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Journal of Aquatic Sciences and Oceanography

RESEARCH ARTICLE

Using Artificial Feeds for the Culture of the Sea Urchin *Paracentrotus Lividus* (Echinodermata, Echinoidea)

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Citation: Tarifa G, Sterioti A, Chatzifotis S, Kentouri M (2019) Using Artificial Feeds for the Culture of the Sea Urchin *Paracentrotus Lividus* (Echinodermata, Echinoidea). J Aqua Sci Oceanography 1: 205

Abstract

The sea urchin *Paracentrotus lividus* (Lamarck 1816) is of particular economic interest due to the high commercial and nutritional value of its gonads, which are widely consumed worldwide. Increased demand has led to overfishing of the species and the collapse of natural stocks in areas where it was in abundance. Thus, the need for culture methods with the use of appropriate food has emerged. In the present study, two artificial handmade feeds of different composition concerning the animal/vegetal protein ratio were tested in rearing sea urchins in two different water qualities (forage water - sea water) for three months. Under these conditions, gonadal growth had been satisfactory over this short period of time compared to wild type sea urchins; however their color differed, despite the enrichment of their diet with natural β-carotene product.

Keywords: Sea Urchin; *Paracentrotus Lividus*; Gonad Development; Cultivation; Artificial Feeds

Introduction

Since 1792 sea urchin *Paracentrotus lividus* was already known as a marketable species [1-4]. This species has gained special attention due to the commercial importance of its gonads (both sexes) [5] which are widely consumed and appreciated especially as a seafood delicacy [6]. Moreover, according to the Pacific Urchin Harvesters Association [7] the sea urchin gonads have a particular nutritional value. As a consequence of the increased demand, this species has been intensively harvested in most areas where it lives, especially on the Mediterranean [8-10]. Overfishing of the species has led to the collapse of the natural stocks and the extinction of the sea urchins from areas where they were abundant [6,9,11-19]. The decline in natural stocks, the increased market demand and the high retail price, has created interest for the development of economically viable cultivation methods, and stimulated research into the possibilities and conditions for the integration of sea urchins in aquaculture [2,10,16,17,20-25].

Echinoculture is based on the production of commercially accepted gonads. In order to be marketable, the gonads of the sea urchins must be characterized by the right color, the appropriate taste and texture (firmness), with their quality to determine the price of the final product (up to US$333/kg) [26]. It has been shown that the gonadic growth is strongly correlated with the availability, quantity and quality of the food, however it must be emphasized that each species of sea urchin has its own environmental/chemical cue [27]. It is indicated that diet based on *Laminaria digitata* often results in achieving the desired color in *P. lividus* gonads, however both researchers and producers recognize the need for cost-effective artificial feeds that would enhance the gonadal growth while reducing any negative effects on taste or color.

One of the main challenges in starting a *P. lividus* culture is to determine the most appropriate diet. The development of artificial feeds is necessary in order to meet requirements such as availability, stability in quality and composition, stability in the water and ease of use compared to fresh algae [10]. In their natural environment *P. lividus* prefers plant source of nutrition but generally and especially in the Mediterranean, it is considered as environmentally unsound practice to gather sufficiently large quantities of macro algae to support intensive cultivation [9]. Moreover, artificial feeds generally provide better growth rates than algae, which have the disadvantage of periodic fluctuations in their quality and abundance [28,29], while they present low consumption rates and high absorption capacity.

The gonads are known to have a dual function: in reproduction and storing nutrients [30,31]. Gonadal production depends on a combination of factors such as food intake, the stage of the reproductive cycle, season, and temperature. However, nutrient

consumption [30] is decisive, with artificial foods more efficiently promoting gametocyte proliferation and/or gametogenesis [32], while increased gonadal volume is also due to the storage of lipids and/or carbohydrate in their cells [33]. The reason seems to be that once a percentage of energy is allocated to conservation, the remaining percentage is distributed to reproduction and physical development [34]. Unlike artificial diets, the algae diet is often inadequately nutritious to promote anything other than skeletal development. Researchers have shown that when food does not provide nutrient accumulation in the intestines at adequate levels, there is no subsequent shift of nutrients to the gonads for their development. Also, that artificial food results in significant growth of gonads, greater than observed in the natural environment.

Concerning the formulation of food pellets appropriate for sea urchins, it is important to achieve sufficient consistency and stability in water. If the artificial feed isn't sufficiently solidified; it undergoes deterioration in its quantity and quality, because of the dissolution or oxidation of specific components such as vitamins, minerals, proteins and lipids. This results in feeds with reduced nutritional value [10,35]. In addition, the decomposition of food pellets is faster, therefore it can create water pollution due to the increase of dissolved nitrogenous substances (nitrate, nitrite and ammonia) [34], anoxia conditions and the production of sulfur compounds if the detritus accumulate at the bottom [35], especially in closed systems. This results in increased breathing rates, hence greater energy loss, and constitutes a stress factor capable of inhibiting growth and possibly causing death due to oxygen deficiency [36]. Furthermore, sea urchins display a cyclic feeding behavior, alternating periods of feeding and fasting, which is different between individuals [10,37-39] and they consume the food slowly, resulting in losses during grazing. Also, the sea urchins more readily consume solid food as their chewing system is built for scraping/raking. Therefore, food must be permanently available *ad libitum* and should remain solid for a few days in water, unlike fish pellets that needs to remain intact for only a few minutes, or shrimp food that needs to remain compact for a few hours [10]. Moreover, it is indicated that the shaping of food pellets is also important. Thinner food particles and flattened or cylindrical pellets, which also may drift in inaccessible places such as the corners of the aquaria, are more difficult to be manipulated and consumed by the sea urchins, which are forced to constant repositioning in order to bring the food item in their mouth. So feeding rates seems to be higher on more massive food items [40-42].

After several tests, it was found that the addition of 12.5% gelatin solution to the solid feed mixture (Premium Gel Grado 1, Enologica Vason) as a binder helped to achieve a good consistency and long-term stability of the pellets, which could remain intact in the water for at least 2 days. However, when calculating the energy content of the feed, it is necessary to take into consideration the additional amount of protein.

Two kinds of diet were chosen, an animal based one (fish derived protein: diet A) and a mixed based one (fish derived protein and carbohydrate (wheat): diet M). It was chosen to enrich diets with animal derived protein, because artificial diets which are formulated with higher level of protein than natural algae, allow sea urchins to shunt more energy into gonadal production [43-46]. Many researchers have shown that the second type of diet has very good results in sea urchin's growth, similar to those of a 100% animal based feed, at a lower cost [34]. According to previous investigations on gonad coloration, artificial feeds result in the production of large gonads but pale in color. Thus, in the final mixture of the mixed diet (M), natural beta carotene product (softgels 25,000 IU, Nature's Plus) was added, extruded from the microalgae *Dunaliella salina*. According to literature this results in the best intensity of gonad coloration [47-52], in contrast to synthetic β-carotene [29,51,53,54] especially for *P. lividus*] or astaxanthin that both fail to produce the desired results [29,53,55]. Bright yellow-orange (mango) is considered as the optimum gonad color.

The aim of this study was to monitor gonadal growth and their color in adult sized *Paracentrotus lividus* fed on artificial handmade formulated feeds with varying animal derived protein quantity, in comparison to control groups from the wild.

Material and Methods

The experiment was carried out at the Aquarium of Crete (Cretaquarium-Thalassokosmos) part of the Hellenic Centre of Marine Research (Heraklion-Crete) under controlled conditions. Tanks with a capacity of 150 l were used which were equipped with an extra perforated bottom 1 cm high, so as to avoid the recycling of the feces that end up there. The sea urchins were placed individually into plastic perforated containers after it was confirmed that good circulation of water and sufficient oxygenation was allowed inside them. Thus, each individual was isolated in order to avoid the possibility of competition and injuries [40,41]. Also, each individual had access only to the amount of food they were given and we could visually inspect the food they consumed. For the present study, 90 sea urchins were collected from Elounda (Mirabello Gulf), Crete, by diving in the end of September and 10 more in the end of December to sample as a second control group by the end of the experiment. The seawater temperature at the time of the field sampling was 24.5 °C (September) and 15 °C (December). The sea urchins' reproductive cycle in September-October is mainly in the spent phase although there can be found individuals in the growing phase. It is noted that for Mediterranean populations of *P. lividus* there are two main spawning periods, in spring and autumn. In December-January their reproductive cycle is mainly in spent or recovering phase. The sample size was determined concerning two factors: the number of the tanks available and the density of the sea urchins reared in each tank which it was chosen to remain low in order to avoid possible water pollution by the degradation of the food pellets. The diameter of the selected individuals was 3.5-4 cm, size which is considered as marketable.

The composition of the two different feeds that were used is reported in Table 1. For the preparation, the fish meal powder (LT 999 Fishmeal) and/or the wheat flour were mixed with the vitamin mixture. Then the fish oil and beta-carotene were added. Finally, the gelatin solution was added gradually. The paste was spread on a metal surface, cut into cubes ~ 0.8cm [34,42] and allowed to dry thoroughly, in an air oven at 40 °C for 32 hours (16 for each side). Thereafter, the food pellets were kept in the freezer (-20 °C) until they were used.

Feed composition			
Mixed (M)		Animal (A)	
Wheat	44,7 %		
Fish meal	44,7 %	Fish meal	89,4%
Fish oil	8,9 %	Fish oil	8,9%
Vitamins	1,7%	Vitamins	1,7%
B-carotene	0,24%		

Table 1: The composition of the two artificial feeds

As it is shown in Table 2, the sea urchins collected were allocated in eight groups of ten individuals each, in order to have a low density in each tank and ensure good water circulation. For each diet type used there were two replicate (r1, r2) groups of sea urchins reared in seawater (SW) and two replicate groups reared in forage salt water (FW). There were used two qualities of rearing water, as between seawater and forage water there are differences in pH levels and content in metals. The O2 was maintained at 94 ± 1% saturation in both conditions: forage water (18.8 °C, pH 7,8, 36 ppt) and seawater (21,2 °C, pH 8,1, 38 ppt) (Table 3). Photoperiod was natural with 12h light and 12h dark. Sampling was carried out once a month and the gonadosomatic index (GSI) was calculated and compared to that of wild sea urchins.

Treatments	Rearing water	Individuals	Samplings (months)		
			1st	2nd	3rd
Control 1		10			
M (rep.1)	SW	10	3	3	3
M (rep.2)	SW	10	3	3	
A (rep.1)	SW	10	3	3	3
A (rep.2)	SW	10	3	3	
M (rep.1)	FW	10	3	3	2
M (rep.2)	FW	10	3	3	
A (rep.1)	FW	10	3	3	3
A (rep.2)	FW	10	3	3	
Control 2		10			

Table 2: Experimental set up

	Forage water (FW)	Seawater (SW)
Temperature	18,8 ± 0,7 °C	21,2 ± 0,1 °C
pH	7,8 ± 0,07	8,12 ± 0,03
O_2 saturation	94 ± 1 %	94 ± 1 %
Salinity	36 ppt	38 ppt
Water renewal	~1,7 lt/min	~1,7 lt/min
Photoperiod (Light/Dark)	12 h/12 h	12 h/12 h
NH_3/NH_4^+	0 mg/l	0 mg/l
NO_3^-	0 mg/l	0 mg/l
Density	1,66 kg/m³	1,66 kg/m³

Table 3: Rearing conditions of the sea urchins *Paracentrotus lividus*

In total, 5 samplings were carried out. The first, involved a control group of wild individuals directly after their capture from the shore, at the beginning of the experiment (Control 1). The 2nd sampling included 3 individuals from each replicate after one month of rearing. Correspondingly, the 3rd and the 4th samplings took place with the sea urchins after two and three months of rearing respectively. To confirm that any changes in the GSI were actually attributable to nutrition and not to a different stage of the reproductive cycle, a second sampling of a control group of wild sea urchins was carried out by the end of the experiment, along with the last sampling from the tanks (Control 2).

During sampling, individuals were individually dehydrated by staying on absorbent paper for 15 minutes. Next, they weighted (g), and their diameter and height (cm) of the test without the thorns, were measured with a caliper. Then, the sea urchins were dissected and the gonads were extracted and weighed (g) separately after staying on absorbent paper for 10 minutes. Afterwards, the gonadosomatic index (GSI) was calculated using the following equation:

$$GST(\%) = \frac{(\text{Wet gonad weight})}{(\text{Wet body weight})} \times 100$$

GSI shows the percentage of the ratio of the wet weight of the gonads to the total wet body weight. Although it is affected by the amount of the coelomic fluid and the content in water of the tissues, as well as by the physical development, it remains the most common index used to describe gonadal production (e.g., 56-62 for *P. lividus*). It is noted that gonadosomatic index does not serve as a gonad quality indicator; it is used to estimate the gonadal growth in comparison to wild sea urchins. In order to assess the gonad quality there are multiple factors which should be tested such as color, hardness, resilience, taste. In the context of this work we have focused on gonadal growth and the color of the gonads of cultured sea urchins.

At each sampling, the color of the gonads of each individual was assessed by observation under constant light conditions. It was compared to a color scale (Munsell color chart) and the RGB numbers were noted. After sampling, the extracted gonads were stored in the freezer (-20 °C) for carotenoid analysis.

Statistical analysis

For the statistical analysis on the gonadosomatic index values between the sea urchin groups of each sampling and the control groups, was performed One-Way ANOVA. Tukey Test was used for all pairwise comparisons of the mean responses to the different groups and the identification of statistically significant differences (Figure 3). The effect of the two types of feeds and the two types of rearing water (predictor variables) on the gonadosomatic index (dependent variable) of each sea urchin group per sampling was estimated by Two-Way ANOVA (Figure 4). When average values differed statistically, Tukey test was used to analyze the differences. All results are presented as mean ± standard deviation. The probability of statistical significance was $P \leq 0.05$. The statistical analyses were performed using the SigmaStat program Version 3.5 for Windows.

Carotenoids in gonads

The method followed was the one described by Tsushima & Matsuno [63]. Carotenoids were extracted with acetone ≥ 99.5% as extractive agent as it is well mixed with the water content without emulsifying. The gonads stayed in acetone for about 30 minutes and homogenized with acetone in a mortar. The solution was collected and the process was repeated until the acetone solution was clear (about 3-4 times). The final solution was filtered through 20-25μm pore size cellulose filters (No. 4, 90 mm diameter, Whatman) to remove aggregates and cell debris from the gonads. To rinse the solution of the carotenoid pigments from the acetone, it was gently mixed with hexane (n-hexane > 99%) and water in 1:1 ratio in a separatory funnel until the two phases were separated (Figure 1). The colorless underlying water-acetone phase was discarded and the supernatant hexane layer containing the carotenoids was isolated.

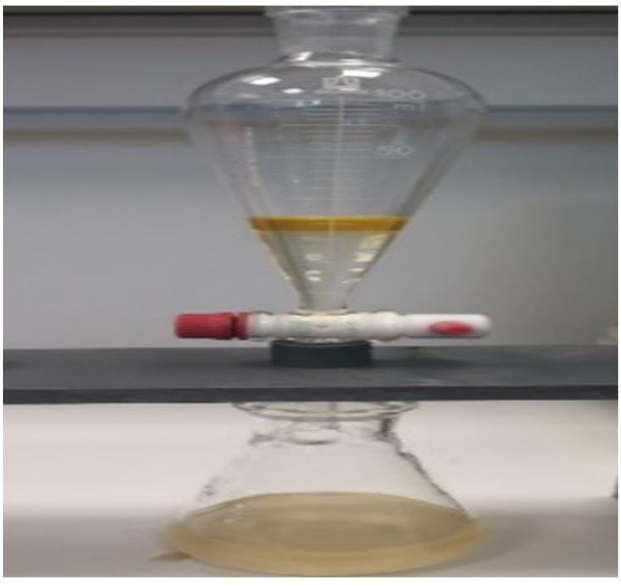

Figure 1: Separation of phases and transfer of the carotenoid pigments to hexane

Subsequently, the hexane solution was concentrated on a rotary evaporator at 40 °C and 30rpm. The carotenoid pigments were rinsed with 3 ml of hexane and placed in amber glass vials. The solution was filtered again using a 0.2μm polytetrafluoroethylene (PTFE) syringe filter (13mm diameter, PTFE CR syringe filters, Gelman).

The absorption of the solutions was measured in a spectrophotometer at 445, 450, 459 and 473nm. Absorption measurement was carried out at these wavelengths in order to estimate the concentration of total carotenoids based on the specific carotenoids of interest here. These carotenoids are echinenone which is the determinant factor for the gonadal coloration, β-carotene as the metabolic precursor of echinenone, and isocryptoxanthin which contributes to the metabolic conversion of β-carotene to echinenone. Previous researches indicate that β-carotene in hexane solvent presents a maximum absorption at 449.9 nm (SCOR data WG 78) and echinenone in hexane solvent presents a peak at 459 nm [64]. According to Plank, *et al.* [65], the measurement of absorption at 445 nm serves to identify echinenone, β-carotene, lutein and zeaxanthin. In the present work satisfactory results were obtained during preliminary tests, so the measurement at 445nm was applied to the samples of the experiment. Furthermore, Shina, *et al.* [66] found a maximum absorption of isocryptoxanthin at 450 and 473 nm.

For the total carotenoid concentration, measured in μg/g of wet gonad weight, the following equation was used:

$$x\ (\mu g/g) = \frac{A * y\ ml * 10^4}{A_{1cm}^{1\%} * B}$$

Where x: the concentration of carotenoids
A : absorption wavelength
y : volume of the solution
$A_{1cm}^{1\%}$: the carotenoid absorption coefficient in the particular solvent (here 2500)
B : weight of the sample

Results

Gonadosomatic Index

Results of the gonadosomatic index calculation from the samplings during the experiment, as well as from the control groups are shown in Figure 2. It seems that the diets with artificial feeds resulted in fast gonadal growth, while the longer the sea urchins were submitted to these diets; the greater was the gonadal growth in contrast to the wild sea urchins.

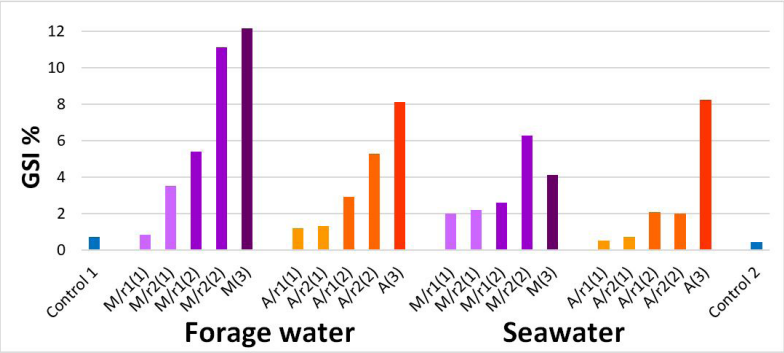

Figure 2: The average sea urchin Paracentrotus lividus GSI (%) per replicate and control groups. M, A: type of the feed, r1, r2: replicates 1 and 2, (n): samplings (1st, 2nd, 3rd month)

Statistical analysis

Results of the statistical analysis for the sea urchins per sampling as a whole in comparison to the control groups are presented in Figure 3.

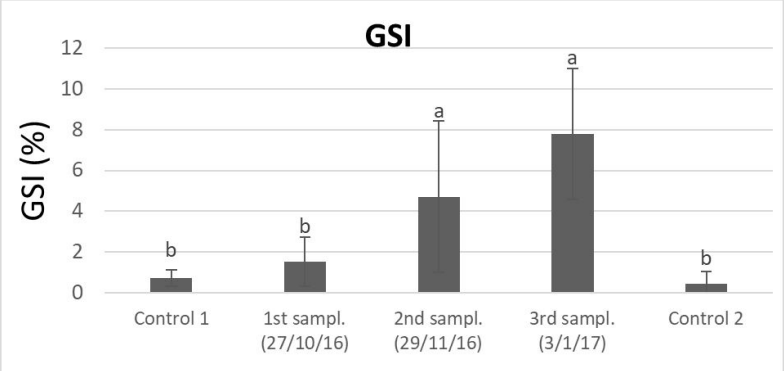

Figure 3: The mean (± SD) gonadosomatic index (GSI) by sampling and control groups. The different grammatical index indicates the statistically significant differences ($P \leq 0.05$) in one-way ANOVA on Ranks

They indicate that there is a statistically significant difference ($P < 0.001$) between the GS index of the sea urchins sampled in the 2nd and 3rd month and the urchins sampled the 1st month and the wild (control groups).

Figures 4 show the results of the GSI statistical analysis between the treatments for each sampling separately, and are mentioned the factors between which there is a statistically significant difference. In the 1st sampling, it is evident that there is a statistically significant difference in the gonadosomatic index between the two types of feed ($P = 0.012$), irrespectively of the type of rearing water. In the 2nd sampling, there is a statistically significant difference ($P = 0.019$) between the two feeds and also between the two types of rearing water ($P = 0.032$). There is no statistically significant difference between the factors of the diet and water type ($P = 0.495$), so the differences

observed between the diets do not depend on the type of rearing water, therefore there is no interaction. In the 3rd sampling, there is a statistically significant difference (P = 0.002) between the two types of rearing water concerning the mixed feed (M), while the same doesn't apply for the fish based feed (A). There are also statistically significant differences between the two feeds in terms of forage water (P = 0.046) and seawater (P =0.029). It seems that the effect of each feed on the GSI depends on the type of rearing water, with a statistically significant interaction (P = 0.008) between the two factors (feeds and water type).

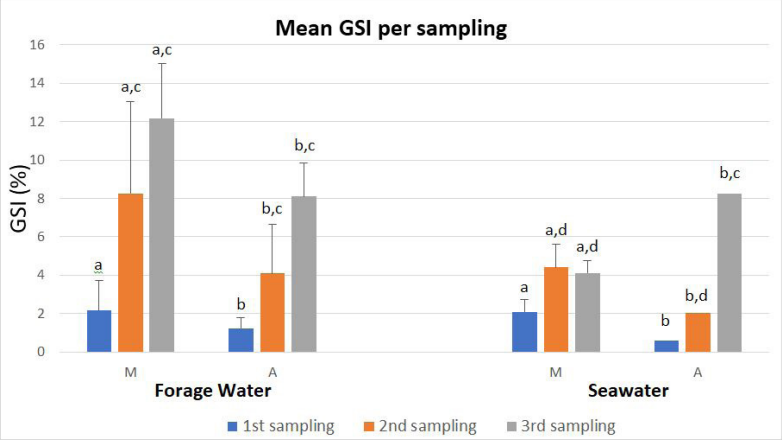

Figure 4: The mean (± SD) gonadosomatic index (GSI) of each sampling, per treatment (diet and water type). The different grammatical index indicates the statistically significant differences (P ≤ 0.05) in the percentage of GSI between factors (Two-Way ANOVA)

Gonad color

Figure 5 shows the percentage of the total number of sea urchins per sampling which had the appropriate gonad color according to the visual observation assessment.

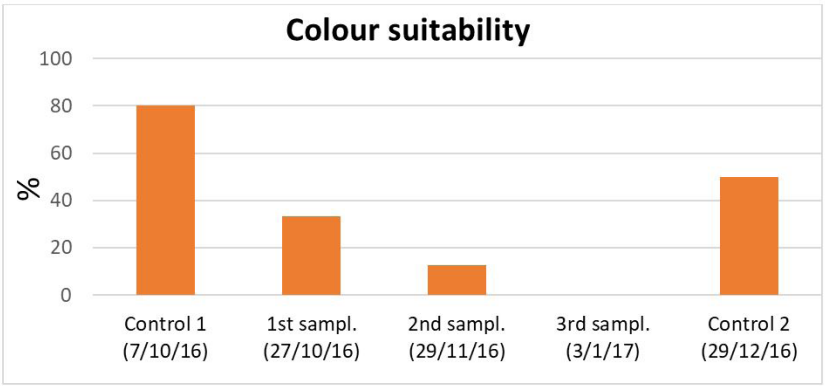

Figure 5: The results of the assessment of the sea urchins characterized by an appropriate color on the gonads, in percent

It seems that as gonadal growth increases, the number of the samples which have the appropriate color is reduced, with the majority of sea urchins' gonads to be pale in color (Figure 6). The results are presented in total per sampling, without separation between the two diets and between the two types of rearing water, because no difference in color levels was observed.

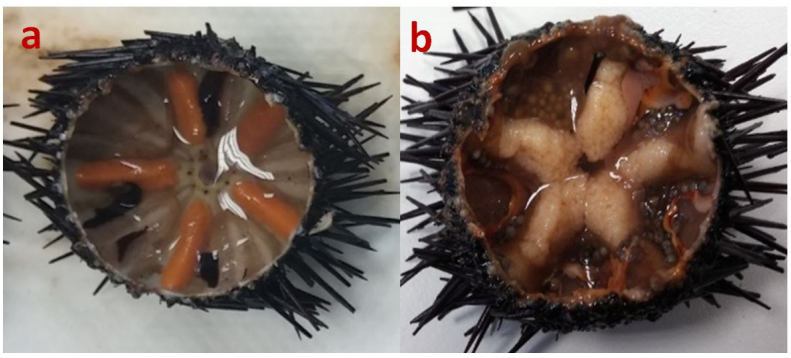

Figure 6: a) The desired color in the gonads, **b)** Commercially inappropriate color

The same pattern is depicted also in the spectrophotometry results (Figures 7a-7d). Generally, no differences appear among the four different wavelengths at which the absorption was measured, while the graphs show the same pattern observed by the visual estimation of the gonad color: the amount of total carotenoids tends to decrease as the period that sea urchins feed on artificial feeds increases.

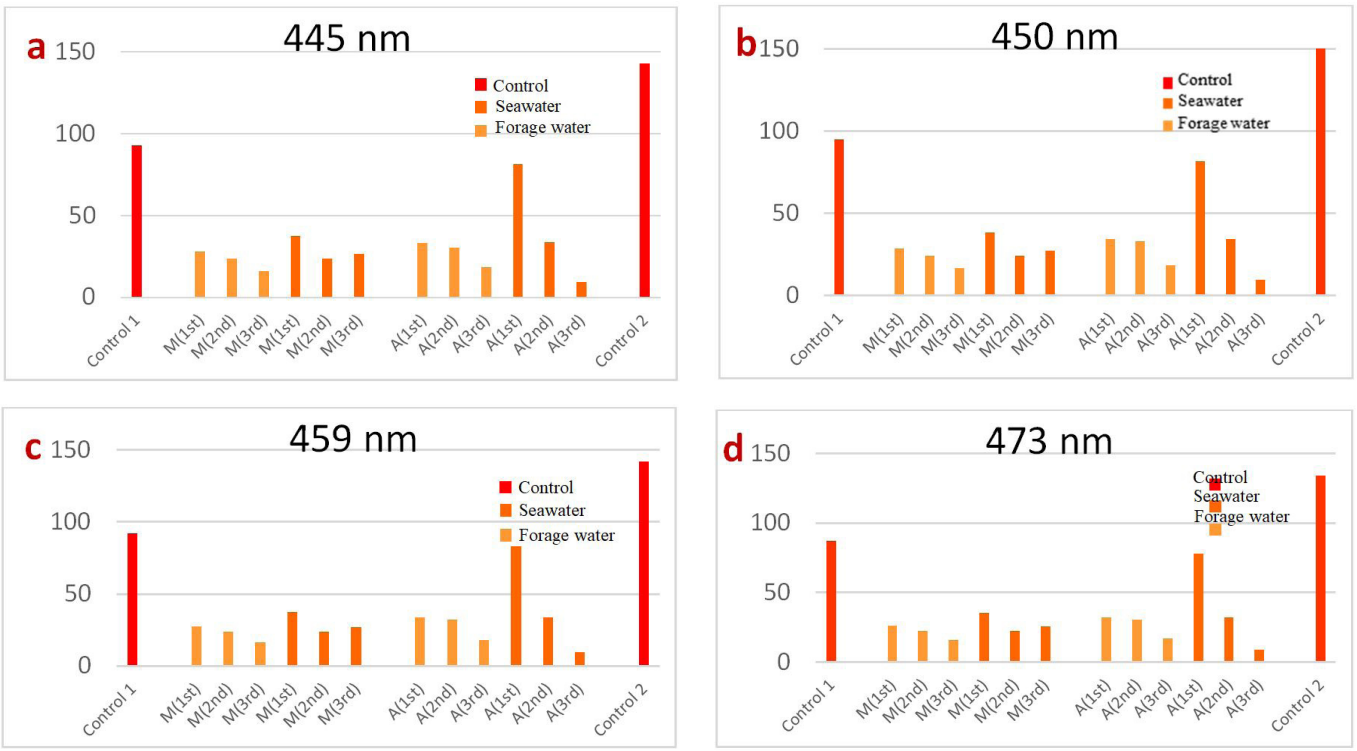

Figure 7a-7d: The total carotenoid content (μg/g) of the gonads at each measured wavelength. M: diet M, A: diet A. The number in brackets corresponds to the sampling

Discussion

Gonadal production generally depends on food ingestion, consumption rate, reproductive cycle, season and temperature [30,31,67-70]. Our results are consistent with those of previous researches on various species of echinoids, in which the gonadosomatic index of sea urchins fed on artificial feeds appears significantly increased compared to sea urchins which feed on algae [27-29,32,46,57,71,72]. In the present study, the gonad mass increased significantly in just three months compared to sea urchins of the wild. Sampling of a second control group by the end of the experiment excluded the assumption that this development was due to a different stage of the reproductive cycle between the first and the last sampling period. Furthermore, there is no inhibition of gonadal growth due to pH levels (forage water) as it has been recorded for other echinoid species such as *Hemicentrotus pulcherrimus* even at pH 7.8 [73,74], possibly because exposure to this pH was not for a long period. On the contrary, there is a decrease on the gonadal growth rate, noticed in the 2nd and 3rd samplings, in sea urchins provided with mixed diet and reared in seawater compared to sea urchins reared in forage water. It is supposed that these differences are attributed to the different temperature between the two types of rearing water, even if both are within the temperature range (18-23 °C) which is considered as optimal for *P. lividus* growth [1, 17,34]. Even though the quantity of food ingested may be the same, it is known that temperature affects significantly the metabolic rate, digestion efficiency and/or the nutrient conversion process, therefore the somatic and gonadal growth [43,61].

Despite the fact that the two diets contained a different percentage of animal (fish) protein, they gave equally satisfactory results in the gonadal growth, while they were both readily consumed by the sea urchins. Therefore, it appears that a mixed carbohydrate-protein diet can give equally desired results, with a 100% animal derived protein diet, at a lower cost. Also, the shape of the pellets (cubes) seems to have served the needs of sea urchins by enabling them to handle it easily [32,42], at least in the special containers where they were placed. It is noted that feed cubes should have been a little larger as several individuals had consumed the total amount before new one was supplied. However, the pale coloration of the gonads makes them commercially undesirable [73,75-77].

Generally, the types and amount of carotenoids in the echinoderm gonads vary greatly with age and location, between and within species. Thus, the gonad color of wild populations of *P. lividus* varies greatly from individual to individual and can range from very pale yellow to dark brown/red [54]. This means that gonads that are deemed to be commercially unacceptable in terms of color can have similar carotenoid (echinenone) content. Therefore, carotenoid content is the determinant factor for the gonad pigmentation, but other factors may also affect the visual perception of coloration [54].

It is obvious that too pale or too dark gonads are undesirable in the market. It is usual a pale off-white gonad color to be observed when aquacultured sea urchins are fed high protein formulated diets [62]. As reported, algae diet has been successfully used in improving the color of the gonads [51,78] and as a part of an artificial feed it has been successful in providing the echinenone precursor, β-carotene [29,51]. Nevertheless, in the present study, despite the enrichment of the M ration with a natural β-carotene product from *Dunaliella salina,* no improvement in color was observed in comparison to feed A which did not contain additional carotenoids. The fact that β-carotene was added in the form of oil rather than powder created concern whether oil could escape more easily in the water when the food cubes were shattered by the sea urchins resulting in non-exploitation of carotenoids. But, analysis of feed cubes showed no loss of carotenoids in the water. Consequently, the following hypothesis can be made: the amount of β-carotene in the feed was not sufficient, the duration of the experiment was too short, the gonad mass increased rapidly, so the addition of β-carotene was not "expressed" in color. In this case the colour of the gonads could be improved over time, when their mass would stabilize.

As mentioned, gonadal quality is determined by many characteristics such as color, texture, firmness and taste which is significantly affected by the amount of nutritive phagocytes (NP) in the gonads [62]. Continuing this work those characteristics will be checked as well, utilizing histology methods and biochemical analysis of the gonads to estimate the proximate composition of protein content, lipid content, fatty acid and carotenoid pigment compositions.

Conclusion

The use of artificial diets in rearing of sea urchins promotes gonadal growth which is the main objective of sea urchin aquaculture. Gonadal development had been satisfactory over a short period of time, compared to the sea urchins in natural environment that fed on algae. Although the two artificial feeds contained different proportion of animal protein, they presented equally satisfactory results in gonadal growth. It seems that the use of a mixed feed based on animal protein and carbohydrates can have the desirable results in gonadal enhancement in a short period of time with a lower cost. Unfortunately, the color of the gonads, which is an important criterion of marketability, was not the desirable (bright yellow-orange), so further improvement in the composition of the feeds is needed in order to acquire the required characteristics.

Aknowledgements

We are grateful for the financial support and the provision of infrastructure by the Cretaquarium, in order to complete our research.

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