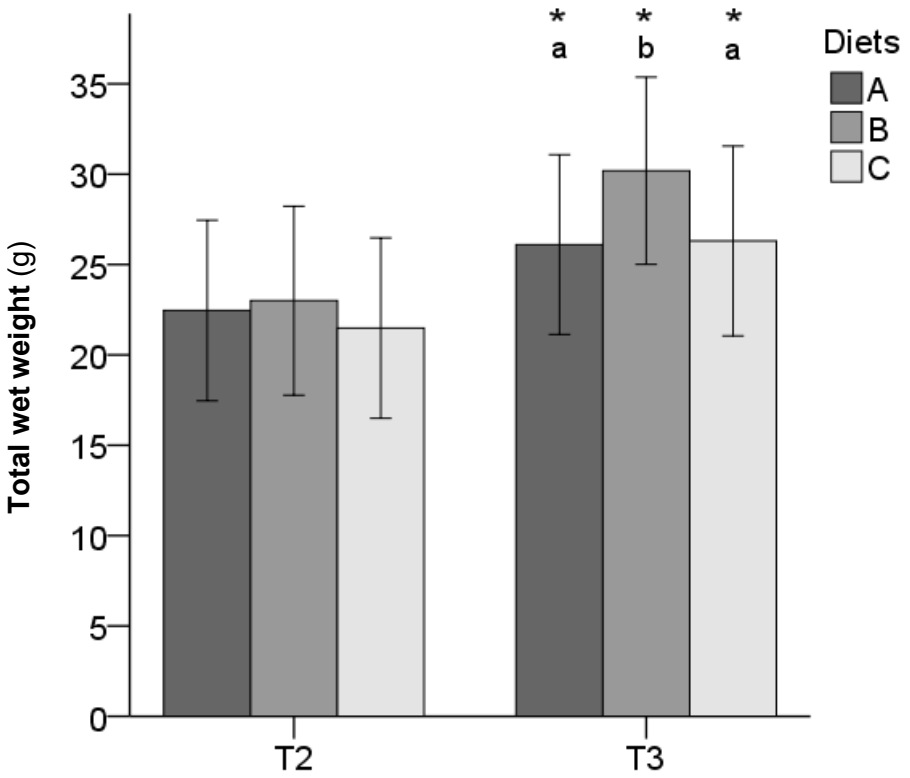

Figure 4 - Total wet weight (g) (mean ± SD) of *Paracentrotus lividus* in the beginning of the feeding trial (T2 - after the sacrifice of 6 individuals per diet) and at the end of the feeding trial of 90 days (T3), with three jellified diets. Diet A: maize & spinach. Diet B: maize, spinach & *Laminaria digitata*. Diet C: maize, spinach & *Cucurbita maxima*. Note: * represents statistically significant differences between time periods for each diet; for each time period, bars sharing the same letter are not significantly different according to Tukey's HSD test.

Finally, regarding the total wet weight gain (mg day^{-1}) in each diet, three independent t-tests determined that the sea urchins were statistically significantly heavier in the three diets, when compared to the beginning of the feed trial. As expressed in figures 4 and 5, individuals fed with diet A, with a total wet weight increment of 40.52 mg day^{-1}, presented a significant weight increase: $t_{(88)} = - 3.469$; $p = 0.001$. Particularly, sea urchins fed with diet

B were also significantly heavier, [$t_{(88)}$ = -6.555; p = 0.000], showing an average total wet weight increment of 79.89 mg day^{-1}. Diet C also resulted in significantly heavier individuals [$t_{(88)}$ = - 4.463; p = 0.000], with an average total wet weight increase of 53.53 mg day^{-1}, when compared to the beginning of the trial.

Regarding the daily growth rates (DGR), the results were the following for each diet: 0.17% day^{-1} (Diet A); 0.30% day^{-1} (Diet B) and 0.22% day^{-1} (Diet C).

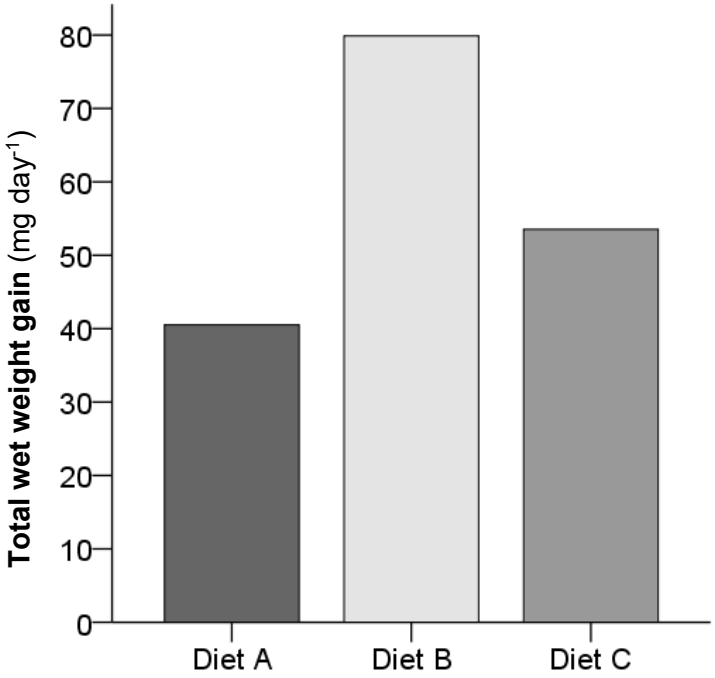

Figure 5 - Total wet weight gain (mg day^{-1}) of *Paracentrotus lividus* fed with three artificial diets over a period of 90 days. Diet A: maize & spinach. Diet B: maize, spinach & *Laminaria digitata*. Diet C: maize, spinach & *Cucurbita maxima*.

3.3 Gonadosomatic index

The gonadosomatic index (GI) values in the end of the trial for each diet, as well as the mean GI value of the 18 sea urchins sacrificed after the starving period, are shown in figure 6. The starving period of 30 days, with all 153 individuals at the same abiotic conditions, led to a GI mean value of 3.33%. In the end of the 90 days feeding trial, the GI was 9.07 ± 2.39 % (diet A); 7.17 ± 1.99 % (diet B) and 7.31 ± 2.26 % (diet C). Therefore, the three diets promoted gonadal growth, however, a one-way ANOVA indicates that there were statically significant differences between the GI of individuals fed with the three different diets [$F_{(2, 132)}$ = 10.283; p = 0.000]. The GI of the sea urchins fed with diet A (maize

& spinach) is significantly higher when compared to the results of diet B (p=0.000) and diet C (p=0.001), as determined by Tukey HSD tests. There were no significant differences in GI between diets B and C (p=0.955).

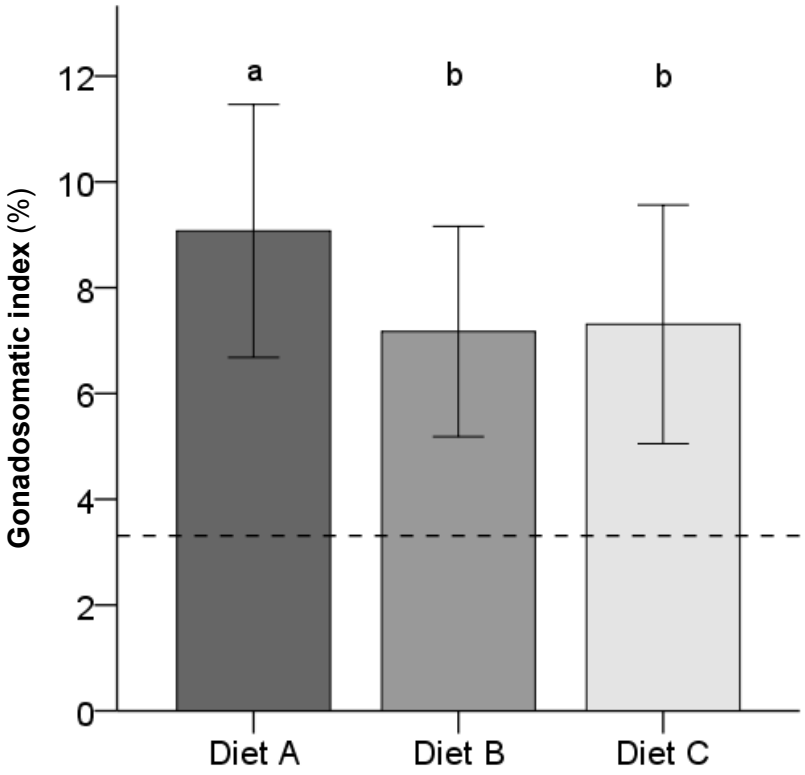

Figure 6 - Gonadosomatic index (%) (mean ± SD) of *Paracentrotus lividus* at the end of the feeding trial of 90 days with three jellified diets. Diet A: maize & spinach. Diet B: maize, spinach & *Laminaria digitata*. Diet C: maize, spinach & *Cucurbita maxima*. The average GI of the 18 sacrificed individuals in the end of the starvation period is reported (3.33%). Note: bars sharing the same letter are not significantly different according to Tukey's HSD test.

3.4 Gonadal wet weight

The gonadal wet weight values in the end of the trial for each diet, as well as the mean gonadal wet weight of the 18 sea urchins sacrificed after the starving period, are shown in figure 7. The starving period of 30 days led to a mean gonadal wet weight of 0,715 g. Similarly to the gonadosomatic index, a considerable increase in the weight of the gonads was recorded, with diet A (maize & spinach) also producing the best results in enhancing gonad growth. A one-way ANOVA determined the existence of statically significant differences between diets [$F_{(2, 132)} = 3.224$; $p = 0.043$]. In this case, the gonads of the sea urchins fed with diet A, with a mean gonadal wet weight of 2.3759 ± 0.7662 g,

are significantly heavier than the gonads of the individuals fed with diet C, with a mean value of 1.9567 ± 0.8298 g (p = 0,033). However, there are no statically significant differences between the gonadal wet weight of individuals fed with diet B (2.1812 ± 0.7527 g) and diet A (p = 0.468) and diet C (p = 0.366), as determined by Tukey HSD tests.

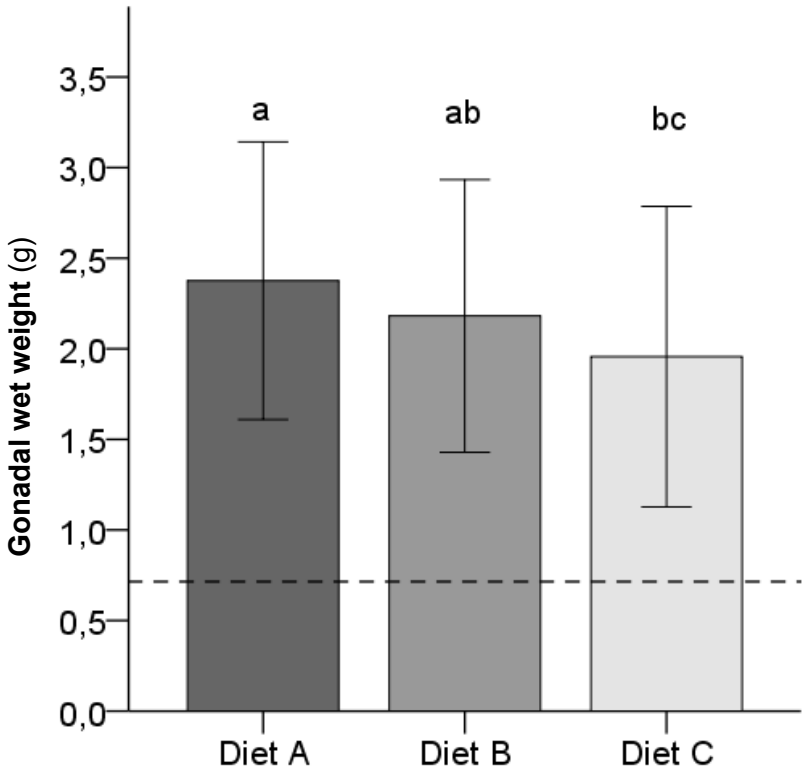

Figure 7 - Gonadal wet weight (g) (mean ± SD) of *Paracentrotus lividus* in the end of the feeding trial of 90 days with three artificial diets. Diet A: maize & spinach. Diet B: maize, spinach & *Laminaria digitata*. Diet C: maize, spinach & *Cucurbita maxima*. The average gonadal wet weight of the 18 sacrificed individuals at the end of the starvation period is reported (0,715 g). Note: bars sharing the same letter are not significantly different according to Tukey's HSD test.

3.5 Feed ingestion

Before analysing the total mean feed ingestion at the end of the trial, there was the need to understand the evolution of feeding behaviour over the three months, as shown in figure 8. In the first month, there were statically significant differences between diets [Kruskal-Wallis H test; $H_{(2)}$ = 13.688; p = 0.001], in which a Games-Howell test showed that the sea urchins fed with diet C were consuming significantly more feed than those fed with diet B (p = 0.001). Regarding the second month, there was also significant differences in the feeding rate [one-way ANOVA; $F_{(2, 84)}$ = 32.104; p = 0.000], as diet C was significantly more consumed than diet A (p = 0.000) and diet B (p = 0.004), and also, diet B was

significantly more consumed than diet A (p = 0.000). However, in the third month, still with statically significant differences [$H_{(2)}$ = 59.086; p = 0.000], diet B was the most consumed. Sea urchins fed with diet A were feeding significantly much less than those fed with diet B (p = 0.000) and diet C (p = 0.000).

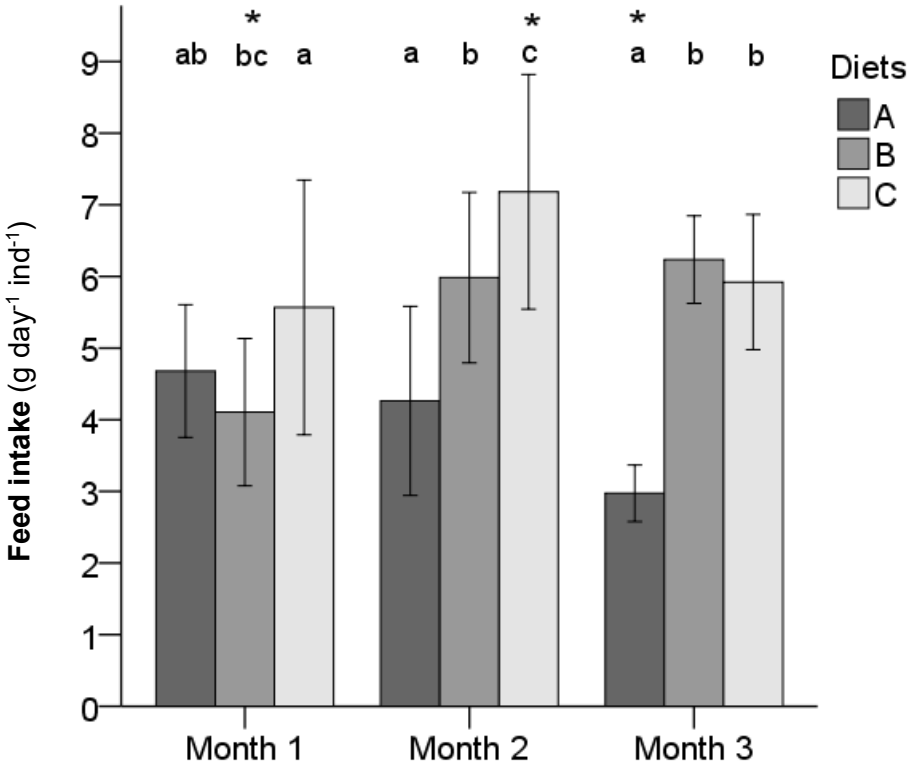

Figure 8 - Feed intake (g day^{-1} ind^{-1}) (mean ± SD) of *Paracentrotus lividus* individuals fed with three artificial diets, in each of the three months of the feeding trial (90 days). Diet A: maize & spinach. Diet B: maize, spinach & *Laminaria digitata*. Diet C: maize, spinach & *Cucurbita maxima*. Note: * represents statistically significant differences between time periods for each diet; for each month, bars sharing the same letter are not significantly different according to Tukey's HSD test.

When analysing the feeding behaviour changes in each diet over time (figure 8), diet A with statically significant differences between the three months [$H_{(2)}$ = 42.144; p = 0.000], was significantly less consumed in the final month, when compared to the first (p = 0.000) and second (p = 0.000). This diet was consumed gradually less over time. Diet B, on the contrary, showing an increasing feeding rate by the sea urchins and having also significant differences between months [$H_{(2)}$ = 42.802; p = 0.000], was less consumed in the first month, compared to month 2 (p = 0.000) and 3 (p = 0.000). Finally, diet 3 [$H_{(2)}$ = 14.046; p = 0.001] was significantly more consumed in the second month, when compared to the first (p = 0.002) and third month (p = 0.002).

The total feed intake, over the three-month trial, represented in figure 9, was the following in each diet: 3.97 ± 1.19 g day^{-1} ind.$^{-1}$ (Diet A); 5.44 ± 1.36 g day^{-1} ind.$^{-1}$ (Diet B); 6.21 ± 1.63 g day^{-1} ind.$^{-1}$ (Diet C). There were statistically significant differences in ingestion, between the three diets [Kruskal-Wallis H test: $H_{(2)}$ = 81.594; p = 0.000], in which a Games-Howell test showed that the consumption of diet C was significantly higher, when compared to diet A (p = 0.000) and diet B (p = 0.002), highlighting the particular preference for the pumpkin containing feed. Similarly, the sea urchins from diet B (containing *L. digitata*) consumed significantly more food than the individuals from diet A (p = 0.000).

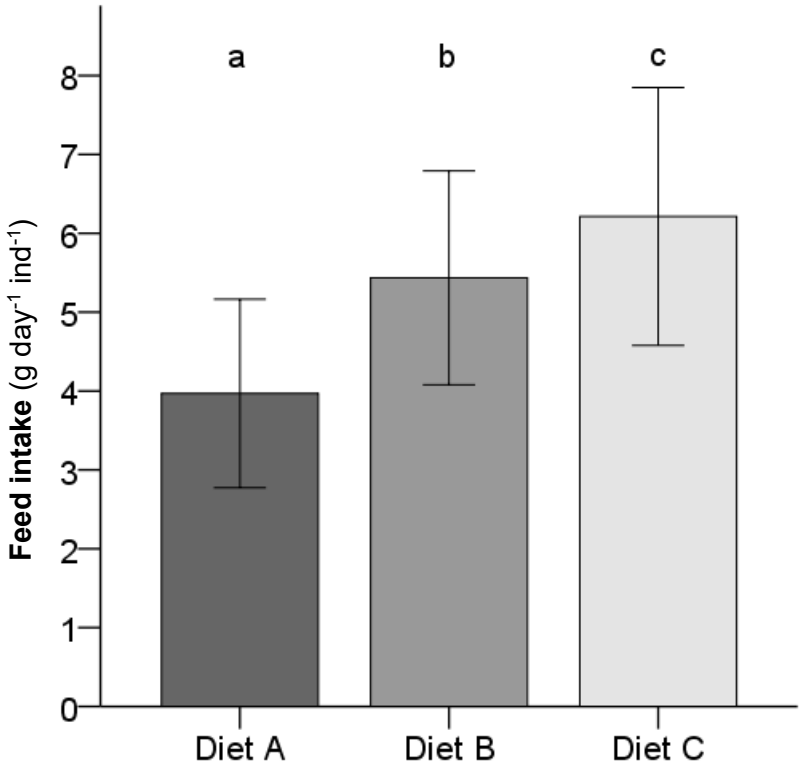

Figure 9 - Feed intake (g day^{-1} individual^{-1}) (mean ± SD) of *Paracentrotus lividus* during 90 days of feeding trial with three artificial diets. Diet A: maize & spinach. Diet B: maize, spinach & *Laminaria digitata*. Diet C: maize, spinach & *Cucurbita maxima*. Note: bars sharing the same letter are not significantly different according to Tukey's HSD test.

3.6 Sex ratio and gametogenic stages

3.6.1 Sex ratio

Regarding the sex ratio, of the 18 individuals sacrificed at the end of the starving period, 12 were females and 6 were males. At the end of the feeding trial, of the total number

of individuals sampled for histology (90 in total, 30 per diet), 44 were females and 46 were males, resulting in a ratio of 0.96 : 1.

3.6.2 Gametogenic stages

Concerning the gametogenic cycle, gonads were classified into 8 stages, according to Spirlet *et al.* (1998a). Figure 10 shows the relative frequencies of the different reproductive stages at the end of the starvation period and at the end of the feeding trial, for each diet. As expected, after the starving period of 30 days, 83.3% of the 18 sacrificed sea urchins were in spent stage, with 22.2% in stage I (spent with relict gametes) and 61.1% in stage II (spent empty). In the remaining individuals, 11.1% were in stage VIII (post-spawned) and 5.6% in stage III (recovery).

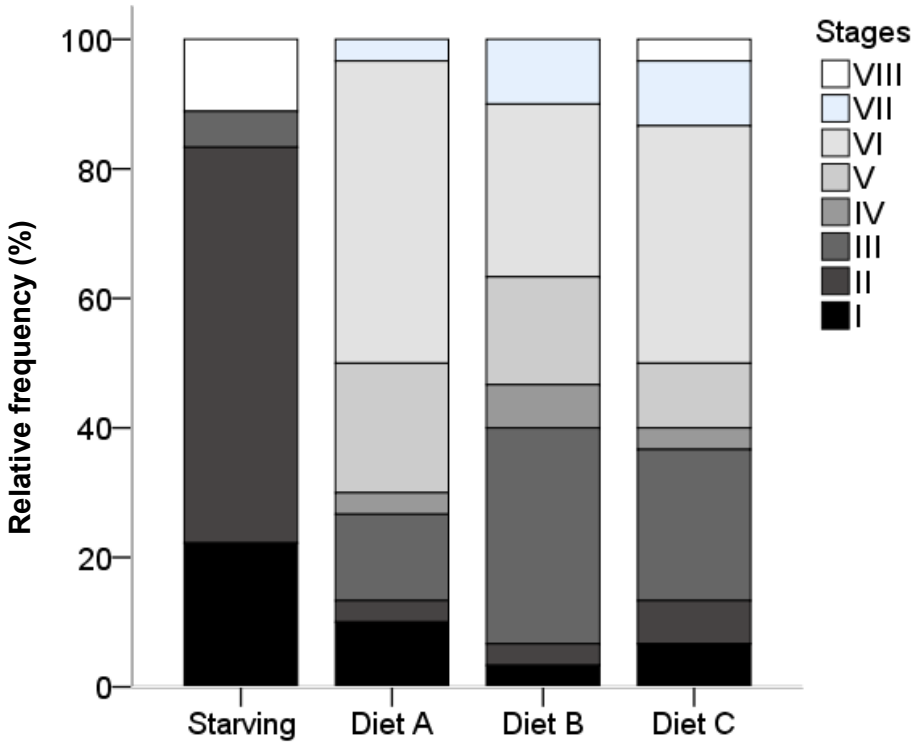

Figure 10 - Reproductive condition of *Paracentrotus lividus* after 30 days of starvation and after 90 days of feeding with three artificial diets. Diet A: maize & spinach. Diet B: maize, spinach & *Laminaria digitata*. Diet C: maize, spinach & *Cucurbita maxima*. Stages: I - spent with relict; II - spent empty; III – recovery; V – growing; V – premature; VI – mature; VII - partly-spawned; VIII - post-spawned.

After 90 days of rearing with three artificial diets, the histological analysis revealed substantial development in the gonads' reproductive condition. Although there is no strong association between diets and the eight gametogenic stages ($\chi^2_{(14)}$ = 10,876; p = 0,696),

the results must be analysed quantitatively. At this time, the number of individuals in spent condition, including stages I and II, was significantly lower in the three diets: 13.3% (diet A); 6.7% (diet B) and 13.3 % (diet C). In particular, diet B was the most successful in promoting the progression from this inactive phase of gametogenesis (spent stage). However, 33.3% of the individuals from diet B was still in recovery stage (stage III), in opposition to diet A, with only 13.3%, and diet C with 23.3%. A stage characterized by small primary sexual cells, but with a meshwork of nutritive phagocytes across the ascinus. Regarding the growing phase (stage IV), relative frequencies are similar between diets: 3.3% (diet A); 6.7% (diet B) and 3.3% (diet C).

All three diets promoted a significant sexual maturation, however, diet A (maize & spinach) was the most successful, with 20% of the sea urchins in premature stage (V), already with mature gametes and able to spawn, and most notably, 46.7% in mature stage (stage VI). Therefore, 66.7% of the total individuals from diet A had mature ova and spermatozoa. It was followed by diet C, with 46.7% containing mature gametes, specifically 10% in premature stage (V) and 36.7% in mature stage (VI). Finally, in diet B, individuals in mature stage (VI) only accounted for 26.7% of the total and 16.7% was in premature stage (V).

Partly-spawned stage (VII) is derived from premature stage, in which occurs spawning of gametes, and few individuals were assigned to this stage: 3.3% (diet A); 10% (diet B) and 10% (diet C).

Figures 11 and 12 illustrate all of the eight gametogenic stages, through histological analysis, of female and male individuals of *P. lividus* from the feeding trial.

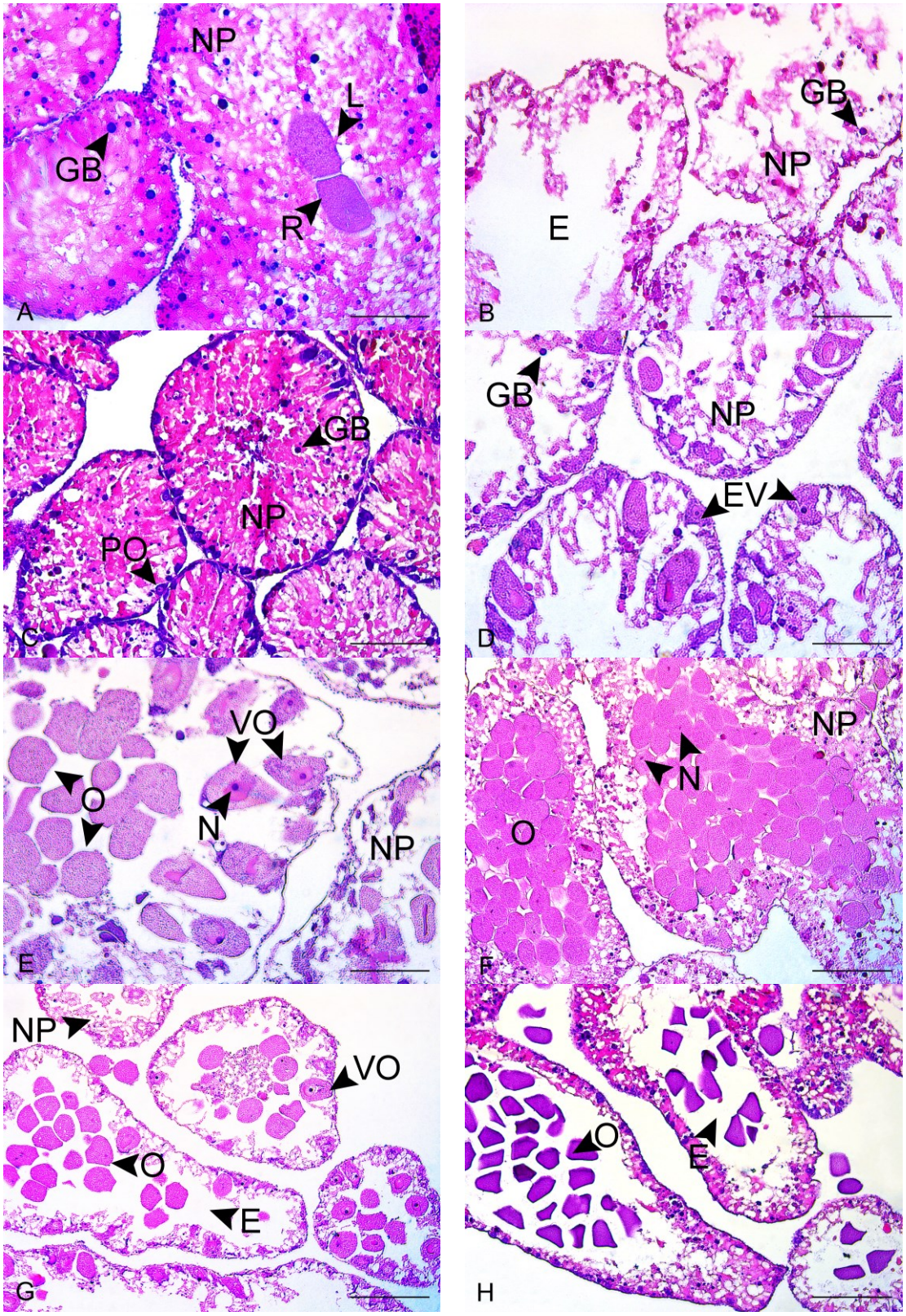

Figure 11 - Microphotographs of histological sections of *Paracentrotus lividus* ovaries, conditioned with three artificial diets over a period of 90 days. Diet A: maize & spinach. Diet B: maize, spinach & macroalga (*Laminaria digitata*). Diet C: maize, spinach & pumpkin (*Cucurbita maxima*). **(A)** Stage I: cross-section through ascinus of late spent ovary with relict ova (R) being lysed and reabsorbed by the already formed meshwork of nutritive phagocytes (NP), with visible globules (GB) resulting from previous lysis of relict ova. **(B)** Stage II: ovary in spent empty stage, with a thin ascinal wall, a pale

meshwork of nutritive phagocytes and empty lumen (E). **(C)** Stage III: recovering ovary with clusters of previtellogenic oocytes (PO) along the ascinal wall; ascini are filled with a meshwork of nutritive phagocytes (NP) containing globules (GB). **(D)** Stage IV: ovary in growing stage with early vitellogenic oocytes in contact with the ascinal wall (EV). **(E)** Stage V: premature ovary containing oocytes at all stages of development, including vitellogenic oocytes (VO) with visible nucleus (N) migrating towards the centre and ova (O) accumulated in the lumen, displacing nutritive phagocytes. **(F)** Stage VI: ovary in mature stage filled with closely aggregated ova and a thin layer of nutritive phagocytes along the ascinal wall. **(G)** Stage VII: partly-spawned ovary with empty spaces (E) resulting from spawned ova; oogenesis is still active, as in stage V, with primary oocytes still maturing and mature ova in the lumen. **(H)** Stage VIII: ovary in post-spawned stage with several empty spaces and the presence of several unspawned ova; the ascinal wall is now almost devoid of sexual cells or nutritive phagocytes. (Haematoxylin-eosin stain. Scale bars: A, B, D, E = 100 µm; C, F, G, H = 200 µm).

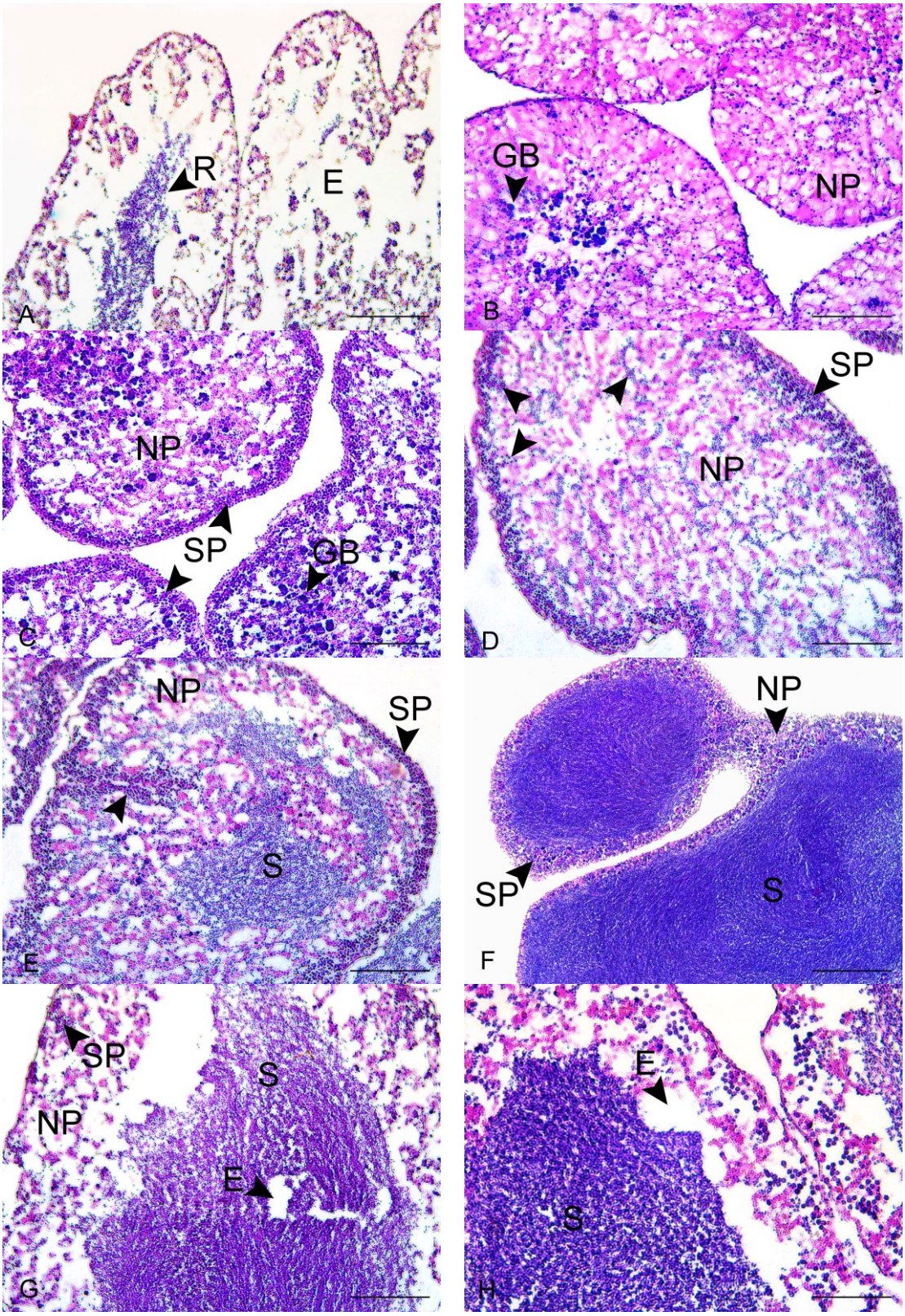

Figure 12 – Microphotographs of histological sections of *Paracentrotus lividus* testes, conditioned with three artificial diets over a period of 90 days. Diet A: maize & spinach. Diet B: maize, spinach & macroalga (*Laminaria digitata*). Diet C: maize, spinach & pumpkin (*Cucurbita maxima*). **(A)** Stage I: cross-section through ascinus of spent testis with relict spermatozoa (R) but mainly devoid of contents, also presenting an empty lumen (E). **(B)** Stage II: testis in spent empty stage, already filled with a meshwork of nutritive phagocytes (NP) containing globules (GB) derived from relict

spermatozoa; almost no spermatogonia is found in the ascinal wall. **(C)** Stage III: recovering testis with a new layer of primary spermatocytes and spermatogonia (SP); several globules visible in the nutritive phagocytes. **(D)** Stage IV: testis in growing stage with a ticker layer of spermatocytes (SP) and columns of spermatocytes are visible projecting centrally (arrows), through the nutritive phagocyte meshwork (NP). **(E)** Stage V: premature testis with a very distinct column of spermatocytes (arrow) and mature spermatozoa (S) already accumulated in the lumen, displacing the nutritive phagocytes (NP). **(F)** Stage VI: mature testis filled with spermatozoa and few nutritive phagocytes confined to the ascinus periphery. Spermatogenesis is now only residual, despite the presence of some spermatocytes. **(G)** Stage VII: testis in partly-spawned stage, similar to stage V, however with spaces (E) in the lumen, as a consequence of released spermatozoa. **(H)** Stage VIII: post-spawned testis with empty spaces between unspawned spermatozoa and the ascinal wall which is almost devoid of spermatogonia or nutritive phagocytes. (Haematoxylin-eosin stain. Scale bars: A, B, C, D, E, G = 100 µm; F = 200 µm; H = 50 µm).

3.6.3 Oocyte diameter

The oocyte/ova diameter measurements in the ovaries of females were the following in each diet: 55.331 ± 22.276 µm (diet A); 46.149 ± 26.529 µm (diet B) and 54.727 ± 26.278 µm (diet C). There were no statistically significant differences between diets, as showed by a one-way ANOVA [$F_{(2, 34)} = 0.512$; $p = 0.604$]. However, the mean values of the oocyte diameters provide little information, therefore, figure 13 represents the oocyte size-frequency distributions in the gonads of females fed with each of the three diets.

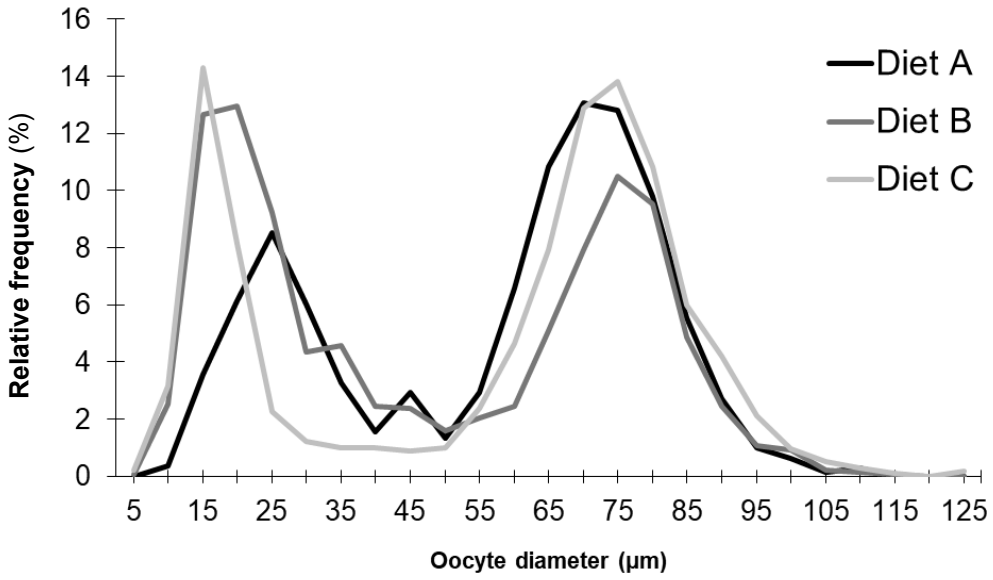

Figure 13 – Oocyte-size frequency distributions of *Paracentrotus lividus* females fed with three artificial diets for 90 days. Diet A: maize & spinach. Diet B: maize, spinach & *Laminaria digitata*. Diet C: maize, spinach & *Cucurbita maxima*.

Despite the similarity of the mean values between diets A and C, the results coincide with the relative frequencies of the gametogenic stages, since diet A led to the major proportion of mature individuals, with large oocytes of 65-85 µm in diameter as the major size-class. It was followed by diet C, with the prevalence of the 70-80 µm size-class, although 22% correspond to the 10-20 µm size-class of small oocytes. Figure 13 reveals the presence of small oocytes in all diets, especially in diets B and C, mainly associated to the recovery stage (III) and growing stage (IV). However, as expected, small oocytes mainly between 10-25 µm in diameter, were the most numerous size-class present in diet B.

3.7 Gonad colour analysis

Regarding the gonads colour analysis, the total CIELAB a∗ values of the sea urchins' gonads from the three diets ranged from 129.96 to 160.94. Through a one-way ANOVA there were detected statically significant differences in gonads colour, between diets [$F_{(2, 132)} = 3.607$; $p = 0.030$]. As shown in figure 14, the gonads of individuals fed with diet C, with an average a* parameter of 144.42 ± 5.45 were significantly more red than gonads of the individuals fed with diet B ($p = 0.042$), these with an average value of 141.47 ± 5.74. However, there were no statically significant differences in colour, between gonads from diet A, with a* value of 141.78 ± 5.98 and gonads from diet B ($p = 0.965$) and diet C ($p = 0.077$), as determined by Tukey HSD tests.

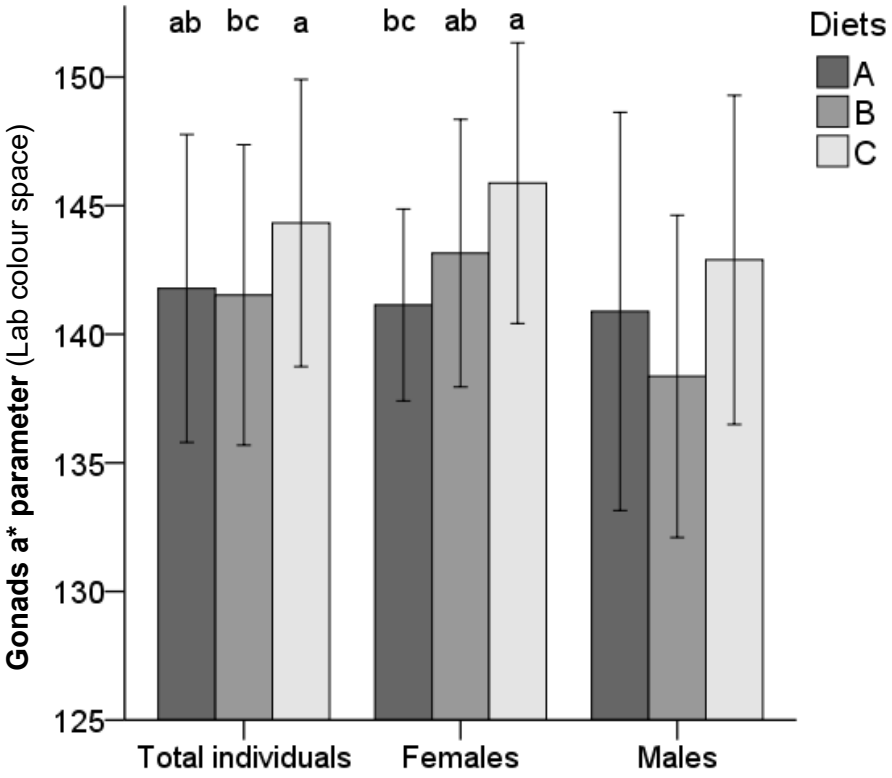

Figure 14 - Gonads a* values (mean ± SD) (Lab colour space) for all *Paracentrotus lividus* individuals fed with three artificial diets over a period of 90 days and for males and females separately. Diet A: maize & spinach. Diet B: maize, spinach & *Laminaria digitata*. Diet C: maize, spinach & *Cucurbita maxima*. Note: in each group, bars sharing the same letter are not significantly different according to Tukey's HSD test.

Furthermore, after sex identification, using the information obtained through the histological analysis, there was also made a colour assessment between *P. lividus* fed with the different diets, separately for males and females. To evaluate the need for this analysis,

there was performed an independent t-test, between males and females, which showed that females, in the end of the feeding trial, had gonads that were statistically significantly more red than male's gonads ($t_{(83)}$ = -2.224; p = 0.029). The mean a* parameter value for all female gonads was 143.55 ± 5.19 and for the males was 140.67 ± 6.98. Therefore, regarding only female individuals, a one-way ANOVA revealed statically significant differences in gonad colour between diets [$F_{(2, 41)}$ = 3.399; p = 0.043], as diet C (maize, spinach & pumpkin *C. maxima*) promoted a more intense colour than diet A (maize & spinach) (p = 0.036). In relation to the male sea urchins, on the other hand, the a* colour parameter did not show statically significant differences between diets [$F_{(2, 41)}$ = 3.399; p = 0.043].

Figure 15 shows female and male gonads from each diet, at the end of the feeding trial. It should be noted that the presented gonads are in different gametogenic stages of the reproductive cycle, hence the presence of released fluid in mature gonads.

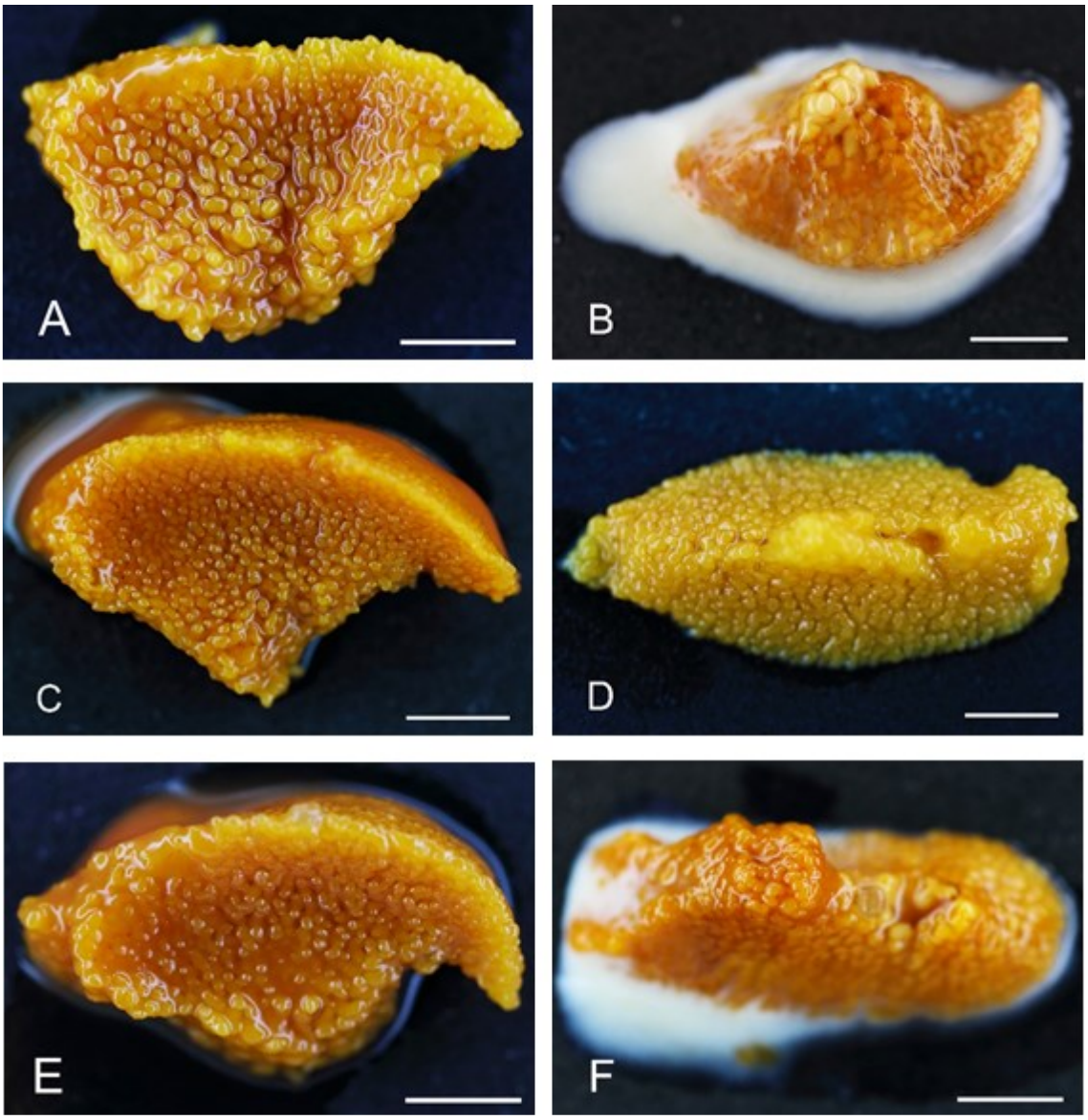

Figure 15 - Macro photographs of gonads from *Paracentrotus lividus* reared with three artificial diets over a period of 90 days: Diet A (maize & spinach); Diet B (maize, spinach & macroalga *Laminaria digitata*); Diet C (maize, spinach & pumpkin *Cucurbita maxima*). **(A)** Ovary in stage VII from diet A. **(B)** – testicle in stage VI from diet A. **(C)** Ovary in stage VII from diet B. **(D)** Testicle in stage III from diet B. **(E)** Ovary in stage V from diet C. **(F)** Testicle in stage VI from diet C. (Scale bars: A - F = 0.65 cm).

4. Discussion

For the development of echinoculture, it is crucial to find new artificial diets that are able to reduce the dependency from wild macroalgae and fishmeal, and also promote optimal growth and reproductive performances. The present study brings new perspectives for the *P. lividus* aquaculture, by comparing the effect of three jellified diets on somatic growth, gonadosomatic index, gonad colouration and gametogenic development, over a period of 90 days.

4.1 Somatic growth

The three rearing diets promoted statically significant differences in the results of somatic growth. The highest growth rate, regarding test diameter and total wet weight, was obtained with diet B (maize, spinach & *L. digitata*). While diet A (maize & spinach) promoted the second highest growth rate in test diameter, diet C (maize, spinach & *C. maxima*) was the second most successful in total weight. Regarding test diameter (mm), the satisfactory growth rates obtained with diets A and C (0.5 and 0.43 mm month^{-1}, respectively) are in line to what Fernandez & Pergent (1998) obtained for the same size classes in *P. lividus* (30 – 45 mm). However, the growth obtained in this study with diet B (1.2 mm month^{-1}) is a particularly remarkable result, more commonly found in smaller sea urchins, with 3 - 25 mm in diameter, in which the energy allocated for somatic growth (test and lantern) is higher. Indeed, growth rate in Echinoidea class varies according to size (Turon *et al.*, 1995; Fernandez & Boudouresque, 2000), being related to the individual's physiology (Lawrence & Lane, 1982), since there is a trade-off in allocating resources to somatic vs. gonadal growth. The published growth curves become flat very early (Lawrence, 2007) and Fernandez & Pergent (1998) inclusively state that 40 mm should be the size limit for the rearing of *P. lividus*, given the marked decrease in the growth rate. Shpigel *et al.* (2005) clearly illustrate this, as no significant test growth was observed in *P. lividus* (40.18 ± 3.08 mm in test diameter) fed different diets for 12 weeks. Cook & Kelly (2007a) obtained the very similar rate of 1.23 mm month^{-1} in test diameter, as their best result, but with juvenile sexually immature individuals, with a mean size of 10.1 mm (Cellario & Fenaux, 1990), reared adjacent to an open-water Atlantic salmon cultivation. Fernandez and Caltagirone (1994) obtained 1.41 mm month^{-1}, also with *P. lividus*, but feeding an artificial diet with fish meal and oil. Cook & Kelly (2009) obtained a growth rate of 1.9 mm month^{-1} fed on *Laminaria spp.*, *ad libitum,* however, with an initial test diameter of 18–20 mm, again a much lower size class compared to the one used in this study.

Regarding total wet weight (g), with the best results also obtained with diet B, Shpigel *et al.* (2004), feeding *P. lividus* with extruded diet, obtained a mean weight gain of 98.5 mg urchin^{-1} day^{-1} in ambient light at 18 ºC and 109.45 mg urchin^{-1} day^{-1} at 20-22ºC. Diet B led to a mean weight gain of 79.9 mg urchin^{-1} day^{-1}, also with ambient light and a mean temperature of 19.24 ± 1.10 ºC. However, the referred study used sea urchins with a slightly lower weight and 35 mm in test diameter, while the present study used several individuals with higher size classes, where the energy allocation to growth is lower. Also, the nutritional factors of extruded feed are very different from the jellified diets used in this experiment. Furthermore, Shpigel *et al.* (2005) obtained their best result of 5.16 g mean increase in total weight, in 12 weeks, with *P. lividus* (40.18 ± 3.84 mm – mean initial test diameter) also fed extruded moist pellets with *Ulva* and *Gracilaria.* In comparison, diet B led to a 7.19 g mean increase in exactly the same time. Similarly, the highest average growth rate achieved by Frantzis & Grémare (1993) was 56.3 mg day^{-1}, with *P. lividus* fed *Rissoella verruculosa.* A superior result when compared to diets A (40.52 mg day^{-1}) and C (53.53 mg day^{-1}), but using sea urchins with only 14-16.4 mm. Additionally, Spirlet *et al.* (2001) achieved their best results in somatic growth (0.39-0.46% day^{-1}) with fresh *Laminaria* sp., identical to their results with extruded feed, thus highlighting the suitability of this macroalga for sea urchins' feed. However, the size class used (15±25 mm) is also significantly lower compared to the present study, highlighting the result from diet B, also containing *Laminaria* sp. (0.30% day-1). Cook and Kelly (2007a) supplemented *Laminaria* spp. to *P. lividus* (37.2 mm mean size) reared next to a mariculture, obtaining a specific growth rate of approximately 0.20% day^{-1}, identical to the results of diets A and C, probably given the similar size class, but still inferior to diet B. Furthermore, Basuyaux and Blin (1998) achieved mean relative growth rates that ranged from 1.1% day^{-1} (6-20 mm size class) to 0.10% day^{-1} (> 40 mm), with *P. lividus* fed a mix of maize and *P. palmata* algae. In the present study, the total initial mean diameter was approximately 36 mm. Therefore, part of the results obtained by those authors for the 30-44 mm class, which range mainly from 0.10-0.19% day^{-1}, are inferior to what was obtained in this trial for total weight: 0.17% (diet A); 0.30% (diet B) and 0.22% (diet C). Particularly, diet B, which promoted a growth of 0.30 % day^{-1}, also containing macroalga (*L. digitata*) and maize, with the addition of spinach, stands out as an improved and more efficient formulated feed, regarding weight gain and test growth, and as a promising alternative in artificial feed for echinoculture.

Somatic growth rates in sea urchins are therefore affected by water temperature and gonadal development, but mainly by the kind of food available, in terms of amount and quality, and also by the ingested organic matter (Rowley, 1990; Frantzis & Grémare, 1993; Fernandez, 1996). The usual variability in growth rate as a consequence of intraspecific

competition (Boudouresque & Verlaque, 2007) was avoided in this experiment, given the daily effort in equally distributing the feed in each tank. Regarding the only article to date referring the use of pumpkin for sea urchins, Luo *et al.* (2014) indicate that *S. intermedius* fed kelp (*Laminaria japonica*) had significantly larger body weight and test diameter than those fed pumpkin (*Cucurbita* spp.), partly corroborating the present work. Although Fernandez & Pergent (1998) obtained the best results in growth with a diet containing fish meal, presumably as a consequence of the higher protein content (Fernandez & Boudouresque, 2000), the present study achieved a significantly higher test diameter growth only with vegetable sources (maize, spinach & *L. digitata*). Furthermore, Fernandez & Caltagirone (1994), Fernandez & Boudouresque (2000) and Shpigel *et al.* (2005) obtained the lowest growth rates with vegetable feed or with macroalgae alone. Indeed, the addition of *L. digitata* in diet B, despite the low concentrations of macro and micronutrients in macroalgae (Cook *et al.*, 2000), compensated the expected lower quantity of food allocated to test growth that is attributed to vegetable food. The process of homogenizing *L. digitata* in the formulation of the agar feed, probably leads to the rupture of cell walls, making proteins and lipids more accessible (Hiratsuka & Uehara, 2007). Moreover, according to Le Gall (1989), *L. digitata* is one of the algae species that provides consumption and growth rates that are acceptable for the aquaculture of *P. lividus*. Given the herbivorous nature of *P. lividus* (Lawrence, 2007) and that fishmeal is a finite and expensive resource, with a high demand (Cummins *et al.*, 2017), this result is considerably positive.

Regarding water temperature, in the natural environment of *P. lividus* in Portugal, the summer water temperatures range from 18-25ºC (Boudouresque & Verlaque, 2007). Le Gall *et al.* (1990) reported that the somatic growth of *P. lividus* was enhanced at a temperature range of 18 - 20 ºC. Fernandez (1996) also obtained the best results with the same temperatures. Furthermore, several studies with this species have used temperatures within this range, including: 20 ± 1 ºC (Spirlet *et al.*, 2001); 20.4 ± 0.7 ºC (Shpigel *et al.*, 2006); 20 ± 1 ºC (Fabbrocini *et al.*, 2012) or 18 ± 1ºC (Fabbrocini *et al.*, 2016). Basuyaux & Blin (1998) indicate 19 °C as an adequate temperature compromise between 24°C, which can be favourable to small urchins and 15 ºC, more favourable to bigger urchins. Shpigel *et al.* (2004) also refer that the range 18-22 ºC enhance both growth and gonadal development, in opposition to temperatures above 24 ºC, in which growth drastically declines. According to Luís *et al.* (2005), who used 18 ± 0.5 ºC in feeding experiments, this temperature is related to late Spring and early Summer conditions, and also the narrow range helps avoid spontaneous spawning and promote gonad maturation. The mean temperatures values in which sea urchins were reared in this experiment are in line with these authors: 19.06 ± 0.75 ºC (diet A); 19.24 ± 1.10 ºC (diet B); 18.98 ± 0.88 ºC (diet C).

4.2 Gonadosomatic index and gonadal weight

Since this study is mainly focused on the reproductive aspect and given that the individuals were wild caught, a probable asynchronous starting condition given the within-population variability (Byrne, 1990), in terms of gametogenesis (mainly between spermatogenesis and oogenesis), could substantially affect the results (Cunningham, 2008; Mercurio & Sugni, 2016). Therefore, starvation is an effective and necessary synchronizing method (Spirlet *et al.*, 2000). Since gonads also represent storage organs, the animal deprived of food is able to model its biochemistry and physiology and induce the reabsorption of the gonadal tissue and mobilize most of their nutrient reserves stored in the nutritive phagocytes (Pearse & Cameron, 1991; Lares & Pomory, 1998; Arafa *et al.*, 2006). Also it may use diverse organic compounds stored in their viscera during these periods to assure metabolic vital functions (Lawrence & Lane, 1982; Leoni *et al.*, 2001). Consequently, there is a decrease in total weight, gonad mass and thus in gonadosomatic index (Spirlet *et al.*, 2001; Tomšić *et al.*, 2015; Sartori *et al.*, 2016). From here, gonads tend to grow and mature in synchronicity, regardless of the treatment (Spirlet *et al.*, 2000).

The starvation period of 30 days, as conducted by Spirlet *et al.* (1998b), led to a mean gonadosomatic index in the sacrificed individuals of 3.33 ± 0.02%. The absence of mortality during starvation in this trial, with the associated water quality, since there is very few organic matter released into the recirculating system, also makes this successful procedure very favourable for the uniform reduction of gonads' weight. Before deciding to start the feeding trial, there was the need to quickly understand the effectiveness of the starvation, despite the homogeneity of the results and still without the histological information of the gametogenic stages. Therefore, when comparing, for example, with Tomšić *et al.* (2015), these authors obtained, after 15 days of starvation, a mean gonadosomatic index (GI) of 5.5% before initiating feeding. Fabbrocini *et al.* (2015) started the experiment with a mean GI of 3.5%. Spirlet *et al.* (2000) attained a GI of approximately 2%, however, in a period of 2 months, which logically increases the probability of sea urchins' death. Sartori *et al.* (2016) with 6 weeks of starving period achieved a mean GI of 4.36%. Consequently, given the similarity with these results, in some cases with a more satisfactory outcome, the feeding trial was immediately initiated. The histological results also corroborated, in a more reliable way, the effectiveness of the method, which is described further in this section.

After the 90 days feeding trial, not only all diets were well accepted, but promoted gonadal growth, especially diet A composed of only maize and spinach. First, it is important to address the importance of presenting gonadosomatic index (GI), as well as gonadal wet

weight. For an echinoculture, GI is the most important factor for evaluating gonadal yield (Fernandez, 1996; Fernandez & Pergent, 1998), since gonads are the desired product. However, in this experiment, somatic growth was significant and non-negligible for all diets, thus GI tends to be biased and underestimate gonadal growth (Spirlet *et al.*, 2001). That is what happened mainly between diets B and C. Diet B was the most successful in promoting somatic growth, specifically total weight, thus leading to the smallest GI of the three diets. However, regarding only gonadal wet weight, diet C was the least productive, as it is detailed in Results.

Given the initial synchrony in test diameter, total weight and GI, the statically significant differences in the final results clearly demonstrate different allocation of nutrients, given the somatic vs. gonadal growth duality (Spirlet *et al.*, 2000). Moreover, energy is first allocated to maintenance, then to the digestive tract and the remaining is shared between gonadal (gametes production) and somatic growth (Calow, 1981; Pearse & Cameron, 1991; Fernandez, 1996). In this context, diet A, with the weakest result in total weight, was the diet with the highest allocation to gonad growth, with a final GI of 9.07 ± 2.39 %, from 3.33% at the beginning (an estimated 172.4% increase) and a mean gonadal weight gain of 232.3%, as calculated from the 18 sacrificed sea urchins after the staving period. Similarly, Sartori & Gaion (2015) with a relatively higher initial GI of 4.36%, achieved the highest GI (19.65%) with *P. lividus* (40-45 mm) also fed with only maize and spinach, although in a more extensive period of 4 months and a diet not created with agar, in which the final percentage of water in the feed is certainly much lower. The superior size class, as previously stated, also accounts positively for a higher gonadal allocation. Equally, Sartori *et al.* (2015) with the same maize and spinach diet obtained 12.84% in GI, with the same initial GI of approximately 4.36%. Additionally, Sartori *et al.* (2016), with the same starting conditions, also reached the highest GI (19.24%) with maize and spinach. Luís *et al.* (2005) obtained as well the best GI in *P. lividus* (11.7%) feeding only dry grains of maize, with also a superior mean size class (> 40 mm), opposed to feeding only a commercial dried *Laminaria ochroleuca* seaweed (8.2 ± 0.5%). In the work of Fernandez & Boudouresque (2000), with a vegetable feed, containing 44.7% of maize, it was achieved one of their best results in GI (10-12%) with 40-45 mm *P. lividus*. Additionally, Tomšić *et al.* (2015) starting with an initial GI of 5.5%, fed *P. lividus* with a diet containing 30% of maize meal and 15% of maize starch, obtaining the highest GI of 9%, in 60 days. Repolho *et al.* (2011) achieved in 12 weeks a mean GI of 10.6% feeding *P. lividus* with only maize grain and 11.67% when feeding an wheat and maize flour mix, however with initial mean test diameters of 4.74 mm and 5.02 mm, respectively. Once again, a significantly superior size class in comparison to this experiment, making the direct comparison of GI much more complex. However, in

summary, the success of diet A (maize & spinach) in promoting gonadal yield is in line to what other authors report, making these accessible ingredients suitable to be further exploited in formulated jellified or extruded diets.

Regarding diet B, as previously referred, given the high allocation to test and total weight growth, it was expected a smaller GI. Nonetheless, it promoted a final mean GI of 7.17 ± 1.99 %, an estimated 115.3% increase, and a mean gonadal weight gain of 205.1%. Once again, it is important to refer that regarding only gonadal yield, this diet produced the intermediate result among the three diets. Spirlet *et al.* (2001) reported that *P. lividus* fed with *Laminaria* sp. rapidly showed somatic growth, but gonadal growth only started after the first month, since there seems to be a period for recovering digestive functions after starving. Tomšić *et al.* (2015) also reported a significantly lower gonadal enhancement in *P. lividus* fed only a macrophyte diet (80% Ulvales order) when compared to other artificial diets. In Spirlet *et al.* (2001), the group of sea urchins fed with fresh *Laminaria* sp. showed a significantly lower GI of 7 – 7.5%, when compared to pellet diets, after three months. A very similar result with diet B (containing *L. digitata*) from the present study, but then again, a simplistic comparison should be avoided given the very different nature of the diets and also diet B's 44% portion of maize and spinach. However, when analysing the gonadal growth (GG) in % per day, evaluated from the initial mean value of gonad weight of the 18 sacrificed sea urchins, diet B promoted a GG of 2.3% day^{-1}, while Spirlet *et al.* (2001) only achieved 0.6% day^{-1} with the *Laminaria* sp. diet. Conversely, as previously indicated, they obtained a higher somatic growth, when compared to diet B, highlighting again the somatic vs. gonadal growth duality. Moreover, Sartori *et al.* (2016) not only registered a lower GI (final 10.25%) in *P. lividus* fed with 9 different macroalgae, but also a regression of gonadal growth from 6th to 9th week. Similarly, Carboni *et al.* (2015) fed during the same three months, fresh *L. digitata* to *P. lividus*, obtaining a final GI of 6-7%, the lowest output when compared to the other formulated diets, more rich in protein, lipids and energy. Fernandez & Pergent (1998) also obtained 7-8% of GI, feeding *Cymodocea nodosa* to individuals of 40-45mm size, while in the 30-35 mm class only achieved a GI of 2-3%, expressing again the heterogeneity in food allocation. Furthermore, while Fabbrocini *et al.* (2012) with agar-diets obtained a lower GI in sea urchins fed on different macroalgae, Fabbrocini *et al.* (2015) starting from a GI of 3.5% (3.33 ± 0.02% in this study) achieved in 14 weeks a GI of just 6.7%, feeding *P. lividus* (30-35 mm, test diameter) with an agar biocomposite, including maize meal (24-25%) and kelp meal (25%). In addition, Shpigel *et al.* (2005) in 12 weeks obtained with *P. lividus* (mean test diameter of 40.18 mm) a GI that is inversely proportional to the amount of time it is fed on algae diet, obtaining the best results with extruded moist pellets. Conversely, Cook & Kelly (2007a) obtained the highest GI of 10.38% with *P. lividus*

(37.2 ± 3.1 mm) fed with *Laminaria* spp., in addition to the salmon farm derived material, thus a rearing condition so different than can easily lead to a biased comparison with this experiment.

Finally, regarding diet C, containing *C. maxima* (50%), it was achieved a final mean GI of 7.31 ± 2.26 %, an estimated 119.5% increase (from 3.3%), and an estimated mean gonadal weight increment of 173.7%. Although this diet promoted the intermediate result in GI, its gonadal wet weight gain was the lowest of the three diets. Interestingly, Luo *et al.* (2014), with due reservations, obtained the same relative results with *S. intermedius*: although sea urchins fed kelp had significantly higher gonad weights than those fed with pumpkin, GI from the pumpkin group was slightly higher. Given that Diet C from this study was also composed of 44% of maize and spinach, which alone promoted the best results in gonadal yield, it seemed to be negatively affected by *C. maxima*, although still achieving the considerable result of 173.7% weight increase in 90 days.

Regarding size class, although Fernandez & Boudouresque (2000) indicate that 35-45 mm in test diameter has the greatest amount of energy allocated for reproduction, high GI values were registered in the 40-70 mm size class, rather than 20-40 mm (Martínez *et al.*, 2003; Sánchez-España *et al.*, 2004). In fact, GI tends to increase with the sea urchin size (Fernandez & Pergent, 1998). The previous comparisons with other authors' work, mainly focused on gonad growth, clearly showed a tendency to also choose slightly superior size classes. In this study, some individuals were even below 35 mm in test diameter. Therefore, in the future, the used size class should be narrowed according to the goal of the investigation, particularly above 40 mm, if focused on reproduction.

It should be noted that not only the accumulation of nutrients by the nutritive phagocyte meshwork, but also the accumulation of gametes contribute to gonad growth and weight, however, they contribute in different degrees. Therefore, given the different outcome in sexual maturation between the 3 diets, thus affecting the ratio between germinative (sexual cells) and somatic (phagocytes) cells, some degree of variance in GI could already be expected (Pearse & Cameron, 1991; Fernandez & Pergent, 1998; Marsh & Watts, 2007; Unuma & Walker, 2009; Fabbrocini *et al.*, 2012).

The growth of the gonads' weight is mainly associated with the ingestion of nutrients and thus the quantity and quality of feed ingested (Lawrence & Lane 1982; Cuesta-Gomez & Sánchez-Saavedra, 2014). In particular, proteins are regarded as the major dietary nutrient with a high nutritional value involved in reproduction, leading to an increase in somatic and gonadal growth rates, the latest through the accumulation of gametes or nutrients (Fernandez & Boudouresque, 2000; Jacquin *et al.*, 2006; Cook & Kelly, 2007a).

Jacquin *et al.* (2006) inclusively state that the ideal protein level to optimize gonad growth in this species is not even reached in natural environment. Conversely, Cook and Kelly (2007b) suggested that *P. lividus*, as an herbivore, might be unable to digest high dietary protein levels, in comparison to more omnivorous species. In fact, according to Marsh & Watts (2007), nutritive phagocytes, during an early growth stage, show a limited capacity for the assimilation of protein, evidencing the large amount of energy that is also required for this process.

Regarding the nutritional profile of maize, it has 8.4% of protein (dry matter), 72% starch and 4.3% of lipid contents, in which most fatty acids are polyunsaturated, namely 45% of linoleic acid (Crampton & Harris, 1969; Luís *et al.*, 2005; Nuss & Tanumihardjo, 2010; Ranum *et al.*, 2014). The high gonadal yield of diet A (maize & spinach) may be partly explained by the significant content of carbohydrates and proteins in maize, when compared to algae and spinach. Spinach (*Spinacia oleracea* L.), in terms of proximate composition (on dry weight basis) only has 2.1-2.6% of protein, 0.38-0.9% of lipids, 0.6% of fibre and 4% of carbohydrates, with water as the main component (91% in wet weight) (Hanif *et al.*, 2006; Biehler *et al.*, 2011). *L. digitata* has an average protein content of 6.9%, with significant seasonal variations (Schiener *et al.*, 2015), 0.2-2% of lipids, 2.3-3.5% of fibre and 9-9.9% of carbohydrates, with 73-90% of water content (Carboni *et al.*, 2015). The chemical composition of *C. maxima* (fresh mesocarp) is: 87.6% moisture content; 1.13-2% protein; 0.42-0.5% of lipid content; 1.1% of fibre and 8-13.3% of carbohydrates (Kim *et al.*, 2012). The 50% content of pumpkin in diet C may explain the low output in gonadal and somatic growth, given the poor content in protein. Although seeds and peel were also included in the formulation, with higher values for all nutrients, except moisture, their percentage in the final feed was much lower when compared to mesocarp.

Schlosser *et al.* (2005) suggested that energy may also play an important role in growth, namely in the GI, as a limiting factor during growing and mature stages. Just like protein content, maize has the highest energy content of 91 Kcal $100g^{-1}$ (fresh weight of canned sweet corn used in this experiment), followed by spinach with 27 Kcal $100g^{-1}$ (fresh leaves), fresh *L. digitata* with 18 Kcal $100g^{-1}$ and fresh pumpkin with 11 Kcal $100g^{-1}$ (Kim *et al.*, 2012; Carboni *et al.*, 2015). Once again, these results support the highest gonadal yield from Diet A (maize & spinach), followed by Diet B (50% *L. digitata*) and finally, Diet C (50% *C. maxima*). Schlosser *et al.* (2005) reported a lower gonadal growth with algal diets, given the poor protein content, that also represent an energy source. If energy plays in fact a more important role, since maize has an energy content similar to prepared diets, but

lower protein content (Luís *et al.*, 2005), it is a very attractive alternative to the expensive extruded diets that normally promote superior gonadal yields (Spirlet *et al.*, 2001).

In summary, since GI or gonadal yield represents the most important factor for an aquaculture of sea urchins, our results are consistent with other studies, in which artificial diets lead to a considerable increase in gonad yield (Fernandez & Boudouresque, 2000; McBride *et al.*, 2004; Fabbrocini & D'Adamo, 2010). Maize, in particular, and also associated with spinach, significantly promotes gonadal growth, in opposition to the effect of algae (*L. digitata*) that favours more somatic growth. Also, as previously stated, the fluctuating availability of macroalgae, their costs and complex logistics, and also the seasonal variations in chemical composition (Cook & Kelly, 2007b; Schiener *et al.*, 2015; FAO, 2016a) make farmers avoid this product (Shpigel *et al.*, 2005). Hence the decision to collect and freeze all the macroalgae needed for this study (Lozano *et al.*, 1995). Furthermore, apart from achieving a considerable gonadal growth rate with alternative feed sources, particularly with diet A, and starting from a very low GI after starvation, the final values are comparable to the natural environment, where the GI ranges between 6% and 12% (Spirlet *et al.*, 2000). According to Spirlet *et al.* (1998a), wild populations of *P. lividus* in Brittany do not exceed 8% of GI.

4.3 Feed ingestion

Regarding ingestion rates, all sea urchins started feeding immediately after the starving month, showing no need for an adaptation period and highlighting the generalist and opportunistic feeding behaviour of *P. lividus* (Boudouresque & Verlaque, 2007). Through the daily recovery and weighing of leftovers, it was noted that feeding in each diet was relatively constant, as reported by Spirlet *et al.* (2001). However, there were significant differences between diets at the end, as Diet C (maize, spinach & *C. maxima*) was the most consumed by the sea urchins over the total period of 90 days, with a mean ingestion value of 6.21 ± 1.63 g $day^{-1}individual^{-1}$. It was followed by Diet B (maize, spinach & *L. digitata*) with a mean feed intake of 5.44 ± 1.36 g $day^{-1}individual^{-1}$ and finally, Diet A (maize & spinach) with 3.97 ± 1.19 g $day^{-1}individual^{-1}$. Furthermore, for each diet, ingestion rates showed significant variations between the three months of the experimental period, and also, as expected, between diets in each month. Monthly variations in feeding rate were already described by other authors (Miller & Mann, 1973; Fernandez & Pergent, 1998; Fernandez & Boudouresque, 2000; Jacquin *et al.*, 2006). They are normally related to abiotic conditions, such as temperature (Miller & Mann, 1973) which in this experiment does not apply once the temperature was controlled over the trial. May be also related to

physiological factors like the reproductive phase in adult individuals, in which for example, the increase of the gonads volume in the coelomic cavity can reduce feeding rate (Lawrence, 1975; Fernandez & Boudouresque, 2000; Chang *et al.*, 2005; Jacquin *et al.*, 2006; Lawrence *et al.*, 2007). Diet A was the only case in which sea urchins consumed progressively less over the three months and it was also the diet that promoted the highest gonadal growth and also maturation, clearly exemplifying the latest assumption. Furthermore, most individuals were initially in spent stage of the reproductive cycle and since energy requirements are much superior in the early stages of gametogenesis, an initial high ingestion rate was expected (McBride *et al.*, 2004; Jacquin *et al.*, 2006; Fabbrocini *et al.*, 2012).

The nutritional condition of the individual also affects feed intake (Lawrence *et al.*, 2003), thus after the starvation period there was expected an initial high ingestion rate, which only happened in the individuals fed with diet A, since feed intake was higher in the first month. In the end of the trial, diets A and C led to many mature individuals (final stages of gametogenesis), partly explaining the final decrease in food intake. Fuji (1967) and De Ridder & Lawrence (1982) inclusively reported very low feeding rates prior to spawning. On the contrary, diet B was progressively more consumed, and coincidentally was the diet with more individuals in early gametogenic stages, thus with higher energetic needs. But also, with the lowest gonadosomatic index, in other words, with more coelomic space, probably leading also to a higher feed ingestion. Moreover, as increasing size of the sea urchin leads normally to an increase in ingestion rate (Frantzis & Grémare, 1993; Fernandez & Boudouresque, 2000), diet B was indeed the diet that promoted a remarkable somatic growth, in terms of test diameter and total weight. Coincidentally, Schlosser *et al.* (2005) also noted an increase in feed ingestion during the trial's final weeks with sea urchins fed macroalgae diets.

The significant differences in mean ingestion values between diets, of the total trial period, are certainly more related to the chemical and physical properties of the feed (Klinger, 1982; Carboni, 2013), although its agar-based formulation was immediately accepted and readily consumed, just as reported by Fabbrocini *et al.* (2015), in which agar biocomposites were actively sought by *P. lividus*. Therefore, palatability and attraction (chemosensory characteristics), key factors of artificial feed, were successfully achieved (Lawrence *et al.*, 2013; Cyrus *et al.*, 2015; Fabbrocini *et al.*, 2015; Cirino *et al.*, 2017), as well as its suitable texture and form, in comparison to fresh algae that might involve more energy to manipulate (Klinger, 1982; Spirlet *et al.*, 2001). The inclusion of triturated *L. digitata* in diet B might had acted as a feeding stimulant, assuming its chemosensory

characteristics were preserved (Klinger & Lawrence 1984; Cyrus *et al.*, 2015). Since the formulation method was the same for the three diets (6% agar), it is easier to focus only on the nutritional aspect of the ingredients. In fact, food type affects ingestion rates in sea urchins, as reported by Vadas (1977), Anderson & Velimirov (1982), De Ridder & Lawrence (1982), Frantzis & Grémare (1993), Fernandez & Pergent (1998), Fernandez & Boudouresque (2000), Cyrus *et al.* (2015) or Vizzini *et al.* (2015). Furthermore, protein levels seem to be also correlated with ingestion rates (Fernandez & Boudouresque, 1998, 2000) and the present experiment is theoretically more in line with the compensatory food intake model (Frantzis, 1992), in which ingestion rates increase with low soluble protein levels, as suggested by Frantzis & Grémare (1993) or Fernandez & Boudouresque (1998, 2000). As was the case in this study, feed rich in carbohydrates can be largely consumed in order to compensate for the low protein content (Miller & Mann, 1973; Carboni, 2013). In fact, not only *ad libitum* feeding tends to increase ingestion (Minor & Scheibling, 1997), but also vegetable feed has the same effect (Fernandez & Boudouresque, 1998). Despite the high ingestion rate of Diet C (6.21 ± 1.63 g $day^{-1}individual^{-1}$), given its expected low protein content, the output in gonadal and test growth was the lowest. Conversely, diet A, containing 47% of maize, the richest compound in protein in this experiment, was the least consumed diet and promoted the highest gonadal yield. Moreover, a diet rich in proteins and lipids also consumes more energy for its assimilation (Marsh & Watts, 2007), and may reduce feed ingestion. In addition, Spirlet *et al.* (2001) obtained a higher intake of fresh *Laminaria* sp. in comparison to the more protein-rich extruded pellets. According to Spirlet *et al.* (1998b), although gonadal growth is correlated to feed intake (Fernandez, 1996), these are not directly proportional and since maximal ingestion is dependent on physical constraints, as the volume of the gut (Frantzis, 1992), the required protein levels for growth and maintenance might not be met.

4.4 Gametogenic stages and oocyte diameter

Regarding the histological analysis, first of all it should be briefly mentioned that the reproductive cycle of *P. lividus* has an annual pattern in the Mediterranean and Atlantic coasts, where active gametogenesis normally occurs from September to May (Byrne, 1990; Lozano *et al.*, 1995; Fabbrocini & D'Adamo, 2010), whereas Sánchez-España *et al.* (2004) in Southern Spain observed a wider period of mature gonads from January to August. Jouhari *et al.* (2014) reported a very similar pattern in the southern Moroccan Atlantic coast. However, in an artificial rearing system, as expected, the annual reproductive cycle of *P. lividus* tends to disappear (Spirlet *et al.*, 1998a; Shpigel *et al.*, 2004), even more when starving is used to eliminate seasonal reproductive conditions. Whereas the influence of

photoperiod is ambiguous (Luís *et al.*, 2005), temperature regulates and enhances the gametogenic cycle (Byrne, 1990; Pearse & Cameron, 1991; Spirlet *et al.*, 2000). The rearing temperature used for this trial seems to be appropriate given that Shpigel *et al.* (2004) indicate the range 18–22ºC as ideal and Spirlet *et al.* (2000) obtained an increasing rate of gametogenesis with increasing temperature until 20ºC. Just as previously stated, the first results from the sacrificed individuals after 30 days of starving could not be properly analysed only with the gonadosomatic index. When analysing the gametogenic stages, the effectiveness of the starvation period as a synchronizing method was definitely corroborated as 83.3% of the individuals were in spent stage (I and II), only with 11.1% in post-spawned stage (VIII) and 5.6% in recovery stage (III), with no individual in active gametogenesis. Fabbrocini *et al.* (2015), after starvation, also initiated trials with 80-90% sea urchins in spent condition, which is characterized by thin ascinal walls and the possible presence of relict gametes in the process of reabsorption. Similarly, Sartori & Gaion (2015) with a longer starvation period of 6 weeks attained 90% of the total individuals in spent stage. Additionally, Spirlet *et al.* (2000) after 2 months of starvation also obtained gonads in the spent stage. Fabbrocini & D'Adamo (2010) in one month reached approximately 90% with spent gonads, some with relict gametes too, and also 5% in recovery stage, as in the present experiment. The direct comparison of relative frequencies with some of these works may be slightly biased, since most authors consider only six stages, according to Byrne (1990), in which for example stage VIII (post-spawned) is not considered and it is probably classified into spent stage. Overall, periods of starvation are natural and usual events to sea urchins (Lares & Pomory, 1998; Arafa *et al.*, 2006) and despite the slow and complex nature of the reabsorption process, the satisfactory obtained results prove the suitability of the method and its chosen duration.

With feeding activity, sea urchins begin to store nutrients in the nutritive phagocytes and producing sexual cells, following the gametogenic cycle (Spirlet *et al.*, 2001). After the feeding trial, the gametogenic development was different among diets, once again highlighting the need for histological analysis, since GI or gonadal growth give few information regarding feed utilization (Schlosser *et al.*, 2005). In contrast, Carboni *et al.* (2013) found no significant difference between treatments and Shpigel *et al.* (2006) had little changes in the gametogenic development, with the majority of individuals in early reproductive stages. In the present study, Diet A (maize & spinach) was the most successful in promoting maturation, with 46.7% in mature stage (VI) and 20% in premature stage (V). It was followed by diet C (maize, spinach & *C. maxima*), with 36.7% in mature stage (VI) and 10% in premature stage (V). On the contrary, most individuals in diet B (maize, spinach & *L. digitata*), 33.3%, were in recovery stage (III). Similarly, Sartori & Gaion (2015) and

Sartori *et al.* (2016) after 3 months of treatment also obtained the highest percentage of mature individuals with maize and spinach, not with a jellified diet though, having around 80% of individuals in active gametogenesis, similarly to diet A, with 73.3%. Once again demonstrating the suitability of these ingredients to provide nutritional and energetic sources for gonadal growth and sexual maturation (Sartori *et al.*, 2016). Moreover, the macroalgae diet (including several species) only promoted 20% of mature organisms in the third month, a very similar result to our diet B (26.7%), and also 60% individuals in active gametogenesis, with the same 60.1% in diet B. However, the same authors observed a much more rapid progression in the sexual cycle from the first month with macroalgae, as mature stages shifted to spawning stages. A simplistic comparison is again difficult to make given the different nature of the diets and the several different species of algae used. Furthermore, Luís *et al.* (2005) also obtained large spawnings with a maize diet (dry grains) during the maturation season. Additionally, Spirlet *et al.* (2001) state that mixed feed may promote a rapid development of gametogenesis, since the sea urchins fed only with fresh *Laminaria* sp. were still mostly between growing and premature stages, just like diet B (containing *L. digitata*) used in this study was the least efficient in promoting maturation. Similarly, Schlosser *et al.* (2005), in a period of 2 months, also obtained the weakest maturation in *P. lividus* with macroalgae, when compared to a prepared diet, although most individuals were just in growing stage. Fabbrocini *et al.* (2015) feeding *P. lividus* with agar biocomposites including several ingredients, during 14 weeks, only reached 15% of the individuals in premature stage and 50% still in recovery stage. A very slow progression in the reproductive cycle, even when compared to diet B. Fabbrocini *et al.* (2012) also using jellified diets, obtained a low gametogenic progression with *G. gracilis* and *Cystoseira* sp. pellets, although *Ulva lactuca* pellets promoted a significant gamete development in 4 weeks.

It should also be mentioned that partly-spawned classification (stage VII) may be sometimes derived from a misjudgement or be open to interpretation. In the process of dissection, almost all premature and mature individuals released gametes, an event that cannot be considered a natural spawning. Later, when analysing the gonad sections, the presence of released gametes outside of the ascini should not immediately lead to considering a stage VII, since, according to Spirlet *et al.* (1998a), this stage corresponds to intermittent spawning that occurs over the spawning season. Furthermore, during the present trial, the presence of gametes in the aboral hemisphere of the individuals was never observed, as reported by Luís *et al.* (2005). Similarly, Carboni *et al.* (2013), although using a lower rearing temperature, also did not registered the partly-spawned stage. Therefore, it

can be assumed that the Effective Accumulated Temperature (EAT) (Liu *et al.*, 2002) was not attained in the present experiment to trigger a natural spawning.

Overall, similarly to what Sartori & Gaion (2015) obtained, the three diets used in the present work, over a period of 90 days, promoted the progression of the reproductive cycle, with more than 60% of the individuals in active phase of gametogenesis (stages IV-VII), although recovery stage (III) could also be considered, since it presents primary oocytes or primary spermatocytes in the ascinal wall, as described by Spirlet *et al.* (1998a). The agar-based biocomposites seemed to promote a quick metabolic reaction, with the progression of the gametogenic cycle, attesting the feed quality and the advantage of choosing and formulating ingredients according to specific nutritional requisites, as reported by Russell (1998) and Fabbrocini (2010). In fact, the most important analysis of these results is related to the desired goal of the rearing: good-quality gonads for the consumer (supress or delay gametogenesis) or the production of mature gonads with viable gametes for seed stock. Therefore, the slower progression in the reproductive cycle promoted by diet B seems to be more appropriate for enhancement and consumption purposes, as synchronization to the desired stage is easier. Since some individuals already had formed mature gametes in this diet, a shorter period of rearing could be applied. Conversely, the high output of gamete production from diet C, and specially diet A, highlights the suitability of maize (Luís *et al.*, 2005), spinach and also pumpkin for the enhancement of gametogenesis. Furthermore, when wild *P. lividus* populations in Portugal are only mature in mid-spring and summer, after accumulating reserves in winter (Gago *et al.*, 2003), these diets allowed to significantly modify the natural reproductive cycle, since the trial ended in early February.

The oocyte diameter-frequency information corroborated the relative frequencies of the gametogenic stages present in each diet. Females from Diet A, which promoted the most significant progress in the reproductive cycle, presented the 60-90 µm size class as the most dominant (56%), mostly corresponding to large ova, as Gonor (1973) reported that mature ovaries have a dominant class of large oocytes. Although Byrne (1990) indicates a large number of ova with 90 µm in diameter in the mature stage, 2% of the measured oocytes from diet A were above this value, as described by Pérez *et al.* (2010) in *Loxechinus albus.* Additionally, Lozano *et al.* (1995), while studying natural populations of *P. lividus*, considered mature ova with diameters above 70 µm, which is also in line with the present results. Given the presence of some females from diet A in the recovery and growing stages, with primary oocytes surrounded by nutritive phagocytes, the size class of 15-30 µm was also abundant, just as Byrne (1990) found primary oocytes of 5-30 µm in the recovery stage and 10-50 µm in growing stage. Regarding Diet B, the majority of individuals was still in

early stages of gametogenic development, thus the dominance (35%) of the 10-25 μm size class, corresponding to primary oocytes attached to the ascinal wall of stages III and IV. However, 28% of the measured oocytes were 65-80 μm in diameter, given also the presence of premature and mature females. Finally, in diet C, there was a prevalence (51%) of 60-85 μm oocytes from the significant percentage of premature/mature females. Still, 22% of the measured oocytes were between 10-20 μm, since maturation in diet C was not so pronounced when compared to diet A. It should be noted that premature (V), mature (VI) and partly-spawned (VII) stages are characterized by the presence of various stages of oocyte development, particularly in stage V. Therefore, given all measurements were pooled, small diameters not only correspond to early gametogenic stages, but also to the primary oocytes still present in pre- and mature stages. Overall, these results highlight the effectiveness of mostly diets A and C in the formation of mature gametes from spent gonads in a period of 90 days.

4.5 Gonad colour analysis

Regarding the colour analysis, the gonads of *P. lividus*, in the wild, range from pale yellow to dark brown, both colours being inappropriate for consumption (Symonds *et al.*, 2007), since a bright yellow-orange range represents the ideal colour for market acceptance and high-priced gonads, as an indication of great quality (Senaratna *et al.*, 2005; Shpigel *et al.*, 2006; Symonds *et al.*, 2007; Carboni, 2013).

The gonads colour derives mainly from carotenoid pigments, which accumulate mainly in those organs (Carboni, 2013; Vizzini *et al.*, 2015) and increasing carotenoid concentration can substantially improve gonadal colour in both sexes (Plank, 2000). In general, echinenone (first isolated from *P. lividus* as β-Caroten-4-one), both α- and β-forms, and also β-carotene represent the principal carotenoids (Lederer, 1935; Goodwin & Taha, 1950; Fox & Hopkins, 1966; Tsushima & Matsuno, 1990; Matsuno & Tsushima, 2001; Symonds *et al.*, 2007), specifically with 9-cis echinenone as the dominant carotenoid in *P. lividus*, representing 51% of the total content, according to Symonds *et al.* (2007). There are evidences that β-echinenone and α-echinenone are derived from dietary β-carotene and α–carotene, respectively, through specific oxidative metabolic pathways (Griffiths & Perrott 1976; Goodwin, 1984; Tsushima *et al.*, 1993; Shpigel *et al.*, 2005; Cook & Kelly, 2007a; Carboni *et al.*, 2015). Given this relation, since Kelp is rich in the precursor β-carotene (McDermid & Stuercke, 2003; Carboni *et al.*, 2015) and since increasing echinenone content is normally correlated with an intense and acceptable gonad colouration (Shpigel *et al.*, 2005, 2006; Suckling *et al.*, 2011), a diet containing macroalgae,

as diet B, is expected to produce a high echinenone content and therefore, decent colouration. This is what Shpigel *et al.* (2006) obtained with six experimental diets, finding a positive correlation between dietary β-carotene concentration and successful gonad colour. The algae diet used by Shpigel *et al.* (2005) produced a dark orange colouration, in comparison to the pale colour of the pellet treatment. Consequently, as reported by Cook *et al.* (1998), Robinson *et al.* (2002) or Shpigel *et al.* (2005), there is often the need to add natural algae to artificially formulated diets, prior to commercialization, in order to reliably achieve an acceptable colouration, inclusively given the poor performance of synthetic pigments from prepared diets. For example, Cook & Kelly (2009) feeding *P. lividus* with *Laminaria* spp. also achieved a gonad colouration classified from acceptable to excellent. On the contrary, Symonds *et al.* (2007) state that echinenone levels are not directly and exclusively related to a market acceptable gonad colour, since the lipid content or lutein (a xanthophyll carotenoid) levels, among other possible factors, may also affect the visual perception of gonad colour. In the present study, diet B (maize, spinach & *L. digitata*) promoted better colour results (higher a* parameter) than diet A (maize & spinach), only when considering female individuals. Indeed, maize is rich in dietary xanthophylls, with lutein as the major carotenoid accumulated and followed by zeaxanthin, a xanthophyll monoester and β-carotene (Janick-Buckner *et al.*, 1999). However, these pigments are more related to fecundity, and have little effect on gonad colour (Tsushima *et al.*, 1993; Kawakami *et al.*, 1998; Plank *et al.*, 2002; Robinson *et al.*, 2004; Lawrence, 2007). Likewise, the major carotenoid in spinach is lutein, followed by β-carotene, violaxanthin and neoxanthin (Bunea *et al.*, 2008). Moreover, in *Laminaria* spp., β-carotene only accounts for 4-6% of total carotenoids, with all-trans fucoxanthin representing 80% (Haugan & Liaaen-Jensen, 1994), a major dietary carotenoid that is discriminated in the gonads and probably does not contribute to a favourable gonad colouration. Additionally, the 9- or 9′-cis forms of carotenoids are absent in natural algae (Symonds *et al.*, 2007). These factors may partly explain why diet C (maize, spinach & *C. maxima*) was the most successful in promoting high *a values in all analysis. In opposition to *L. digitata*, the main carotenoid in pumpkin (>80%) is β-carotene, with lower levels of lutein, lycopene, α-carotene and inclusively, cis-β-carotene (Seo *et al.*, 2005). Luo *et al.* (2014), although achieving the highest a* values feeding *S. intermedius* with kelp, reported that individuals from the pumpkin treatment also attained a very acceptable gonad colour with slight differences from the kelp treatment. Additionally, Vizzini *et al.* (2015) observed the most intense gonad colour in *P. lividus* fed on lettuce (*Lactuca sativa*) or beet (*Beta vulgaris*) and surprisingly, the least attractive colour in the macroalga *Ulva lactuca* treatment. In our study, diet B also led to the lowest a* values when considering males or total individuals. Furthermore, Senaratna

et al. (2005) found that three pelleted diets, including one based on vegetable protein, resulted in commercially acceptable gonad colouration, without significant differences from the macroalga *Ulva australis* treatment or the wild population. Despite the different nature of diets and species, when compared to the present study, it also highlights the potential of alternative feed sources for sea urchins, in order to produce a quality product. Additionally, it should also be noted that the different reproductive stages between diets may also affect gonad colour, since according to Symonds *et al.* (2007), low levels of echinenone and therefore a paler colouration, coincide with pre- and during spawning, which may have influenced individuals mainly from diets A and C, with several mature stages. Consequently, it can be further speculated that the already superior results from diet C could be enhanced with earlier reproductive stages. Furthermore, Fabbrocini *et al.* (2012) reported that the used agar pellets were weakly metabolized by *P. lividus* and thus having no effect on gonad colour, thus the jellified diets used in present study are also expected to not have negatively affected gonad colouration. Just as the type of protein source also does not seem to affect colour (Senaratna *et al.*, 2005; Woods *et al.*, 2008).

The general analysis using all individuals is relevant for this study, since in rearing aquaculture systems the sex ratio of sea urchins is usually unknown. However, apart from the colour differences promoted by the three diets, there was also statically significant differences between males in females, with females showing higher a* values. According to Symonds *et al.* (2007), female gonads not only contain a much higher lutein and isozeaxanthin content, but also exclusively contain keto-carotenoids, possibly contributing to colouration. Additionally, these authors obtained an inferior β-carotene:echinenone ratio in female gonads, which also may imply a more intense colour in female individuals. However, account must be taken that male gonads discharge a white fluid, while the female gonads emission is orange/red (Lustres-Pérez *et al.*, 2010). Though the areas of the gonads covered in fluid were always avoided in the analysis, these major differences may have a marked effect on the visual perception of gonad colouration. Zhao *et al.* (2010) also obtained with *S. intermedius* higher a* parameter values in females, just as Phillips *et al.* (2009), who rated ovaries of *Evechinus chloroticus* significantly higher in terms of uniformity of colour.

Overall, as applied in the salmon aquaculture, gonad colour in sea urchins can be easily modified through the use of different dietary carotenoids (Carboni, 2013), in order to reach the echinenone concentrations found in wild populations (Carboni *et al.*, 2015). Since echinenone is not commercially available to be used in a formulated feed for sea urchins (Symonds *et al.*, 2007) and there is the need to reduce the dependency from wild

macroalgae (Carboni, 2013), this study emphasizes the efficacy of accessible vegetable sources, particularly pumpkin, for colour enhancement. In aquaculture, the need for diverse feed sources (Carboni *et al.*, 2015) in order to attain not only acceptable colour, but also somatic or gonadal growth, can be easily facilitated through rotational feeding regimes (Shpigel *et al.*, 2005), which allows to easily alternate between different diets, according to the availability of the ingredients and the nutritive requirements of the species.

5. Conclusions

The results obtained in this study demonstrate the suitability and efficiency of alternative feed sources for the rearing of adult *P. lividus* individuals in Recirculating Aquaculture Systems (RAS). First of all, the required period of one month of starvation was, once again, proven to be a simple and effective method to obtain sexual synchronization between individuals. During the feeding trial of 90 days, all of the three artificial diets promoted somatic and gonadal growth, as well as the progression in the reproductive cycle from spent stage, without mortality. The formulated diets were promptly accepted by the sea urchins and easily manipulated. However, diet B (maize, spinach & macroalga *Laminaria digitata*) was the most successful regarding test diameter and total weight increase. Conversely, diet A (maize and spinach), as the least consumed diet, promoted the highest gonadosomatic index and gonadal wet weight. Furthermore, diet C (maize, spinach and pumpkin *C. maxima*) led to the most intense gonad colouration, bringing new perspectives regarding the inclusion of carotenoids in a future commercial diet. Regarding the reproductive cycle, while diet A was the most efficient in promoting a rapid sexual maturation, allowing to obtain gametes in the winter, which is very unlikely in wild populations, diet B proved to be more suited for consumption purposes, given the slower progression in the gametogenic cycle. However, the nutritive requirements of each reproductive or life stage should be studied in more detail, in order to maximize the production according to the desired goal. Furthermore, it should also be noted that given the nature of the experimental jellified diets created for this study, with high percentages of water, the results should be analysed in the context of this experiment. In a future extruded commercial diet for *P. lividus*, including these ingredients, the aspects regarding growth, maturation and colouration could certainly be much more optimized. Above all, the present results highlight the promising use of land vegetables and possible sub-products of this industry in artificial diets for a large scale echinoculture, contributing to the circular economy and also preserving the natural populations.

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