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"Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída."

(Ioannis Emmanouil Choulis, n° 70009)

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Abstract

Sea urchins' gonads are considered as a delicacy, with certain countries such as Japan, Honk Kongand United States, having the highest import rate worldwide. The rising demand has led to an unsustainable exploitation of the wild sea urchins' stocks. This situation led to the development of echinoculture, covering the ongoing demand and alleviating the fishing pressure. Past studies have shown encouraging results on the somatic and gonadal development, when the feed availability isn't a limiting factor. In this study, 308 individuals of *Paracentrotus lividus* were tested in four (4) distinguished methods: a) natural (non-manipulated) photoperiod and temperature, corresponding to the experiment's time period, with natural diet (*Ulva* spp.*)*, b) natural photoperiod and temperature with a formulated diet (pellets), c) manipulated photoperiod and temperature with natural diet (*Ulva* spp.), corresponding to the environmental conditions starting from January until June and d) manipulated conditions with pellets as feed. Data regarding monthly: feed intake, gonadosomatic index and the state of the gonad maturation, were gathered and statistically analyzed. GSI was increased in all the groups regardless of the treatment. Significant differences were found in the sea urchins fed with pellets which had the highest final GSI, compared to the sea urchins fed with *Ulva* spp. There was no correlation between GSI and the natural or manipulated environmental conditions. The sea urchins in manipulated photoperiod and temperature, after the spawning period in December 2021 – January 2022, had advanced in their gametogenic cycle, compared to the sea urchins in natural environmental conditions. Overall, the experiment verified past studies in were by manipulating the rearing conditions (photoperiod, water temperature), the gametogenic cycle of *P. lividus* progressed faster, supporting the world echinoculture industry.

KEYWORDS: *P. lividus,* Reproduction, Photoperiod, Temperature, Echinoculture

Resumo

A indústria da aquacultura tem sido capaz de cobrir a crescente procura global de produtos da pesca. Em 2018, a indústria aquícola mundial atingiu o recorde de 114,5 milhões de toneladas de peso vivo, com valor de mercado de 263,6 milhões de dólares. O crescimento da aquacultura é notável, partindo de 25,7% em 2000 e chegando a 45% em 2018. É evidente que os produtos da aquacultura têm grande impacto nas estratégias globais de segurança alimentar e nutrição.As gónadas dos ouriços-do-mar são consideradas uma iguaria, sendo o Japão, Honk Kong e Estados Unidos, os países com maior taxa de importação em todo o mundo. Do ponto de vista nutricional, este produto é considerado uma valiosa fonte de proteínas, ácidos gordos (nomeadamente os poliinsaturados, PUFA), carbohidratos e carotenóides. O aumento da procura destes organismos para consumo levou a uma exploração insustentável das populacões de ouriços-do-mar selvagens. Esta situação levou ao desenvolvimento da equinocultura, cobrindo a procura e aliviando a pressão da pesca. Pesquisas anteriores mostraram resultados animadores sobre o desenvolvimento somático e das gonadas, quando a disponibilidade alimentar não é um fator limitante. Além disso, parâmetros físicos como o fotoperíodo e temperatura podem afetar positivamente o crescimento das gónadas, com a temperatura a apresentar maior significância neste aumento. Neste estudo, são investigadas as condições ambientais e de alimentação adequadas na equinocultura de *Paracentrus lividus*, em condições de laboratório no período de outubro de 2021 a fevereiro de 2022. No ensaio, 308 indivíduos de *P. lividus* foram sujeitos a 4 tratamentos: a) fotoperíodo (não manipulado) e temperatura naturais, durante o período de tempo do ensaio, com dieta natural (*Ulva* spp., NUC), b) fotoperíodo e temperatura natural, com dieta formulada ("pellets", NPC), c) fotoperíodo e temperatura manipulados (simulando as condições ambientais de janeiro a junho) e com dieta natural (*Ulva* spp., MUC), e d) condições manipuladas com "pellets" como ração (MPC). Para as condições ambientais naturais, o fotoperíodo foi de 9,4L:14,6D a 10,4L:13,6D, enquanto a temperatura variou entre 13,92-19,22°C. Em relação às condições ambientais manipuladas, o fotoperíodo variou entre 10,1L:13,9D e 13,5L:11,5D. A temperatura para as condições manipuladas foi de 14,58-18,01 °C. Para cada tratamento foram estabelecidas três (3) replicados, em tanques com dimensões de 0,45*0,7*0,45 m totalizando um volume de 100 L. Os ouriços-do-mar foram colocados dentro de caixas plásticas de dimensões 0,57*0,38*0,08 m, com uma rede plástica divisora no meio para diferenciar os ouriços-do-mar consoante o seu regime de alimentar. A temperatura da água foi regulada com o uso de refrigeradores-aquecedores programados e o fotoperíodo com o uso de uma cubertura preta e iluminação temporizada. A renovação da água foi de aproximadamente 112,5 L/h, com arejamento garantido com pedras difusoras. A temperatura da água e os níveis de oxigénio foram medidos diariamente com o auxílio de uma sonda multi-paramétrica da Estação Piloto de Piscicultura de Olhão (EPPO). Os "pellets" utilizados neste ensaio, foram desenvolvidos e fabricados pela SPAROS, I&D (Olhão, Portugal), enquanto que a *Ulva* spp. foi recolhida de um tanque de terra nas instalações da EPPO. Os ouriços-do-mar foram alimentados uma vez a cada 3-4 dias e a quantidade de ração foi adaptada de acordo com a biomassa total existente. A amostragem mensal foi realizada determinando o peso total de cada replicado. Foram recolhidos 5 indivíduos para avaliação gonadossomática e para processamento histológico e posterior análise. O peso total das gónadas foi registado. Os estados de desenvolvimento das gónadas de *P. lividus* foram identificados de acordo com a escala de maturação de Byrne (1990) em seis estados: recuperação (I), crescimento (II), prematuro (III), maduro (IV), postura (V) e pós-postura (VI).

Mensalmente foram recolhidos e analisados estatisticamente os seguintes dados: consumo de ração, índice gonadossomático e estado de maturação das gónadas. Os dados foram testados para normalidade (Shapiro-Wilk) e para variâncias iguais (teste de Levene) antes de compará-los para diferenças significativas. Em continuação para as medidas de consumo de ração e GSI, o teste t de Welch Two Sample e o teste não paramétrico de Kruskal-Wallis foram usados com um contraste adicional post hoq ANOVA Tukey, quando o teste não paramétrico foi significativo ($p<0,05$). Os ouriços-do-mar alimentados com ração granulada apresentaram um consumo de ração menor em comparação com os ouriços-do-mar alimentados com *Ulva* spp., sendo o consumo médio máximo de ração de 0,427 e 1,406 g/dia/SU, respectivamente. Este resultados pode ser atribuído ao fato de que os ouriços-do-mar, devido a um mecanismo de adaptação, poderem consumir menos ração e atingir o crescimento adequado quando a ração é rica em proteína bruta. Não houve diferenças estatisticamente significativas em relação ao consumo de ração e às condições ambientais (naturais e manipuladas). O Indice Gonadossomático (IGS) inicial foi de 0,63 ± 0,44%. Sendo que os ouriços-do-mar alimentados com ração apresentaram maiores valores de IGS, quando comparados ao grupo com *Ulva* spp. O maior valor final foi medido nos ouriços-do-mar alimentados com pellets em condições manipuladas (16,81 ±1,82%), enquanto o menor foi observado nos ouriços-do-mar alimentados com *Ulva* spp. em condições manipuladas (11,08 ±4,41). Os fagócitos nutritivos nas gónadas podem acumular os nutrientes de forma mais eficiente, quando a dieta contém altas percentagens de proteína. Não se verificou efeito significativo das condições ambientais naturais ou manipuladas no IGS. Após o período de desova (dezembro de 2021 a janeiro de 2022), o IGS não diminuiu significativamente, o que pode ser atribuído ao duplo papel das gónadas como órgãos reprodutivos e armazenamento de nutrientes. Em relação à maturação das gónadas os dados foram analisados qualitativamente. A amostragem inicial foi composta por 8 indivíduos, tendo-se verificado que todos eram imaturos. A razão sexual (masculino:feminino) foi de 1:1. Durante o período de dezembro de 2021 a janeiro de 2022, através da análise histológica verificou-se a ocorrência de desova não registada. A manipulação das condições ambientais (temperatura, fotoperíodo) promoveram um aceleramento do ciclo gametogenético. Durante o mês de janeiro de 2022, para o tratamento com as condições ambientais manipuladas para os dois regimes alimentares (*Ulva* spp. e ração) tiveram menos individuos em avançado estado de maturação. Assim, para os grupos alimentados com *Ulva* spp. (MUC) os estados de desenvolvimento encontrados foram (I, II, III e VI), enquanto que para o grupo alimentado com ração (MPC) os estados foram (I, II e VI). Em contraste, para o mesmo mês houve mais estados presentes para o tratamento com condições ambientais naturais no regime alimentar *Ulva* spp (NUC). Nos grupos (NUC) e (NPC), os estados de maturação presentes foram I-IV e II-VI, respectivamente. Com a manipulação das condições ambientais, os ouriços-do-mar conseguiram progredir mais rapidamente e ter um fenómeno de "estratificação" menos evidente durante o mês de janeiro, promovendo a sincronização das gónadas. Esta técnica de reprodução pode produzir resultados positivos na produção indústrial de ouriços-do-mar. Trabalhos futuros deverão ser concentrados no impacto das condições ambientais (vários regimes de temperatura e fotoperíodo), em comparação com condições constantes, para facilitar a anáolise de dados.

PALAVRAS-CHAVE: *P. lividus,* Reprodução, Fotoperíodo, Temperatura, Equinocultura

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LIST OF UNITS AND ABBREVIATIONS

BW	Body weight
DM	Dry Matter
EPPO	Estação Piloto de Piscicultura de Olhão
GSI	Gonadosomatic Index
IPMA	Instituto Português do Mar e da Atmosfera
MPC	Manipulated Pellets Conditions
MUC	Manipulated Ulva Conditions
N	Number of individuals
NPC	Natural Pellets Condition
NUC	Natural Ulva Conditions
S	Spermatozoa
SD	Standard Deviation
SU	Sea urchin

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1 INTRODUCTION

1.1 State of Aquaculture

For the past decades fisheries and aquaculture have managed to cover the growing global demand for fish and fish products. For the time period 1990-2018, the consumption of fish and fish products have been observed to have risen about +122%. In the same period global fisheries production managed to present a stability and a small increase (+14%), even with the decreasing of available fish stocks, while the worldwide aquaculture sector achieved an immense expansion of +527% (FAO, 2020). Specifically, fisheries increased production, with marine capture fisheries, up to 84.4million tons in 2018 (81.2 million tons, 2017), where the leading countries were, China, Indonesia,Peru, India, Russian Federation, USA and Vietnam, accounting for almost the 50% of the total global captures. On the other hand, the worldwide aquaculture production, had an average increase 5.3 % per year for the time period of 2001-2018 (**Fig. 1.1**), starting with a percentage of 25.7% in 2000 and rose up to 45.0% until the 2018 (FAO, 2020).

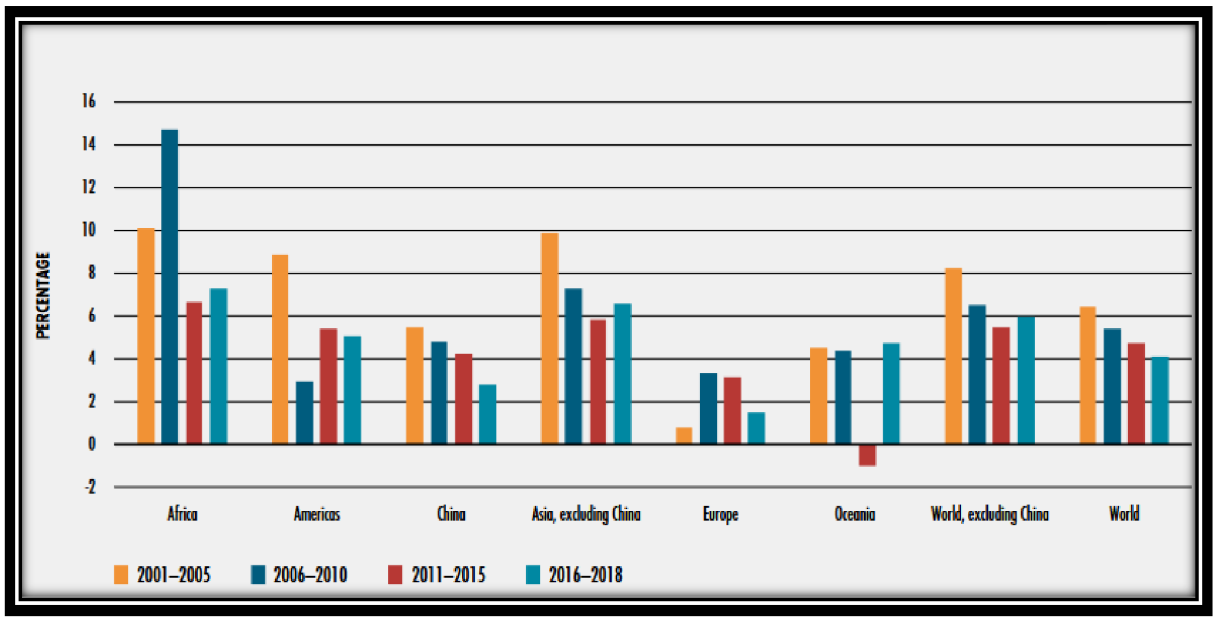


Figure 1.1 Annual growth rate of aquaculture fish production quantity (Source: FAO 2020)

Moreover, the primary sector of fisheries and aquaculture occupy a significant amount of people, with 20.5 million people engaging the aquaculture sector and approximately 39 million people being employed in the fisheries business, in 2020 (**Fig. 1.2**). Distinctively, in 2018, the world aquaculture production reached a substantial record of 114.5 million tones in live weight, which had a market value of 263.6 million USD.

	1995	2000	2005	2010	2015	2018
			(thousands)			
Fisheries and aquaculture						
Africa	2 812	3 348	3 925	4 483	5 067	5 407
Americas	2 072	2 239	2 254	2 898	3 193	2 843
Asia	31 632	40 434	44 716	49 427	49 969	50 385
Europe	476	783	658	648	453	402
Oceania	466	459	466	473	479	473
Total	**37 456**	**47 263**	**52 019**	**57 930**	**59 161**	**59 509**
Fisheries						
Africa	2 743	3 247	3 736	4 228	4 712	5 021
Americas	1 793	1 982	2 013	2 562	2 816	2 455
Asia	24 205	28 079	29 890	31 517	30 436	30 768
Europe	378	679	558	530	338	272
Oceania	460	451	458	467	469	460
Total	**29 579**	**34 439**	**36 655**	**39 305**	**38 771**	**38 976**
Aquaculture						
Africa	69	100	189	255	355	386
Americas	279	257	241	336	377	388
Asia	7 426	12 355	14 826	17 910	19 533	19 617
Europe	98	104	100	118	115	129
Oceania	6	8	8	6	10	12
Total	**7 878**	**12 825**	**15 364**	**18 625**	**20 390**	**20 533**

NOTE: The regional and global totals have been adjusted in some cases as a result of extended work on the dataset to revise historical data and improve the methodologies applied for estimations.

Figure 1.1 World employment in the aquaculture sector 1995 - 2018 (Source: FAO, 2020)

Recognized as one of the healthiest types of food worldwide, fish covered approximately the 20% of animal protein intake for 3.3 billion people in 2017. Furthermore, the per capita fish consumption from 1961 to 2018 rose from 9.0 kg to 20.5 kg (**Fig. 1.3**). The global expansion of aquaculture, has made fish consumption available to locations, regions and even countries whoseaccess to farmed species was limited or non-existent and in conjunction with low market prices, improved nutrition. It is self-evident, that fish and fish products possess a key

role for national to global food security and nutrition strategies, thus contributing in the elimination of malnutrition and hunger (FAO, 2020).

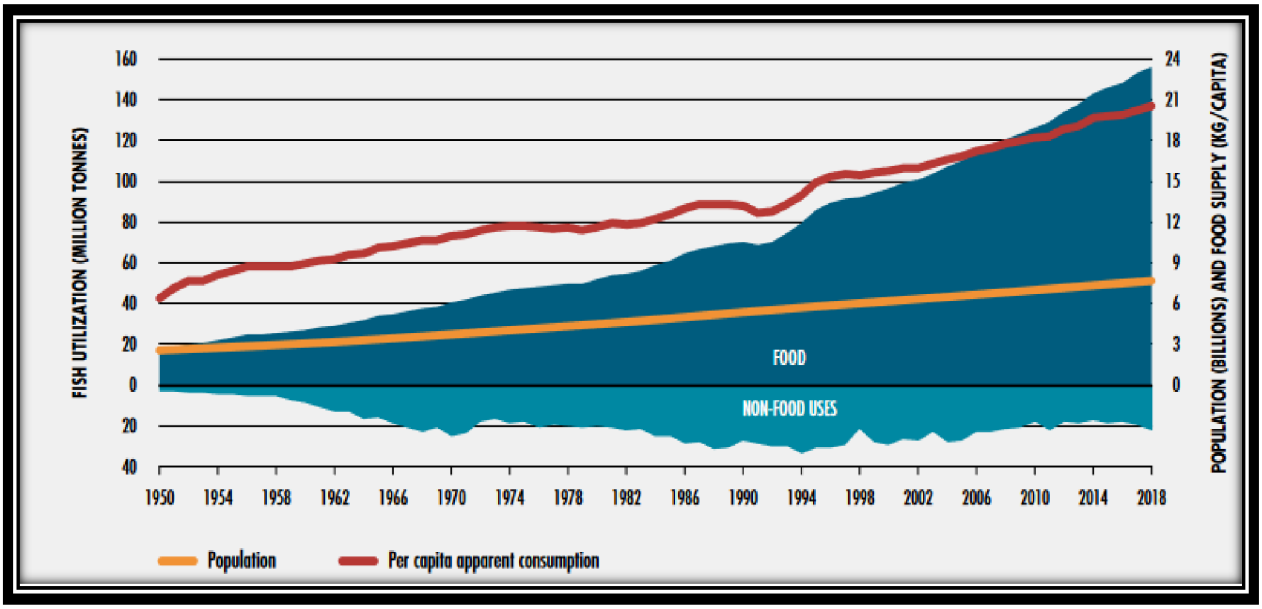


Figure 1.2 Per capita fish consumption (Source: FAO, 2020)

1.2 Description of species

Paracentrotus lividus, as known as purple sea urchin (Lamarck, 1816), is an edible macroalgivore echinoid species of the *Parechinidae* family. The largest individuals have a diameter of 7.5 cm (Boudouresque & Verlaque, 2013; Koehler & Bonnet, 1925; Lozano et al., 1995), rendering it as a significant large species of sea urchin. Its construction is that of a flattened pentameric radial greenish "test", clothed with long pointed spines, which, regardless of the common name of "purple sea urchin", have high color variability: black-purple, purple, red-brown, dark brown, yellow-brown, light brown or olive green. The color of the sea urchin doesn't show a correlation to depth or size (Boudouresque & Verlaque, 2013; Gamble, 1966). The test, in which the podium is located, is composed by 10 ambulacral and 10 interambulacral plates, where the spines are located **(Fig. 1.4)**, showing an alteration pattern between them (Carboni, 2013). The sea urchin's passive and active defense system, apart from the spines (Guidetti & Mori, 2005), includes small wrench or claw-shaped

appendages, called pedicellariae. Those wrench-shaped appendages have movable jaws, assisting them to place and hold or remove debris (e.g., pebbles, shells, macroalgae) or repel predators (Flammang, 2020; Verling et al., 2002). Furthermore, the tube feetform small arc arrangements with groups of 5-6 each and along with the spines, serve a significant role to the substrate adhesion as well as to the locomotor performance of the sea urchin (Domenici et al., 2003; Guidetti & Mori, 2005; Pizzolla, 2007)**.**

Aristotle's lantern, serves as an additional common feature among most of the Echinoid species. Its complex pentasymmetric structure, is found in the mouth region, consisting of 5 mobile plates in a circular arrangement, with each one of the containing a tooth. Being interconnected with more than 40 ossicles, moved by powerful muscles, this formation by protracting and retracting, functions as a feeding system for the sea urchin as well as a strategy, to protect themselves from predators and waves, by digging holes in the substrate (Barnes, 1987; Devin, 2002; Tortonese, 1965).

1.3 Distribution & Habitat

P. lividus populations are distributed throughout the Mediterranean Sea, the North East coast of Atlantic, the Canary Islands, the Azores and from Scotland and Ireland to South of Morocco, (Araújo et al., 2020; Bayed et al., 2005; Boudouresque & Verlaque, 2013; Ciriminna et al., 2020). It is commonly found in regions with the seawater temperature ranging from 10 to 15°C in winterand 18 to 25°C in summer, such as the western sector of Mediterranean off the coast of Portugal (Boudouresque & Verlaque, 2013). It shows high sensitivity in extreme salinity variations, with the low lethal values being 15-20 PSU and the high values 39-40 PSU. As a subtidal species *P. lividus*, lives in mean low-water mark down and intertidal pools, up to 10-20 m depth, with a fewexceptions, where isolated individuals are found in depths up to 80 m (Boudouresque & Verlaque, 2013; Crook et al., 2000; Girard et al., 2012; Tortonese, 1965)**.** Furthermore, in the open sea, individuals occur on solid rocks and in meadows of seagrasses such as *Posidonia oceanica* and *Zostera marina* (Boudouresque & Verlaque, 2013; Ebling et al., 1966; Tortonese, 1965), although it is quite uncommon to be found on sandy bottoms (Boudouresque & Verlaque, 2013). Certain physical and biological factors: wave hydrodynamics, heterogeneity of habitats, benthic morphology, nutrition, mortality, available photoperiod, predation and larval survival; the distribution and

the density of this species are extremely variables in space and time (Boudouresque & Verlaque, 2013; Guidetti et al., 2003).

1.4 Reproduction

P. lividus is a in species, since its fertilization occurs directly in the watercolumn after the emission of the gametes (McEdward & Miner, 2001a, 2001b)**.** Its reproductive apparatus, being composed by 5 gonads, adheres to the aboral portion of the test (carapace) and each one is connected to a gonopore via a *gonoduct*. When the gonads reach maturity, they appear bright yellow, orange or reddish, increase in their volume by spreading from the aboral pole almostto the Aristotle's lantern (Araújo et al., 2020). According to Ruocco et al. (2020), *P. lividus'* reproductive period occurs from October until May,even though past studies indicate that, even with a few small seasonal variations, spawning takes place all-year round (Boudouresque & Verlaque, 2013). This may be a strategy, aiming to distribute the risk of loss of planktonic stage larvae throughout the year (Pinna, 2014). Water temperature between 13 and 16°C, may also provide an approximate indication of the starting time of gametogenesis.

Gonad maturation occurs *in vitro* in specimens with a test diameter between 13 and 20 mm, and atleast at the age of 5 months (Cellario & Fenaux, 1990)**,** while *in situ*, possibly due to food availability, maturation occurs rather later. However, according to Boudouresque & Verlaque (2013), sexual maturity occurs in the third year of life with a test diameter of 20 – 25 mm (Grosjean,2001). There have been studies in which, somatic and gonadal growth is increased, when the food availability is not a limiting factor (Gago et al., 2001; J. Lawrence et al., 1991)**.** However, additional physical parameters such as light regime and temperature can positively affect gonadal growth (Sartori et al., 2015), with temperature showing higher significance in the gonadal growth's enhancement (Spirlet et al., 2000, 2001).

1.5 Feeding profile

P. lividus consumes several species of macroalgae such as *Ulva* spp*., Laminaria ochroleuca, Fucus vesiculosus,* and *Sargassum muticum* (Cardoso et al., 2020). Although it has been proven that *P. lividus* prefers feeding on *Ulva* spp., it cannot be used as a main echinoculture feed, because of several disadvantages.

The immense transportation and storage cost, as well as the significant variability in its nutritionalprofile throughout the seasons and location, renders the use of macroalgae as a tremendous investment, even for large-scale echinocultures (Candeias-Mendes et al., 2020; Ciriminna et al., 2020; Fernandez & Boudouresque, 2000; Santos et al., 2020; Schlosser et al., 2005)**.**

To the contrary, formulated diets (pellets) have shown significantly lower costs regarding, the transportation and storage, while also can have their nutritional profiles controlled throughout the year (Fernandez & Boudouresque, 2000)**.** Furthermore, several studies have indicated that inert diets fed to sea urchins, can contribute in higher digestible energy intake (DE), resulting in increased somatic and gonadal growth, than that of sea urchins with macroalgae for feed (Cyrus et al., 2014; Schlosser et al., 2005). An additional parameter to consider is the palatability of the feed. By incorporating macroalgae in the formulated diet, there is a significant increase in the consumption rate from the sea urchins, without any repercussion in the nutritional profile of the pellet (Cyrus et al., 2014). The formulated diet used in the trial was incorporated with *Ulva* spp*.,* since it has shown a greater preference from sea urchins apart from several red and brown macroalgae (Cardoso et al., 2020; Cyrus et al., 2014).

1.6 Economic & nutritional value

P. lividus presents a substantial economic interest, considering the fact that its gonads are regardedas a delicacy (also known as "roe") from a plethora of regions such as Mediterranean, South American and Asian countries (Araújo et al., 2020; Candeias-Mendes et al., 2020; Ciriminna et al., 2020; Cyrus et al., 2015; Mendes et al., 2019; Santos et al., 2020). Japan, a country with over125 million people as its population, combined with the utilization of sea urchin's gonads ("uni")in their culture and gastronomy, has established a significant share in the global imports. In 2020 it is estimated that Japan holds almost 63% in the global share of imports resulting in a value of $102M (Tridge, 2022, **Table 1.1**). Russia on the other hand, is currently the highest export share worldwide, 48.9% with a total production volume of 8,349 metric tons and a value of $78.25M (*Sea Urchins Production in Russia - Markets, Suppliers and Exporters*, n.d.; Tridge, 2022)**.** In Europe, using the same source, the countries that have a significant percentage in the importationare France, Spain and Italy, with 1.45%, ≈ 0.42% and ≈ 0.4%, respectively. In addition, Spain has the highest percentage of exports in

the E.U. being 1.47% of the global production, corresponding to approximately $2.4M. Prices for both live sea urchins and processed sea urchin roe are highly dependent on a number of

Table 1.1 Countries with the highest share (%) and value (USD $) in Export (left) and Import (right) of sea urchins (Source: Tridge.com)

Country	Share in Export Value 2020	Export Value 2020, USD
Russia	48.9%	$78.25M
Japan	16.94%	$27.11M
United States	9.5%	$15.21M
Canada	9%	$14.40M
China	4.89%	$7.83M
Mexico	2.73%	$4.36M
Peru	2.13%	$3.40M
Spain	1.47%	$2.36M
Australia	1.15%	$1.83M
Portugal	≈ 0.68%	$1.09M

Country	Share in Import Value 2020	Import Value 2020, USD
Japan	62.73%	$102.01M
Hong Kong	11.02%	$17.92M
United States	7.58%	$12.32M
South Korea	5.1%	$8.29M
Singapore	3.68%	$5.98M
Taiwan	3.47%	$5.64M
France	1.45%	$2.35M
Macao	≈ 0.98%	$1.59M
Canada	≈ 0.9%	$1.46M
China	≈ 0.54%	$881.89K

factors, these include (Ásbjörnsson, 2011; Carboni et al., 2012; P. James et al., 2017; Sun & Chiang, 2015): appearance; color and quality; species, season and region of harvest; flavors and textures; demand and distribution; form and processing. Sea urchins can be sold at a price between 0.3 € and 3 €, or about 20€ per kilo (Candeias-Mendes et al., 2020; Carboni et al., 2012; Hagen, 1996). Although, since the gonads can be considered as an expensive type ofseafood, they have attained market values of even 280€ per kilo (Stefánsson et al., 2017).

From a nutritional perspective, sea urchins are considered a valuable source of proteins, fatty acids (in particular polyunsaturated fatty acids, PUFA), carbohydrates and carotenoids (Camacho et al., 2018; Mol et al., 2008; Rocha et al., 2019; Zhou et al., 2018). Seafood supplies the human body with essential fatty acids (EFAs), such as n-3 and n-6 PUFAs, since it can't synthesize them on its own. PUFAs such as eicosapentaenoic acid (EPA, 20:5 ω3) and docosahexaenoic acid (DHA, 22:6ω3) are linked to decreased morbidity and mortality from

cardiovascular and other diseases (Candeias-Mendes et al., 2020). Furthermore, literature suggests that by consuming sea urchins, there are overall benefits as antioxidant effects and antitumor, anti-inflammatory and antimicrobialproperties (Matveeva et al., 2021).

1.7 Fishing increase and overexploitation of wild populations

In order for the demand gap to be fulfilled, there has been a radical increase in the capturing of wild sea urchins. During this attempt, the global catch increased approximately 2.5 times from 48.000 tons to 120.000 tons for the time period 1982-1995, with the volume overexploitation of multiple wild stocks have occurred (Andrew et al., 2002; Candeias-Mendes et al., 2020; Carboni et al., 2012; Mendes et al., 2019; Yeruham et al., 2015). By exploiting the sea urchin populations, without proper fisheries management when the recruitment rate is low, in combination with global phenomena such as environmental destruction and global warming, the community seizes to reproduce (Mendes et al., 2019; Santos et al., 2020; Spirlet et al., 2001).

1.8 Aquaculture

As the overexploitation of sea urchins continued, the idea of echinoculture was logically encouraged (Araújo et al., 2020). Hence, several research groups worldwide aim to eliminate the bottlenecks limiting the echinoculture. For instance, sea urchins' gonads adopt a bitter taste and unpleasant texture during the spawning period, while the gonadal index is significantly low during a few months throughout the year. Along these lines the commercially viable exploitation has a narrow time window to take place (Carboni et al., 2012). By adapting the sea urchins in an aquaculture environment, not only the demand gap would be met, but also the fishing pressure would be significantly alleviated (Ciriminna et al., 2020). Additionally, *P. lividus* is the main sea urchin species for the European echinoculture, while also considered as a target species in the diversification of the aquaculture (Carboni et al., 2012; Grosso et al., 2021; Santos et al., 2020), providing further research opportunities. Moreover, sea urchins' gonads have a significant contribution as model organisms for ecotoxicology and embryology (Cirino et al., 2017; Ettensohn, 2017; McBride, 2005). Small-sized wild juveniles are preferred for the aquaculture, seeing that their growth is faster and less cost-intensive (Baião et al., 2019). The research on the specific mariculture sector includes: abiotic factors (temperature, salinity, photoperiod), rearing conditions (e.g., water circulation), feeding requirements, effects of the feed on organoleptic characteristics, somatic growth, reproduction - gonadal development and husbandry (Baião et al., 2019; Rocha et al., 2019; Santos et al., 2020; Shpigel et al., 2018).

1.9 Aim of the thesis

The objective of this experiment was the development of a procedure, which allows the manipulation of the period of the gonadal development, in such a way, that it is possible to obtain mature gonads outside the normal spawning period. In this sense, the influence of abiotic factors will be tested, such as photoperiod and temperature, as well as the role of food in gametogenesis**.**

2 MATERIALS & METHODS

2.1 Location and duration of the experiment

The present trial took place in the Pilot Fish Farming Station in Olhão (EPPO), Portugal. The duration of the experiment was set for the time period 26 October 2021 until 15 February 2022.

2.2 Collection, acclimatization, and maintenance of sea urchins

F1 generation *P. lividus* juveniles produced at the EPPO facilities were used for this trial. For acclimatization purposes, the juveniles were kept in indoor fiberglass raceways tanks of 300-L until April of 2021. From then on, the sea urchins were moved in earth ponds around the EPPO area until July of 2021, when they were finally moved in outdoor 3800-L fiberglass tanks, until late of October 2021. Their feed was *Ulva* spp. until the launching of the experiment, in order to avoid any nutritional differences. For the purposes of the trial, 308 individuals of *P. lividus* were used, with minimum, maximum and average weight, 9.2 g, 48.8 g and 20.36 ±6.5g, respectively.

2.3 Methods

Four (4) cultivation methods were tested and compared in the present experiment (**Fig. 2.1**):

1) Natural (non-manipulated) photoperiod and temperature with a natural diet (*Ulva* spp.). Under these conditions the sea urchins will be subject to the sea water temperature and photoperiod at the time (10/21 to 02/22), (NUC).
2) Natural (non-manipulated) photoperiod and temperature with a formulated feed (by SPAROS I&D), (NPC).
3) Manipulated photoperiod and temperature with a natural diet (*Ulva* spp.). In this case, the normal environmental conditions present in the period from January to June (Shpigel et al., 2004) will be created (normal period of gametogenic development), (MUC).
4) Manipulated photoperiod and temperature with a formulated feed (by SPAROS I&D), (MPC).

2.4 Oxygen, temperature and photoperiod

With use of air lift and the daily monitoring from the EPPO stuff the Dissolved Oxygen saturation levels had an average value of 90%, in all the tanks regardless of the treatment.

According to the established research plan, 3 tanks were set to simulate natural environmental parameters (October-February), while 3 additional tanks were simulated in order for the water temperature (°C) and photoperiod (L: hours of light, D: hours of darkness) parameters to resemble those of January to May, a time gap of 3 months. The manipulated photoperiod varied between 10.1L:13.9D and 13.5L:11.5D. The temperature for the manipulated conditions was 14.58-18.01 °C. As for the normal conditions, the photoperiod was 9.4:14.6D to 10.4L:13.6D, while the temperature varied between 13.92-19.22°C (**Table 2.1, Figure 2.1)**.

Table 2.1 Environmental parameters (±SD) of the tanks for the duration of the experiment (October 2021 – February 2022).

Month	Natural Photoperiod (hours of light, hh:mm)	Manipulated Photoperiod (hours of light, hh:mm)	Natural Temperature (°C)	Manipulated Temperature (°C)
Oct-21	10:45 ±0:02	10:15 ±0:02	19.22 ±1.46	18.01 ±1.03
Nov-21	10:11 ±0:14	10:50 ±0:17	15.97 ±2.02	14.58 ±1.56
Dec-21	9:40 ±0:03	12:00 ±0:21	14.85 ±1.26	14.54 ±1.72
Jan-22	9:57 ±0:02	13:10 ±0:19	13.92 ±0.87	15.70 ±1.76
Feb-22	10:36 ±0:09	13:57 ±0:08	15.42 ±0.48	17.66 ±0.35

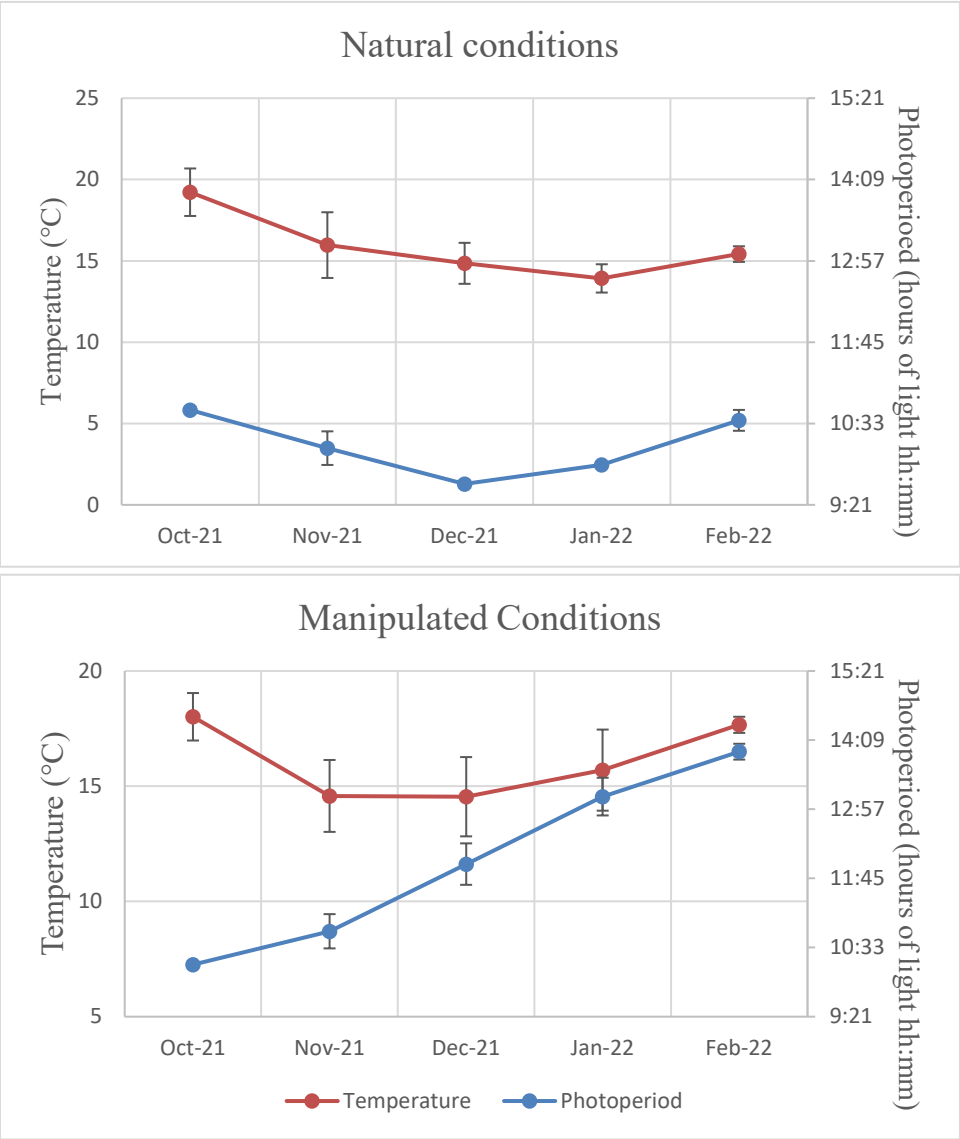


Figure 2.1 Mean temperature and photoperiod (±SD) for the natural (top) and manipulated(bottom) environmental conditions over 4 months.

2.5 Experimental Design

For each method, three (3) replicates were established. A total of six (6) 110-L tanks with dimensions 0.45*0.7*0.45 m were used. In rectangular PVC plastic boxes of 0.57*0.38*0.08 m dimensions, 50 individuals were placed. The mesh size was appropriate in order for the sea urchins to attach themselves on the surface, the feed to be kept in the box and the feces to be able to fall from the cage. On top of each box another equal box was placed preventing the sea urchins to escape. In order to distinguish the pellets- and algae-feeding sea urchins, a

plastic net divider was placed in the middle of each cage (**Fig. 2.2**, **Fig. 2.3**). Temperature configuration was managed with the use of chillers-heaters, while the photoperiod was controlled by covering the tanks with a black veil and illuminating them with headlights connected with a timer. Temperature and photoperiod were set according to the observed environmental conditions for the desired time period for both the normal and manipulatedtanks. The water renewal was approximately 112.5 L/h and the oxygenation of the tank was adequate with a use of porous stone in which air was blown by means of a pump (*air lift*). Temperature and oxygen levels were measured daily by EPPO staff, using the HANNA HI98196 multiparameter.

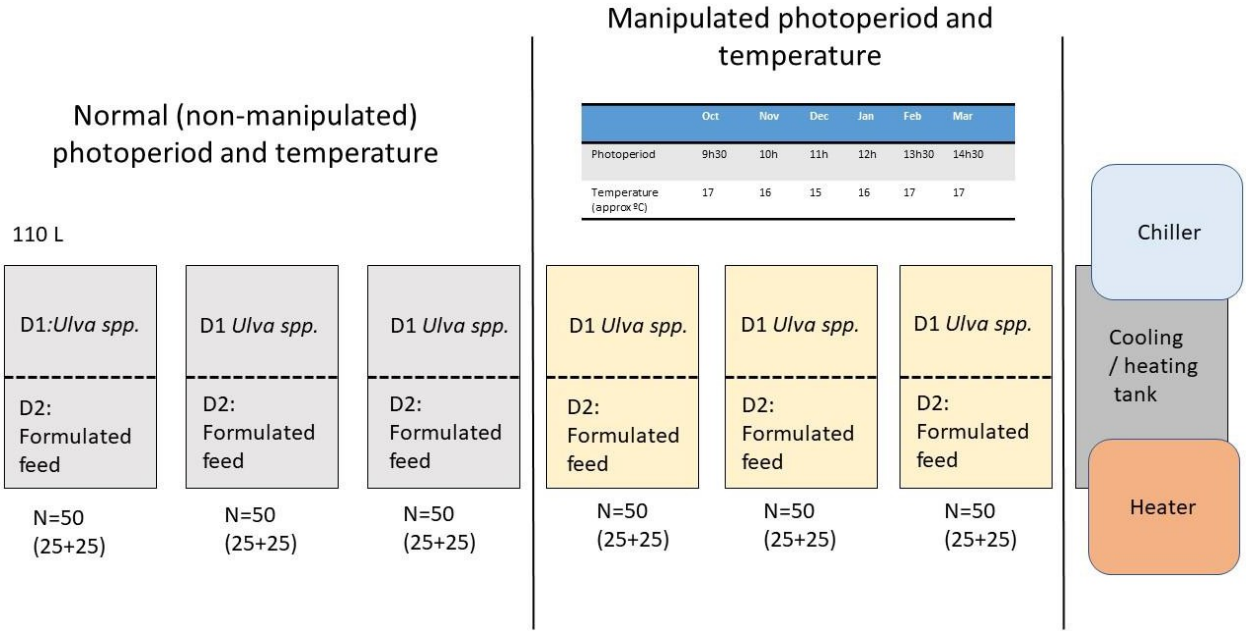


Figure 2.2 Experimental design of the replicates according to the methods used

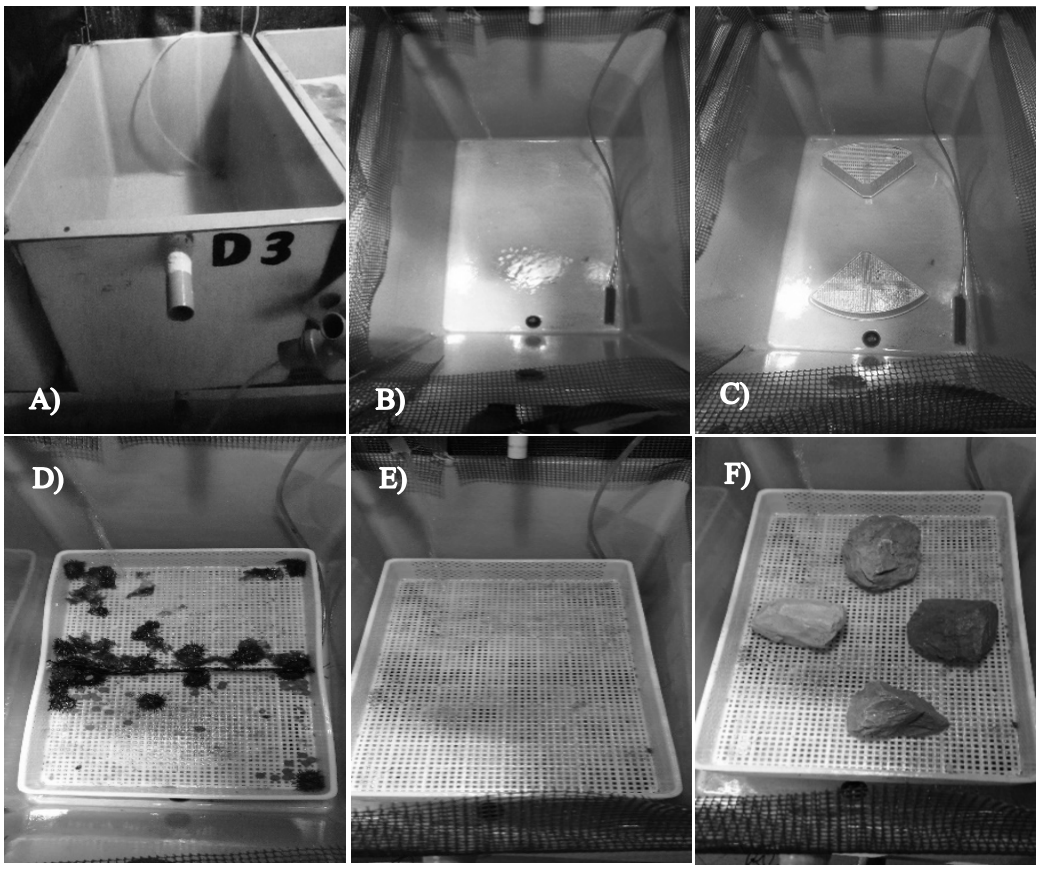


Figure 2.3 Set up of the experimental tankσ. A) Overview of the tank type, B) Bottom floor of the tank, C) Placement of plastic props to elevate the box from the bottom floor, D) Placement of the box containing the sea urchins with the plastic divider in the middle, to distinguish the different feeding regimes, E) Placement of an additional plain plastic box to prevent sea urchins from escaping, F) Placement of multiple rocks to keep the plastic boxes combined.

2.6 Feed supply

In the trial 2 types of feed were utilized; pellets and algae. The pellets used as feed for the experiment were developed and manufactured by SPAROS, I&D (Olhão, Portugal), provided to the EPPO facilities for echinoculture research. In order to be acceptable from the sea urchins, resulting to an increase of feed consumption, the pellets have a disk shape, while also contain *Ulva* spp (Cyrus et al., 2014, 2015; **Table 2.1**). The algae used as feed was *Ulva* spp. because of its extended use in similar experiments (Candeias-Mendes et al., 2020; McBride, 2005; Raposo et al., 2019)(**Table 2.2**). *Ulva* spp. was collected, when needed, from an earth pond used by EPPO for culturing this algae species, transferred to an isolated tank and once the preferable amount of algae was rinsed with freshwater, it would be given to the sea

urchins. The feeding procedure of the sea urchins occurred once per 3-4 days and the amount of feed was adapted according to the remaining total biomass of each replicate. The undigested food was removed and depending on the type of feeding regime, it had a different approach to weight it. For *Ulva* spp, the leftovers were placed into fireproof glass containers, then into the furnace in order to dehydrate it and then weighed. For pellets, the remaining disks were counted and with average weight of 0.3354 ± 0.0303 g per pellet the leftover quantity was calculated.

Table 2.2 Ingredients and proximal composition of formulated diet fed to Paracentrotus lividus.

Ingredients and proximal composition	**Pellets (% of total dry matter)**
Fish gelatine	5.00
Macroalgae (*Ascophyllum nodosum*)	20.00
Macroalgae (*Ulva* spp supplied by IPMA)	20.00
Wheat gluten	7.50
Corn gluten meal	17.00
Wheat meal	10.00
Vitamin and mineral premix	2.00
Antioxidant	0.40
Monocalcium phosphate	3.00
Calcium carbonate	5.00
Beta-carotene 10%	0.50
Algae biomass (*Schizochytrium* 16% DHA)	9.60
Total	**100.00**
Protein	32.62
Fat	5.19
Gross Energy	15.75
Ash	21.00

Table 2.3 Proximal composition of fresh Ulva spp. (% DM).

Proximal composition of ***Ulva* spp**.	
Protein (%)	22.13 ± 0.08
Fat (%)	1.63 ± 0.10
Gross Energy (%)	11.03 ± 0.28
Ash (%)	30.34 ± 0.04

2.7 Data acquisition and Analysis

2.7.1 Biometric and Histological Data

Monthly, biometric sampling was carried out, by measuring the total weight of each side of each basket in all the replicates with a KERN PRS / PRJ precision and analytical balance. 5 individuals from each side (**Fig. 2.3**), were placed for 5-10 minutes in an ice bath (-1°C) for anesthetization/euthanization. Using a bended pair of dissection scissors, an incision in the muscles surrounding the Aristotle's lantern, helped in creating a circular opening at the bottom of the sea urchin. Using tweezers and slight jets of seawater, the internal organs and feces were removed. For determining the gonadosomatic index (GSI), the gonads were measured.

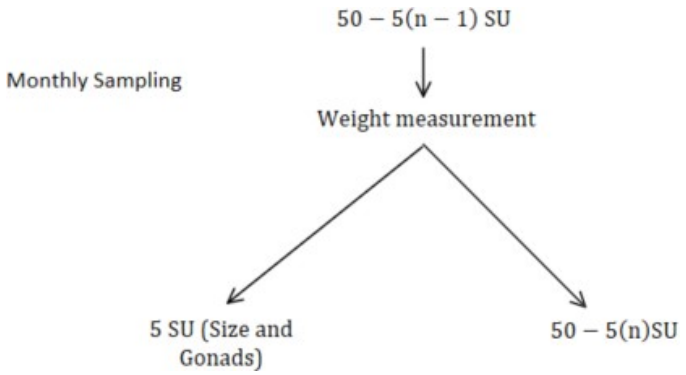


Figure 2.2.4 Monthly sampling of Paracentrotus lividus individuals.

For histological purposes one gonad per dissected individual was placed in a numbered tissue embedding cassette (10 gonads per tank * 6 tanks = 60 gonad samples each month). The cassettes were maintained in 10% formaldehyde for 48h for fixation. In continuation,

they were transferred to 70% ethanol for storage until paraffin histology was done in IPMA Matosinhos at Porto, Portugal. The samples would be dehydrated with ethanol and then with the use of the intermediate agent Xylol, the samples were subjected to paraffin followed by the inclusion of the samples. the inclusion. Histological sections with 4µm were produced and stained according to the Hematoxylin Eosin (H&E) protocol, which included coloring with Mayer's Hematoxylin, rinsing in water and a swift dip to differentiation solution (0.5% HCL in 80% ethanol) and coloring with 1% eosin in distilled water solution, a process done with an automatic slide stainer (Model Shandon Varistain 24-4, Thermo Scientific, Nanjing, China). Mounted slides were scanned with a Hamamatsu NanoZoomer C13140-01 and images were visualized with the NDPview software. The monitoring of the gametogenic state will be done through observation under a microscope, using the Byrne maturation scale (Byrne, 1990), according to which, the gametogenesis stages of ovaries and testes are divided into six stages: recovery (I), growing (II), premature (III), mature (IV), spawned (V), and spent (VI).

2.7.1.1 *Histology of the ovaries*

Stage I: recovery stage

Stage I ovary includes primary oocytes (5 to 30 µm diameter) and early ovary clusters along the acinal wall. Nutritive phagocytes, the non-germinal companion cells, form a mesh across the ascinus, giving the ovary avacuoled appearance. The ovary can contain unspawned ovaries in the lysis process and may be absorbed by the phagocytes.

Stage II: growing stage

At the beginning of vitellogenesis, primary oocytes increase in size (10 to 50µm diam) and become less and less basophilic, gaining a light purple tone. They are connected on the ascinal wall, surrounded by nutritive phagocytes. Groups of early primary oocytes may exist. The nutritional phagocytes pack the ovary and relict oocytes can still be present.

Stage III: premature stage

Vitellogenesis continues, with oocytes in all stages of development (10 to 90m diameter) present in the ovary. The shape of the large primal oocytes is oval and are directed to the center of the ascinus. When vitellogenesis is initiated, the nutrient phagocytes move from

the central position through large oocytes and their size decreases. When the primary ovaries reach their maximum size, they mature quickly and the ovary accumulates in the ovary lumen.

Stage IV: mature stage

The ovaries are intensely packed with ova reaching size of 90 μm, while some oocytes with size of 10 μm are located on the ascinal wall. The nutritive phagocytes can eitherbe in the form of a mesh network covering the oocytes or can be completely absent.

Stage V: partly spawned stage

The ovaries are loosely packed in the space emptied from the spawned ova. There are cases where oocytes are contained in the ovaries at all stages as mentioned in the Stage III, while on the other hand, ovaries may contain a significant number of ova as mentioned in the Stage IV. Consequently, the females may appear to begin spawning from the Stage III and IV.

Stage VI: spent stage

The ascinal wall in the spent ovaries is thin and can appear to be empty except several relict oocytes. Ovaries may contain unspawned ova or can be completely empty of oocytes. The remaining ova and oocytes are subjected to reabsorption and phagocytosis.

2.7.1.2 *Histology of testes*

Stage I: recovery stage

The ascinal wall in this stage is thin containing spermatogonia and primary spermatocytes. Nutritive phagocytes form a meshwork across the ascinus and relict spermatozoa can be present.

Stage II: growing stage

The ascinal layer increases in depth while columns of spermatocytes are directed to the center. The ascinus is filled with nutritive phagocytes.

Stage III: premature stage

Spermatocytes are displayed along the ascinal wall and spermatozoa are accumulated in the lumen. The phagocytes still appear, although they are displaced from the center by the spermatozoa.

Stage IV: mature stage

In stage IV the mature testes are filled with spermatozoa and the nutritive phagocytes are located only at the periphery.

Stage V: partly spawned stage

This stage is similar to the one of IV, with the differentiation that there are spaces in the ascinal lumen with less concentrated spermatozoa. The ascinal wall is rendered thin.

Stage VI: spent stage

The thin ascinal walls contain a pale meshwork of nutritive phagocytes around the periphery. They are devoid of contents, although relict spermatozoa can exist.

2.7.2 Analytical methods

For this work the gonadosomatic index, as well as the state of gonad maturation through histology were monitored monthly. Feed consumption was also monitored, by determining the food provided and the food not consumed.

The following formulas were used:

Feed intake (g dry feed/urchin/day) = $Dry\ feed\ input\ (g) - Dry\ feed\ leftovers\ (g)$

Gonadosomatic Index (GSI) = $\frac{Gonad\ wet\ weight\ (g)}{\text{Total wet weight (g)}} \times 100$

2.7.3 Statistical analysis

Using the statistical R® software and its "R Studio" package, the acquired data was tested for normality of distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test). Then additional tests were used in order determine the existence of significant differences between the natural and manipulated conditions, as well as between the use of algae or pellets as feed. Specifically, for the relationship between feed intake with feed regime (*Ulva* spp., pellets) and feed intake with environmental conditions (natural, manipulated), Kruskal Wallis non-parametric test and Welch Two sample t-test were used, respectively. The results for the mentioned tests were presented as Kruskal-Wallis chi-squared = value; p-value and Welch t=

value; df (degrees of freedom)= value; p-value, respectively. Consecutively, the relationship of GSI with feed regime and environmental conditions was examined using Kruskal Wallis non-parametric test and Welch Two sample t-test, respectively. Statistically significant differences were considered at p-value ≤ 0.05. On the occasion were the Kruskal Wallis test gave p-value <0.05, an ANOVA post hoq Tukey contrasts Comparisons of Means was applied, to showcase the comparisons between each pair of levels of the independent variables. The maturation stages data were analyzed qualitatively, through the acquired histological photos.

3 RESULTS

3.1 Feed intake

For the duration of the experiment, the total amount of feed provided to the sea urchins in each feeding session followed a declining trend, in order to minimize the leftovers. The mean feed ingestion over the 112-days trial depending on the treatment, is presented in **Table 3.1 and Figure 3.1**. Due to the trial starting on the 26th of October, the data regarding the feed intake during October were implemented in November. The group with SU fed with pellets in natural environmental parameters (NPC**)**, had the lowest total feed ingestion (2.611^{b} ± 0.14 g/SU). Significant differences (Kruskal -Wallis chi - squared = 25.332, p-value <0.001) were found in relation to the food regimes of the SU and not depending on the treatments regarding the environmental parameters (t = 0.053174, df = 45.999, p-value = 0.9578 >0.05). The maximum average amount of *Ulva* spp and pellets consumed per individual were 1.406^{a} g/day/SU and 0.427^{b} g/day/SU, respectively.

Table 3.1 Feed ingestion (g/day/SU ± SD) of P lividus reared with 4 different treatments regarding environment parameters and feed regimes. Note: common subscripts are of no significant differences between the treatments during the trial.

	Ulva spp		*Pellets*	
Month	Natural (g/day/SU)	Manipulated (g/day/SU)	Natural (g/day/SU)	Manipulated (g/day/SU)
Nov-21	0.343^{a} ±0.009	0.354^{a} ±0.001	0.181^{b} ±0.019	0.156^{b} ±0.014
Dec-21	0.194^{a}	0.194^{a}	0.106^{b} ±0.012	0.098^{b} ±0.006
Jan-22	0.658^{a} ±0.001	0.660^{a} ±0.002	0.211^{b} ±0.06	0.189^{b} ±0.012
Feb-22	1.167^{a} ±0.141	1.406^{a} ±0.032	0.304^{b} ±0.113	0.427^{b} ±0.082
Sum	7.086^{a} ±0.4	7.840^{a} ±0.49	2.406^{b} ±0.09	2.611^{b} ±0.14
Min	0.194^{a}		0.098^{b} ±0.006	
Max	**1.406^{a}** ±0.032		**0.427^{b}** ±0.082	

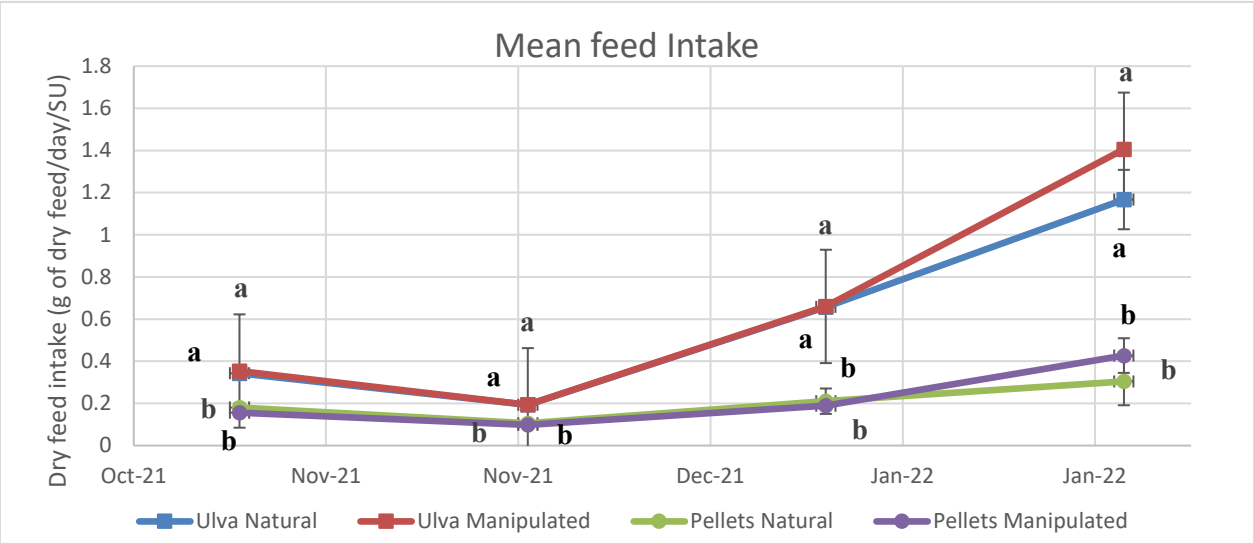


Figure 3.1 Scatterplot presenting the feed intakes (g/day/SU, mean ± SD) of Paracentrotus lividus reared in 4 different treatments regarding environmental parameters (temperature & photoperiod) and feed regime. Letters a & b indicate the significant differences between the 2 feed regimes.

3.2 Gonadosomatic Index

The monthly variations in GSI of *P. lividus* as a function of the treatments are presented in **Figure 3.2.** In the beginning of the experiment (26/10/2021) GSI of the first sampling was 0.63 ±0.44 %, as the basis of the measurements, thus no significant differences between the methods in October 2021. Significant differences (Kruskal-Wallis chi-squared = 33.411; df = 3; $p<0.001$) were observed between the treatments regarding the feed that was given (**Table 3.2**). At the end of the experiment the SU fed with pellets, in natural and manipulated environmental conditions (NPC, MPC), produced values of 16.64^{b} ±3.31 and 16.81^{b} ±1.82 (%), respectively, while the SU fed with *Ulva* spp. in the same natural and manipulated conditions (NUC, MUC), reached values of 12.00^{a} ±3.06 and 11.08^{a} ±4.41 (%), respectively. There were no significant (t = -0.2852; df = 237.82; p-value = 0.7757 > 0.05) differences for the GSI values in regards of the natural and manipulated environmental parameters (temperature & photoperiod).

Table 3.2 Monthly mean GSI values (%) (±SD) of Paracentrotus lividus reared in 4 different treatments over a period of 112 days. N= 15. Letters a, b and c represent the significance ($p<0.05$) between the individuals under different treatments. Values with common superscripts are of no significant difference ($p>0.05$). Results during Oct-2021 were the same across the methods with no significant differences, since it was the beginning of the trial.

Month	*Natural Ulva* spp *(%)*	*Natural Pellets (%)*	*Manipulated Ulva* spp *(%)*	*Manipulated Pellets (%)*
Oct-21	$0.63^{c} \pm 0.44$	$0.63^{c} \pm 0.44$	$0.63^{c} \pm 0.44$	$0.63^{c} \pm 0.44$
Nov-21	$1.36^{a} \pm 1.92$	$5.26^{b} \pm 2.50$	$2.15^{a} \pm 0.90$	$5.07^{b} \pm 3.28$
Dec-22	$4.38^{a} \pm 2.27$	$8.28^{b} \pm 2.37$	$5.11^{a} \pm 2.78$	$9.25^{b} \pm 2.92$
Jan-22	$11.55^{a} \pm 3.67$	$17.29^{b} \pm 3.07$	$9.74^{a} \pm 2.52$	$15.50^{b} \pm 3.83$
Feb-22	$12.00^{a} \pm 3.06$	$16.64^{b} \pm 3.31$	$11.08^{a} \pm 4.41$	$16.81^{b} \pm 1.82$

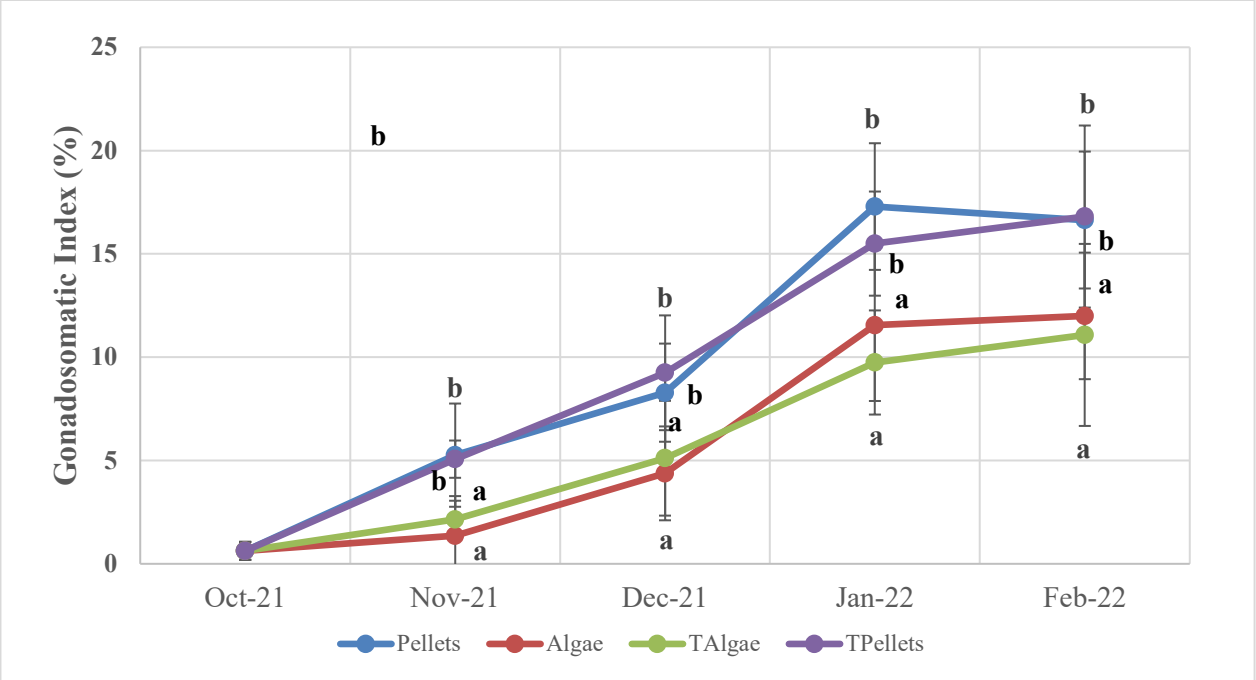
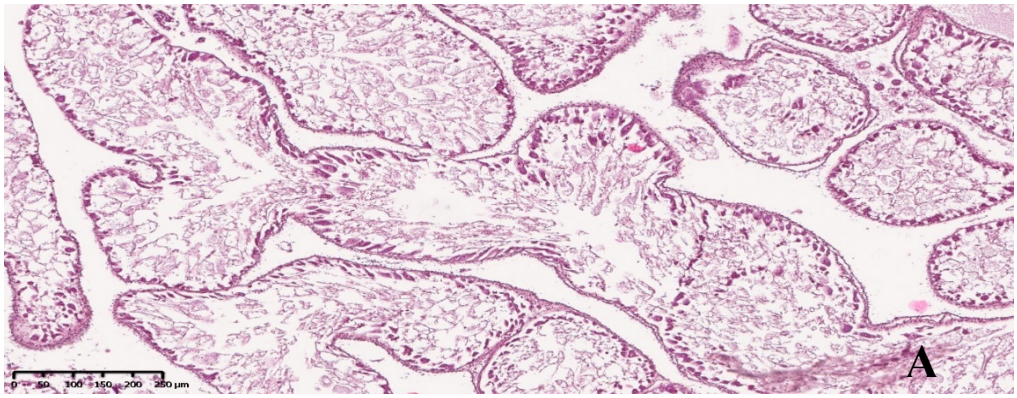
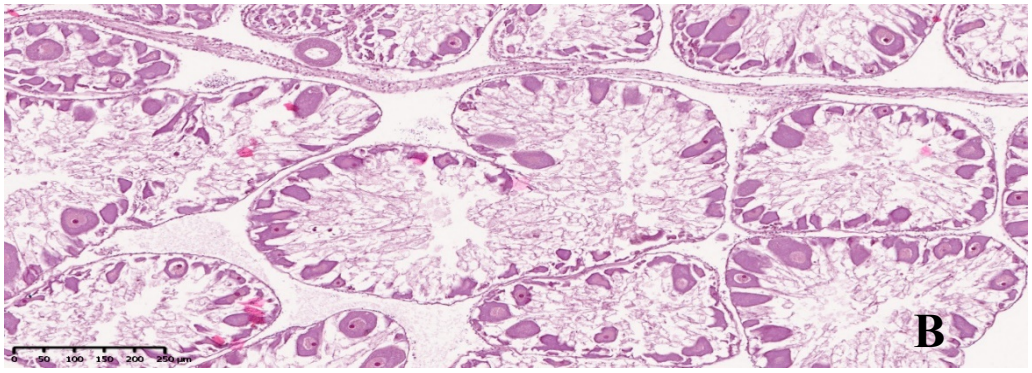


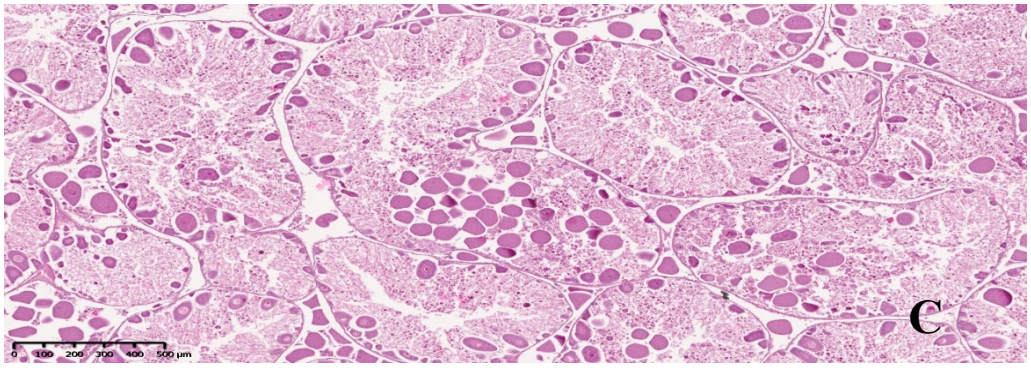
Figure 3.2 Scatterplot of GSI (%) (mean ± SD) of Paracentrotus lividus reared with four different treatments (Natural- Ulva spp, Natural-Pellets, Manipulated- Ulva spp, Manipulated-Pellets) over a period of 112 days. Letters a & b indicate the significant differences between the 2 feed regimes

3.3 Gonadal development

In the beginning of the trial 8 SU were sacrificed from which 3 (37.5%) were male, 4 females (50%) and one unidentifiable (12.5%) leading to a sex ratio of 0.75:1 (male: female). The maturity stage of those individuals was established, so they were categorized as immature. At the end of the experiment out of 239 samples, 122 (51%) were Male and 117 (49%) were female, resulting in a ratio of ~1.:1. During December - January, through the monthly histological analysis, a progress from stages II and III to V, VI and I was observed, indicating that spawning took place. **Figure 3.3** and **Figure 3.4** showcase the histological sections of *P. lividus* ovaries and testis.







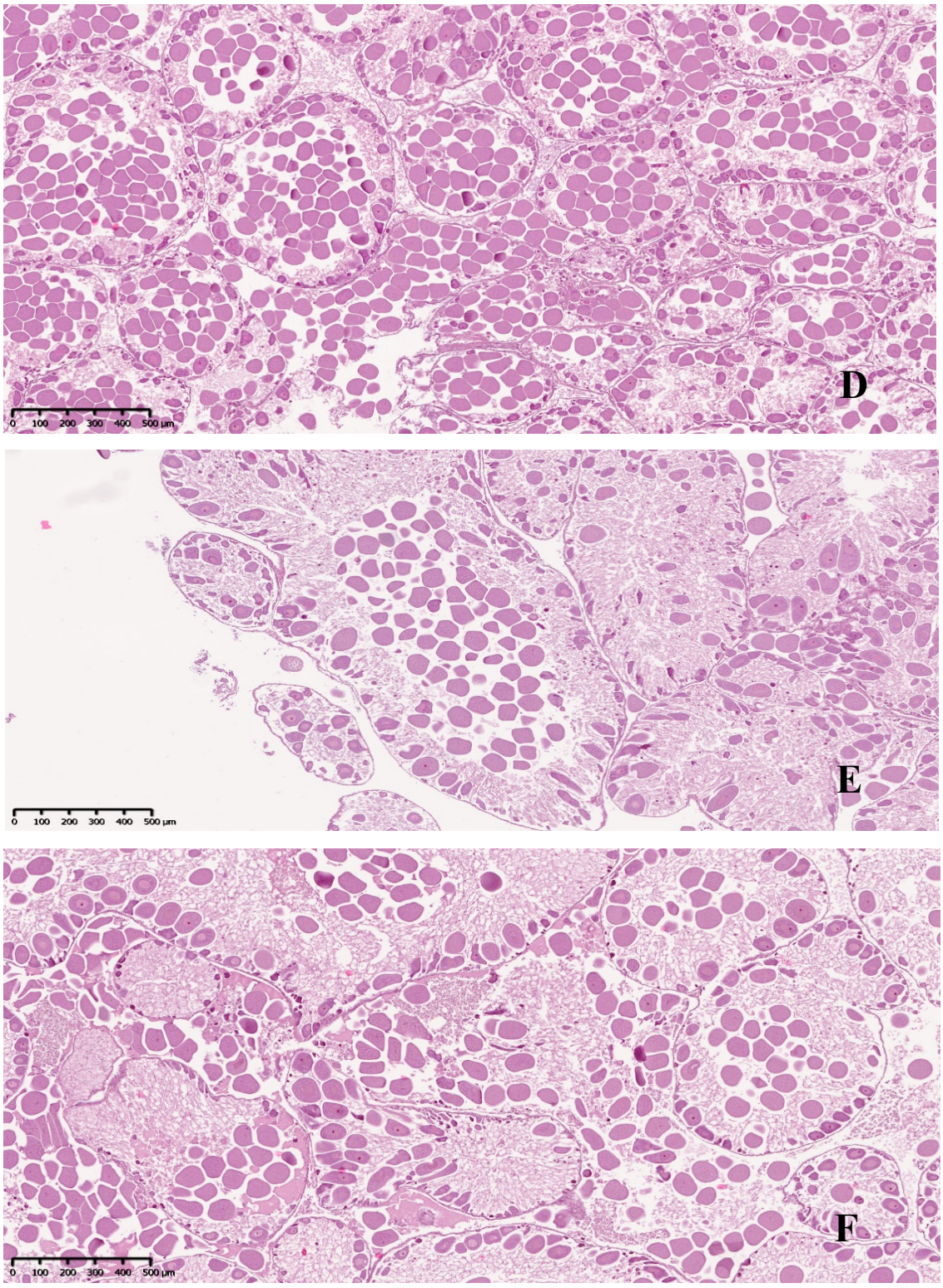
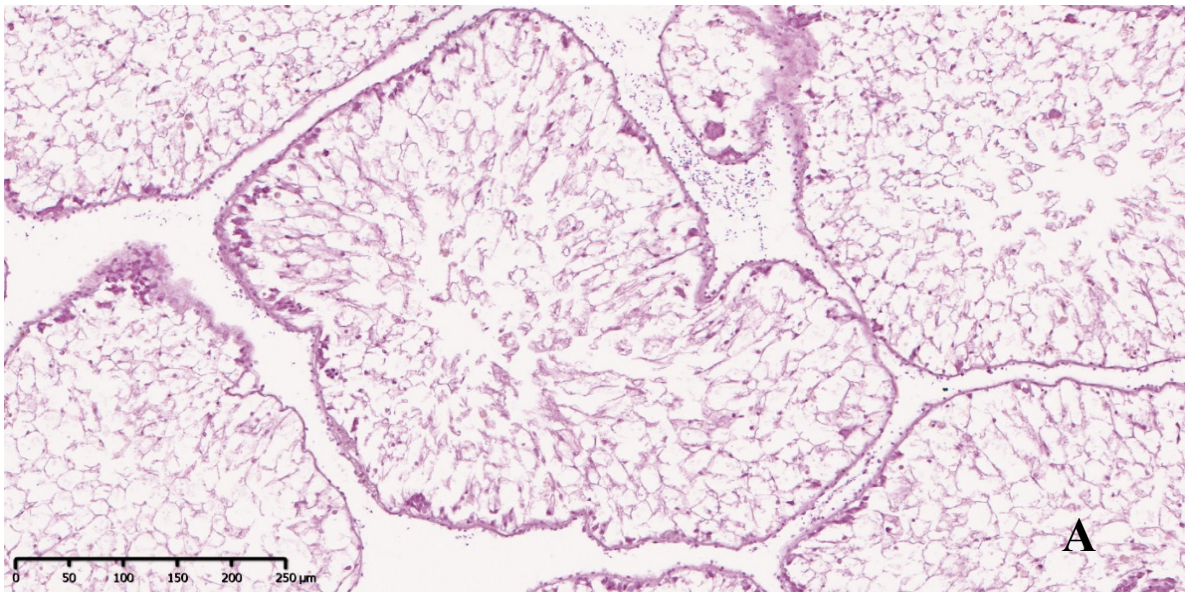


Figure 3.3 Paracentrotus lividus. Histological sections of ovaries acquired in the duration of the trial. (A) Stage I, (B) Stage II, (C) Stage III, (D) Stage IV, (E) Stage V, (F) Stage VI.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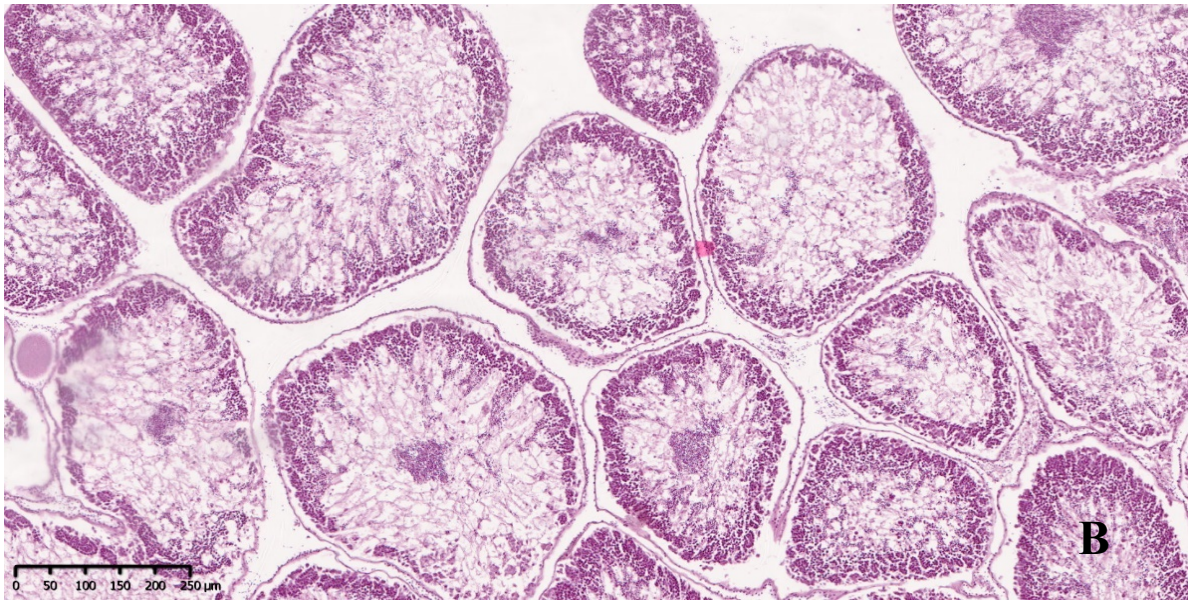
A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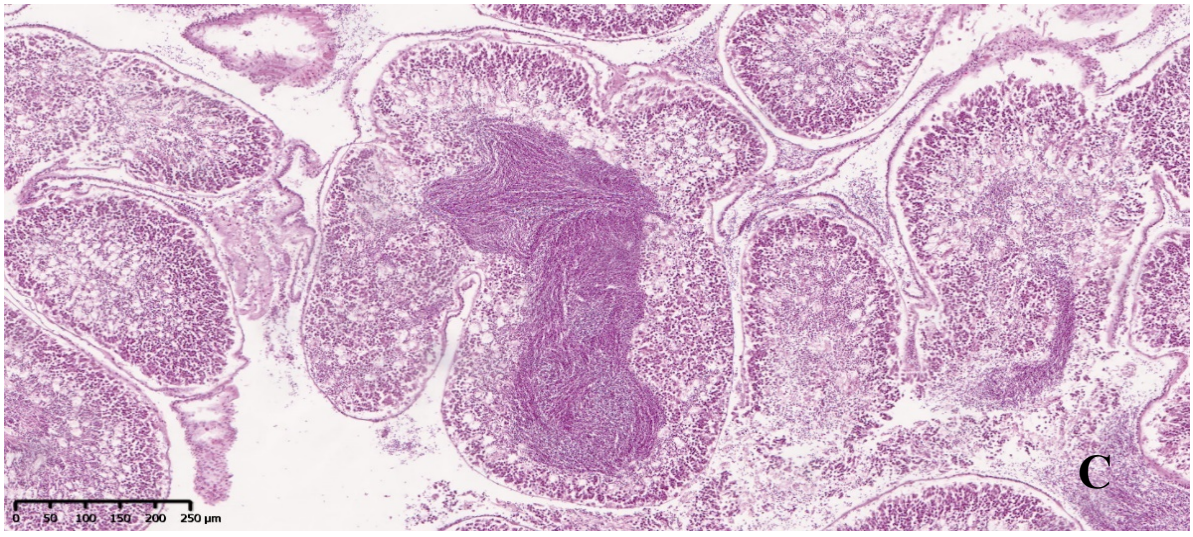
0 50 100 150 200 250 µm
C

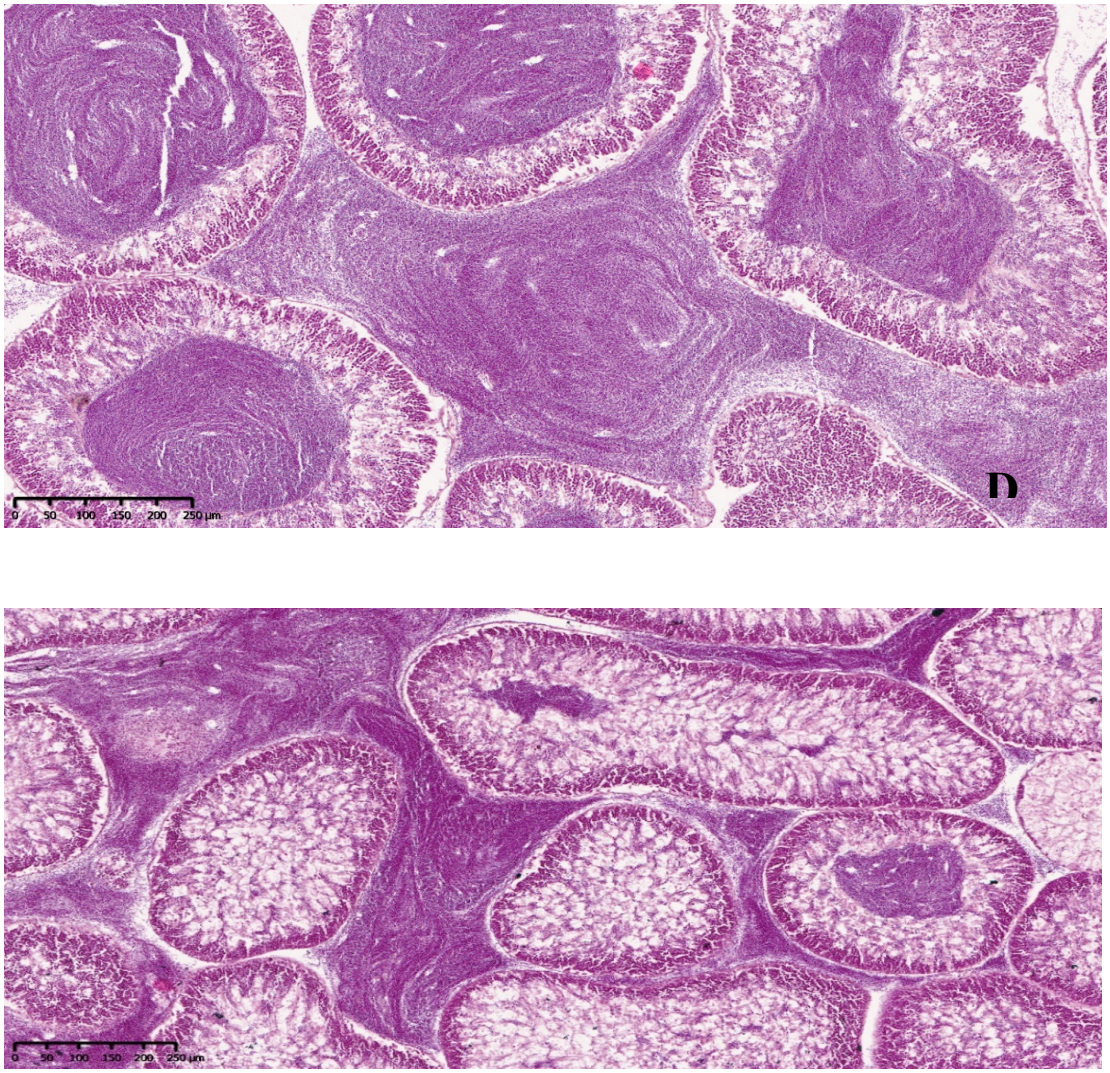

Figure 3.4 Paracentrotus lividus. Histological sections of testis acquired in the duration of the trial. (A) Stage I, (B) Stage II, (C) Stage III, (D) Stage V, (E) Stage VI. There were no cases of a male individual of Paracentrotus lividus in the Stage IV

E

3.3.1 Natural conditions versus manipulated conditions. Diet: Ulva spp.

Difference in the maturity stages depending on the environmental parameters, were observed in January and February (**Fig. 3.5**). During January the boxes in manipulated conditions with *Ulva* spp. feed regime (MUC), had 60% and ~27% of SU, in the spent and recovery stages (VI and I), respectively, while the natural conditions had 0% and 13% in the same gametogenic developmental stage, respectively. In February the number of SU in spent and recovery stage (stages VI and I) in MUC conditions were ~27% and ~67% respectively, while in NUC were 73% and ~7%, respectively (**Table 3.3**).

Table 3.3 Monthly percentages (%) of each gametogenic stages of Paracentrotus lividus reared in Natural and Manipulated environmental parameters under Ulva spp. feeding regime.

Ulva spp.

Conditions	Stage	Nov	Dec	Jan	Feb
Natural	I	92.86%	66.67%	13%	6.67%
	II	7.14%	26.67%	40%	0%
	III	0%	6.67%	20%	6.67%
	IV	0%	0%	13%	0%
	V	0%	0%	13%	13%
	VI	0%	0%	0%	73%
Manipulated	I	100%	46.67%	26.67%	66.67%
	II	0%	46.67%	6.67%	0%
	III	0%	6.67%	6.67%	6.67%
	IV	0%	0%	0%	0%
	V	0%	0%	0%	0%
	VI	0%	0%	60%	26.67%

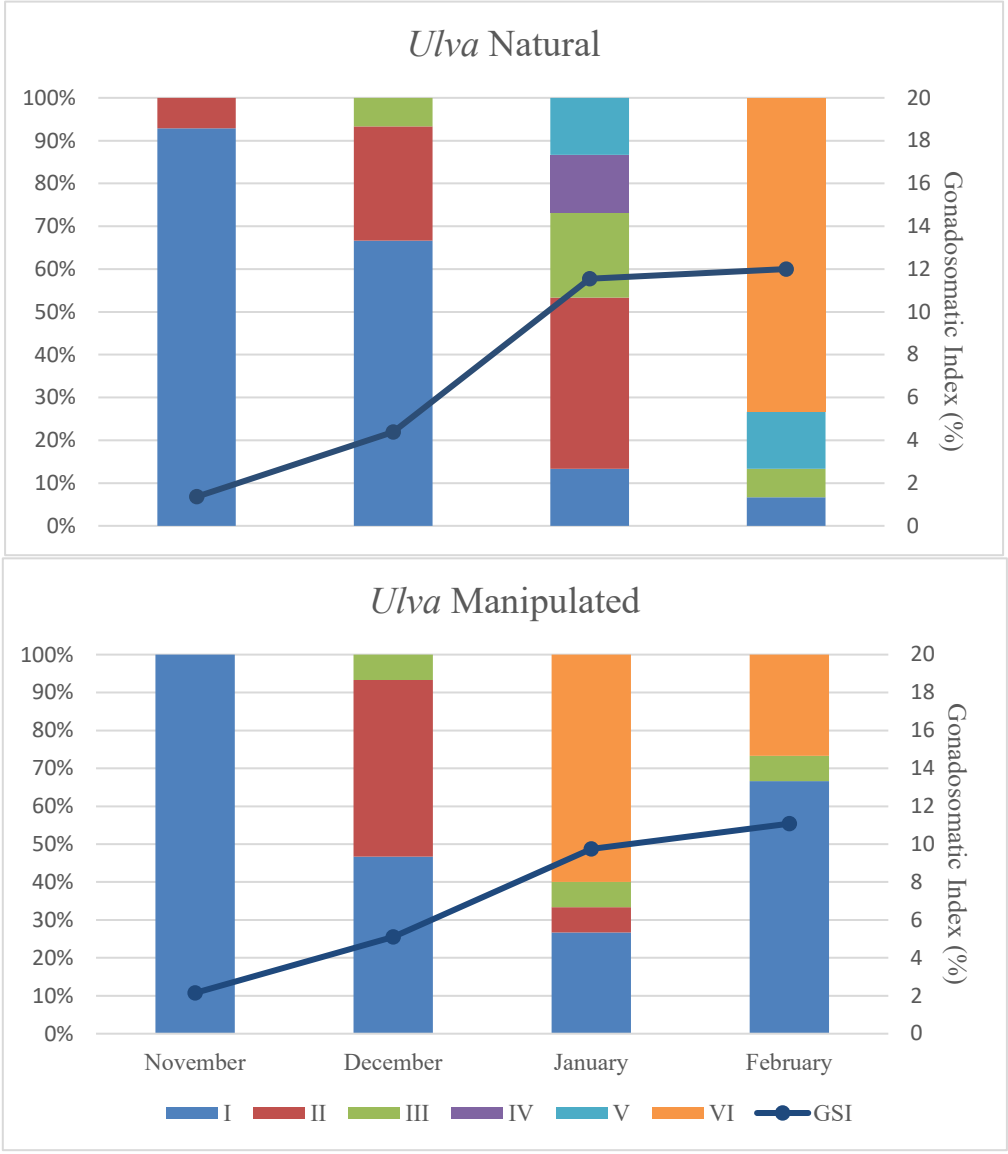

Figure 3.5 Temporal trends of the relative frequency for each gonad stage and of the gonadosomatic index (GSI) of Paracentrotus lividus fed with Ulva spp. reared in natural(top) and manipulated (bottom) environmental conditions for each sample over a period of 112 days.

3.3.2 Natural conditions versus manipulated conditions. Diet: formulated feed

Regarding the sea urchins which had pellets as feed source, fluctuations were observed in December, January and February (**Table 3.4**, **Fig. 3.6**). During December the SU in the MPC group showed an increased percentage in stages II and III, ~33% and ~13%, respectively, in comparison with the individuals in NPC in the same stages (27% and 7%, respectively). In January 60% of the MPC individuals were in the spent stage (VI), while the NPC tanks had 20% of the individuals in the spent stage. In the last month of the trial (February), both NPC

and MPC groups showed the same percentage for the spent stage (VI), being at 60% of the sample, but the MPC group had increased percentage of individuals being in the recovery stage (I), being 33% contrary to the 20% of the tanks in natural environmental conditions.

Table 3.4 Monthly percentages (%) of each gametogenic stages of Paracentrotus lividus reared in Natural and Manipulated environmental parameters under Pellets feeding regime.

Pellets					
Conditions	Stage	Nov	Dec	Jan	Feb
Natural	I	66.67%	66.67%	0%	20%
	II	33.33%	26.67%	20%	0%
	III	0%	6.67%	46.67%	0%
	IV	0%	0%	6.67%	0%
	V	0%	0%	6.67%	20%
	VI	0%	0%	20%	60%
Manipulated	I	80%	53,3%	26.67%	33.33%
	II	13.33%	33.3%	13.33%	0%
	III	6.67%	13.3%	0%	0%
	IV	0%	0%	0%	0%
	V	0%	0%	0%	6.67%
	VI	0%	0%	60%	60%

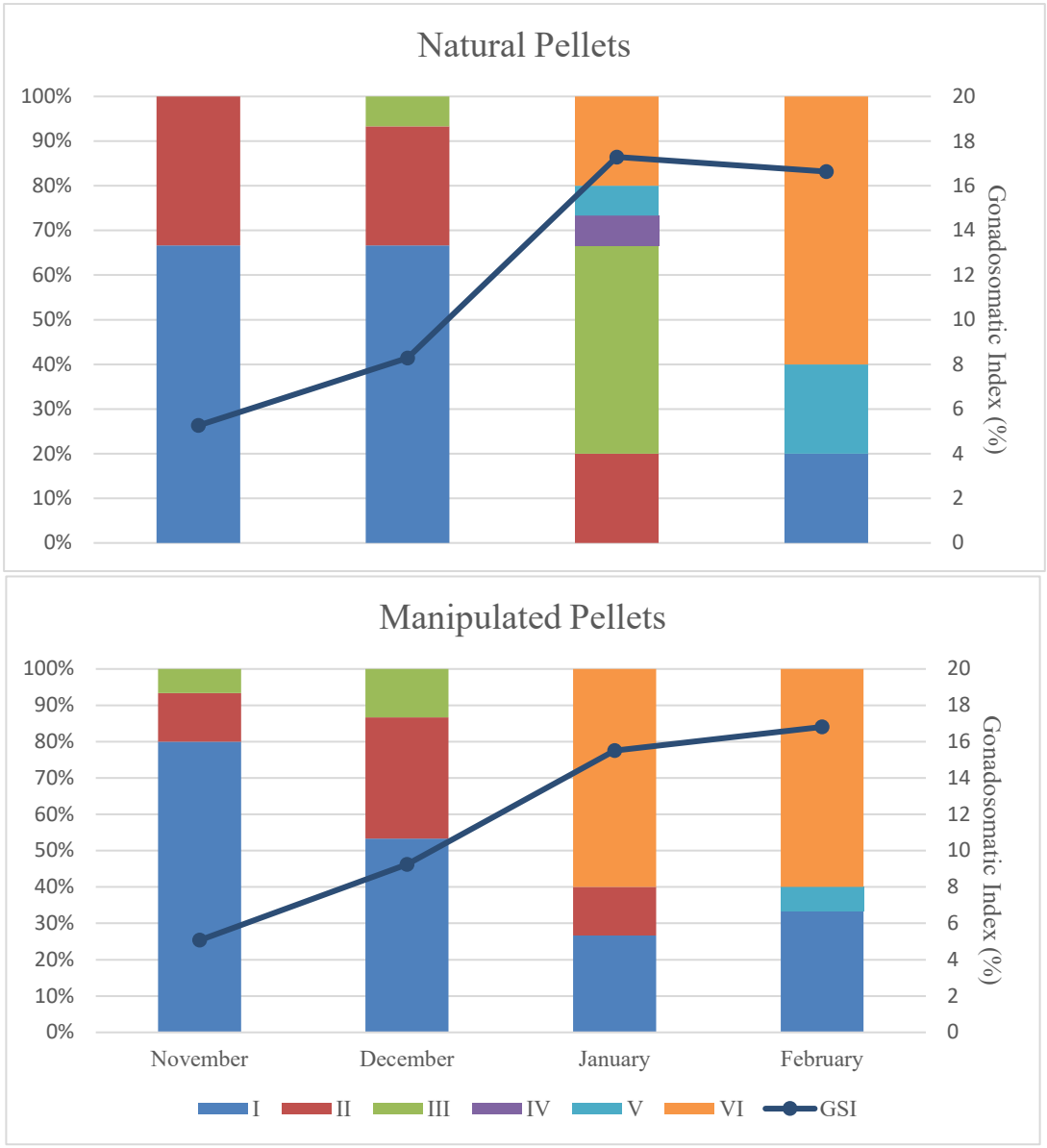

Figure 3.6 Temporal trends of the relative frequency for each gonad stage and of the gonadosomatic index (GSI) of Paracentrotus lividus fed with pellets reared in natural(top) and manipulated (bottom) environmental conditions for each sample over a period of 112 days.

3.3.3 Natural conditions versus manipulated conditions

In January 2022, for the Natural *Ulva* spp. Conditions (NUC) and Natural Pellets Conditions (NPC) groups, the maturation stages present were I-IV and II-VI, respectively. In contrast, for the same month there were less stages present for the manipulated conditions for both Manipulated *Ulva* spp. Conditions (MUC) (I, II, III, VI) and Manipulated Pellets Conditions (MPC) (I, II, VI) groups (**Table 3.3, Table 3.4**). Furthermore, the distinguish in the treatment effect is more notable during

February 2022, where the manipulated groups have more cases in the recovering stage (I) than the natural groups which had more cases in the spent stage (VI). According to the results so far, it was established that regardless of the food source (*Ulva* spp. and pellets), the manipulated conditions were more efficient than the natural conditions, in order to promote the gametogenic cycle growth (**Table 3.5, Figure 3.6**).

Table 3.5 Overall monthly percentages of each gametogenic stages of Paracentrotus lividus reared in Natural and Manipulated environmental conditions.

Conditions	*Stages*	*Nov*	*Dec*	*Jan*	*Feb*
Natural	I	79.31%	66.67%	6.67%	13.33%
	II	20.69%	30%	30%	0%
	III	0%	3.33%	33.33%	0%
	IV	0%	0%	10%	0%
	V	0%	0%	10%	20%
	VI	0%	0%	10%	66.67%
Manipulated	I	86.67%	46.67%	26.67%	53.33%
	II	10%	40%	10%	0%
	III	3.33%	13.33%	3.33%	3.33%
	IV	0%	0%	0%	0%
	V	0%	0%	0%	0%
	VI	0%	0%	60%	43.33%

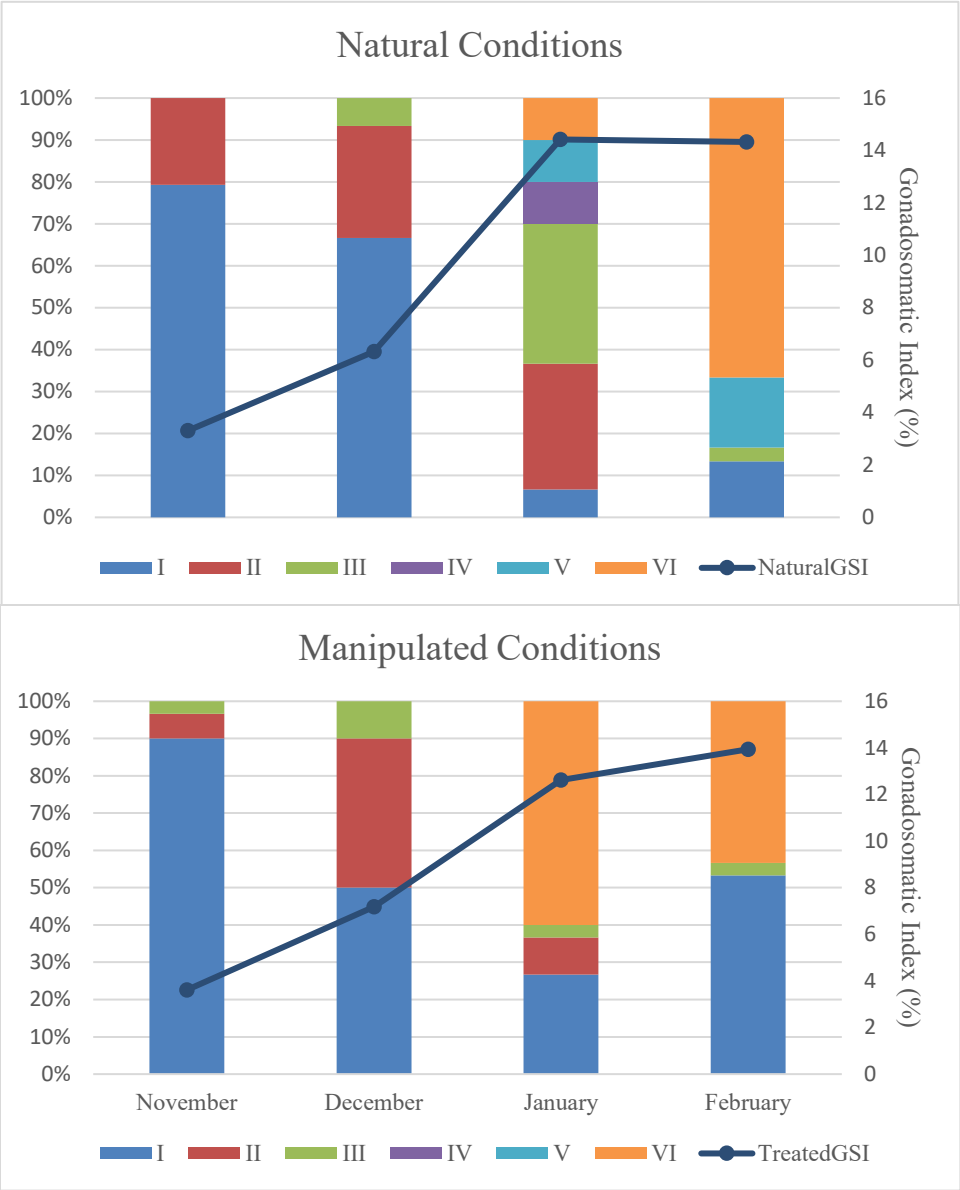

Figure 3.7 Temporal trends of the relative frequency for each gonad stage and of the gonadosomatic index (GSI) of Paracentrotus lividus in both feeding regimes reared in natural(top) and manipulated (bottom) environmental conditions for each sample over a period of 112 days.

4 DISCUSSION

The experimental data presented in this study provide clear evidence on the effect of the abiotic parameters on the gonadal development of *P. lividus*. Feeding regime showed substantial influence on the GSI and feed intake of the sea urchins (SU).

4.1 Gonadal development

Studies have been conducted in order to describe the relationship between the gametogenic cycle and the abiotic factors (temperature, photoperiod), with several of them supporting that temperature has greater impact on the gametogenic cycle than photoperiod (Amri et al., 2017; Fenaux, 1968; González-Irusta et al., 2010; Machado et al., 2019; McCarron et al., 2010; Santos et al., 2020; Shpigel et al., 2004; S. Siikavuopio et al., 2006; Soualili & Guillou, 2009; Spirlet et al., 2000). Gonadal development was promoted with the manipulated temperature-photoperiod in both feed regimes *(U*lva spp., pellets). Advanced maturation was observed in the manipulated environmental conditions groups during Jan-Feb 2022. As the structure of the experiment was to simulate the continuous fluctuations of the environmental conditions, it is not clear which abiotic parameter is the most influential variable, rather the combined impact of the parameters was examined. According to the literature, the only studies that may be in agreement with the present experiment, are those from Grosjean and Jangoux, (1998) and Shpigel *et al.*, (2004), in which the range of 18-20°C or 18-22°C, respectively, was suggested for enhancement of gonadal development.

4.2 Gonadosomatic Index

At the end of the trial gonad weight was increased in all the tanks, regardless of the treatment, due to the *ad libitum* feed regime, with similar results observed in Kayaba *et al.*, (2012). The abiotic parameters (temperature, photoperiod) did not affect the GSI ($p>0.05$). This fact comes into contradiction with the literature, were the abiotic conditions have an important role in the GSI. Studies have shown that both rearing temperatures (James et al., 2007; Santos et al., 2020; Shpigel et al., 2004; S. Siikavuopio et al., 2006; Siikavuopio et al., 2008; Spirlet et al., 2000) and photoperiod (Shpigel et al., 2004; Spirlet et al., 2000) have an effect on the gonad weight. Contrastingly, Grosjean & Jangoux (1998), object on the photoperiod parameter, while it has been suggested that the food quality and availability is far more important factor than the water temperature regarding the gonad growth of the sea urchins (Barker et al., 1998; Klinger et al., 1988; Lawrence et al., 1997; Mcbride et al., 1997). Common element in the former studies has been the fact that the abiotic parameters which were tested, were kept constant throughout the trial. In contrast the abiotic parameters in the present study, were set to simulate the continuous fluctuations of the environment.

Several authors (Boudouresque & Verlaque, 2001; Cuesta-Gomez & Sánchez-Saavedra, 2016; Lourenço et al., 2020; Volpe et al., 2018), were found that artificial feed is the most influential factor for the gonad weight. Similar results have found in the present study where sea urchins' which fed pellets (NPC, MPC), produced higher GSI, than the SU fed with *Ulva* spp (NUC, MUC). It was noteworthy that NPC and MPC groups were able to almost quadruple their GSI in just one month. This can be attributed to the enhanced nutritional value (Vizzini et al., 2015) of the pellets mainly in the form of crude protein. The results from the present study concur with the literature, in which the gonad yield of *P. lividus* is enhanced thanks to the formulated diets provided when compared to the natural feed (Baião et al., 2019; Lawrence et al., 2013; Lourenço et al., 2020; Vizzini et al., 2018; Woods et al., 2008). Dietary protein, contributes in a significant degree, in the gonad growth, through the accumulation of nutrients in the phagocytes (Lourenço et al., 2020).

GSI did not decrease on February 2022 after the spawning period, except the NPC group. Interestingly enough, this phenomenon has been observed in similar studies (King et al., 1994; Lozano et al., 1995; Machado et al., 2019; Sellem & Guillou, 2007), in which the gonads have been attributed with the double role of being reproductive organs and nutrient storage. An extended trial period might have shown conclusive results in this observation.

4.3 Feed intake

Feeding intake was closely dependent on the feeding regime and in a smaller degree on the maturation stage of the SU. Groups of SU fed reared with pellets in both natural and manipulated environmental conditions (NPC, MPC), had significantly lower feed intake than the SU reared with *Ulva* spp. in the same conditions (NUC, MUC). This result may be due to the relationship that sea urchins have between feed quality and feed intake, in which they present higher ingestion rates when the dietary levels of crude protein in the feed provided are low and vice versa (Marsh et al., 2013). This way, the groups fed with pellets, rich in protein concentration, had to consume less feed, in order to achieve the required protein levels (Lourenço et al., 2020). In December, regardless of the treatment, all groups had decreased feed intake, while after the spawning period (Jan-Feb 2022), the intake increased. This observation concurs with several studies (Marsh et al., 2013; Scheibling & Hatcher, 2001), in which the developmental stage of the nutritive phagocytes in both ovaries and testes are dependent on the feeding rates. Additional studies (Bayed et al., 2005;

Jacquin et al., 2006; S. Siikavuopio et al., 2006), suggest that abiotic factors (including temperature) have an impact on the metabolic rates, even though this was not observed in the present study. There was no substantial evidence, indicating that the environmental conditions affected the feeding rates.

5 CONCLUSIONS

Overall, this study confirms the positive effects of the abiotic variables, temperature and photoperiod on the gonad maturation process of *P. lividus*. By manipulating the environmental parameters, the sea urchins were able to progress faster after their spawning period, compared to the natural conditions. Also, the feeding regime can be considered as an important factor for the gonadal growth of the sea urchins. The consequent increase of the GSI in sea urchins fed with pellets is attributed to the high protein contents of the diet which promotes the nutrient accumulation in the phagocytes. Furthermore, with protein-rich diet, the sea urchins achieve their protein intake for their growth, with significantly lower amount of feed. Future works could induce in an effort, either on an extended period of time under the same experimental plan to observe an additional spawning period, or the effects of manipulated constant environmental variables, on the gametogenic cycle combined with the feeding regimes used in this trial.

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