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Ecologia e Gestione Sostenibile delle Risorse Ambientali - XXX Ciclo

**CHANGES IN GONAD CONDITIONS OF THE SEA URCHIN *PARACENTROTUS LIVIDUS* IN
RESPONSE TO ANTHROPIC, SEASONAL, ENVIRONMENTAL, PHYSIOLOGICAL AND
DIETARY FACTORS: SUGGESTIONS FOR AN IMPROVEMENT OF THE ACTUAL HARVESTING
PRACTICES AND ECHINOCULTURE PRODUCTION**

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ABSTRACT

Sea urchin roe represents a gourmet delicacy in several parts of the world. In the last decades, the increasing market demand of sea urchin roe and the consequent continuous catches of commercial stocks have gradually led to the impoverishment of wild resources. The variability of some gonad features, e.g. yield and quality, may have direct consequences on fishing, concentrating it in areas where conditions are expected to be most favorable. Understanding which factors are involved in these changes is essential to make the fishing activities more sustainable.

The general aim of my PhD thesis was to enhance the existing knowledge of the biology of the sea urchin *Paracentrotus lividus* which is heavily fished along the Sardinian coasts, as well as to give suggestions for the improvement of current management practices for Sardinian fishery. Secondly, I aimed to provide some useful information for the development of echinoculture as it is not well-developed throughout the European area.

In chapter 2, published in *PeerJ*, 2017, 5: e3067; DOI 10.7717/peerj.3067, I studied the effect that fishing may have on sea urchin populations, comparing the reproductive output of two populations which are subject to different fishing pressures. Data suggest that human predation affects the population structure of the high-pressure zone causing the loss of the largest and most fertile individuals (TD>60 mm). This implies a smaller gonad yield of the commercial-sized individuals in that zone and a reduction in the number of reproductive events. However, the survival of the population living in the highly-fished zone does not seem to be endangered due to the high number of individuals of intermediate size (40-50 mm) which are fertile and effectively contribute to the gamete production. This suggests that the potential reproductive contribution of a population depends on the density of fertile individuals and not on their size.

In chapter 3, I investigated the seasonal changes of gonad yield (throughout two sampling years) and color (for one year) in adult (≥ 45 mm) *P. lividus* in two different habitats, i.e. a rocky bed dominated by seaweeds (RB) and a *Posidonia oceanica* meadow over a sandy bottom (PM). Results show that habitat and seasonality have a direct influence on yield and color. Yield is generally higher in the population from PM, probably due to the effect of the seagrass leaves which protect sea urchins from waves. Color is better in individuals from RB probably because of a higher quantity or quality of pigments in the seaweeds eaten. In RB, the best compromise for harvesting profitable gonads was reached in summer and autumn, when gonads are

sufficiently large, and their color is appealing to consumers. In PM these quality parameters are reached in spring. Diversifying harvesting grounds according to the quality characteristics of gonads may be part of the solution to the overfishing of certain coastal areas. In this work, the *P. oceanica* meadow is considered a favorable alternative substrate for harvesting.

In chapter 4, published in *Marine Environmental Research* 2016, 113: 124-133, I collaborated with some researchers from the Porto Conte Ricerche Laboratory, to investigate the influence of seasonal and environmental patterns on the lipid fraction of *P. lividus* (≥ 50 mm) gonads living on rocky bottoms at Su Pallosu Bay (central-western Sardinia) and Tavolara-Punta Coda Cavallo Marine Protected Area (north-eastern Sardinia). Lipid content is affected by season, with the lowest values corresponding to the period of maximum gonad maturity i.e. from December to April due to the consumption of fatty reserves during gametogenesis. Photoperiod is more important than seawater temperature in triggering and regulating fat utilization and storage in *P. lividus* gonads. Fatty acid profiles are influenced by the area of origin, with gonadic MUFAs as the main discriminative fatty acids for the two areas sampled. On the contrary, long chain PUFAs such as ARA and EPA, normally involved in reproductive or water temperature adaptation processes, are mainly responsible for the seasonal clusterization.

In chapter 5, published in *Journal of Proteomics* 2016, 144: 63-72; DOI 10.1016/j.jprot.2016.05.035, I participated in a project together with some researchers of Porto Conte Ricerche Laboratory, in which gonads at various developmental stages were analyzed by gel-based and by shotgun proteomics. A detailed characterization of the proteome changes occurring in gonads of both sexes during maturation was achieved (dataset deposited in the ProteomeXchange Consortium via the PRIDE partner repository, code: PXD004200). Results show that the gonad protein profile is different between sexes and between the developmental stages. There are significant modifications in numerous proteins involved in nutrient accumulation in nutritive phagocytes (e.g. toposome), as well as in gamete maturation (e.g. ribosomal proteins, maternal butanol extracted protein, metallothioneins, T complex proteins, mitochondrial and flagellar proteins).

Finally, in chapter 6, I compared the effect of two macroalgal diets (the chlorophyta *Ulva lactuca* and the rhodophyta *Gracilaria gracilis*) on the gonad yield and color of adult *P. lividus* (41-45 mm test diameter), taken from a rocky bottom in winter. Both diets produced a similar, significant increase in gonad yield after three months (2.34 ± 0.51 in gonads from urchins fed *U. lactuca* and 2.88 ± 0.73 in gonads from urchins fed *G. gracilis*), despite the higher quantity of

red algae ingested daily (0.88 ± 0.01 g and 1.35 ± 0.03 g, for *U. lactuca* and *G. gracilis* respectively) and the lower absorption efficiency of the green algae (two-fold lower than the efficiency of the red algae). None of the color components (lightness, redness, yellowness) differed significantly from the initial sample (i.e. urchins from the same locality starved for two weeks), however the gonads of urchins fed *U. lactuca* were slightly yellower than those of urchins fed *G. gracilis*. All results being equal, but considering the lower Feed Conversion Ratio and total wet feed intake in urchins fed *U. lactuca*, it resulted economically more convenient to feed reared sea urchins with *U. lactuca*.

Overall, this project provides information on the characteristics of sea urchin gonads in relation to numerous extrinsic and intrinsic factors. Sea urchin gonads are subjected to continuous changes in size, color and chemical composition, thus making their market value variable. The origin of this variability is not yet well-known and published results are contradictory. Therefore, more research is required in order to optimize harvesting activities, both in the wild and in aquaculture plants, and to promote sustainable management of wild resources.

RIASSUNTO

Le gonadi di riccio di mare rappresentano una prelibatezza ricercata in molte zone del mondo. Negli ultimi decenni, la crescente richiesta di mercato di gonadi di riccio e le conseguenti continue catture degli stock commerciali hanno gradualmente portato all'impoverimento delle risorse selvatiche. La variabilità di alcune caratteristiche gonadiche, per esempio la biomassa e la qualità, possono avere conseguenze dirette sulla pesca che, pertanto, si concentra in aree dove tali caratteristiche sono considerate più favorevoli. Comprendere quali fattori siano implicati in questi cambiamenti è essenziale per rendere le attività di pesca più sostenibili.

L'obiettivo generale della mia tesi di dottorato è incrementare le conoscenze attualmente esistenti sulla biologia del riccio di mare *Paracentrotus lividus*, fortemente pescato lungo le coste sarde, e dare suggerimenti per un miglioramento delle attuali pratiche di gestione della pesca del riccio in Sardegna. In secondo luogo, mi sono posta l'obiettivo di fornire alcune informazioni utili per lo sviluppo dell'echinocoltura, che ancora non è particolarmente sviluppata in tutta l'area europea.

Nel capitolo 2, pubblicato su *PeerJ*, 2017, 5: e3067; DOI 10.7717/peerj.3067, ho studiato l'effetto che la pesca può avere sulle popolazioni del riccio di mare, comparando l'output riproduttivo di due popolazioni soggette a differenti pressioni di pesca. I dati suggeriscono che il prelievo ha un impatto sulla struttura di popolazione della zona a forte pressione di pesca a causa della perdita degli individui più grandi e fertili ($TD > 60$ mm). Il prelievo determina anche una minore massa gonadica degli individui di taglia commerciale così come la riduzione del numero di eventi riproduttivi. Tuttavia, la sopravvivenza della popolazione nella zona fortemente pescata non sembra minacciata per l'alto numero di individui di taglia intermedia (40-50 mm) che risultano essere fertili e che effettivamente contribuiscono alla produzione dei gameti. Questo suggerisce che il potenziale contributo riproduttivo di una popolazione dipende dalla densità degli individui fertili e non dalla loro taglia.

Nel capitolo 3, ho indagato sulle variazioni stagionali della biomassa (durante due anni di campionamento) e del colore delle gonadi (per un anno) di individui adulti (≥ 45 mm) di *P. lividus* in due habitat differenti, un fondale roccioso dominato da macroalghe (RB) e una prateria di *Posidonia oceanica* su fondo sabbioso (PM). I risultati mostrano che habitat e stagionalità hanno un'influenza diretta sulla biomassa e il colore. La biomassa è maggiore in PM probabilmente per l'effetto delle foglie della fanerogama che protegge i ricci dalle onde. Il

colore è migliore in RB probabilmente per una maggiore quantità o qualità di pigmenti presenti nelle macroalghe assunte nella dieta. In RB, il miglior compromesso per raccogliere gonadi redditizie viene raggiunto nella stagione estiva ed autunnale, quando le gonadi sono sufficientemente grandi e il colore è gradevole per il consumatore, mentre in PM viene raggiunto in primavera. Diversificare i substrati di raccolta a seconda delle caratteristiche qualitative delle gonadi può contribuire a ridurre la sovrapesca in alcune aree costiere. In questo lavoro, la prateria di *P. oceanica* rappresenta un substrato di raccolta altrettanto favorevole alternativo al classico substrato roccioso.

Nel capitolo 4, pubblicato su *Marine Environmental Research* 2016, 113: 124-133, ho collaborato con alcuni ricercatori di Porto Conte Ricerche, per indagare sull'influenza di modelli stagionali ed ambientali sulla frazione lipidica delle gonadi di *P. lividus* (≥ 50 mm) di fondali rocciosi della baia di Su Pallosu (Sardegna centro-occidentale) e dell'Area Marina Protetta di Tavolara-Punta Coda Cavallo (Sardegna nord-orientale). Il contenuto lipidico è influenzato dalla stagione, con i valori più bassi in corrispondenza del periodo di massima maturità gonadica ovvero tra Dicembre ed Aprile, dovuto al consumo delle riserve lipidiche durante la gametogenesi. Il fotoperiodo risulta più influente della temperatura dell'acqua nell'innescare e regolare l'utilizzo e l'immagazzinamento dei grassi nelle gonadi di *P. lividus*. Il profilo degli acidi grassi è influenzato dall'area di prelievo, con i MUFA gonadici come maggiori acidi grassi discriminanti per le due aree d'indagine; al contrario i PUFA a lunga catena come ARA ed EPA, generalmente coinvolti in processi riproduttivi o di adattamento termico, sono maggiormente responsabili per il raggruppamento stagionale.

Nel capitolo 5, pubblicato su *Journal of Proteomics* 2016, 144: 63-72; DOI 10.1016/j.jprot.2016.05.035, ho partecipato ad un lavoro con i ricercatori di Porto Conte Ricerche, in cui gonadi in vari stadi di sviluppo sono state analizzate con metodiche di proteomica su gel o shotgun. E' stata ottenuta una caratterizzazione dettagliata dei cambi proteici che avvengono nelle gonadi di entrambi i sessi lungo la maturazione (il dataset è stato registrato nel ProteomeXchange Consortium tramite il partner di deposito PRIDE, codice: PXD004200). I risultati mostrano che il profilo proteico gonadico è diverso tra i sessi e tra gli stadi di sviluppo, con modificazioni significative in numerose proteine coinvolte nell'accumulo di nutrienti all'interno dei fagociti nutritivi (ad esempio, il toposoma), così come nella maturazione dei gameti (ad esempio, proteine ribosomiali, proteine del tipo maternal butanol extracted, metallotioneine, proteine del T complex, proteine mitocondriali e flagellari).

Infine, nel capitolo 6, ho comparato l'effetto di due diete macroalgali (la clorofita *Ulva lactuca* e la rodofita *Gracilaria gracilis*) sulla massa gonadica e il colore in individui adulti (41-45 mm di diametro) di *P. lividus*, raccolti da un fondale roccioso nella stagione invernale. Entrambe le diete producono un aumento significativo della massa gonadica dopo tre mesi (2.34 ± 0.51 nelle gonadi dei ricci alimentati con *U. lactuca* e 2.88 ± 0.73 nelle gonadi dei ricci alimentati con *G. gracilis*), nonostante una maggiore quantità di *G. gracilis* ingerita giornalmente (0.88 ± 0.01 g) rispetto a *U. lactuca* (1.35 ± 0.03 g) e una minore efficienza di assorbimento dell'alga verde (due volte inferiore dell'efficienza dell'alga rossa). Nessuna delle tre componenti del colore (L^* , a^* , b^*) differisce significativamente dal campione iniziale (cioè ricci provenienti dalla stessa località di raccolta tenuti a digiuno per due settimane), tuttavia le gonadi dei ricci alimentati con *U. lactuca* sono lievemente più gialle di quelle dei ricci alimentati con *G. gracilis*. A parità di risultati, ma considerando i più bassi Feed Conversion Ratio e consumo totale di alimento umido nei ricci alimentati con *U. lactuca*, è economicamente più vantaggioso alimentare ricci allevati con *U. lactuca*.

Complessivamente, questo progetto ha fornito informazioni sui cambiamenti delle gonadi di riccio di mare in relazione a numerosi fattori estrinseci ed intrinseci. Le gonadi di riccio di mare sono soggette a continue modifiche in dimensioni, colore e composizione chimica, che fanno variare il loro valore di mercato. L'origine di questa variabilità è ancora scarsamente conosciuta e i risultati pubblicati sono contraddittori. Pertanto, ulteriori indagini sono necessarie in prospettiva di ottimizzare le attività di raccolta, sia in natura che in impianti d'acquacoltura, e di promuovere una gestione sostenibile delle risorse selvatiche.

1. GENERAL INTRODUCTION

Sea urchin roe has been eaten for several centuries in many parts of the world, particularly in Japan, Chile and several European (Atlantic and Mediterranean) countries. Particularly in Japan, sea urchin consumption has increased along the demand for this luxury food in the last decades, making this country the largest importer and consumer of sea urchins. In fact, 68% of the world's production is consumed here (FAO Fisheries commodities production and trade 1976-2013, 2016). The average price of roe, on the Japanese market, ranges from € 20/kg for local production (fresh animals considered as the top quality), to € 8/kg for fresh imported echinoids (Hagen, 1996). In Sardinia the average price of fresh roe is about € 80/kg, which is more than four times the Japanese price (Cau *et al.*, 2007). This demonstrates the high profitability of this relatively small-scale fishery. Gonad yield, as well as water content, texture, firmness, color, taste and proximate composition are important quality factors in sea urchin roe marketing and they strongly influence the price (Spirlet *et al.*, 2000; Unuma, 2002; McBride *et al.*, 2004).

Sea urchin fishery is characterized by very poor sustainability: fishing can be a very profitable business during the first years of activity in a new fishing ground. Later, wild populations begin to decline due to their slow growth rate (Grosjean, 2001) and due to the high efficiency and selectivity of the fishing techniques used. Animals are easily picked at shallow depths either by hand or with simple equipment (Andrew *et al.*, 2002; Addis *et al.*, 2009). Consequently, in several areas of the USA, Ireland, France and Spain, sea urchin fisheries have declined considerably and their stocks are currently considered over-exploited (Andrew *et al.*, 2002).

In the last decades, aquaculture has been considered a necessary alternative to sea urchin fisheries, due to the overexploitation of wild resources and the constantly increasing demand for high quality gonads available on the market throughout the year (Grosjean, 2001; Pearce, 2010). In fact, echinoculture could play a significant role in fulfilling the gap between the growing market's supply and demand (Andrew *et al.*, 2002; Carboni *et al.*, 2013). Sea urchin aquaculture represents about 1% of the world's aquaculture production. China is the world's largest producer (99.4%), followed by Russia (0.6%) and Ireland (0.02%) (FAO Aquaculture production, 2017).

In the last years, the interest in the sea urchin species *Paracentrotus lividus* (Fig. 1) has grown. This is primarily due to its role in maintaining the equilibrium of sublittoral communities of

rocky reefs through its grazing activity (Sala and Zabala, 1996; Sala *et al.*, 1998) as well as to the economic value of its roe (Palacín *et al.*, 1998).



Figure 1 – Adult sea urchin *Paracentrotus lividus* (from: www.alchetron.com).

P. lividus gonads (Fig. 2) are reddish-orange thanks to the presence of carotenoids and are marketed fresh, frozen and pasteurized. The highest demand is during the cold season (autumn-winter), when the gonads reach their maximum size and their color is intense enough to deserve the appellation "red gold".



Figure 2 - *P. lividus* roe (from: www.frescopesce.it/alla-scoperta-del-prelibato-riccio-di-mare/).

This is the main echinoid species consumed in Mediterranean and Atlantic Europe (Boudouresque and Verlaque, 2007), with France being one of the largest consumers of sea

urchins (around 100 t per year, Hagen, 1996). According to FAO, France's capture production in the last 65 years has been over 11,000 tons, which represents 80% of the total European catches (Fig. 3, FAO global capture production 1950-2015, 2017).

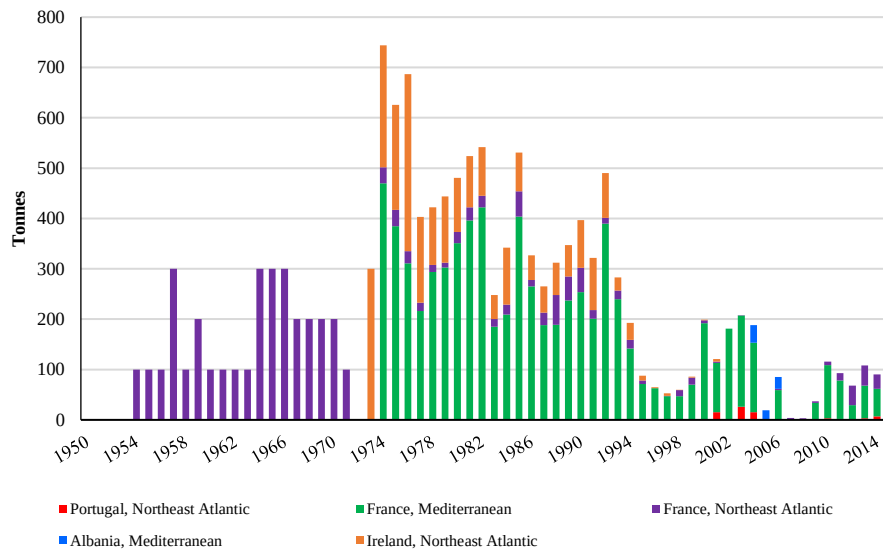


Figure 3 - Capture of *P. lividus* in Europe from 1950-2015 according to FAO Capture Production (release date 2017).

In Italy, sea urchin harvesting occurs mainly in the South and on the main islands (Guidetti *et al.*, 2004; Pais *et al.*, 2007; Addis *et al.*, 2009). In Sardinia, the practice of fishing for sea urchins underwent a marked increase about thirty years ago, at the end of the '80s. Due to the increasing economic importance of the fishery, starting in 1994, the Department for Environmental Protection of the Autonomous Region of Sardinia has introduced several limitations on fishery operations. A fishing season (November-April) has been scheduled, a limited number of licenses are issued (189 licenses), daily individual quotas have been fixed (1500 sea urchins d⁻¹), as well as a minimum size limit of the test diameter (50 mm excluding spine; RAS, 2016). Since 2011, every authorized fisherman is supposed to complete a log-book containing data on sea urchin captures. Nonetheless, no reliable data on Sardinian catches exist. A study conducted by researchers from the Department of Animal Biology and Ecology of the University of Cagliari speculates that the annual quota harvested by fishermen exceeds 32,000,000 units (Cau *et al.*, 2007). On the other hand, the Fishing and Aquaculture Department of the Autonomous Region of Sardinia, according to the log-books concerning the 2009/2010 Sardinian fishing season, reported the number of harvested sea urchins to be approximately 4,000,000 (Pulina, 2014) which is 8-fold lower than the number reported by Cau *et al.* (2007). In any case, the

Department itself considers the self-certification of fishermen to be unreliable (Campolmi, personal communication).

Studies performed in northern Sardinia highlighted that this continuous harvesting is exerting negative effects on sea urchin populations, such as decreases in density, size and average biomass (Pais *et al.*, 2007; Ceccherelli *et al.*, 2011; Pais *et al.*, 2012). These results, indicating an over-fishing of wild sea urchin stocks, are consistent with those obtained by several researches in other areas (Carter and VanBlaricom, 2002; Bertocci *et al.*, 2014), and suggest that there is a risk of collapse of the sea urchin stocks in Sardinia. In order to ensure an immediate plan for conservation of the species and fishing sustainability, it would be appropriate to adapt the sea urchin exploitation levels to the carrying capacity of the wild resource while combining this with economic profitability and social benefits (Secci, 2013). A deep understanding of the status of sea urchin fisheries is necessary to sustain harvesting. Data on population dynamics and knowledge of the reproductive cycle and adaptation to change in different environmental conditions are particularly relevant (Sellem and Guillou, 2007).

In order to support production in spite of exploitation of wild stocks, *P. lividus* is becoming an important model organism for the emerging aquaculture industry in Europe (Phillips *et al.*, 2009; Angioni and Addis, 2014). Usually the focus has been on an increase in gonad yield (Liyana-Pathirana *et al.*, 2002) with less emphasis placed on quality parameters such as appearance, color, texture and flavor, despite the strong influence of these factors on market price (Yokota *et al.*, 2002; Phillips *et al.*, 2009). However, recent studies have focused not only on the optimization of culture methods for larvae, but also for adults (Grosjean *et al.*, 1998), and on nutritional requirement/roe enhancement for the production of high quality gonads (Carboni *et al.*, 2013a; Shpigel *et al.*, 2006).

1.1. PARACENTROTUS LIVIDUS

1.1.1. Morphology and structural organization

The regular echinoid *P. lividus* (Lamarck, 1816) belongs to the phylum Echinodermata (class Echinoidea, order Diademantoida). The name which has been assigned to the group is of Greek origin and refers to the fact that these animals are covered with spines. These spines are not

very long, but they are strong and sharp and located throughout the body, implanted on tubercles.

Echinoderms are deuterostome, with a well-developed coelom. The cavities are lined with peritoneum and the coelomic fluid plays an important role in the circulatory system. A rigid shell (dermal skeleton), called “test”, composed of fused plates of calcium carbonate, contains the viscera. The test can reach a maximal diameter of 75 mm (Boudouresque and Verlaque, 2007). The body is spherical and slightly flattened, covered in spines. The color varies from green to violet, to reddish to brown, but this does not depend on the size of the individuals or the depth of their habitat (Boudouresque and Verlaque, 2007).

Sea urchins have a pentamerous structure. Each section consists of two zones: in the ambulacral areas there are very specialized organelles, the tube feet, which have locomotive and tactile functions; the interambulacral zones are devoid of tube feet. The mouth and the anus are located on two opposite poles of the body. The oral area always faces downwards, resting on the substrate. The mouth lies in the center of the oral zone, and is surrounded by lips of a softer tissue, with numerous small, bony pieces embedded in it (peristome). The mouth is made up of five calcium carbonate teeth and is commonly known as Aristotle's Lantern.

The mouth opens into a long, simple intestine which ends in an anus (periproct) located in the aboral area of the test. The periproct is surrounded by five genital plates, with a small gonopore directly connected to a gonad through a gonoduct (Fig. 4).

An aquifer system and a non-centralized nervous system are present in the sea urchin. Both are composed of a ring around the mouth from which radial channels depart. The radial channels of the aquifer system run along the entire ambulacral zones, from which the tube feet (podia) originate, going outward through small breaches of the test. Water is forced into the tube feet when the muscles contract or relax, causing the extension or the retraction of podia.

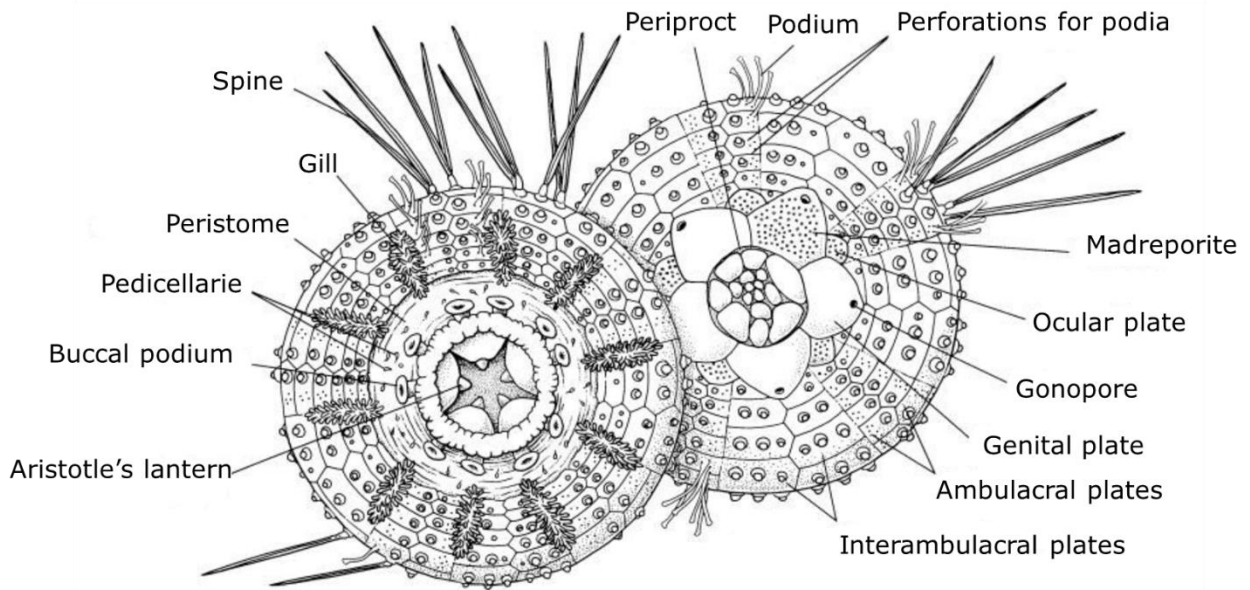


Figure 4 - External anatomy of the sea urchin *P. lividus*. On the left, the oral view; on the right, the aboral view (modified after Reid W.M., in: Ruppert and Barnes, 1994).

Although there is no specialized respiratory system, there are five pairs of gills around the mouth. In addition, the aquifer system also plays an important role in respiratory exchanges, along with the tube feet which increase the exchange surface.

The five gonads are located in interambulacral areas and are directly connected with the genital plates by a gonoduct (Fig. 5).

1.1.2. Distribution and habitat

P. lividus is distributed throughout the Mediterranean Sea and in the North-Eastern Atlantic, from Scotland and Ireland to southern Morocco and the Canary Islands, including the Azores (Boudouresque and Verlaque, 2007). It is particularly common in the Mediterranean Sea where it inhabits the subtidal rocky seabed areas and meadows of *Posidonia oceanica*, between the limits of the low tide and a depth of 10-20 meters (Gamble, 1965; Tortonese, 1965; Allain, 1975; Règis, 1978; Harmelin *et al.*, 1980; Crook *et al.*, 2000). In coastal lagoons (Thau and Urbinu lagoons in the Mediterranean; Archachon Bay, Atlantic Ocean, France), *P. lividus* can even live either on muddy substrates or on coarse sand (Allain, 1975; San Martín, 1987; Fernandez *et al.*, 2003).

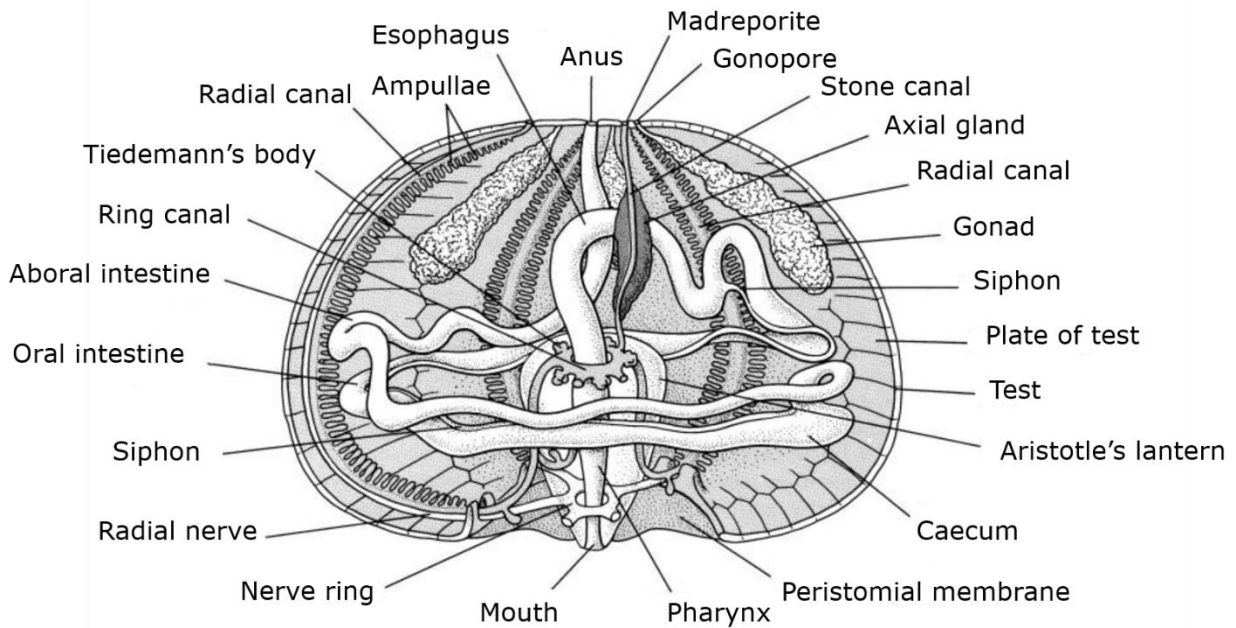


Figure 5 - Internal anatomy of *P. lividus* (modified after Reid W.M., in: Ruppert and Barnes, 1994).

Although present in both Mediterranean and Atlantic coastal lagoons, *P. lividus* is sensitive to high and low salinity. Long-term exposure to salinity lower than 15-20 ‰ or higher than 39-40 ‰ causes the death of the organism (Allain, 1975; Pastor, 1971; Le Gall *et al.*, 1989). It is particularly abundant in areas where the water temperature in winter varies between 10 and 15°C and in summer between 18 and 25°C. The northern and southern limits of the natural range of *P. lividus* are bound by an isotherm of 8°C in winter and 28°C in summer.

1.1.3. Predation

The main natural predators of *P. lividus* in the Mediterranean Sea are the seabream *Diplodus sargus*, *Diplodus vulgaris*, *Sparus aurata*, the wrasses *Labrus merula* and *Coris julis*, the crustacean decapod *Maja crispata* and the gastropod *Hexaplex trunculus*. Seabreams can feed on individuals with a test diameter of up to 5 cm, while *C. julis* and *Thalassoma pavo* generally feed on individuals with a diameter of less than 1 cm (Tertschnig, 1989; Sala, 1996, 1997; Guidetti *et al.*, 2005; Heureu *et al.*, 2005). The starfish *Marthasterias glacialis* can eat sea urchins with a test diameter of up to 63 mm (Boudouresque and Verlaque, 2007). In coastal areas where *D. sargus* and *D. vulgaris* populations are subjected to an intense fishing effort, the predation of sea urchins is due to *T. trunculus* (43%) and to other species of fishes (the remaining 57%). In contrast, in marine protected areas, fishes are the main predators of *P.*

lividus (Sala and Zabala, 1996), even if gastropods can be particularly effective inside *P. oceanica* meadows (Farina *et al.*, 2016).

The Atlantic situation is slightly different, in fact, the main predators are starfishes and crustaceans. Crabs (e.g. *Cancer pagurus*, *Necora puber*, *Maja brachydactyla* and *Carcinus maenas*) are able to feed on individuals of any size class, while *Homarus gammarus* can prey on individuals of *P. lividus* with a test diameter larger than 6 cm. (Muntz *et al.*, 1965; Ebling *et al.*, 1966; Kitching and Ebling, 1967, Neil and Pastor, 1973; Kitching and Thain, 1983; Bernádez *et al.*, 2000).

1.1.4. Densities

The density of *P. lividus* varies for different geographic areas and generally ranges from a few to a dozen individuals per square meter (Boudouresque and Verlaque, 2007). However, very high densities (>50-100 individuals for square meter) may occur in shallow water environments, on rocky substrates with a low slope, in intertidal pools and in polluted environments (Kempf, 1962; Pastor, 1971; Crapp and Willis, 1975; Harmelin *et al.*, 1981; Delmas and Régis, 1986; Delmas, 1992). In general, sea urchin populations have a patchy spatial distribution (Barnes and Crook, 2001; Guidetti *et al.*, 2003; Addis *et al.*, 2009). Small individuals can also be found under larger ones, as a protective mechanism against predators (Tegner and Dayton, 1977; Grosjean, 2001).

1.1.5. Feeding habits

Two important sources of information about the diet of *P. lividus* are aquarium experiments and *in situ* observations through analysis of gut contents.

P. lividus is basically a herbivore (Mortensen, 1943; Kitching and Ebling, 1961; Kempf, 1962; Ebling *et al.*, 1966; Neil and Larkum, 1966; Neill and Pastor, 1973; Verlaque and Nédélec, 1983b; Verlaque, 1987a, 1987b). In the Mediterranean Sea, *P. lividus* has been clearly recognized as a keystone species due to its ability, when at high densities, to transform macroalgal-dominated communities into barren areas characterized by increased cover of bare substrates and encrusting coralline algae. This reduces biodiversity and alters ecosystem functions (Sala and Zabala, 1996).

Several species are clearly preferred as food, e.g. *Rissoella verrucolosa* (Rhodobionta), *Cymodocea nodosa* (Magnoliophyta), *Cystoseira amentacea*, *Padina pavonica* and *Undaria*

pinnatifida (Ochrophyta). Other species such as *Asparagopsis armata*, *Gelidium spinosum* (Rhodophyta), *Anadyomene stellata*, *Caulerpa taxifolia*, and *Flabellia petiolata* (Chlorophyta) are strongly avoided (Traer, 1980; Cuomo *et al.*, 1982; Nédélec, 1982; Kitching and Thain, 1983; Verlaque and Nédélec, 1983a, b; Verlaque, 1984; Zupi and Fresi, 1984; Knoepffler-Péguy *et al.*, 1987; Shepherd, 1987; Verlaque, 1987a, b; Frantzis *et al.*, 1988; Odile *et al.*, 1988; Fernandez, 1989; Rico, 1989; Boudouresque *et al.*, 1993; Knopffler-Péguy and Nattero, 1996; Lemée *et al.*, 1996; Aubin, 2004). *P. lividus* consumes all the parts of the seagrass *P. oceanica* with some level of preference, precisely living leaves with and without epiphytes, dead leaves, rhizomes and roots (Traer, 1980; Verlaque and Nedelec, 1983a; Traer, 1984; Zupi and Fresi, 1984; Shepherd, 1987).

Food choice usually depends on the relative abundance of available items (Chiantore *et al.*, 2008; Privitera *et al.*, 2008). Selectivity is high under conditions of high food supply, but declines as the grazing pressure increases and disappears when grazing pressure becomes extreme (Boudouresque and Verlaque, 2007). In addition, food preferences are size dependent. Recruits (1 mm in diameter) graze on encrusting algae such as Corallinaceae and Peyssonneliaceae. Subsequently (3-7 mm) they eat filamentous algae (Ceramiaceae, Rhodomelaceae and Ectocarpaceae). Juveniles, between 7 and 10 mm, eat coarsely-branched algae (e.g. *Halopteris scoparia*, *Padina pavonica*, *Cystoseira* spp., *Corallina elongata*), while from 10 mm diameter upwards, brown algae and leaves of *P. oceanica* are dominant in its diet (Verlaque and Nedelec, 1983b; Verlaque 1984, 1987b; Fernandez, 1989; Rico, 1989).

P. lividus has a generalist and opportunistic behavior in food consumption, especially under conditions of limited resources. Photosynthetic unicellular organisms, sponges, hydroids and copepods can also be found in gut contents (Mortensen, 1943; Tortonese, 1965; Pastor, 1971; Neill and Pastor, 1973; Régis, 1978; Délmas and Régis, 1986; Fernandez, 1990; Mazzella *et al.*, 1992). Even acts of cannibalism were recorded in exceptionally dense populations. In aquariums, small individuals of 2-3 cm in diameter may be preyed upon by larger ones that have been starved (Pastor, 1971).

Food consumption rate is calculated using the repletion index (RI, Nedelec, 1983) i.e. the proportion of the gut contents relative to the total weight of the test. In wild populations of *P. lividus*, the peak of RI is reported just before or during the spawning period (Bayed *et al.*, 2005). Only weak relationships have been observed between gonad index and repletion index (Régis, 1978; Semroud and Kada, 1987).

1.1.6. Growth

Somatic growth of *P. lividus* can be influenced by water temperature, the type of available food, and gonad development (Fernandez, 1996), although seasonal variations in growth rate seem to be mainly dependent on water temperature. Growth increases proportionally with increasing temperature between 7 and 18°C, but the optimum condition for growth is between 18 and 22°C. Over 22°C, growth begins to slow down, until stopping completely when the temperature exceeds 28°C (Le Gall *et al.*, 1990). In the Mediterranean, the highest growth occurs when the water temperature is between 12 and 18°C (spring). Growth hardly occurs in autumn and almost never in winter (Azzolina, 1988; Fernandez and Caltagirone, 1994; Turon *et al.*, 1995; Shpigel *et al.*, 2004). Studies on growth conducted in wild populations have shown that the commercial size (i.e. 50 mm in test diameter) is reached in 4-6 years (Crapp and Willis, 1975; Haya de la Sierra, 1990; Gago *et al.*, 2003; Tomšić *et al.*, 2010; Ouréns *et al.*, 2013).

1.1.7. Reproduction

P. lividus has separate sexes and there is no sexual dimorphism. Cases of hermaphroditism have been observed (Drzewina and Bohn, 1924; Neefs, 1937; Byrne, 1990).

In laboratory conditions, sexual maturity is reached in individuals ranging in size between 13 and 20 mm and/or after 5 months of age (Fenaux L. in Azzolina, 1987; Cellario and Fenaux, 1990). However, in wild population, sexual maturity can be reached later. Limiting conditions, such as scarce availability of food or unfavorable environmental conditions, can decrease the size at sexual maturity (Lozano *et al.*, 1995). The size at sexual maturity can vary between geographic areas, perhaps due to different energetic investments in reproduction or to different growth rates (see Ouréns *et al.*, 2011).

Gonad growth occurs when food availability (Lawrence *et al.*, 1992; Gago *et al.*, 2003) and the organic matter ingested are high (Frantzis and Grémare, 1992). Temperatures between 18 and 22°C, paired with a short photoperiod, seem to enhance gonad development (Shpigel *et al.*, 2004).

High gonad indices were found in low-density populations, probably because of a lack of competition for the food source (Guettaf and San Martín, 1995). On the contrary, in Spain (Catalonia), gonad indices were found to be higher in high-density populations living in shallow habitats characterized by high hydrodynamics and few algal species than in stable environments

of deep waters characterized by low-population density and dominated by preferred species (Lozano *et al.*, 1995). These results suggested a greater investment by *P. lividus* in reproduction in limited food conditions.

The reproductive cycle of *P. lividus* has been studied in detail by several authors. It is influenced by several environmental factors such as water temperature (Byrne, 1990; Lozano *et al.*, 1995), photoperiod (Byrne, 1990; Lozano *et al.*, 1995; Shpigel *et al.*, 2004), hydrodynamic conditions (Guettaf *et al.*, 2000), and trophic availability (Regis, 1979; Fenaux, 1968, Lozano *et al.*, 1995; Guettaf *et al.*, 2000).

P. lividus has an annual reproductive cycle which can differ conspicuously between neighboring localities (Lozano *et al.*, 1995; Sánchez-España *et al.*, 2004). Gonadosomatic index (GSI), which is defined as the proportion of the gonads relative to the total weight of the test (Sánchez-España *et al.*, 2004), is commonly used to describe the seasonal evolution of the reproductive cycle. It is an indicator that can be easily estimated but, if used alone, could lead to the wrong conclusions. Indeed, it is known that somatic cells within the germinal epithelium store nutrient reserves, and under stressful situations, the energy accumulated in the gonads can be allocated to other compartments (Walker *et al.*, 2007; Mercier and Hamel, 2009), causing drops in the GSI that are not strictly linked to reproductive events. In the Atlantic Ocean, the reproductive cycle of *P. lividus* has a clear pattern and a single annual spawning is reported by most of the authors who based their estimates on the GSI and histological studies (see references in Ouréns *et al.*, 2011). Only Crapp and Willis (1975) found more than one spawning season in an Irish population, basing their statements on GSI evaluation. On the contrary, in Mediterranean populations, the GSI does not show a clear peak during the year, and therefore it does not allow for a clear identification of the reproductive pattern (Lozano *et al.*, 1995; Sellem and Guillou, 2007). Most of the authors who employed the GSI or who monitored larval and recruit abundances, identified two annual spawning periods: a more intense episode in spring and another in autumn (Semroud and Kada, 1987; Pedrotti and Fenaux, 1992; Pedrotti, 1993; Semroud, 1993; Fernandez and Boudouresque, 1997; López *et al.* 1998; Guettaf *et al.*, 2000; Tomas *et al.*, 2004). Among the authors that include histological analyses, Lozano *et al.* (1995), Martínez *et al.* (2003), Sánchez-España *et al.* (2004) and Sellem and Guillou (2007) recorded a single spawning season, while Fenaux (1968) and Leoni *et al.* (2003) reported two. It is still not known whether the gonads undergo a new gametogenic cycle between the two annual spawning periods reported in most of the Mediterranean regions, or if both reproductive

events are a consequence of a short inactive episode within the breeding season, as detected for an Atlantic population (González-Irusta *et al.*, 2009). Coupling the estimation of GSI and histology techniques is surely the most suitable tool to determine the reproductive cycle of *P. lividus* in the Mediterranean with certainty.

During spawning events, male and female *P. lividus* aggregate and simultaneously release their gametes (Cherbonnier, 1954). Fertilization is external, and gametes are emitted by the genital pores disposed around the anus. Spawning episodes do not always involve all individuals of a population.

Water temperature seems to play a key role in determining the start of the spawning events. In the case of two spawning events, the first occurs when the temperature rises to a critical level, and the second when it returns to that level (Fenaux, 1968; Byrne, 1990; Pedrotti, 1993; Bayed *et al.*, 2005). The first release can also be triggered by the day length (about 15 h of daylight) rather than by water temperature, while the end of spawning events seems to be controlled merely by temperature (Spirlet *et al.*, 1998, 2000).

Variability in the number of spawning events was observed between localities and habitats within the same region (Guettaf, 1997). Lozano *et al.* (1995) stated that spawning only occurs during spring and early summer, although the presence of larvae and individuals at post-metamorphosis in autumn suggests the presence of a spawning event in late summer. Therefore, considering all the variables that affect the release of gametes, spawning can occur almost year-round, although at very low levels. This behavior could be a strategy to ensure greater reproductive success of the species (Boudouresque and Verlaque, 2007).

1.1.8. Gametogenesis and larval developing

During the annual gametogenic cycle, gonads of both males and females undergo a predictable series of structural changes (Walker, 1982; Pearse and Cameron, 1991; Walker and Lesser, 1998; Walker *et al.*, 2005). These changes are classified according to the activities of the two major cellular populations that compose the germinal epithelium: gametogenic cells (oocytes to ova in the ovary, or spermatocytes to fully differentiated spermatozoa in the testis) and nutritive phagocytes. The latter are somatic cells within the germinal epithelium, which store extensive nutrient reserves before gametogenesis begins.

According to Byrne (1990), it is possible to identify six stages of *P. lividus* oogenesis.

Stage I: recovery stage

Ovaries contain primary oocytes (5 to 30 μm diameter) and clusters of early oocytes along the acinal wall. Nutritive phagocytes form a meshwork across the ascinus, giving the ovary a vacuolated appearance. The ovary may contain unspawned ova in the process of lysis and be engulfed by the phagocytes (Fig. 6a).

Stage II: growing stage

With the beginning of vitellogenesis, the primary oocytes increase in size (10 to 50 μm diameter) but remain attached to the ascinal wall and are surrounded by nutritive phagocytes. Clusters of early primary oocytes may be present. The ovary is packed with nutritive phagocytes (Fig. 6b).

Stage III: premature stage

Oocytes at all stages of development (10 to 90 μm diameter) are present in the ovary. The nutritive phagocytes are displaced from their central position by large ovoid oocytes, which detach from the ascinal wall and project towards the centre of the ascinus. Here they undergo maturation and become ova which begin to accumulate in the lumen of the ovary (Fig. 6c).

Stage IV: mature stage

Ova (90 μm) are closely packed in the ovaries. Few oocytes (10 to 60 μm diameter) are present along the ascinal wall, while the nutritive phagocytes are either absent or form a thin, pale meshwork around the small oocytes (Fig. 6d).

Stage V: partly-spawned stage

Spawning has taken place and ova do not appear as closely packed as in the previous stage. Ovaries may appear extremely different from each other. In some cases, there may be oocytes at all stages of development (as in stage III), with a small number of ova at the onset of spawning and primary oocytes replace ova as they are shed.

In other cases, ovaries have a large store of ova at the onset of spawning and there are few or no primary oocytes to replace them as they are shed. In these ovaries, vitellogenesis is mostly finished and the nutritive phagocytes are reduced or absent (Fig. 6e).

Stage VI: spent stage

Spent ovaries have thin ascinal walls and appear empty except for relict oocytes. The number and type of oocytes present in the ovary at the end of the breeding season is variable. Some ovaries contain unspawned ova, whereas others are largely devoid of them. Any vitellogenic oocytes and ova present in the ovary are eventually resorbed and phagocytosis of relict oocyte material may be evident. A pale meshwork of nutritive phagocytes around the periphery, that may have started to sequester reserves for the next oogenic cycle, may be present. Clusters of primary oocytes can occur along the ovary wall (Fig. 6f).

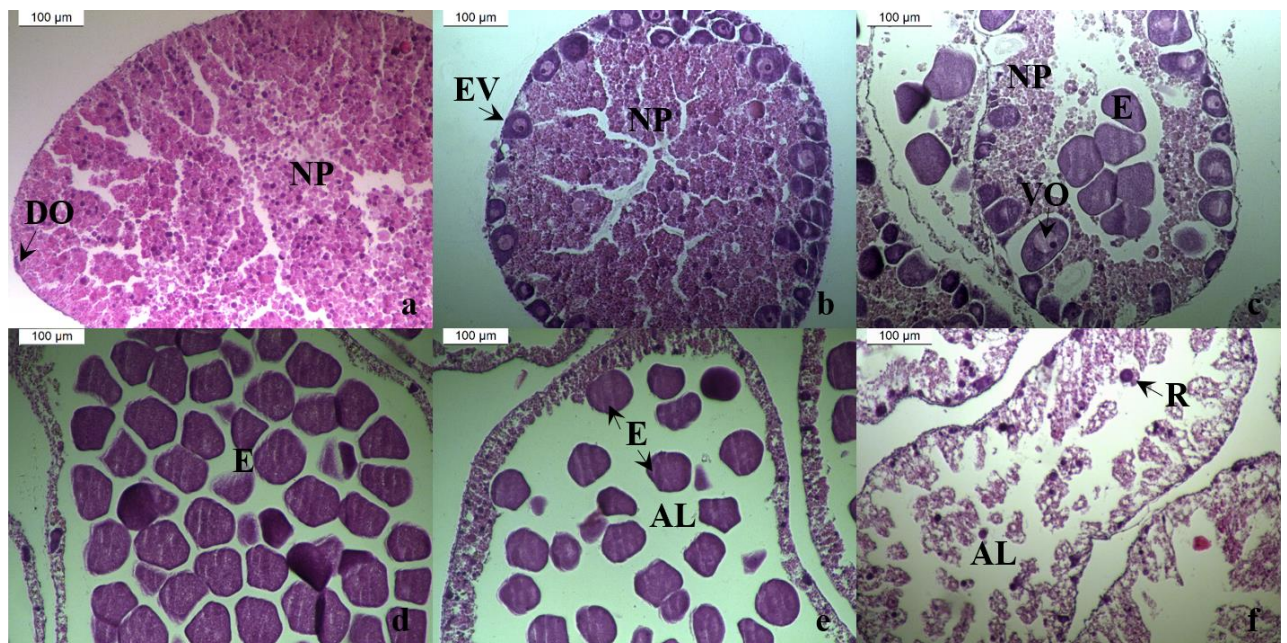


Figure 6 - Gametogenic stages in *P. lividus* females. a) recovery; b) growing; c) premature; d) mature; e) partly spawned; f) spent. DO: developing oocytes; NP: nutritive phagocytes; EV: early vitellogenic oocytes; VO: vitellogenic oocytes; E: mature eggs; AL: ascinal lumen; R: relict eggs.

For the testes, as described for the ovaries, six different stages can be identified (Byrne, 1990).

Stage I: recovery stage

The ascinal wall is lined with a thin layer of spermatogonia and primary spermatocytes. Nutritive phagocytes form a meshwork across the ascinus. Relict spermatozoa may be present (Fig. 7a).

Stage II: growing stage

The layer of germinal cells increases in depth as columns of spermatocytes project centrally. The ascinus is filled with nutritive phagocytes (Fig. 7b).

Stage III: premature stage

Spermatozoa begin to accumulate in the lumen of testes, whereas nutritive phagocytes are displaced from the centre and localized along the ascinal wall (Fig. 7c).

Stage IV: mature stage

Mature testes are packed with spermatozoa and the nutritive phagocytes are limited to the periphery (Fig. 7d).

Stage V: partly spawned stage

Spermatozoa are less concentrated and there are empty spaces in the lumen generated by spawned gametes. The ascinal wall appears thin and spermatozoa may be present in the gonoduct (Fig. 7e).

Stage VI: spent stage

Spent testes have thin ascinal walls, with a pale meshwork of nutritive phagocytes along them. Testes appear to be devoid of contents, except for few relict spermatozoa (Fig. 7f).

Once released into the water column, eggs are promptly fertilized by sperm and gradually develop into free-swimming planktotrophic larvae characteristic of echinoids: the echinopluteus. This larva develops 4, 6 and 8 arms supported by calcareous skeletal rods. After 2-4 weeks, the echinopluteus will develop a rudiment of an epidermic invagination inside the wall, located on the right-hand side of the body. When the larva becomes competent, it seeks a solid substrate on which to settle and metamorphose. Metamorphosis lasts less than one hour: the echinoid rudiment is evaginated and most larval tissues are reabsorbed. The postlarva resembles a miniaturized adult but has no mouth or anus, and is thus endotrophic. After a week, the postlarva undergoes some major changes and becomes an exotrophic juvenile with a fully developed digestive tract (Grosjean, 2001).

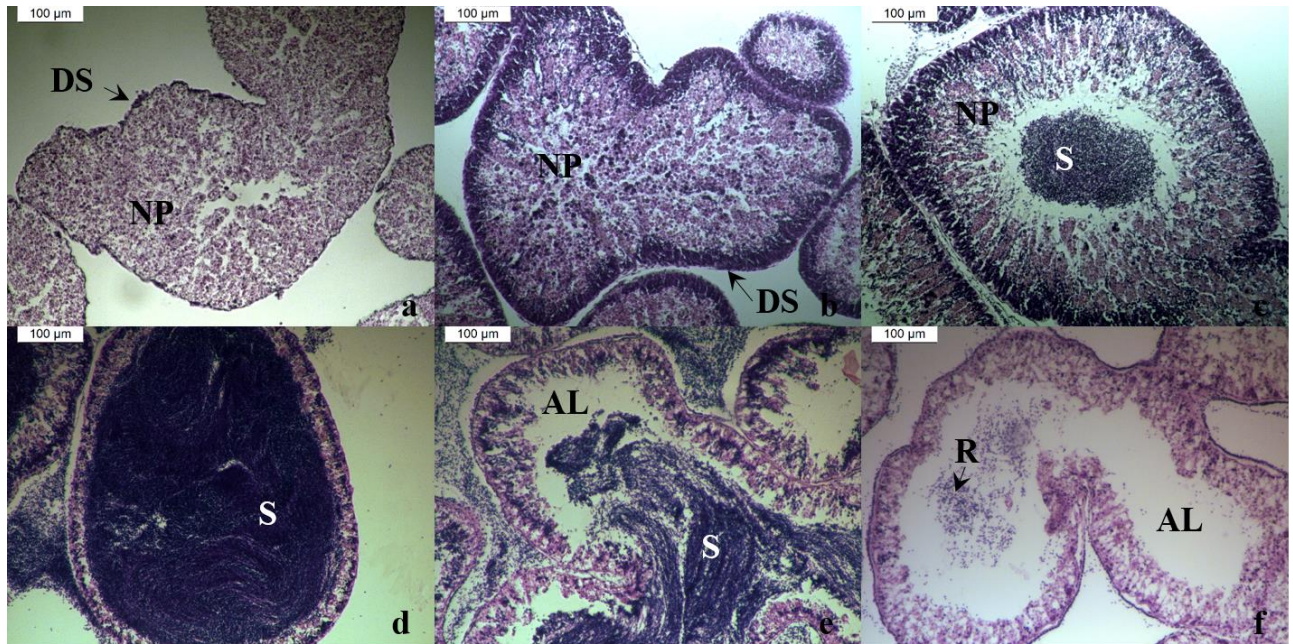


Figure 7- Gametogenic stages in *P. lividus* males. a) recovery; b) growing; c) premature; d) mature; e) partly spawned; f) spent. DS: developing spermatocytes; NP: nutritive phagocytes; S: mature sperms; AL: ascinal lumen; R: relict spermatozoa.

1.2. CONSEQUENCES OF INTENSIVE SEA URCHIN HARVESTING

In the last decades, the growing market demand for sea urchin gonads has been inexorably leading to the impoverishment of wild stocks.

Negative effects of continuous harvesting of *P. lividus* were documented in highly fished areas in terms of reduction of the total density of populations (Gianguzza *et al.*, 2006; Pais *et al.*, 2007, 2012) and biomass (Pais *et al.*, 2007, 2012), as well as in the overall abundance of large-sized individuals (Ceccherelli *et al.*, 2011; Pais *et al.*, 2012), when compared to a protected condition (e.g. a marine protected area).

Since harvesting is generally practiced during the period of maximum gonad development and maturity, the systematic removal of sea urchins leads to the loss of a considerable amount of fertile and sexually mature individuals that contribute to the local recruitment pool by releasing gametes into the water column (Levitan and Sewell, 1998).

Large-scale removal of echinoids on temperate reefs may have ecological impacts, such as the intense development of brown algae beds which leads to substantial changes in relative

abundance of fish and benthic invertebrates (Bell *et al.*, 2014). This could have direct negative consequences on sea urchin recruitment. In fact, several authors demonstrated that post-settlement mortality due to predation is significantly higher within kelp forests (McNaught and Steneck, 1998; McNaught, 1999; Balch and Scheilbling 2001) because of the increased presence of natural sea urchin predators, e.g. fishes like *Coris julis*, *Diplodus spp.* and *Sparus aurata*, gastropods and the sea star *Marthasterias glacialis* (Guidetti *et al.*, 2005; Peharda and Morton, 2006; Bonaviri *et al.*, 2009), for the Mediterranean.

In coral reefs, changes in the abundance of echinoids have been associated with blooms of benthic algae and, ultimately, limited coral recruitment and survival (Hughes *et al.*, 1987, McManus *et al.*, 2000, Williams and Polunin, 2001). For example, in Kenyan lagoons the massive growth of the brown algae *Sargassum latifolium* and the increased thickness of the algal turf considerably reduced the hard coral cover, thus reducing the coral diversity (McClanahan *et al.*, 1996).

In conclusion, the productivity of the sea urchin fishery is damaged directly by intensive harvesting activity and indirectly by ecological imbalances.

1.3. GONAD QUALITY

From a commercial standpoint, high quality sea urchin gonads are characterized by a large size, coupled with a number of sensory characteristics preferred by consumers (i.e., taste, texture, color, and firmness) (Walker *et al.*, 2015). Gonad quality is influenced by gender (Lee and Haard, 1982; Murata *et al.*, 1998; Murata *et al.*, 2002), diet (availability and quality of foods) (Shpigel *et al.*, 2006; Siikavuopio *et al.*, 2007; Woods *et al.*, 2008), size (Blount and Worthington, 2002; Pearce *et al.*, 2004; Agatsuma *et al.*, 2005; Woods *et al.*, 2008), geographic location (Blount and Worthington, 2002), season and reproductive period (Blount and Worthington, 2002; Unuma, 2002) and environmental conditions (James *et al.*, 2007; James *et al.*, 2008). Quality can also be affected by proximate composition of gonads (McEdward and Chia, 1991; Arafa *et al.*, 2012).

Obtaining high-quality sea urchin gonads begins with the divers. The ability to harvest sea urchins containing quality gonads requires harvesting expertise as well as a knowledge of the sea urchin's environmental and feeding conditions (Liyanapathirana, 2001).

Understanding which factors may contribute to produce high-quality sea urchin gonads would benefit world fisheries and aquaculture (McBride *et al.*, 2004). Knowing how different aspects affect the quality of sea urchin gonads is relevant for fishery managers and aquaculturists in order to improve the management of sea urchin fisheries and for optimising the production of desirable sensory characteristics of gonads.

1.3.1. Gonad yield

Gonad size is typically quantified in terms of the gonadosomatic index (GSI), with a higher index indicating larger gonads. The GSI, assessed as gonad wet weight/total wet weight $\times 100$, is the standard measure for determining the market value of sea urchin gonads and for evaluating the relative success of methods intended to increase gonad yield.

The rate of increase and the maximum GSI depend mainly upon the sea urchin species, the reproductive cycle, the size of the individual, and the availability and quality of feed.

After spawning (i.e. at the end of gametogenesis), the GSI is at its lowest value in all sea urchins. As gonad rebuilding occurs (pre-gametogenesis) and gametogenesis begins, the GSI increases (Fig.8). The most appreciated gonads for marketing purposes are those in which the phagocytes have reached their maximum size, before gametogenesis has fully progressed, (Unuma, 2002; Walker *et al.*, 2007). At this stage, the phagocytes have accumulated the necessary substances to be used in the maturation of gametes, and the gonads have high levels of proteins, carbohydrates and lipids.

Seasonal variations in reproductive condition are in turn influenced by a range of exogenous factors, such as photoperiod and temperature, which are able to control sea urchin gonad growth and development (Byrne, 1990; Spirlet *et al.*, 2000; Garrido and Barber 2001; Kelly, 2001; Shpigel *et al.*, 2004; Dumont *et al.*, 2006; Siikavuopio *et al.* 2006; James *et al.*, 2007). When food availability is unlimited, temperature is the most important factor for gonad growth rate in sea urchins, whereas contrasting data for the influence of photoperiod on gametogenesis are reported (Spirlet *et al.*, 2000; Shpigel *et al.*, 2004). Byrne (1990) and Spirlet *et al.* (2000) demonstrated that the gonad growth of *P. lividus* occurs during the coldest months of the year when days are the shortest, suggesting that both parameters are responsible for gonad growth.

Body size may have some influence on gonad yield (Gonor, 1972). Generally, individuals with sizes ranging from 40 to 70 mm have higher GSI values than individuals belonging to the class

size of 20-40 mm (Martínez *et al.*, 2003; Sánchez-España *et al.*, 2004). Gonad size increases volumetrically with sea urchin test diameter (Mita *et al.*, 2007). In overfished areas, where the average size of individuals is reduced by fishing pressure (Guidetti *et al.*, 2004; Pais *et al.*, 2007; Pais *et al.*, 2012), the mean GSI is lower than in strictly regulated locations (Loi *et al.*, 2015).

Sea urchin gonads, besides their reproductive role, act as the main organ for nutrient storage (Gonor, 1973; Siikavuopio *et al.*, 2006). The amount of nutrient intake, and subsequent gonad growth, depends on food quantity and quality, and on the rate of consumption, digestion, and absorption (Lawrence, 1975; Lares and McClintock, 1991; Lawrence *et al.*, 2003). Several studies have shown a strong, positive correlation between GSI and the availability of food (Fuji, 1960; Ebert, 1968; Garrido and Barber, 2001; Gonor, 1973; Lawrence *et al.*, 1992; Spirlet *et al.*, 1998; James *et al.*, 2007). Urchins harvested from barrens (i.e. grounds lacking macroalgae) usually have very low GSI (Unuma *et al.*, 2015). Such urchins have long been proposed for use in short-term aquaculture, where they are fed *ad libitum* with preferred algal species or formulated diets until the gonads have increased in size (Bridger *et al.*, 1998; Lawrence *et al.*, 1997; James *et al.*, 2004). For this reason, dietary proteins have been identified as the main contributing factor for gonad enhancement (de Jong-Westman *et al.*, 1995; McBride *et al.*, 1998; Pearce *et al.*, 2002a, b; Hammer *et al.*, 2004; Pearce *et al.*, 2004; Cook and Kelly, 2007). In wild conditions, sea urchins must ingest and process large amounts of macroalgae and/or seagrasses to meet their nutritional requirements for protein (Hammer *et al.*, 2006). This could explain why macroalgal diets alone typically produce urchins with lower growth rates and smaller gonads when compared to urchins fed animal-derived, formulated diets (Cook *et al.*, 1998; Fernandez and Boudouresque 1998, 2000).

1.3.2. Taste, texture and firmness

The taste of gonads is related to the animal's diet. Macroalgal feeds produce gonads with a better taste, sweeter than those of sea urchins feed with prepared diets (Pearce *et al.*, 2002; Pearce *et al.*, 2004). Increasing the protein level in the diet worsens the flavor (Pearce *et al.*, 2002). Amino acid profiles of gonads from wild Japanese sea urchins (*Heliocidaris crassispina*, *Strongylocentrotus intermedius*, *S. nudus*, *S. pulcherrimus*) suggest that good flavor may be associated with high levels of certain amino acids such as alanine, arginine, glutamic acid, glycine, lysine, serine, and taurine (Komata *et al.*, 1962; Hirano *et al.*, 1978) while a bitter flavor is associated with high concentrations of valine and pulcherrimine (Hoshikawa *et al.*,

1998; Murata *et al.*, 2001). Even gender and the developmental stage of the gonads have an effect on taste. Phillips *et al.* (2009) reported that roe from male sea urchins had a sweet taste and a milky flavor, while roe from female sea urchins had a bitter and sour taste, with an herbaceous, metallic flavor. For a sweet taste, gonads should be developed but not too mature, since a high gamete to NP ratio makes gonads watery and bitter (Phillips *et al.*, 2009; Unuma, 2002) (Fig. 8).

Texture affects gonad appearance and is usually evaluated according to the visibility of individual gonad follicles (smoothness) and segment halves. The textural properties and hence the firmness, defined as the force required to compress a sea urchin gonad (Cyrus *et al.*, 2014), are very important as gonads that remain intact during processing and packaging are considered a high quality marketable product. Experimental studies on firmness conducted in the last decades are contradictory. According to Pearce *et al.* (2002) and Azad *et al.* (2011), a prepared diet produces firmer gonads with better texture than a diet of kelp. In another study, Pearce *et al.* (2004) stated that gonad firmness increases with increasing urchin size and that gonads of urchins fed a prepared diet are significantly softer (less firm) and have more water than those fed kelp.

Hammer *et al.* (2006) found that gonad texture is directly influenced by protein content in the diet. This is negatively affected by high levels of protein, which cause the promotion of gamete growth and a reduction of the volume of nutritive phagocytes in the gonads. In fact, the reproductive stage of urchins can affect roe firmness. Roe is most valuable prior to and in the early stages of gametogenesis, when they are large but still firm in texture (Liyanapathirana, 2001; Agatsuma *et al.*, 2004) (Fig.8).

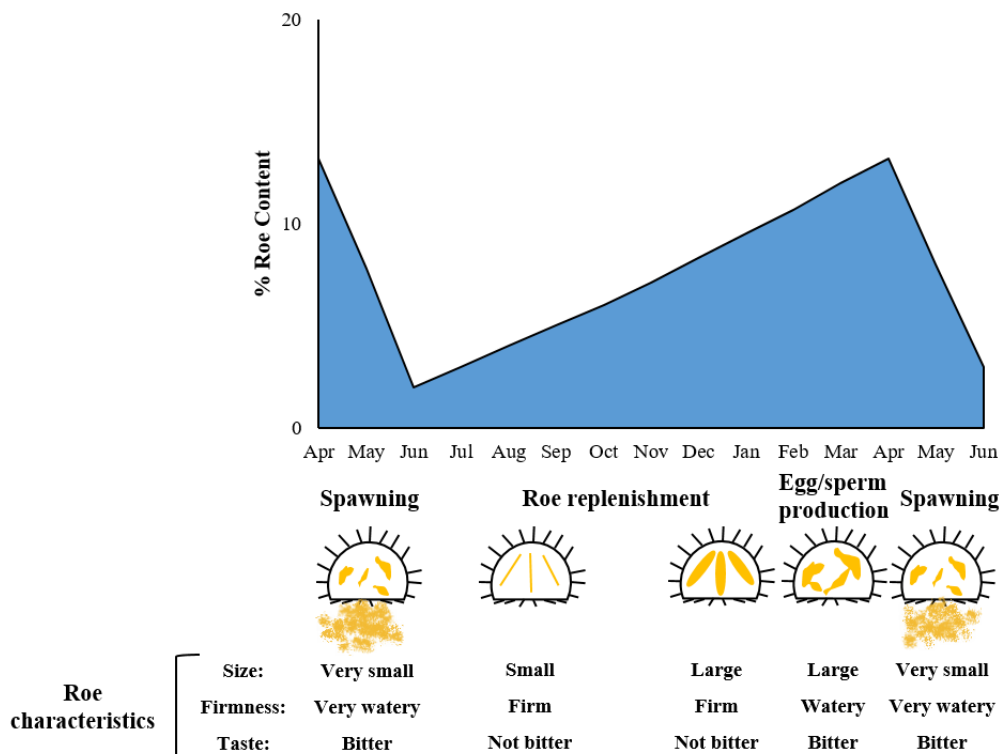


Figure 8 - Roe content and characteristics in sea urchins over time. Timings are based roughly on the European edible sea urchin *P. lividus* in Ireland (image revised from <https://seaurchinaquaculture.wordpress.com>).

1.3.3. Color

In natural populations, the color of the roe is dependent on the quality and quantity of feed, gender, and time of harvest. Depending on the local market preference, acceptable gonad color can range from a bright yellow to a dark orange or almost red (Dincer and Cakli, 2007). Lighter, pale-colored or dark-brown gonads are not as valued (Azad *et al.*, 2011). The brown gonad colorization sometimes observed in wild sea urchins is correlated with aging and with a decreasing gonad production determined by low availability of food (Agatsuma *et al.*, 2005). A pale off-white gonad color is often observed when aquacultured sea urchins are fed high protein formulated diets (Barker *et al.* 1998; Watts *et al.* 1998; Robinson *et al.*, 2002; Shpigel *et al.*, 2005; Eddy *et al.* 2012).

The color of sea urchin gonads has been traditionally measured using some form of “match-by-eye” technique (James, 2007). However, there are intrinsic difficulties with this subjective technique. Robinson *et al.* (2002) described a more accurate technique for breaking color down into three components in a three-dimensional measurement system similar to that developed by the *Commission Internationale de l’Eclairage* in 1976. This technique called “CIE (L*a*b*)

1976” uses a spectrophotometer to take measurements on the X axis or hue (a^*) (extends from green on the negative side to red on the positive one) and the Y axis or chroma (b^*) (extends from blue on the negative side to yellow on the positive one). These two axes will define any color. The intensity or lightness of the color is determined by the Z axis (L^*), which extends from black on the negative side to white on the positive one. The numerical values derived from this technique provide an objective measure of the variance within a sample and therefore can be statistically evaluated (Robinson *et al.*, 2002).

The yellowish-orange color in the sea urchin gonad is the most acceptable color for the market. It is principally due to the echinone pigment (Griffiths and Perrot, 1976; Tsushima, 2007), which is synthesized from β -carotene (Shina *et al.*, 1978; Goodwin, 1984; Matsuno and Tsushima, 2001) obtained through dietary intake (Lawrence, 1975; Watts *et al.*, 1998). The issue of color can be addressed by using carotenoids in formulated feeds. Sea urchins fed high protein formulated diets often produce gonads that are large (i.e. with high GSI) but pale (McBride *et al.*, 1997; Lawrence *et al.*, 1997; Barker *et al.*, 1998; Walker and Lesser, 1998; Watts *et al.*, 1998; Robinson *et al.*, 2002; McBride *et al.*, 2004; Pearce *et al.*, 2004; Shpigel *et al.*, 2005; Eddy *et al.*, 2012). Such gonads are commercially unacceptable (Lawrence *et al.*, 1995; Robinson and Colborne, 1998; Watts *et al.*, 1998; McLaughlin and Kelly, 2001). Natural algal diets or addition of natural β -carotene product, derived from spray-dried algae *Dunaliella salina*, to manufactured diets, resulted in an intensification of gonad color (McBride *et al.*, 1997; Robinson *et al.*, 2002; Pearce *et al.*, 2004).

1.3.4. Chemical composition

Sea urchin gonad composition may exhibit a wide variability, which can affect gonad quality. The factors that affect gonad composition can be endogenous, exogenous, or both. The endogenous factors are associated with the animal’s life cycle and are genetically controlled. The exogenous factors, both environmental (e.g. temperature, salinity) and dietary (e.g. diet composition, feeding frequency, ration level), have also been reported to influence the proximate composition of gonads (Shearer, 1994; Arafa *et al.*, 2012).

Sea urchin roe is made up of water (about 80%), protein (12%), lipids (2-3%, Dincer and Cakli, 2007; Mol *et al.*, 2008), carbohydrates (3%), mineral elements (e.g. P, Na, K), and vitamins (Yokota, 2002).

Protein profiles change throughout the reproductive cycle, according to the maturation stage and the sex (Sewell *et al.*, 2008), thus influencing the sensory quality (e.g. color, appearance, taste) of roe. Monitoring the changes in the quality may enhance the collection of better and more profitable gonads. For example, an exhaustive knowledge of the protein profiles can have useful implications in improving the establishment of regulatory time spans for wild stock catches, as well as in monitoring development cycles and quality of roe in aquaculture plants.

Among amino acids, the major components of roe are glycine, glutamic acid, asparagic acid, arginine, lysine and leucine (Yokota, 2002).

It has been demonstrated that gonad fatty acids differ according to the sex of individuals, since they are related either to specific requirements of males and females during gametogenesis, and/or to differences in the lipid composition of gametes present in mature testes and ovaries (Martinez-Pita *et al.*, 2010a). Additionally, it is known that dietary lipids have a key role in characterizing the fatty acid profile of sea urchin gonads (Carboni *et al.*, 2013; Castell *et al.*, 2004; Martinez-Pita *et al.*, 2010a, b).

Among lipids, saturated fatty acids are mainly represented by palmitic acid (16:0, Yokota, 2002); among the unsaturated ones, which represent almost the 74% of the total fatty acids, the monounsaturated palmitoleic acid (16:1), and the polyunsaturated (PUFA) arachidonic (20:4 n-6), eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic (DHA, 22:6 n-3) acids are dominant (Martinez-Pita *et al.*, 2010b, Arafa *et al.*, 2012).

1.4. AIMS OF THE WORK

Nowadays, the growing demand for sea urchin roe and the consequent continuous catch of wild stocks is inexorably leading to the collapse of wild resources. In light of these reasons, the strict regulation of the harvest of wild *P. lividus* stocks is an urgent matter. In addition, improving aquaculture methods to culture sea urchins with increased gonad yield and quality is also of paramount importance.

The aims of this thesis are to enhance the knowledge of the biology of the species *P. lividus* living along Sardinian coasts, to encourage an improvement in current management practices

(e.g. fishing periods, substrates) for sea urchin fishery in Sardinia and to implement the aquaculture practices which would allow the production of high-quality roe.

In particular, I focused my work on several aspects linked to sea urchin gonads, such as the gonad changes in response to different fishing pressures, habitats and growing areas, as well as dietary factors and seasons.

My experimental work has been conducted on the following topics:

1. the effect of different harvesting pressures on gonad yield and reproductive output of two sea urchin populations;
2. the gonad quality (i.e. gonad yield and color) of two sea urchin populations living in different habitats (rocky bottom vs. *P. oceanica* meadow) and how it changes throughout seasons;
3. the influence of season and growing area on the lipid fraction of *P. lividus* gonads;
4. the proteome changes occurring in *P. lividus* gonads of both sexes during maturation;
5. the effect of two macroalgal feeds, over a period of 12 weeks, on gonad yield and color in *P. lividus* adults.

1.4.1. Hard time to be parents? Sea urchin fishery shifts potential reproductive contribution of population onto the shoulders of the young adults.

Commercial fishing is certainly the main cause of the collapse of sea urchin populations. A remarkable reduction in sea urchin populations can have drastic ecological consequences. One example is the rapid development of macroalgae that leads to substantial changes in the abundance of fish and other benthic invertebrates. In addition, the systematic removal of individuals drastically reduces wild stocks and changes growth, production and recruitment of populations. One of the most striking effects of sea urchin fishing is the rapid decrease of total densities and abundances of populations, as well as the reduction of mean size of individuals. Moreover, the decline of sea urchin populations due to harvesting could be even more significant because of the loss commercial-sized adults which are sexually mature and are fundamental for the maintenance of the local recruitment pool.

The edible sea urchin *P. lividus* is subjected to strong fishing pressure in the whole Mediterranean area, especially in the south of Italy. In this work, conducted in two zones of

Sardinia which have different harvesting pressures, I tested the hypothesis that the contribution of the commercial-size class to reproductive potential drastically decreases under high fishing pressure and, as a result, the risk of a population collapse increases.

1.4.2. The influence of habitat and season on the gonad quality of *Paracentrotus lividus* (Echinodermata, Echinoidea) in a Sardinian coastal area (Western Mediterranean).

P. lividus roe represent a very appreciated delicacy in many parts of the world. The marketability of roe depends on its quality, especially on gonad yield and color. Both characteristics may vary according to seasonal (seawater temperature, photoperiod) and habitat features (hydrodynamism, food quality and quantity), thus influencing the gonad quality. This variability in roe condition has direct consequences on fishing effort, heavily concentrating it in areas where the conditions are expected to be most consistent, leading to the depletion of wild sea urchin stocks.

In Sardinia, *P. lividus* plays an important economic role. Its harvesting is carried out mainly on rocky bottoms, and allowed by law from November to April, when gonads are supposed to be large and economically most profitable. Scarce attention is actually given to gonad color as an attribute of quality. Even in the Mediterranean area, the continuous catches have seriously damaged sea urchin population. Therefore, diversifying harvesting sites and regulating the harvesting season has become an urgent matter. This should be done after identifying which habitats and seasons would be the best for harvesting the most profitable high-quality gonads.

With this perspective, in this work I compared the gonad yield and color of adult Sardinian *P. lividus* living at two sites characterized by different habitats, in different seasons. Results were discussed with consideration given to factors which can influence gonad characteristics such as reproductive state, feeding intensity and diet.

1.4.3. Influence of seasonal and environmental patterns on the lipid content and fatty acid profiles in gonads of the edible sea urchin *Paracentrotus lividus* from Sardinia.

P. lividus gonads represent a valued gourmet delicacy, and their commercial value is generally associated with their size, freshness, color and texture. Sensory parameters characterizing sea urchin gonads are likely to be simultaneously affected by either environmental, seasonal or physiological factors such as hydrodynamism, temperature, photoperiod, diet and reproductive cycle. These in turn affect metabolic processes and chemical composition.

Gonads go through a periodical alternation of their structure at the cellular level, with the ratio of germinative (ova and spermatozoa) and somatic (nutritive phagocytes) cells varying seasonally throughout the reproductive cycle. This reflects the dual function of gonads as storage tissue and reproductive organ.

Considering that an analytical parameter that can predict the expected periodical changes occurring in sea urchin gonads is still lacking, this paper discusses an investigation of the lipid fraction of *P. lividus* gonads sampled monthly from two different areas of the Sardinian coasts over one year. Firstly, the model of the periodic fluctuation of total lipids of sea urchin gonads was provided. Secondly, the importance of the different environmental and physiological factors in differentiating the composition of gonad lipids was highlighted through a multivariate analysis of the composition of gonad fatty acids.

1.4.4. Proteomic changes occurring along gonad maturation in the edible sea urchin *Paracentrotus lividus*.

The increasing demand of sea urchin gonads has resulted in commercial overexploitation of the wild population. For this reason, *P. lividus* is becoming an important candidate for the emerging aquaculture industry in Europe. The focus has mainly been on an increase in gonad yield with less emphasis placed on quality parameters such as appearance, color, texture and flavor, despite the strong influence of these factors on the market price.

Gonad quality is strongly influenced by the sex and reproductive stage. Large, sweet, compact gonads are generally preferred. Sensory attributes are positively related to the ratio of nutritive phagocytes to gametes which varies during the reproductive cycle. Although they are larger, gonads at advanced maturation stages have less desirable attributes, such as being more watery and bitter. This is especially true for females.

Currently, the identification of gender and maturation stage of gonads in echinoids is carried out by histology. Histological techniques, however, imply a long time frame for obtaining information that may be useful in shorter times from gonad collection. More rapid and precise procedures are needed for improving the definition of regulatory time frames for wild sea urchin catches, as well as for monitoring development cycles and quality in sea urchin aquaculture plants.

A detailed description of the proteomic makeup of echinoid gonads, and of how it changes during sexual maturation, might contribute to the identification of potential markers of

gametogenesis essential for monitoring the reproductive process, promoting more conscious harvesting and farming activities. Up to now, few studies have investigated the proteome of echinoderm gonads, and a complete dataset of proteins expressed at different developmental stages is not yet available.

In this study, wild *P. lividus* was collected along the coasts of Sardinia, and the gonads were analyzed by gel-based and shotgun proteomics in the recovery, pre-mature, mature, and spent stages. A comprehensive description of the proteome changes occurring in the gonads of both sexes during maturation was provided.

1.4.5. The influence of macroalgal diets on gonad yield and color of adult *Paracentrotus lividus*.

Due to the increasing demand for sea urchin roe, in recent times plenty of studies have been conducted to test different feeds with the aim of improving both gonad production and color in echinoids. While hyperproteic manufactured feeds enhance the gonad growth more efficiently than macroalgae, algal-based diets allow to obtain a brighter, more reddish/yellow gonad color.

Although the production costs of artificial diets are quite moderate, the massive production of macroalgae is still not economically feasible. However, many countries have been recently trying to reduce costs through the combination of seaweed cultures with the farming of commercial species in IMTA (Integrated Multi-Trophic Aquaculture) systems. Three of the emerging species in the aquaculture industry are the sea urchin *P. lividus* and the seaweeds *Ulva lactuca* and *Gracilaria spp.*

In this study, I evaluate the effect of *U. lactuca* and *G. gracilis* on gonad conditions of adult *P. lividus*, for three months. Results are discussed considering the feed intakes and absorption efficiencies of the two diets, as well as the food conversion ratios.

Chapter 2. Hard time to be parents? Sea urchin fishery shifts potential reproductive contribution of population onto the shoulders of the young adults

2. HARD TIME TO BE PARENTS? SEA URCHIN FISHERY SHIFTS POTENTIAL REPRODUCTIVE CONTRIBUTION OF POPULATION ONTO THE SHOULDERS OF THE YOUNG ADULTS

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2.1. Abstract

In Sardinia, as in other regions of the Mediterranean Sea, sustainable fisheries of the sea urchin *Paracentrotus lividus* have become a necessity. At harvesting sites, the systematic removal of large individuals (diameter ≥ 50 mm) seriously compromises the biological and ecological functions of sea urchin populations. Specifically, in this study, we compared the reproductive potential of the populations from Mediterranean coastal areas which have different levels of sea urchin fishing pressure. The areas were located at Su Pallosu Bay, where pressure is high and Tavolara-Punta Coda Cavallo, a marine protected area where sea urchin harvesting is low.

Reproductive potential was estimated by calculating the gonadosomatic index (GSI) from June 2013 to May 2014 both for individuals of commercial size (diameter without spines, $TD \geq 50$ mm) and the undersized ones with gonads ($30 \leq TD < 40$ mm and $40 \leq TD < 50$ mm). Gamete output was calculated for the commercial-size class and the undersized individuals with fertile gonads ($40 \leq TD < 50$ mm) in relation to their natural density (gamete output per m^2).

The reproductive potential of populations was slightly different at the beginning of the sampling period but it progressed at different rates with an early spring spawning event in the high-pressure zone and two gamete depositions in early and late spring in the low-pressure zone. For each fertile size class, GSI values changed significantly during the year of our study and between the two zones. Although the multiple spawning events determined a two-fold higher total gamete output of population (popTGO) in the low-pressure zone, the population mean gamete output (popMGO) was similar in the two zones. In the high-pressure zone, the commercial-sized individuals represented approximatively 5% of the population, with almost all the individuals smaller than 60 mm producing an amount of gametes nearly three times lower than the undersized ones. Conversely, the high density of the undersized individuals released a similar amount of gametes to the commercial-size class in the low-pressure zone.

Overall, the lack of the commercial-size class in the high-pressure zone does not seem to be very alarming for the self-supporting capacity of the population, and the reproductive potential contribution seems to depend more on the total density of fertile sea urchins than on their size. However, since population survival in the high-pressure zone is supported by the high density of undersized sea urchins between 30 and 50 mm, management measures should be addressed to maintain these sizes and to shed light on the source of the larval supply.

2.2. Introduction

Commercial fisheries are one of the main causes of the deterioration of marine communities (Jackson *et al.*, 2001). The systematic removal of fishery resources drastically reduces natural stocks and changes growth, production and recruitment of target species (Pinnegar *et al.*, 2000). In extreme cases, overfished populations are still present in the community but no longer interact significantly with other species (Estes *et al.* 1989). This phenomenon can lead to a simplification of trophic webs with dramatic consequences for marine ecosystems (Pauly, 1995; Myers and Worm, 2003; Coll *et al.*, 2008a; Coll *et al.*, 2009a; Coll *et al.*, 2009b; Lotze *et al.*, 2011; Navia *et al.*, 2012; Tunca *et al.*, 2016).

One of the clearest examples reported for several temperate coastal systems is the overexploitation of target species involved in the typical tri-trophic interaction “fish-sea urchins-macrophyte”. Trophic relationships can be altered by overfishing predatory fishes. This triggers an uncontrolled proliferation of sea urchins which then leads to an overgrazing of algal cover (Sala *et al.*, 1998; Steneck *et al.*, 2002; Steneck *et al.*, 2004). Although this is the main effect of overfishing on many Mediterranean coasts (Sala and Zabala, 1996; Sala *et al.*, 1998), trophic imbalances have also been found to act in the opposite direction. Indeed, over the last 2-3 decades, the general decline of natural fish stocks has led to a focus on new target species, often further down the food web.

In many regions sea urchin harvesting was added to higher-trophic-level fisheries (Anderson *et al.*, 2011). This is the case of some regions in the South of Italy where the edible sea urchin *Paracentrotus lividus* (Lamarck, 1816) is subjected to high fishing pressure (Tortonese, 1965; Guidetti *et al.*, 2004; Pais *et al.*, 2007). The most striking effect of sea urchin fishing is the rapid decrease in commercial resources in terms of total density and abundance (Andrew *et al.*, 2002; Bertocci *et al.*, 2014). A remarkable reduction in density and mean size of the sea urchin population would have dramatic consequences for the whole benthic community. For instance, the removal of hundreds of thousands of sea urchins from the temperate reef has coincided with the rapid development of brown algae that has led to substantial changes in the abundance of fish and benthic invertebrates (Bell *et al.*, 2014).

Furthermore, the decline of sea urchin populations due to harvesting by humans could be even more significant because of the loss of sexually mature individuals that contribute to the local recruitment pool (Levitan and Sewell, 1998). Indeed, gonads are proportional to a sea urchin's body-size and are more mature and developed in large sea urchins (Mita *et al.*, 2007).

The gonadosomatic index (GSI) is generally used to evaluate the reproductive features of echinoids such as fluctuations in gonad size and spawning periods (Spirlet *et al.*, 1998; Shpigel *et al.*, 2004; Gianguzza *et al.*, 2013) and these relate to the reproductive potential of the individual (Brewin *et al.*, 2000). Fertile size classes can produce more than one cohort of mature gametes in a single breeding season (Mita *et al.*, 2007) and the reproductive cycle generally has one or two seasonal GSI peaks (see reviews in Boudouresque and Verlaque, 2007 and Ouréns *et al.*, 2011). Sometimes there can be continuous spawning events of lower significance and with strong dependence on the variability of the gametogenesis (Boudouresque and Verlaque, 2007). Generally, GSI shows considerable spatial and temporal variability as a result of extrinsic factors such seasonal changes in photoperiod, water temperature and phytoplankton blooms (see Ouréns *et al.*, 2011 and references therein). Food quality and availability (Byrne, 1990; Minor and Scheibling, 1997; Brady and Scheibling, 2006; Scheibling and Hatcher, 2007) as well as hydrodynamic conditions (Lozano *et al.*, 1995; Meidel and Scheibling, 1998; Guettaf *et al.*, 2000; Sellem and Guillou, 2007; Gianguzza *et al.*, 2013) can also influence the reproductive cycle and fecundity of populations.

In Sardinia (Italy, Western Mediterranean), commercial fishing of the sea urchin *P. lividus* is limited by law to specimens larger than 50 mm test diameter (TD), from November to April. However, despite regional decrees concerning fishing periods, minimum size and catch quotas per day per fisherman, the harvesting of *P. lividus* is intensively practiced. Removal by occasional recreational fishermen occurs throughout the year because of the long tourist season (Pais *et al.*, 2007). The systematic removal of the largest sea urchins may decrease the number of fertile individuals that release gametes into the surrounding environment. This leads to a population collapse, as reported for some overfished areas (Pais *et al.*, 2007).

The aim of this work is to assess the reproductive potential of populations living in two zones of Sardinia which have different harvesting pressure and to compare, through the evaluation of the GSI over one year, the annual gamete output (Brewin *et al.*, 2000) of the commercial-size class (diameter without spines, $TD \geq 50\text{mm}$) and the fertile, undersized one ($40 \leq TD < 50\text{ mm}$) of both populations. Our hypothesis supports that, under high fishing pressure, the contribution of the commercial-size class to reproductive potential drastically decreases and, as a result, the risk of a population collapse increases.

2.3. Materials and methods

2.3.1. Study sites and sea urchin sampling

The reproductive potential of the *P. lividus* populations was examined in two zones of Sardinia that differ in sea urchin fishing pressure (Fig. 9). These two zones were selected because they are at two extremes as regards the exploitation of sea urchins in Sardinia. Su Pallosu Bay, located along the Sinis peninsula (central-western Sardinia, 40° 03' N; 008° 25' E), is subjected to very high pressure (HP zone) which is widespread across the entire bay. Harvesting can be practised through scuba diving from November to April by 189 professional fishermen authorized by regional decree. Each one of them, when helped by an assistant, is allowed to collect up to 3000 sea urchins ($TD \geq 50$ mm) per day, according to the regional decree n. 1967/DecA/67 issued by the Regione Autonoma Sardegna (RAS, 2013).

On the contrary, the marine protected area of Tavolara – Punta Coda Cavallo (north-eastern Sardinia, 40° 53' N; 009° 40' E) is considered a low-pressure zone (LP zone). This marine reserve was legally established in 1997 and has been considered well-enforced since 2003 (Di Franco *et al.*, 2009). This is one of the few zones in Sardinia where sea urchin fishing is strongly restricted (there are only few authorized fishermen and no more than 500 catches per day for an overall maximum of 300,000 sea urchins harvested each year) and the populations are well preserved (Guala *et al.*, 2011). Although no data are available on annual catches, Su Pallosu Bay is among the most exploited in the whole region and the theoretical number of sea urchins that can be legally harvested in one year (calculated according to the number of licenses, allowed catches and fishing days) is over 300 times greater than that of Tavolara – Punta Coda Cavallo.

Sea urchin specimens were collected from two areas of high harvesting pressure (A and B) at Su Pallosu Bay, and low harvesting pressure (C and D) at Tavolara MPA. In each zone, the two areas were 1-3 km apart.

Sampling (approved by the Regione Autonoma Sardegna through the release of the fishing license for scientific purposes n. 9727/AP SCIE/N.7 03/06/2016) involved all sea urchin size classes that have gonads and contribute to the reproductive potential of the population. Specifically, once a month, we collected 10 individuals of the commercial size (CS, $TD \geq 50$ mm), 5 undersized individuals (US, $40 \leq TD < 50$ mm) and 5 smaller, undersized sea urchins (Small-US, $30 \leq TD < 40$ mm) for an entire year.

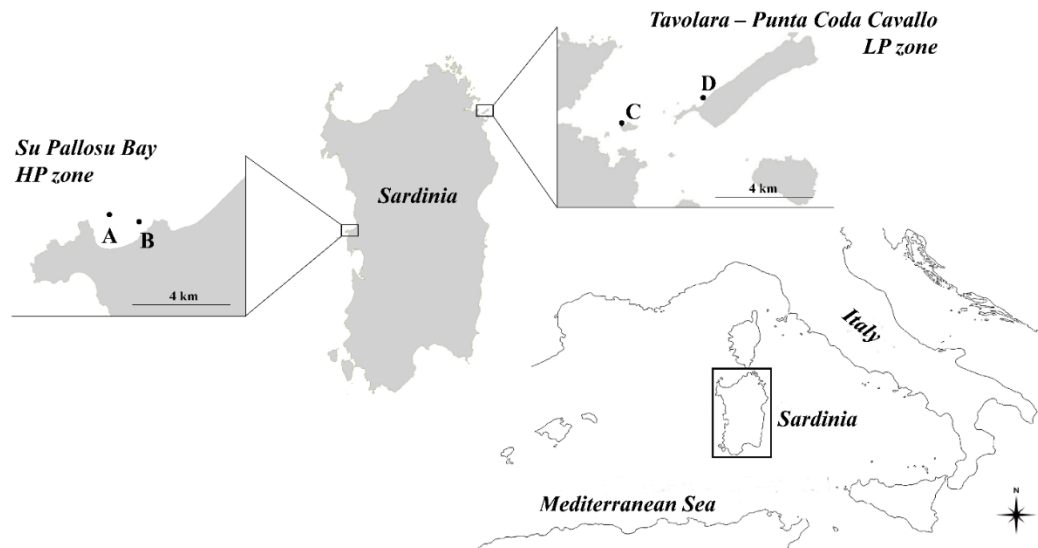


Figure 9 - Map of the study zones. Su Pallosu Bay, in the Sinis Peninsula is considered a high-pressure zone (HP zone); Tavolara – Punta Coda Cavallo marine protected area is considered a low pressure zone (LP zone). Sampling areas, named A and B for Su Pallosu Bay, C and D for Tavolara – Punta Coda Cavallo, are represented by the black dots.

The sea urchins were collected from both zones by scuba diving over a rocky bottom covered in photophilic algal communities at a depth of five meters. Sea urchin samples were wrapped in cloth soaked in salt water, stocked in iceboxes and immediately transported to the laboratory.

2.3.2. Environmental features

Despite the differences in position along the Sardinian coast and in wind exposure, sampling was performed in areas with similar environmental features (i.e. depth, slope, shelter from the waves), on rocky bottoms.

Differences in food availability, potentially able to affect sea urchin gonad growth and consequently fecundity (Byrne, 1990; Minor and Scheibling, 1997; Brady and Scheibling, 2006; Scheibling and Hatcher, 2007), were assessed as temporal changes of algal cover between zones. Digital photographs were taken over three PVC quadrats of 50 x 50 cm which were randomly placed on the sea bottom in each area. This was done three times (July 2013, January and May 2014) during the surveyed year. Image analysis was carried out using Seascape® software (Segmentation and Cover Classification Analyses of Seabed Images, Teixidó *et al.*, 2011) to detect the percentage cover of conspicuous algal taxa or morphological groups. A

permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001a) was done, on the basis of a Bray-Curtis dissimilarity matrix calculated from square-root transformed data (Primer-E 6 Permanova®), to estimate the variability of assemblages between the two zones over time. A 3-way model was used with Time (random, 3 levels) and Zone (fixed, 2 levels, Su Pallosu Bay vs. Tavolara – Punta Coda Cavallo) as crossed factors, and Area (2 levels) as a random factor nested in Zone. P-values were obtained through Monte Carlo random draws from the asymptotic permutation distribution (Anderson, 2005) and a pairwise test was used to discriminate between various levels of significant factors. A non-metric Multi-Dimensional Scaling (nMDS) ordination was used as a graphical representation of data.

In order to investigate any potential thermic anomalies, seasonal variations of the coastal water temperature were monitored with the “Mediterranean Sea – High Resolution and Ultra High Resolution L3S Sea Surface Temperature” product (<http://marine.copernicus.eu/web/69-myoccean-interactive-catalogue.php>). Daily sea surface temperatures (SST) were extrapolated from the catalogue after choosing an intermediate point between the two sampling areas within the zones, then mean monthly temperatures were obtained and used to represent the annual trend.

2.3.3. Gonadosomatic Index and fertility

The gonadosomatic index (GSI) was examined every month from June 2013 to May 2014 (with the exception of November due to adverse weather conditions) for all of the three sampled size classes. Sea urchins were allowed to drip dry for some minutes and then weighed. The test diameter (TD) without spines was measured and the gonads were successively extracted and weighed as well.

GSI was calculated by the formula: $GSI = [\text{gonad wet weight} / \text{total wet weight}] \times 100$ as reported by Lawrence *et al.* (1965).

The fertility was tested according Brundu *et al.* (2016) during the months of maximum gonadal development (i.e. from December to April). Gonads from females were extracted and gently shaken in filtered seawater to allow the mature ova to come out. Sperm was then added and finally, the fertility of the individuals was assessed as a percentage of the effectively fertilized eggs after the appearance of the fertilization membrane (fertilization was considered successful if it took place in at least 80% of the eggs according Falugi and Angelini, 2002). The percentage of achievement of the first larval stage (development of four-arm echinopluteus) was also

measured. The fertility test was carried out on the US individuals but not on Small-US specimens because of the paucity of their gonads (see “Results”). Nor was it done on the commercial-sized individuals (CS) because they were assumed to be fertile (Ouréns *et al.*, 2011).

Monthly mean values of GSI were calculated for the two zones and areas of sampling. Since sex ratio was nearly 1:1 for both sampling zones (Data S8), females and males were pooled together to obtain a single mean GSI value per month. A four-way ANOVA (using Statistica 6.0, Statsoft Inc.) was performed to highlight the differences in GSI values in different months (fixed and orthogonal factor, 11 levels), zones (fixed and orthogonal factor, 2 levels), areas (random and nested in zones, 2 levels), and fertile size classes (fixed and orthogonal factor, 2 levels, CS and US). Eight replicates for each size class were haphazardly selected from among those available in order to get a balanced design. Cochran’s C test was used to check for the assumption of homogeneity of variances and a posteriori SNK tests were performed to find alternative hypotheses (Underwood, 1997).

2.3.4. Population structure and potential reproductive contribution

Abundance and size-frequency distribution of the populations were estimated for both zones by counting all sea urchins found in the PVC plots and measuring them with calipers. The plots were 50 x 50 cm and were placed randomly as many times as necessary to cover two replicates of 25 m² in each area (100 m² for each zone).

Sea urchin abundance was estimated as the total density (individuals m⁻²) and the density of each 10 mm size class: $TD < 10$ mm, $10 \leq TD < 20$ mm, $20 \leq TD < 30$ mm, $30 \leq TD < 40$ mm, $40 \leq TD < 50$ mm and $50 \leq TD < 60$ mm, $TD \geq 60$ mm. Size-class densities were then translated into frequency percentages and used to compose the population structure for each zone. The gamete output and the spawning magnitude of each spawning event were calculated for the fertile CS and US classes according to Brewin *et al.* (2000). The highest and the lowest mean monthly GSI recorded during the year of sampling corresponded to the period just before the beginning (pre-spawning) and after the end (post-spawning) of the spawning events. We defined the mean individual gamete output (IGO), in units of gamete wet weight per urchin per spawning event ($\text{g g}^{-1} \text{ se}^{-1}$), as the difference of the mean monthly pre-spawning GSI and the mean monthly post-spawning GSI. The spawning magnitude was defined as the percentage ratio of the mean individual gamete output and the mean monthly pre-spawning GSI.

For each spawning event, we calculated the gamete output per m^2 (GO, $\text{g g}^{-1} \text{m}^{-2} \text{se}^{-1}$), released by fertile size classes in both zones, as their IGO multiplied for the respective natural density. The total gamete output (TGO) and the mean gamete output (MGO), used to estimate the reproductive contribution of each size class per m^2 per year, were defined respectively as the sum and the average of GO ($\text{g g}^{-1} \text{m}^{-2} \text{yr}^{-1}$). Finally, the potential reproductive contribution of the whole population in both zones (popTGO and popMGO, respectively) were calculated for the investigated year as the sum of the contributions of both fertile size classes.

2.4. Results

2.4.1. Environmental features

Multivariate analysis on algal cover has detected a significant interaction between Time and Zone. More specifically, pairwise tests highlighted that algae where sea urchins live changed significantly over time but not between the two zones (Table 1).

Table 1 - PERMANOVA on the percentage cover of conspicuous algae. Results of PERMANOVA analysis on square root transformed data. P-values were obtained using 9999 Monte Carlo samples from the asymptotic permutation distribution. Significant results are in bold. DF = degrees of freedom; MS = mean sum of squares; F = F-statistics; p = p-value.

Source of variation	df	MS	F	p
Time = Ti	2	7344.3	7.1242	0.0003
Zone = Zo	1	1545.9	0.53823	0.8649
Area(Zone)= Ar(Zo)	2	1242.9	1.2057	0.3343
Ti x Zo	2	3544.6	3.4383	0.0108
Ti x Ar(Zo)	4	1030.9	3.1397	0.0001
Residual	24	328.35		
Pairwise tests (Ti x Zo)				
July '13: High-pressure zone, Low-pressure zone 0.0677				
January '14: High-pressure zone, Low-pressure zone 0.0668				
May '14: High-pressure zone, Low-pressure zone 0.3645				

The nMDS showed that plots of the HP zone were interspersed on the graph with those of the LP zone (Fig. 10).

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Moreover, the water temperature changed over time but no difference was observed in the annual trend between the two zones studied (Fig. 11).

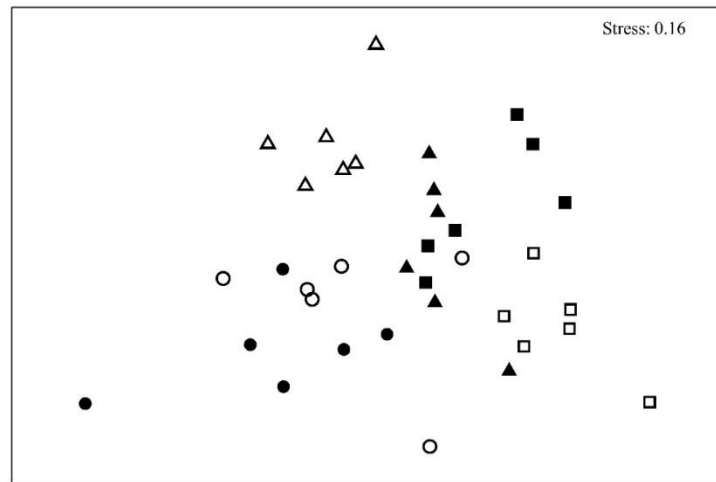


Figure 10 - nMDS plot of algal cover. Non-metric MDS plot of algal cover for all replicate quadrats. White plots = high-pressure zone (Su Pallosu Bay); black plots = low-pressure zone (Tavolara – Punta Coda Cavallo); triangles = July '13; squares = January '14; circles = May '14

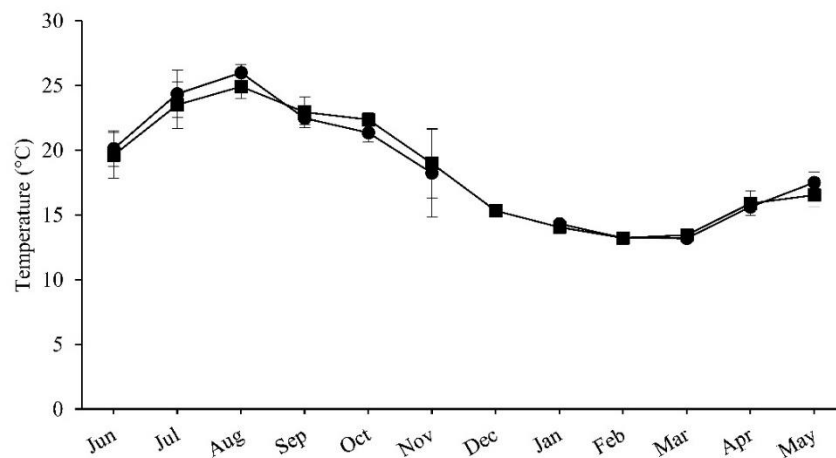


Figure 11 - Seasonal SST pattern. Mean values ($\pm$ standard deviation) of water temperature (SST) registered from June 2013 to May 2014. Squares = high-pressure zone (Su Pallosu Bay); circles = low-pressure zone (Tavolara – Punta Coda Cavallo).

2.4.2. Gonadosomatic Index and fertility

At each area, 220 specimens of the commercial-size class (CS) and 110 undersized and smaller undersized individuals (US and Small-US) were randomly collected over a year to compare

reproductive potential between populations. Regarding CS individuals, sampled sea urchins with $TD \geq 60$ mm were 24 of 440 (5.5%) in the HP zone and 219 of 417 (53%) in the LP zone. Fertility tests showed that 100% of the undersized individuals (US) checked were fertile and contributed to the reproductive potential of the populations, with high percentages of fertilized eggs (ranging from 87 to 96%) and developing larvae (ranging from 95 to 100%) (Table 2).

Table 2 - Results of fertility tests. Mean percentage ($\pm$ standard deviation) of fertilized eggs and four-arm echinoplutei obtained from tests of fertility performed on US individuals from both sampling zones during the months of maximum gonadal development. na= no count of larvae was done.

		Dec	Jan	Feb	Mar	Apr
High-pressure zone	% of fertilized eggs	90.3 $\pm$ 5.7	87.3 $\pm$ 10.4	92.8 $\pm$ 4.7	96.0 $\pm$ 0.8	91.8 $\pm$ 6.0
	% of four-arm echinoplutei	na	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	99.6 $\pm$ 0.5	94.8 $\pm$ 8.2
Low-pressure zone	% of fertilized eggs	89.8 $\pm$ 5.4	93.9 $\pm$ 0.0	90.5 $\pm$ 6.1	92.9 $\pm$ 0.0	92.3 $\pm$ 0.5
	% of four-arm echinoplutei	na	100.0 $\pm$ 0.0	99.6 $\pm$ 0.5	99.3 $\pm$ 0.0	98.6 $\pm$ 2.1

Conversely, the Small-US individuals had reduced gonads (Fig. 12) and, even during the period of maximum development, they never released gametes, therefore their contribution to the reproductive potential of the population can be considered negligible.

The GSI trend over the year was generally higher for CS individuals than US ones for both zones (Fig. 12). At the HP zone, we recorded a single large spawning period from March to May for both fertile classes (see Fig. 12a). In pre-spawning time, GSI values reached $6.6 \pm 0.3\%$ (mean $\pm$ standard error) and $4.4 \pm 0.4\%$, while in post-spawning time values were $1.3 \pm 0.2\%$ and $1.6 \pm 0.1\%$ for the CS and US classes respectively (Fig. 12a).

At the LP zone, a spawning event was observed twice (Fig. 12b). The first one was recorded from June to December with a pre-spawning GSI of $6.7 \pm 0.3\%$ and $5.2 \pm 0.5\%$, and a post-spawning GSI of $2.5 \pm 0.3\%$ and $1 \pm 0.2\%$ for the CS and US classes respectively. The second spawning event occurred from February to April with a pre-spawning GSI of $5.4 \pm 0.4\%$ and a post-spawning GSI of $2.5 \pm 0.2\%$ for the CS class. From February to May we observed a pre-spawning GSI of $4 \pm 0.6\%$ and a post-spawning GSI of $1.2 \pm 0.2\%$ for the US class (Fig. 12b).

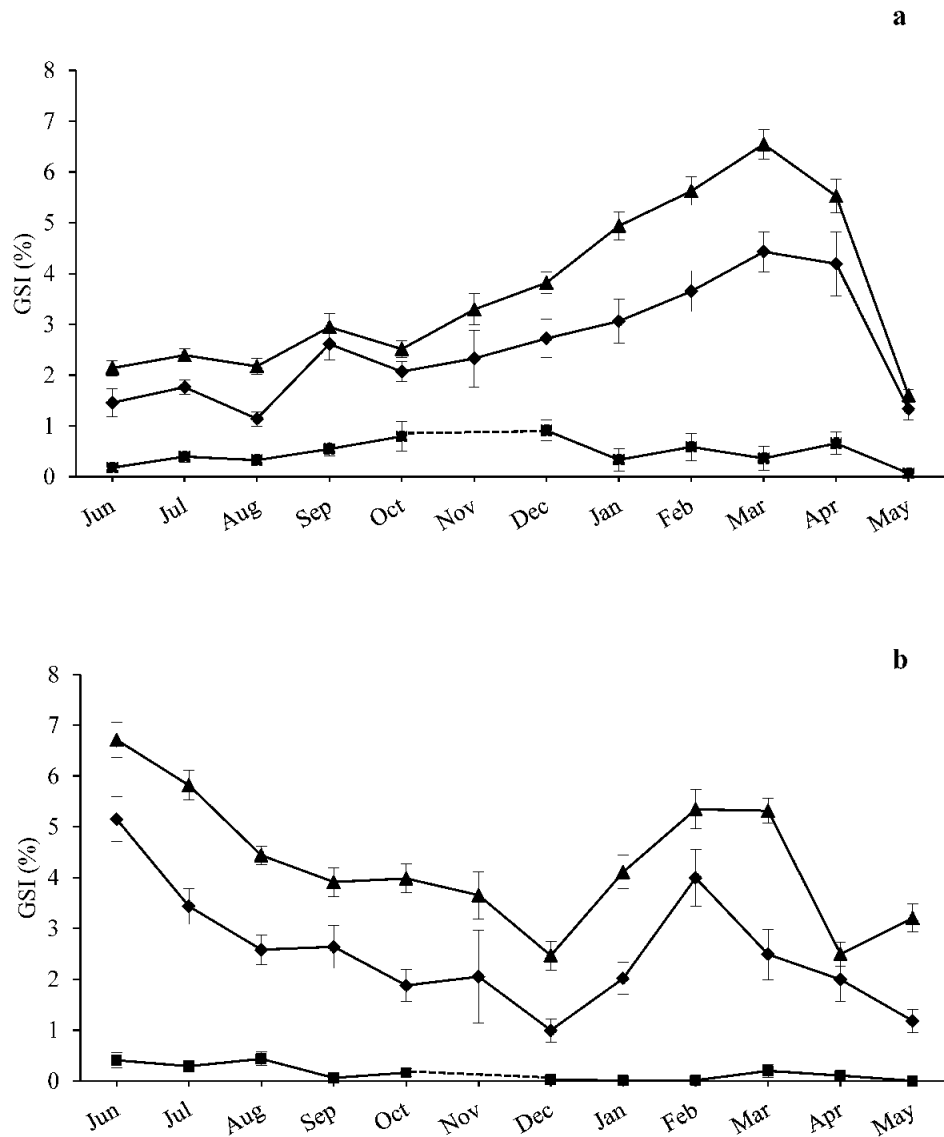


Figure 12 - Annual trend of gonadosomatic index at sampling zones. GSI is represented as mean $\pm$ standard error for the three size classes examined (CS, US, Small-US) at a) high-pressure zone (Su Pallosu Bay) and b) low-pressure zone (Tavolara – Punta Coda Cavallo). No distinction between sexes was done in the graph, since females and males belonging to the same size class were pooled together. Observation began in June 2013 and ended in May 2014. However, November 2013 was not sampled due to general bad weather conditions and GSI data of CS and US classes were estimated from different years (i.e. November 2007 for Tavolara – Punta Coda Cavallo and November 2015/16 for Su Pallosu Bay). GSI data for Small-US class was not available for this month; the dotted line for the Small-US class represents an approximation of the expected values of GSI for November. Triangles = CS; rhombuses = US; squares = Small-US.

ANOVA highlighted significant differences during the year and between the different size classes, while there were no major statistical differences found between sampling areas and zones. The CS class had higher GSI values in the LP zone while no differences were found for

the US individuals (Table 3). A significant interaction between Zone and Size Class was detected, and the GSI of the CS class was significantly higher than that of the US individuals for both zones. A significant interaction was also found between Month and Size Class with SNK pointing out significantly higher GSI values for the CS individuals during the whole sampling year excluding April (Table 3).

2.4.3. Population structure and potential reproductive contribution

Size class distribution was consistently different between the two zones (Fig. 13). In the HP zone, sea urchin density was almost two-fold higher than in the LP zone: 10 ± 1.4 and 5.4 ± 0.5 individuals per m^2 respectively. Sea urchins with TD ranging from 0 to 20 mm were 1.7 ± 0.1 per m^2 and 1.1 ± 0.6 per m^2 in the HP and LP zones respectively, and they represent 17% and 21% of their populations. The most abundant size classes were those ranging from 20 to 50 mm diameter (77%) with a density of 3 ± 0.6 and 3 ± 0.3 individuals per m^2 for the Small-US and US classes respectively ($30 \leq \text{TD} < 40$ mm and $40 \leq \text{TD} < 50$ mm). The proportion of individuals of the CS class with respect to the entire population was 6% (0.6 ± 0.2 individuals per m^2) and all the individuals were included in the range of $50 \leq \text{TD} < 60$ mm (Fig.13). In the LP zone, the individuals between 20 and 50 mm represented 28% of the population while the CS class was 52% with 1 ± 0.4 and 2 ± 0.2 individuals per m^2 for $50 \leq \text{TD} < 60$ mm and $\text{TD} \geq 60$ mm respectively (Fig. 13).

In relation to the population structure, the reproductive contribution was compared between the two zones, but no comparisons were made between the sampling areas because no differences were found (see Table 3). The potential reproductive contribution was calculated according to the number of spawning events during the surveyed year and the natural density of the fertile size classes (US and CS). In the HP zone, a single spawning event occurred and the spawning magnitude for the year was 73% on average (Table 4) with an individual gamete output (IGO) of 0.03 and $0.05 \text{ g g}^{-1} \text{ se}^{-1}$ for the US and CS individuals, respectively (Table 4). The gamete output (GO), calculated in relation to the natural density of this zone, was $0.08 \text{ g g}^{-1} \text{ m}^{-2} \text{ se}^{-1}$ for the US sea urchins (2.7 ± 0.3 individuals per m^2) and $0.03 \text{ g g}^{-1} \text{ m}^{-2} \text{ se}^{-1}$ for the CS class (0.6 ± 0.2 individuals per m^2). Because of the single spawning event, the total gamete output per m^2 (TGO) overlaps the mean gamete output per m^2 (MGO) (Table 4). Accordingly, the total gamete output of the whole population (i.e. sum of TGO of the two fertile size classes) corresponded to the mean gamete output for m^2 (popMGO) with a value of $0.11 \text{ g g}^{-1} \text{ m}^{-2} \text{ yr}^{-1}$ (Table 4).

Table 3 - Results of four-way ANOVA. Analysis of variance was performed to test the effects on Gonadosomatic Index of Month, Zone and Size class (orthogonal fixed factors) and Area (random nested factor in Zone). Bold value is statistically significant at $p < 0.05$. SNK tests were conducted for comparisons of significant interactions. DF = degrees of freedom; MS = mean sum of squares; F = F-statistics; p = p-value.

Source of variation	df	MS	F	p
Month = Mo	10	933.74	2.94	0.0193
Zone = Zo	1	11.883.74	14.26	0.0635
Area(Zone)= Ar(Zo)	2	833.46	2.27	0.1039
Size class = Sc	1	117763.64	362.25	0.0027
Mo x Zo	10	597.33	1.88	0.1104
Mo x Ar(Zo)	20	317.95	0.87	0.6301
Mo x Sc	10	2000.32	7.12	0.0001
Zo x Sc	1	13457.07	41.39	0.0233
Sc x Ar(Zo)	2	325.10	0.89	0.4126
Mo x Zo x Sc	10	648.71	2.31	0.0535
Sc x Mo x Ar(Zo)	20	281.10	0.77	0.7552
Residual	616	366.70		

Transformation: none; Cochran's test $C = 0.0664$, $p < 0.05$

SNK

Zo x Sc: High-pressure zone (US < CS)

Low-pressure zone (US < CS)

US (High-pressure zone = Low-pressure zone)

CS (High-pressure zone < Low-pressure zone)

Mo x Sc: April (US = CS)

Other months (US < CS)

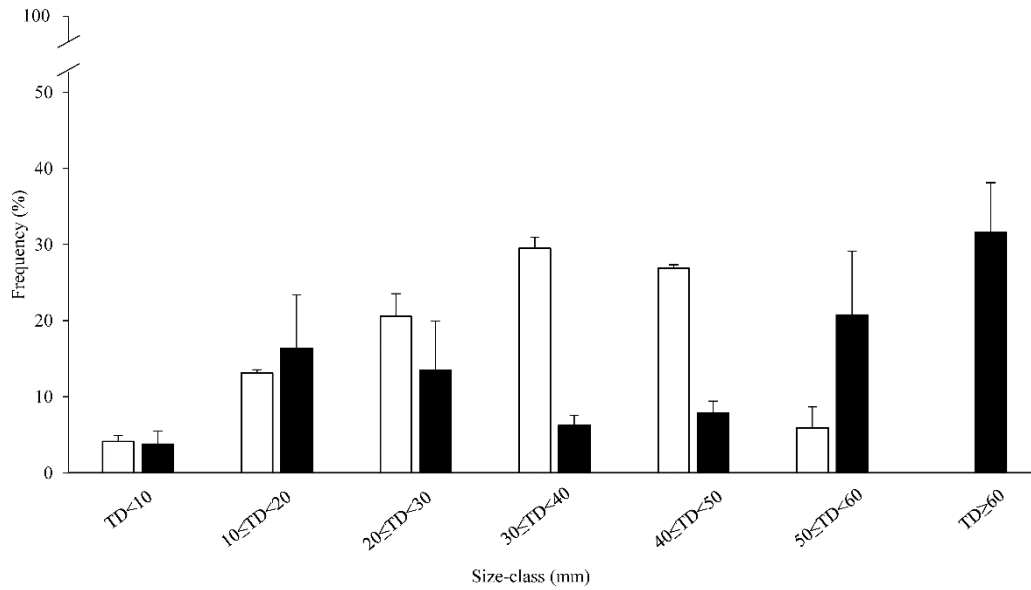


Figure 13 - Size-frequency distribution (%) of sea urchin populations at sampling zones. The range of the size classes is 10 mm of test diameter without spines (TD). Commercial size classes under fishing pressure are those larger than 50 mm test diameter. White bars = high-pressure zone (Su Pallosu Bay); black bars = low-pressure zone (Tavolara – Punta Coda Cavallo).

Conversely, in the LP zone, two spawning events were observed (Fig. 12b). Spawning magnitude varied from 54 to 81% with higher values for US individuals (Table 4). IGO was similar for both size classes with values ranging from 0.03 to 0.04 g g⁻¹ se⁻¹ according to the spawning period. The GO of the US individuals was 0.02 g g⁻¹ m⁻² se⁻¹ during the first spawning event and 0.01 g g⁻¹ m⁻² se⁻¹ during the second one, while it was 0.11 and 0.08 g g⁻¹ m⁻² se⁻¹ for the CS class. Total gamete output (TGO) and mean gamete output (MGO) of the US individuals, whose density was 0.4 ± 0.1 individuals per m², were 0.03 and 0.01 g g⁻¹ m⁻² yr⁻¹ respectively. Meanwhile they were 0.19 and 0.10 g g⁻¹ m⁻² yr⁻¹ for the CS class, whose natural density was 2.7 ± 0.3 individuals per m². Consequently, the total gamete output of the whole population was estimated to be 0.22 g g⁻¹ m⁻² yr⁻¹ and the total mean gamete output was 0.11 g g⁻¹ m⁻² yr⁻¹ (Table 4).

Table 4 - Summary table of potential reproductive contribution results. Timing and extent of mean monthly Gonadosomatic Index, Spawning Magnitude, and Gamete Output for US ($40 \leq TD < 50$ mm) and CS ($TD \geq 50$ mm) class, for spawning event and in relation with their natural density, and for the whole populations at high-pressure (Su Pallosu Bay) and low-pressure (Tavolara – Punta Coda Cavallo) zone.

IGO = Individual Gamete Output per spawning event; GO = Gamete Output per m^2 ; TGO = Total Gamete Output per m^2 ; popTGO = Total Gamete Output of the whole population per m^2 ; MGO = Mean Gamete Output per m^2 ; popMGO = Mean Gamete Output of the whole population per m^2 .

		High-pressure zone		Low-pressure zone			
Size class		US	CS	US		CS	
Spawning period	Start	Mar '14	Mar '14	Jun '13	Feb '14	Jun '13	Feb '14
	End	May '14	May '14	Dec '13	May '14	Dec '13	Apr '14
Mean monthly GSI (%)	Pre-spawning	4.4	6.6	5.2	4.0	6.7	5.4
	Post-spawning	1.3	1.6	1.0	1.2	2.5	2.5
IGO ($g\ g^{-1}\ se^{-1}$)		0.03	0.05	0.04	0.03	0.04	0.03
Spawning magnitude (%)		70.5	75.8	80.8	70.0	62.7	53.7
Natural density (ind m^{-2})		2.7	0.6	0.4		2.7	
GO ($g\ g^{-1}\ m^{-2}\ se^{-1}$)		0.08	0.03	0.02	0.01	0.11	0.08
TGO ($g\ g^{-1}\ m^{-2}\ yr^{-1}$)		0.08	0.03	0.03		0.19	
popTGO ($g\ g^{-1}\ m^{-2}\ yr^{-1}$)		0.11		0.22			
MGO ($g\ g^{-1}\ m^{-2}\ yr^{-1}$)		0.08	0.03	0.01		0.10	
popMGO ($g\ g^{-1}\ m^{-2}\ yr^{-1}$)		0.11		0.11			

2.5. Discussion

The results showed a considerable difference between the two zones in population structure and in abundance of sea urchins, as well as in the gonadosomatic index trend throughout the surveyed year. As consequence, also the potential reproductive contribution differed between the two populations.

While recruits (TD < 20 mm) had the same proportion of the population structure, the density of the fertile undersized individuals (US) was 4.5-fold higher at the HP zone than at the LP one, and the density of the commercial size (CS) was ~ 7-fold lower. Furthermore, within the commercial size, a large percentage was composed of individuals ≥ 60 mm at the LP zone, while these large-sized individuals were nearly absent at the HP zone (none have been detected during the sampling for the estimation of the population structure, only 24 throughout the annual sampling for the GSI assessment). Both populations showed a spawning event at the end of winter or at the beginning of spring, which lasted until April-May for the two fertile size classes examined (US and CS). Moreover, a second relevant peak of GSI was registered in the LP zone which was demonstrated to be concomitant with a spawning event from June 2013 (Siliani *et al.*, 2016) by histological analysis. This is consistent with observations from other areas of the Mediterranean where one or two annual spawning periods were commonly identified, regardless of the proportion of size classes shaping the population structure (Fenaux, 1968; Semroud and Kada, 1987; Pedrotti and Fenaux, 1992; Pedrotti, 1993; Semroud, 1993; Lozano *et al.*, 1995; Fernandez and Boudouresque, 1997; López *et al.*, 1998; Guettaf *et al.*, 2000; Leoni *et al.*, 2003; Martínez *et al.*, 2003; Sánchez-España *et al.*, 2004; Tomas *et al.*, 2004; Sellem and Guillou, 2007).

The differences identified in the reproductive potential between the populations were not ascribed to different environmental features since the sampling zones were similar in terms of sheltering from waves and there were no differences found for either water temperature or algal assemblages. Therefore, since fecundity depends on food availability and other environmental characteristics (Minor and Scheibling, 1997; Scheibling and Hatcher, 2007), the two populations could be considered similar in their ability to produce offspring.

At both zones, GSI of the Small US individuals was lower than 1% throughout the year, therefore its contribution to the reproductive potential of the population was considered

negligible. On the contrary, GSI of the two fertile size classes (US and CS) changed significantly over the year and between zones. Specifically, GSI of the CS class was significantly higher than those of the US individuals and it was significantly higher in the LP zone than in the HP zone.

Growth rate is one of the main factors that may affect the size-frequency distribution of a sea urchin population (Dix, 1972; Barry and Tegner, 1990), and it can differ among populations (e.g. Sellem *et al.*, 2000; Turon *et al.*, 1995). However, a previous study conducted in the same zones by Loi *et al.* (2013) showed that the growth rates of the two populations were comparable (Fig. 14). Definitively, as widely described in many other locations of the Atlanto-Mediterranean areas (Guidetti *et al.*, 2004; Gianguzza *et al.*, 2006; Pais *et al.*, 2007; Ceccherelli *et al.*, 2009; Bertocci *et al.*, 2014), our study clearly points out how human predation adversely affects the population structure in the HP zone, truncating the adult cohorts with $TD \geq 50$ mm. The high presence of commercial-size adults ($TD \geq 50$ mm) at the LP zone, and in particular the high number of individuals larger than 60 mm, confirmed this evidence. Furthermore, these results are in agreement with previous studies on population structure performed at Su Pallosu Bay (Guala *et al.*, 2006) and Tavolara – Punta Coda Cavallo (Guala *et al.*, 2009) (Fig. 15).

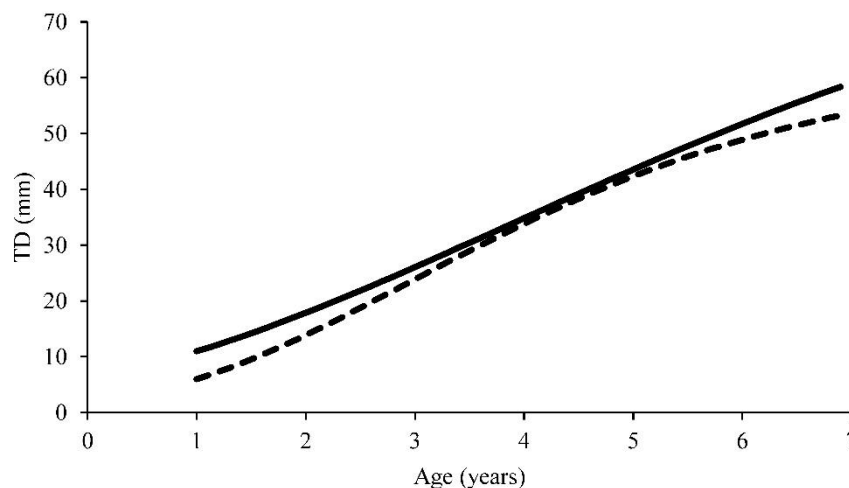


Figure 14 - Growth curves plot displayed in previous studies. Comparison of growth rate between the populations living in the zones examined. The growth rates were compared using the non-parametric Wilcoxon test. P-value = 0.31 (n.s.). Modified from Loi *et al.* (2013). Dotted line = Sinis; continuous line = Tavolara – Punta Coda Cavallo.

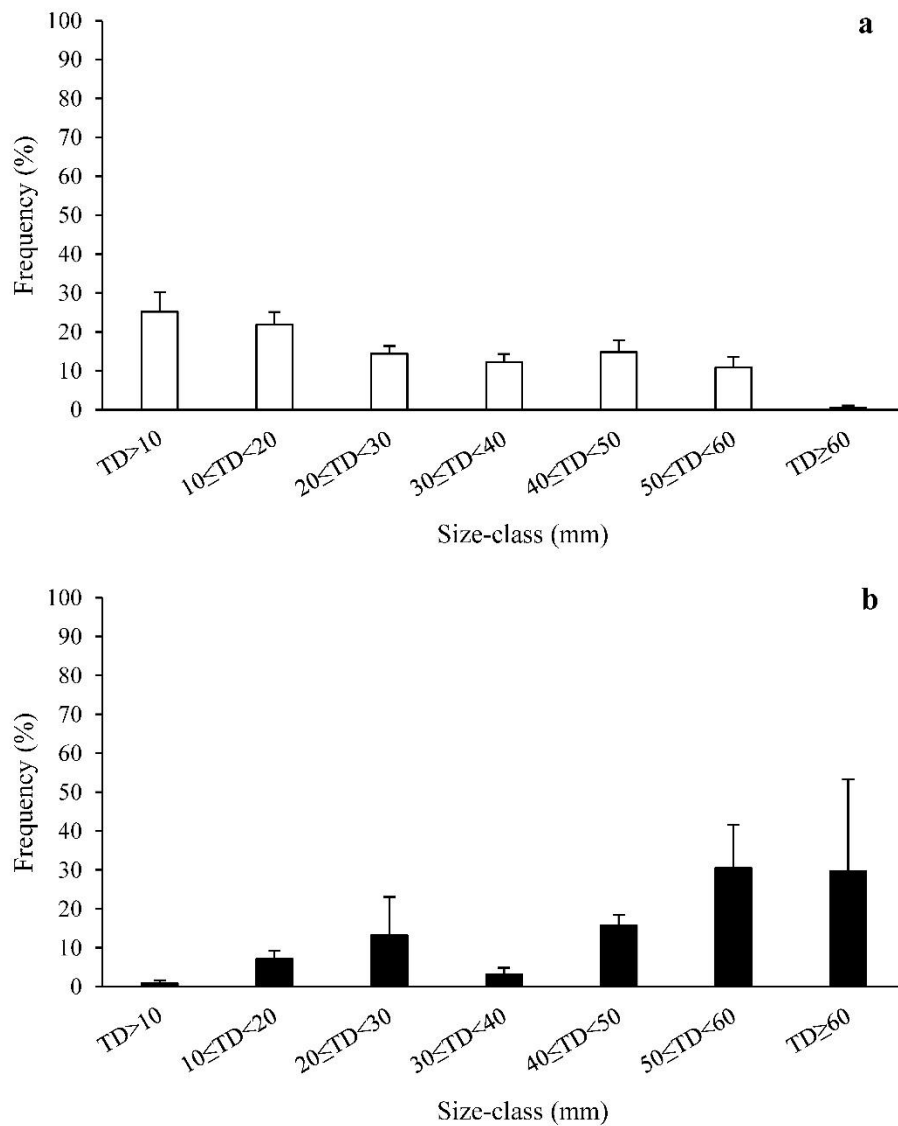


Figure 15 - Population structure plots displayed in previous studies. Size-frequency distribution (%) of sea urchin populations at a) Su Pallosu Bay (Guala et al., 2006), and b) Tavolara - Punta Coda Cavallo MPA (Guala et al., 2009).

More interestingly, our results suggest the existence of a strong connection between the contribution to the reproductive potential of the fertile size classes and the pressure of commercial harvesting. Since the mean density of the US individuals at the HP zone was similar to the mean density of the CS sea urchins at the LP zone, and vice versa, the mean gamete output of the population (popMGO) was similar at the two zones but the contribution of the two fertile size classes was specular. The US individuals were therefore the main producers of gametes at the HP zone while the CS ones were likely annihilated by harvesting. On the other

hand, at the LP zone, the main producer was the CS class probably because the US individuals were strongly reduced by fish predation as a response to protection measures (Sala, 1997; Hereu *et al.*, 2005; Parravicini *et al.*, 2010).

In contrast, the total gamete output of the population (popTGO), which represented the annual reproductive contribution of populations throughout the year, was two-fold higher at the LP zone. Mita *et al.* (2007) suggested that gonad size increases volumetrically with sea urchin test diameter, with the largest body size implying the most mature and developed gonads (and the consequent possibility to produce more than one cohort of gametes). Effectively, in marine reserves, more fertile gametes are produced by the largest sea urchins compared to sites where large sea urchins are lacking (Lundquist, 2000). Moreover, the very important effects related to the individual's age and size are proven by studies conducted on different fish species that have demonstrated how a greater age diversity in a spawning stock may extend spawning both temporally and spatially. This, in consequence, increases the chances that more offspring encounter favourable conditions for development (Secor, 2000; Berkeley *et al.*, 2004b; Fiorentino *et al.*, 2008).

Despite the fact that a single year of study is not enough to unambiguously establish the regularity of reproductive episodes throughout time, the number of spawning events we observed at the two zones are consistent, respectively, with previous GSI studies conducted at Tavolara – Punta Coda Cavallo (Guala *et al.*, 2009) and in a location in the Sinis peninsula, contiguous to Su Pallosu Bay (Baroli *et al.*, 2006), that is intensely affected by fishing (Marra *et al.*, 2016) (Fig. 16).

Thus, since the popMGO strongly depended on the density per m² of fertile individuals regardless of their size class, it would be plausible that the continuous catch of commercial-sized sea urchins has led to a reduction of spawning events due to a considerable decrease of the body size of the fertile individuals. Hence, it might be reasonable to suppose that spawning events were favoured by the well-structured spawning stock and larger sea urchins which are typical of protected populations.

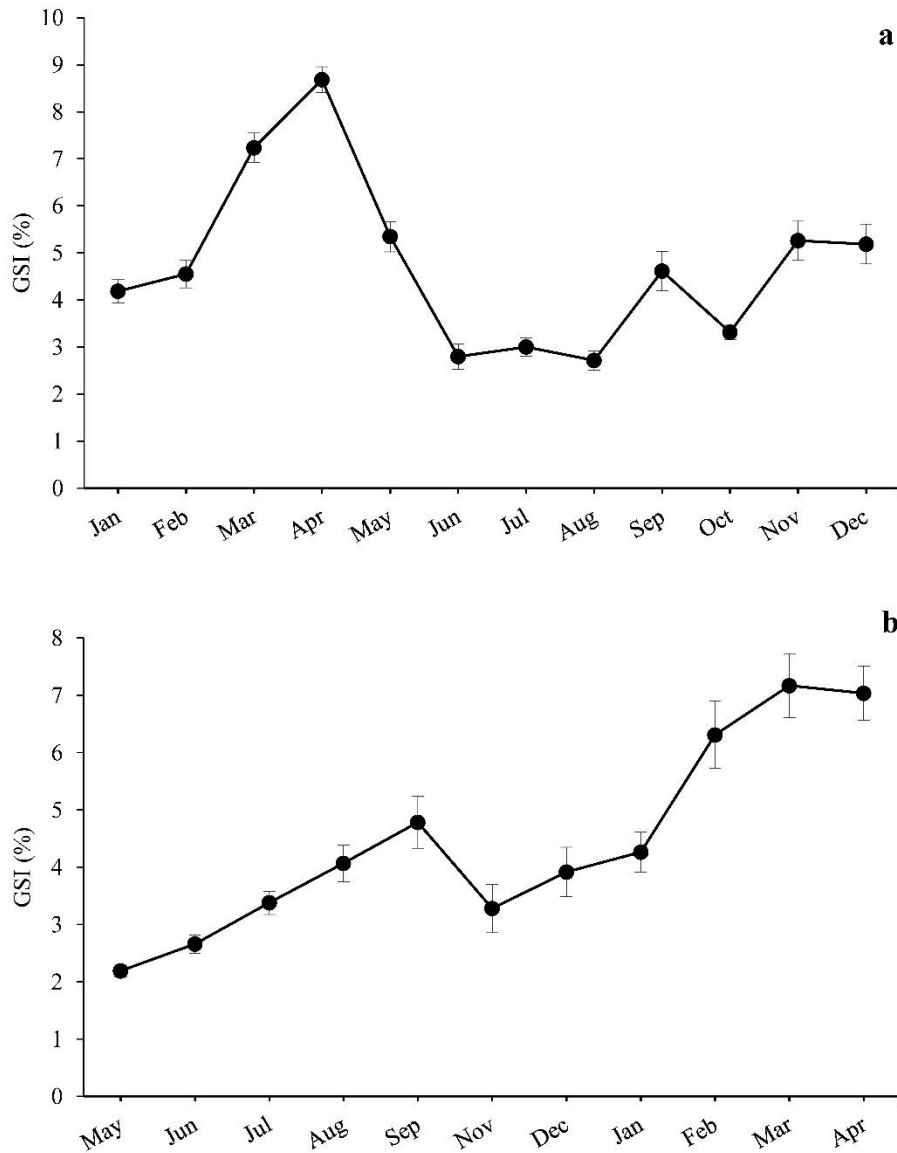


Figure 16 - GSI charts displayed in previous studies. GSI trend of sea urchin population at a) a location (Seu) in the Sinis peninsula, contiguous to Su Pallosu Bay (Baroli *et al.*, 2006), and b) Tavolara – Punta Coda Cavallo (Guala *et al.*, 2009).

In general, sea urchin population dynamics is driven by various ecological processes that operate on different spatial and temporal scales. Larval supply fluctuates widely from region to region as it is associated with oceanic currents (Fenaux *et al.*, 1988; Prado *et al.*, 2012). The success of settlements is influenced by local constraints linked to habitat (e.g. adult abundance, presence of crustose algae, substrate rugosity) (Boudouresque and Verlaque, 2007; Oliva *et al.*, 2016). Finally, predation becomes the prevalent mechanism of sea urchin population control after settlement and serves as a critical bottleneck for urchin populations (Guidetti, 2004; Hereu *et al.*, 2005; Farina *et al.*, 2009; Farina *et al.*, 2014; Boada *et al.*, 2015). In our case, the lack of

predatory fish in the HP zone (Marra *et al.*, 2016; Oliva *et al.*, 2016) and the high pressure of harvesting are likely to shift the whole potential reproductive contribution onto the young adults. On the contrary, in the LP zone, it mostly depended on commercial-sized individuals since predation in marine reserves significantly affects sea urchins until they reach the size of 50 mm TD (Guidetti, 2004).

Therefore, we could state that the self-sustenance of the sea urchin populations did not change between the two zones, despite the different harvesting pressure. The high number of recruits at the HP zone ensured an availability of new juveniles for the immediate future and suggested an important larval input of which the origin still has to be investigated. However, the two spawning events at the LP zone suggested a more abundant and successful gamete production than in the population which was under strong fishing pressure. This is probably due to a well-structured spawning stock which enhanced the resilience of the pristine population. Hence, in equal recruitment conditions, when the density of fertile individuals decreased (e.g. as a result of intense commercial harvesting or natural predation) the mean reproductive contribution consequently diminished.

Because of over-exploitation of *P. lividus* populations in several Mediterranean areas (Pais *et al.*, 2012; Bertocci *et al.*, 2014) and their ecological (Sala *et al.*, 1998) and economic relevance (Palacín *et al.*, 1998), sea urchin fisheries need urgent and effective regulation. Our findings may have important implications for creating a management of sea urchin fisheries that is effective in terms of the sustainable conservation of resources.

In a marine reserve context, where sea urchin populations have a normal bimodal structure (Brundu *et al.*, 2013), the opening of commercial harvesting should be avoided (despite the insistence of local fishermen) to prevent the depletion of fertile individuals, as a result of the synergistic action with the natural predation, and the breakdown of the population. On the contrary, in areas affected by fishing pressure, larger individuals are lacking (Baroli *et al.*, 2006), but the survival of the fertile, intermediate-size classes, which therefore support the mean gamete output of the whole population, is guaranteed by laws (as harvesting is authorized only for individuals ≥ 50 mm) and by the absence of predatory fishes, which in turn are targeted by commercial fishing.

Our present findings suggest that the reproductive potential contribution of the population does not depend on the size of fertile individuals but on their density. For this reason, the harvesting

Chapter 2. Hard time to be parents? Sea urchin fishery shifts potential reproductive contribution of population onto the shoulders of the young adults

of individuals between 40 and 50 mm should be avoided particularly in over-fished locations, since they are the only ones capable of generating new life, as far as we know without more information on the origin of the larvae present.

Acknowledgements

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3. THE INFLUENCE OF HABITAT AND SEASON ON THE GONAD QUALITY OF *PARACENTROTUS LIVIDUS* (ECHINODERMATA, ECHINOIDEA) IN A SARDINIAN COASTAL AREA (WESTERN MEDITERRANEAN)

Not submitted

3.1. Abstract

Paracentrotus lividus gonads represent a much appreciated delicacy in Mediterranean countries and particularly in Sardinia, and the continuous market demand has led to the depletion of populations in some areas. The marketability of roe is unavoidably linked to its quality, which in turn depends on several factors such as gonad yield and color. Both may vary greatly according to seasonal and habitat features such as seawater temperature, photoperiod, hydrodynamic conditions, food availability and quality. Identifying the season and habitat in which harvesting high-quality gonads with the minimum fishing effort seems to be crucial for increasing the marketability of the product and promoting the sustainable use of sea urchin resources.

We conducted a two-year-long study in a central-western Sardinian coastal area (Su Pallosu Bay), where we chose two sites characterized by different habitats. These were a rocky bottom (RB) and a *Posidonia oceanica* meadow (PM) in which we monitored the gonad yield and color in adult *P. lividus* with relation to habitat and season.

Gonad yield was assessed by the Gonadosomatic Index (GSI), while colorimetric analyses were conducted to evaluate gonad color. The three components of color, L* (lightness), a* (redness) and b* (yellowness) were numerically quantified through a colorimeter. In addition, to identify any possible connections with yield and color, we monitored three parameters: i) the reproductive cycle, using histological analyses; ii) the feeding intensity, using the Repletion Index (RI); iii) and the diet, by observing the gut contents.

GSI was significantly higher at the PM site, with a peak repeated every spring before a spawning event. Conversely, during the first year at RB, the peak was registered in winter, whereas in the following year no clear peaks emerged, with stable GSI values throughout the first part of the year and a decrease in winter. As RI was comparable between the sites

throughout the years, the gonad investment at RB did not correlate with the feeding intensity. Probably, the higher hydrodynamics to which sea urchins are subjected at this site acted as a limiting factor for GSI, thus directing part of the energies derived from ingested food to the maintenance of test rather than to reproduction. Regarding roe color, the gonads from RB showed higher L^* and b^* values than gonads at PM, while no differences between sites were detected for a^* . At RB, the highest values for L^* were obtained in spring, for a^* in summer and autumn, for b^* from spring to autumn. The consumption of a larger variety of seaweeds by the urchins at RB probably caused the absorption of a higher quantity and/or quality of carotenoids and hence the production of brighter and yellower gonads.

Our results suggest that diversifying harvesting grounds in different seasons, according to the desired quality characteristics of gonads, could be most profitable for fisheries and represents one of the possible strategies for the sustainable harvesting of wild resources.

3.2. Introduction

The edible sea urchin *Paracentrotus lividus* (Lamarck, 1816) is the most common echinoid species in sublittoral habitats in the Mediterranean Sea (Lozano *et al.*, 1995; Boudouresque and Verlaque, 2007;), and inhabits rocky seabed areas and meadows of *Posidonia oceanica* up to a depth of 40 m. The increasing interest in this species is primarily due to its role in maintaining the equilibrium of sublittoral communities of rocky reefs (Sala *et al.*, 1998) and to its economic value (Palacín *et al.*, 1998). Its gonads (also named “roe”) are very appreciated in many European countries, so that fisheries have drastically depleted the populations in some Atlanto-Mediterranean areas (Régis, 1986; Zavodnik, 1987; Byrne, 1990; Barnes and Crook, 2001; Bertocci *et al.*, 2012).

The factors that affect gonad quality represent a crucial concern for aquaculture and fishery (Kinoshita *et al.*, 2013). The marketability, and consequently the price of urchin gonads is linked to several characteristics, i.e. their yield and sensory parameters (Unuma, 2002; McBride *et al.*, 2004) such as freshness, firmness, taste and especially color (Shpigel *et al.*, 2006; Carboni *et al.*, 2015).

Gonad yield varies seasonally and according to the variation of environmental features (Byrne, 1990; Gago *et al.*, 2001; Sánchez-España *et al.*, 2004; Bayed *et al.*, 2005; Arafa *et al.*, 2012b). In particular, it seems that seawater temperature (Byrne, 1990; Bayed *et al.*, 2005; González-Irusta *et al.*, 2009), photoperiod (González-Irusta *et al.*, 2009), phytoplankton availability (Lozano *et al.*, 1995; Bayed *et al.*, 2005; González-Irusta *et al.*, 2009), and habitat features such

as algal production (Bayed *et al.*, 2005), food availability (Guidetti *et al.*, 2003; Sánchez-España *et al.*, 2004), and hydrodynamism (Sellem and Guillou, 2007) are of crucial importance. Nevertheless, contradictory results have been obtained from field data regarding the effects of habitat on *P. lividus* gonadosomatic index (GSI). Guettaf (1997) found no differences in GSI in populations from *P. oceanica* meadows, barren grounds or dense algal stands. Chiantore *et al.* (2008) detected no habitat effects on the reproductive conditions (GSI) of sea urchins from a barren ground and a rocky bottom in well-vegetated conditions. Several authors (San Martín 1995; Fernandez and Boudouresque, 1997; Ouréns *et al.*, 2011; Boudouresque and Verlaque, 2007; Sellem and Guillou, 2007) showed that the GSI in individuals from seagrass meadows was larger than those from pebbly or rocky bottoms. On the contrary, Addis *et al.* (2014) demonstrated that GSI was larger on rocky bottoms than in *Posidonia* beds, in a study that lasted one month.

Profitable gonad color can range from a light yellow to a dark orange or almost red (Robinson *et al.*, 2002). Gonad color changes seasonally (Agatsuma, 1998; Robinson *et al.*, 2002), being dependent on reproductive cycle, food composition and grazing activity (Kinoshita *et al.*, 2013; McBride, 2004; Pearce *et al.*, 2002b). Roe color can be quantitatively measured using the international standard developed by the *Commission Internationale de l'Eclairage* (CIE), which breaks it down into three components (L*, a*, b*) in a three-dimensional measurement. The use of colorimetric methods to measure the color of sea urchin roe in relation to nutrition or environmental parameters has been widespread for experimental studies on sea urchins held in cages or tanks, fed with artificial or natural feed (McBride *et al.*, 2004; James, 2006; Shpiguel *et al.*, 2006; Cook and Kelly, 2007; Woods *et al.*, 2008; James and Heath, 2008; Phillips *et al.*, 2009; Azad *et al.*, 2011; Carboni *et al.*, 2013b). Very few studies on gonad color have been conducted considering sea urchins in the wild environment (Phillips *et al.*, 2010; Addis *et al.*, 2014), and these were usually limited to a single sampling. Phillips *et al.* (2010) conducted a color analysis on gonads from *Evechinus chloroticus* harvested on rocky bottoms in the northern and southern islands of New Zealand. They found significant differences in color between the two areas ascribed to the ingested diet (*Ecklonia radiata* vs *Macrocystis pyrifera*). On the other hand, Addis *et al.* (2014) did not find any difference between gonad color of wild *P. lividus* harvested from two different habitats (rocky substrate and a *P. oceanica* meadow).

In Southern Italy, and particularly in Sardinia, fishing of *P. lividus* plays a significant socio-economic role (Addis *et al.*, 2009, 2012; Pais *et al.*, 2007). Here, the continuous catches, even if regulated by a regional decree, have caused the overfishing of many shallow rocky reef

populations (Pais *et al.*, 2007). At Su Pallosu Bay (central-western Sardinia) the sea urchin fishing season is legally open from November to April, when gonad yield is supposed to be high. Although no particular limitation on the fishing ground exists (hard seabeds or *P. oceanica* meadows), fishermen currently focus their efforts on harvesting sea urchins exclusively on rocky substrates. This has led to a decrease in the commercial-sized individuals on Su Pallosu Bay rocky bottoms, as shown in a previous work conducted by our group (Loi *et al.*, 2017). Given the great relevance of *P. lividus* in Sardinia, it is of paramount importance to increase the existing knowledge of the most profitable habitats and harvesting period in order to obtain high-quality gonads.

For this purpose, we studied a sea urchin population living in a heavily fished area located in central western Sardinia. We chose two sites which were characterized by different habitats (a rocky bed dominated by seaweeds and a *P. oceanica* meadow on sandy bottom) in order to monitor the changes in gonad quality (yield and color) in a 2-year study. We then related them to season, reproductive cycle and dietary factors.

Our final goal was to provide new information which can be useful in improving the harvesting practices (timing, duration of harvesting, habitat) of *P. lividus* resources, possibly encouraging the increase of the economic value of sea urchin roe in Sardinia.

3.3. Materials and methods

3.3.1. Study site and sampling

Adult *P. lividus* were collected from Su Pallosu Bay (40°03' N; 008°25' E), located in the northern part of the Sinis peninsula (central western Sardinia) and exposed to prevailing winds blowing from North-west (Fig. 17). Samplings were conducted at two sites, which were 500 meters apart, characterized by different substrates. The first site (hereafter named RB) was situated in the external part of the bay and was characterized by a rocky seabed covered with a photophilic algal community dominated by *Padina pavonica*, *Dictyota dichotoma*, *Halopteris scoparia*, *Codium spp.*, *Halimeda tuna* and *Laurencia spp.* The second site (named PM) was a more interior *P. oceanica* meadow standing on a sandy bottom.

From April 2015 to March 2017, 20 sea urchins larger than 45 mm (average test diameter of 52 ± 0.16 mm and 54 ± 0.20 mm in RB and PM, respectively) were collected monthly from both sites by scuba divers at a depth of five meters. After sampling, sea urchins were stocked in iceboxes and immediately transported to the laboratory.



Figure 17 - Study sites. RB: rocky bottom; PM: *P. oceanica* meadow.

3.3.2. Gonadosomatic Index and histological analyses

In the laboratory, sea urchins were weighed and the test diameter without spines was measured. The gonads were then extracted and weighed. The Gonadosomatic Index (GSI) was calculated for all samples from each site by the formula: $GSI = [\text{gonad wet weight} / \text{total wet weight}] \times 100$ as reported by Lawrence *et al.* (1965). Since sex ratio was nearly 1:1 for both sampling sites, females and males were pooled together to obtain a single mean GSI value per month.

Their reproductive state was determined through histological analyses, which were performed monthly on 12 individuals chosen randomly among the sea urchins sampled (in each site). One of the gonads was fixed in 10% formalin, then dehydrated, embedded in paraffin, sectioned at 7 μm and stained with hematoxylin/eosin. The sections were analyzed under a binocular microscope, and classified according to the six stages described by Byrne (1990): recovery (stage I), growing (stage II), premature (stage III), mature (stage IV), partly spawned (stage V), spent (stage VI).

3.3.3. Gonad color

The same sea urchins used for the histological analysis were used for determining the color of the gonads. A colorimetric analysis was performed with a digital colorimeter (Chroma meter CR-400; Konica Minolta, Tokyo, Japan), following the guidelines of the Commission Internationale de l'Eclairage (CIE) $L^*a^*b^*$ color space (CIELAB) and Addis *et al.* (2014). The colorimeter, equipped with a pulsed xenon lamp as a light source, measures color with six silicon photocells and a standard luminant D65. The color formula defines color in terms of

three coordinate values (L^* , a^* , b^*), which locate the color of an object within a three-dimensional color space. The L^* component represents lightness, a^* is the component from green (negative values) to red/magenta (positive values) and b^* is the component between blue (negative values) and yellow (positive values) (CIE 2008). Hereafter, we define a^* as redness and b^* as yellowness, since we always obtained positive values during all the measurements. After the calibration of readings against a white plate, each gonad sample was measured four times and the mean value was used for calculations. Colorimetric analyses were performed during the second sampling year (from April 2016 to March 2017).

3.3.4. Repletion Index and diet

The feeding intensity, calculated for all the individuals from each site, was monitored monthly by an assessment of the Repletion Index (RI), using the formula $RI = [\text{gut content wet weight} / \text{total wet weight}] \times 100$ (Nedelec, 1983).

Diet was analyzed through the observation of gut contents. The digestive tract, excluding Aristotle's lantern, was separated from the gonads, wet weighed, fixed and conserved in a solution of 4% formaldehyde. Gut contents were observed monthly for each site in 10 individuals randomly selected among those used for the histological and colorimetric analyses of gonads. The gut content was spread evenly in a Petri dish for observation through a stereoscopic microscope, and analyzed by the "contact method" described by Jones (1968) and Nedelec and Verlaque (1984). The analysis of 100 contacts, each one corresponding to a specified food item, gives a good estimation of the digestive content of a sea urchin (Paul *et al.*, 1983). Each food item recognized was assigned to a specific group, as done by Chiantore *et al.* (2008) and Privitera *et al.* (2008). Starting from the taxonomic groups they used in their analysis, five main groups were created: Phaeophyceae (including Fucales, Dictyotales and Other Brown Erect Algae), Chlorophyta (including Green Algae), Non-calcified Rhodophyta (including Red Erect and Filamentous Algae), Calcified Rhodophyta (including Red Articulated and Encrusting Algae) and Magnoliophyta (including Seagrasses). Since animal taxa represented only 1% of the total gut contents, they were considered non influential and were excluded from the main groups and from further analysis. The mean percentage of contribution of each group was first calculated monthly and then the results were merged into seasons.

3.3.5. Environmental parameters

Water temperature was constantly measured by a HOBO datalogger (Onset Computer Corporation, Pocasset, Massachusetts, USA) positioned at a depth of 5 meters at the RB site.

This site was considered representative of the whole bay. For August 2016, due to the loss of the datalogger, we provided a mean sea surface temperature obtained from the data collected on www.eurometeo.com. The photoperiod was determined for the entire sampling period from the US Navy Observatory data (<http://aa.usno.navy.mil>). We chose an intermediate point between the two sites.

3.3.6. Statistical analyses

A three-way ANOVA was performed to point out the differences in GSI and RI values between sites (fixed and orthogonal factor, 2 levels, RB and PM), seasons (fixed and orthogonal factor, 4 levels, spring, summer, autumn and winter), and dates (random and nested in the former factors, 6 levels). The replicates (sea urchins) used for the analysis were 20 for each date.

Separated ANOVAs (three-way ANOVAs) were carried out to highlight the differences in L^* , a^* and b^* values between sites (fixed and orthogonal factor, 2 levels, RB and PM), seasons (fixed and orthogonal factor, 4 levels) and dates (random and nested in the former factors, 3 levels), using 12 replicates for each date.

Prior to statistical analyses, normality and homogeneity of variance were checked for all data (Kolmogorov-Smirnov test and Cochran's test, respectively). If data were not normal nor homoscedastic even after transformations, we considered ANOVA to be robust enough to depart from these assumptions since our sample was large (Box, 1954; Underwood 1997).

When overall significant differences were detected, a posteriori pair-wise comparison of means was performed using the Student-Newman-Keuls comparison test (SNK; Zar, 1989). Differences were considered significant at $p < 0.05$. All analyses were run with the GMAV software package (University of Sydney, Australia).

3.4. Results

3.4.1. GSI and reproductive cycle

The reproductive cycle followed quite a different pattern at the two sites. During the first sampling year, a GSI peak was observed at the RB site in winter (in March 2016, 5.45 ± 0.49 , mean $\pm$ standard error) (Fig. 18a). GSI persisted with similar values during the following period (spring, summer and autumn 2016) decreasing in winter 2017 when it reached the value of 2.71 ± 0.21 (Fig. 18b).

Conversely, at the PM site, the GSI peaks were observed for both sampling years during spring season only (5.71 ± 0.23 in May 2015 and 6.19 ± 0.36 in April 2017, respectively) (fig. 18a). GSI

decreased during summer, while a gradual increase was observed in the autumn of both sampling years (Fig. 18b).

At both sites, the GSI peaks corresponded to the period of maximum gonad development (mature stage) or the initial spawning period (partly-spawned stage) (Fig. 19).

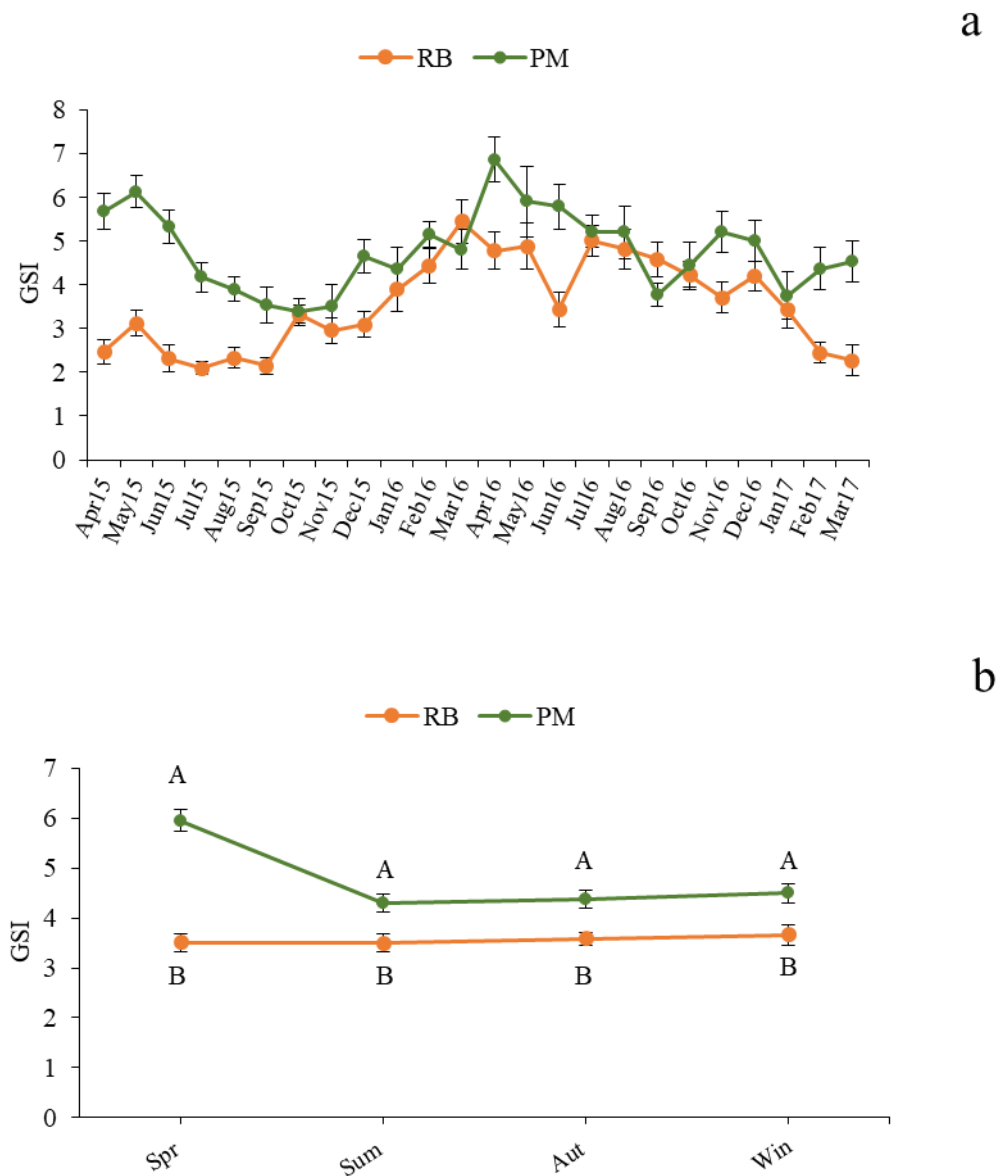


Figure 18 - Monthly (a) and seasonal (b) GSI trends at the two study sites, during the two sampling years. Capital superscripts indicate significant differences between sites.

The gametogenic cycles followed a similar trend at both sites, and no relevant interannual differences were observed (Fig. 19). Generally, gonads were in the recovery stage at the end of

spring until the first part of autumn, when they began to develop until reaching the fully mature stage during the winter season. Spawning normally began in spring at both sites (Fig. 19).

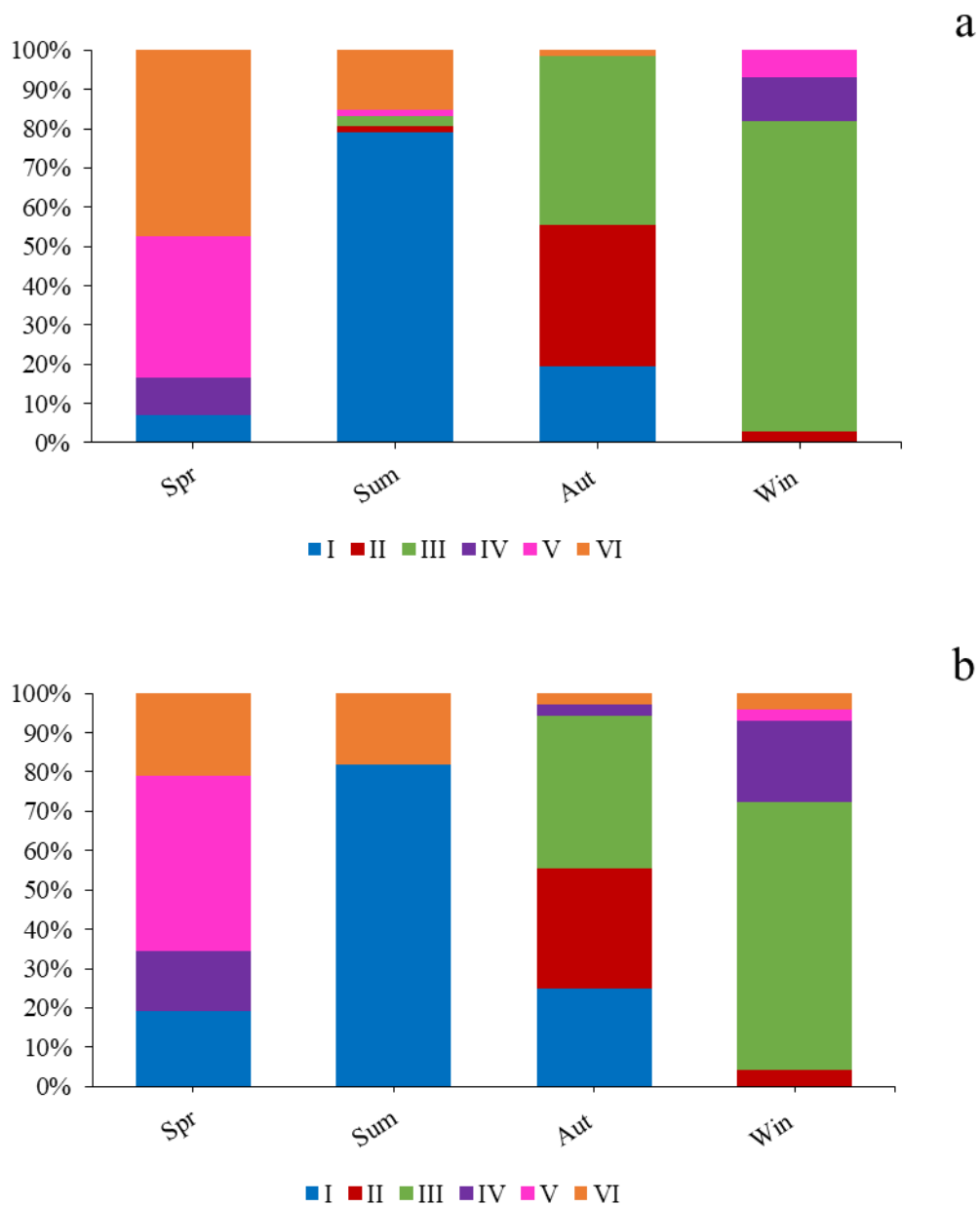


Figure 19 - Gametogenic cycles at the RB (a) and PM (b) sites, during the two sampling years.

The period of maximal gonad growth coincided with decreasing sea temperatures and a day length of approximately 12 hours at most (Fig. 20).

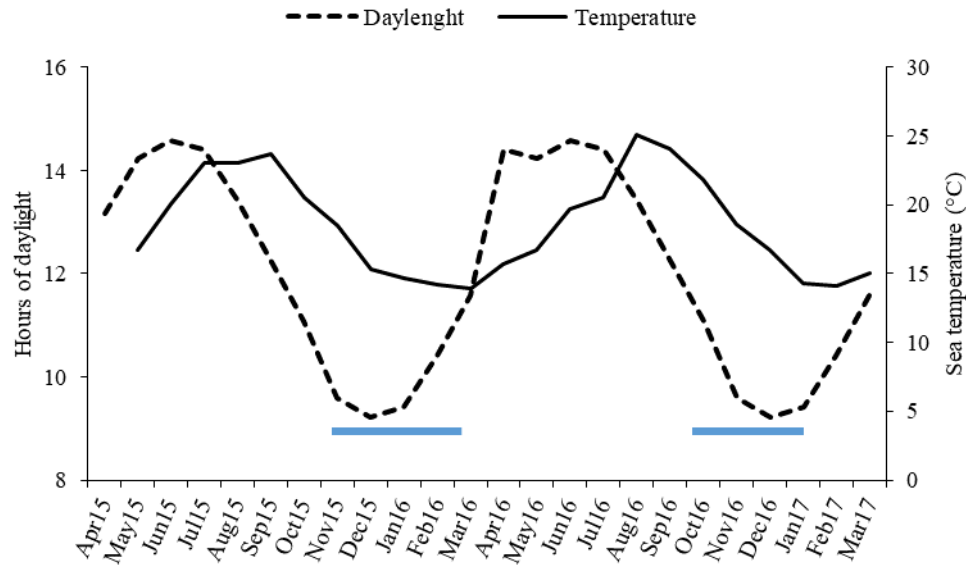


Figure 20 - Photoperiod (expressed as hours of daylight) and water temperature at Su Pallosu Bay from April 2015 to March 2017. The blue horizontal bar indicates the period of maximum gonad growth.

Analysis of variance highlighted a significantly different GSI between sites, while no differences arose among seasons (Tab. 5). SNK results showed that GSI was higher at the PM site (Tab. 5).

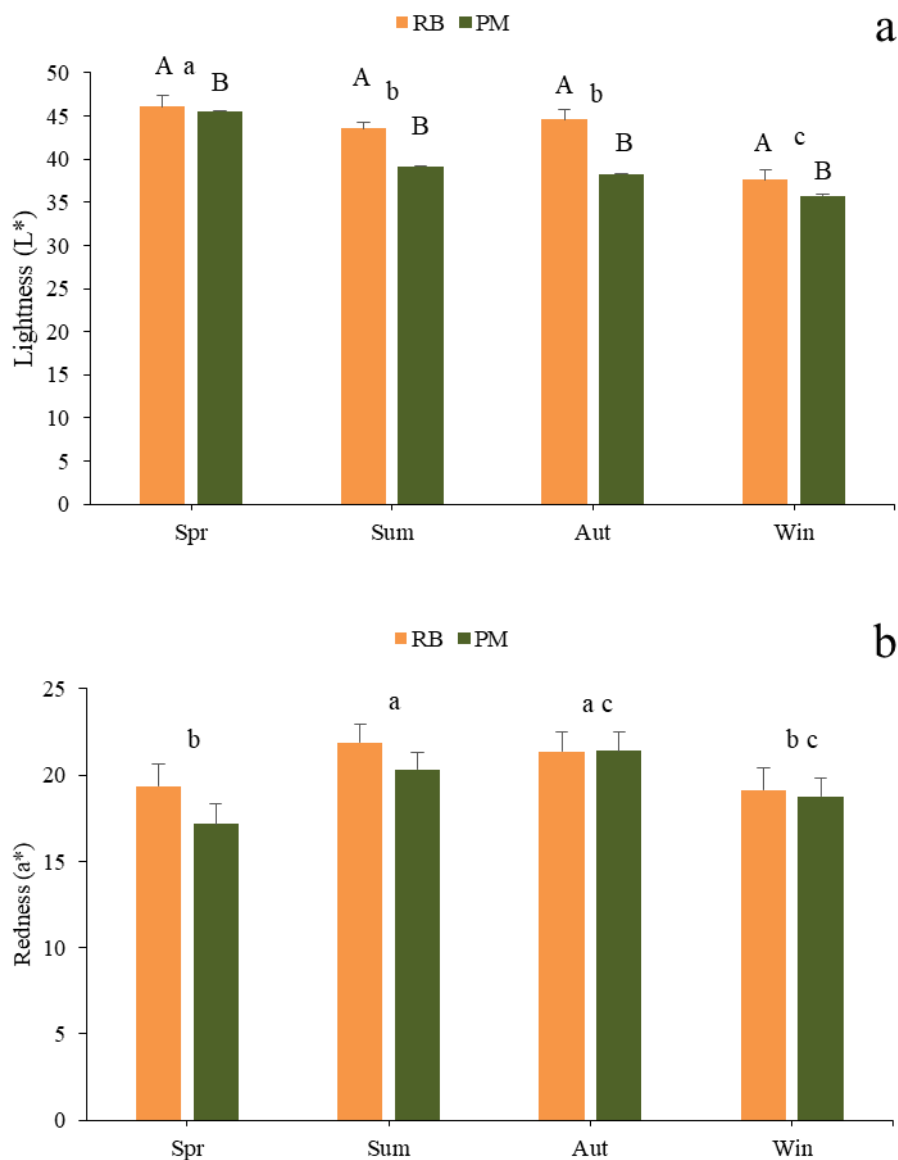
Table 5 - Results of three-way ANOVA on GSI. Analysis of variance was performed to test the effects on gonadosomatic index of Site and Season (orthogonal fixed factors) and Date (random nested factor). The values in bold are statistically significant at $p < 0.05$. SS = sum of squares; DF = degrees of freedom; MS = mean sum of squares; F = F-statistics; P = p-value.

Source of variation	SS	DF	MS	F	P
Site = Si	356,83	1	356,83	21,34	0,0000
Season = Se	102,80	3	34,27	2,05	0,1224
Date(Site x Season) = Da(Si x Se)	668,94	40	16,72	4,93	0,0000
Si x Se	120,85	3	40,28	2,41	0,0812
RES	3096,21	912	3,40		
TOT	4345,63	959			
Cochran's Test C = 0,0810 ($p < 0.01$), transf.: none					

3.4.2. Gonad color

A slight difference in the values of gonad color was observed between sites, with some seasonal fluctuations (Fig. 21). Significantly higher L^* (lightness) values were measured at the RB site; L^* changed according to season and resulted higher in spring (46.08 ± 1.34 and 45.55 ± 0.13 , at

the RB and PM site respectively) than for the remaining part of the year. The winter season was characterized by the lowest values (37.61 ± 1.19 and 35.76 ± 0.14 , respectively) (Fig. 21a and tab. 6). Redness (a^*) showed similar levels and trends between sites, with higher values during summer (21.88 ± 1.07 and 20.35 ± 0.45 , at the RB and PM site, respectively) and autumn (21.39 ± 1.13 and 21.42 ± 1.05 , at RB and PM respectively) (Fig. 21b and tab. 6). Yellowness (b^*) was higher from spring to autumn but showed the lowest values during the cold season (winter), with significantly higher values at the RB site particularly during summer and autumn (31.05 ± 1.06 and 32.07 ± 0.87 , respectively) (Fig. 21c and tab. 6).



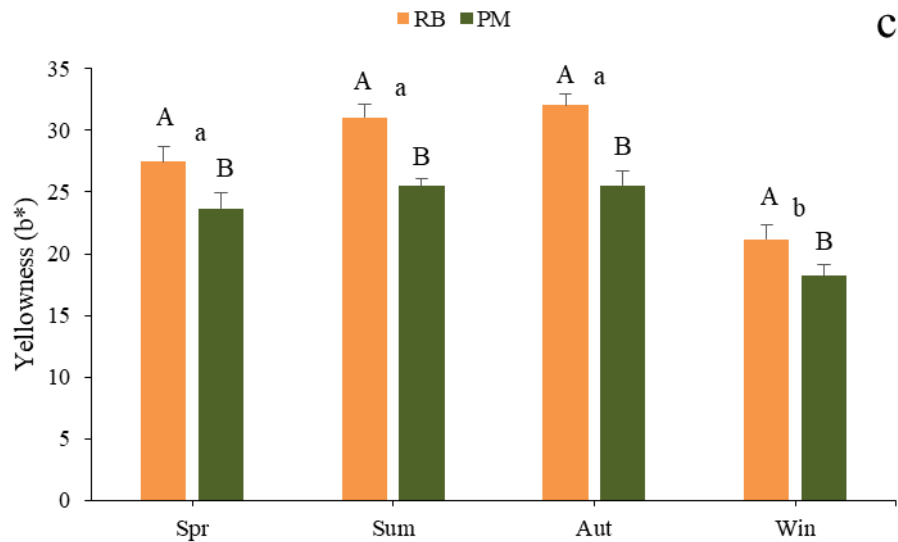


Figure 21 - Gonad L* (a), a* (b) and b* (c) values obtained by colorimetric lectures in different seasons, at both sampling sites. Capital superscripts indicate significant differences between sites; lowercase superscripts indicate significant differences between seasons.

Table 6 - Results of three-way ANOVA on gonad L*, a* and b*. Analysis of variance was performed to test the effects of Site and Season (orthogonal fixed factors) and Date (random nested factor) on the three components of gonad color. Values in bold are statistically significant at $p < 0.05$. SS = sum of squares; DF = degrees of freedom; MS = mean sum of squares; F = F-statistics; P = p-value.

	L*					a*					b*				
Source of variation	SS	DF	MS	F	P	SS	DF	MS	F	P	SS	DF	MS	F	P
Site = Si	773,85	1	773,85	13,28	0,0022	74,27	1	74,27	2,20	0,1574	1594,15	1	1594,15	12,54	0,0027
Season = Se	3002,84	3	1000,95	17,18	0,0000	525,41	3	175,14	5,19	0,0108	3775,77	3	1258,59	9,90	0,0006
Date(Site x Season)	932,08	16	58,25	1,06	0,3891	539,99	16	33,75	0,74	0,7566	2033,31	16	127,08	3,76	0,0000
Si x Se	364,52	3	121,51	2,09	0,1424	57,17	3	19,06	0,56	0,6461	142,75	3	47,58	0,37	0,7726
RES	14441,05	264	54,70			12113,93	264	45,89			8928,89	264	33,82		
TOT	19514,33	287				13310,77	287				16474,87	287			
Cochran's Test C = 0,0876 ns						Cochran's Test C = 0,1013 ns					Cochran's Test C = 0,1152 (p < 0.05), transf.: none				

3.4.3. Repletion Index and diet

RI trend was similar between sites during the entire sampling period (Fig. 22).

RI values rose in function of the reproductive seasons (i.e. winter – spring) (Fig. 22); the lowest values corresponded to the less-mature gametogenic stages (Fig. 19). ANOVA highlighted a peak during spring, independently of the site (Tab. 3).

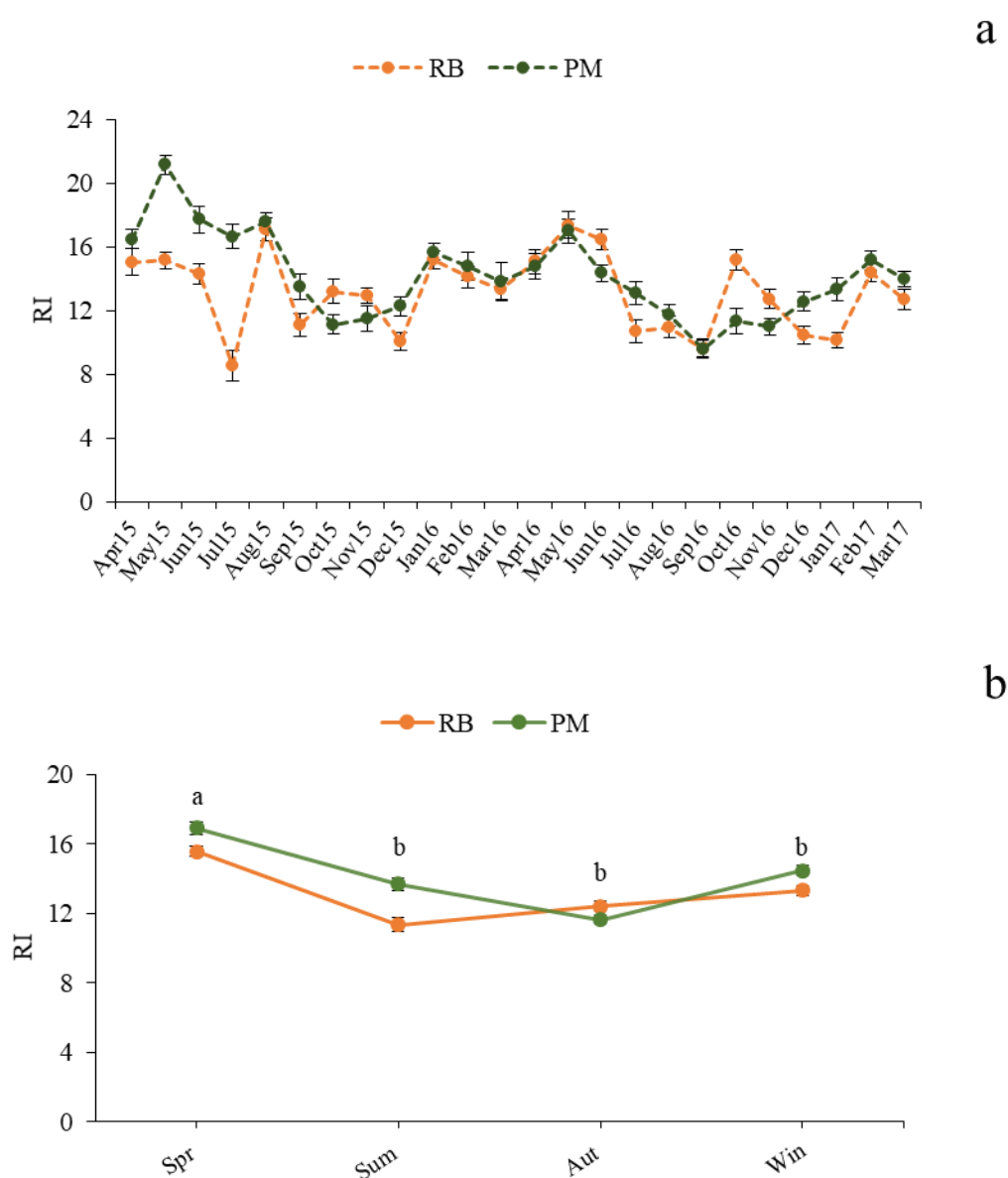


Figure 22 - Monthly (a) and seasonal (b) RI trends at the two study sites, during the two sampling years. Lowercase superscripts indicate significant differences between seasons.

Table 7 - Results of three-way ANOVA on RI. Analysis of variance was performed to test the effects of Site and Season (orthogonal fixed factors) and Date (random nested factor) on gonadosomatic index. Values in bold are statistically significant at $p < 0.05$. SS = sum of squares; DF = degrees of freedom; MS = mean sum of squares; F = F-statistics; P = p-value.

Source of variation	SS	DF	MS	F	P
Site = Si	244,72	1	244,72	2,96	0,0931
Season = Se	2561,64	3	853,88	10,33	0,0000
Date(Site x Season) = Da(Si x Se)	3307,84	40	82,70	8,82	0,0000
Si x Se	309,12	3	103,04	1,25	0,3059
RES	8550,07	912	9,38		
TOT	14973,39	959			
Cochran's Test C = 0,0574 ns					

The analysis of gut contents revealed a different percentage of contribution for the 5 main groups between sites. At the RB one, the majority of the food items found belonged to Phaeophyceae (38%) and was mainly composed of *D. dichotoma*, *P. pavonica*, *H. scoparia* and *Sphacelaria* spp., consumed especially during spring and summer. Non-calcified Rhodophyta (principally *Laurencia* spp. and filamentous species such as *Polysiphonia* spp.) represented 29% of the total food items, and were most frequent during autumn and spring. The Magnoliophyta group (21%) was represented exclusively by *P. oceanica* leaves, and was more frequent during summer and autumn. Chlorophyta (mainly *Caulerpa cylindracea*, *H. tuna* and *Codium* spp.) and Calcified Rhodophyta (mostly *Amphiroa* spp., *Jania rubens*, *Lithophyllum* spp., *Hydrolithon* spp. and *Peyssonnelia* spp.) were found but uncommon (5 and 7%, respectively) (Fig. 23a).

At the PM site, the most frequent group over the whole period was Magnoliophyta (53%, exclusively *P. oceanica*), which was particularly abundant in summer and autumn. This was followed by Non-calcified Rhodophyta (mainly filamentous species, 19%) and Calcified Rhodophyta (12%, mostly *Hydrolithon* spp.) (Fig. 23b). The algae belonging to these groups were regularly found during the year as epiphytes on the leaves of *P. oceanica*. Phaeophyceae represented 9% of the total gut contents and were mostly found during spring. Chlorophyta

corresponded to 6% of the total gut contents and were mainly found during the autumn season (Fig. 23b). *C. cylindracea* was the most represented species probably because of its abundant growth in the meadow during that period.

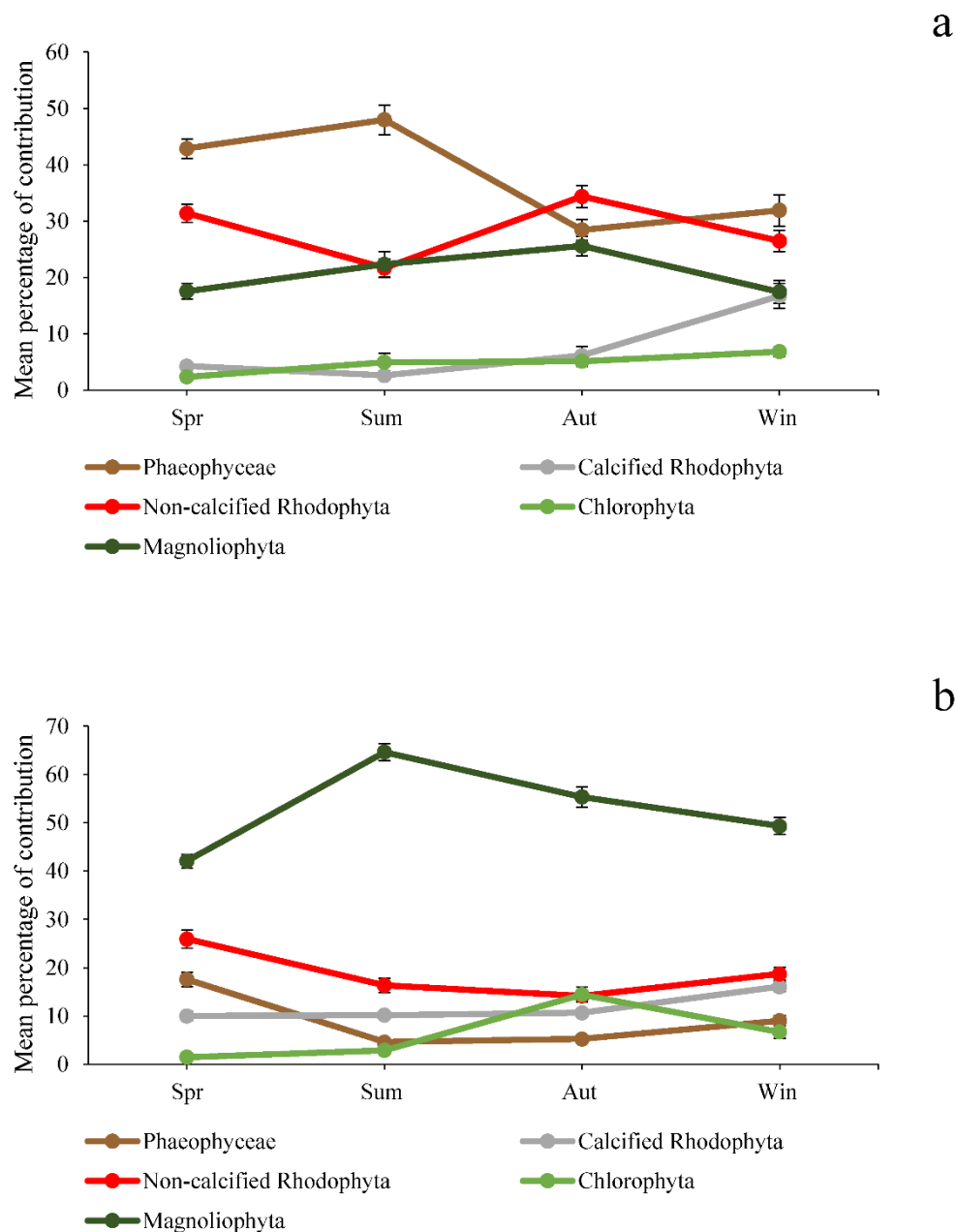


Figure 23 - Temporal trends in abundances (% mean $\pm$ se) of the different groups present in the gut content of the sea urchins harvested at the RB (a) and PM (b) site.

3.5. Discussion

Habitat has been extensively recognized as an important factor in the gonad growth of Mediterranean populations (Ouréns *et al.*, 2011). Our results suggest an influence of habitat on gonad yield. Habitat and season both influenced gonad color.

The two sites were quite different in terms of GSI. All the main GSI peaks were the prelude of a spawning event, as confirmed by the histological analyses. Each increase or drop in GSI values outside the strictly reproductive period (e.g. October 2015, July and December 2016 at the RB site, September and November 2016, January 2017 at the PM site) were considered to be changes in the gonad reserves (Sellem and Guillou, 2007).

The GSI peak at the PM site was registered yearly in the spring season. Conversely, at the RB site the highest values were observed in winter during the first year. The trend from June 2015 to March 2016 at RB was similar to what was described previously by our group (Loi *et al.*, 2017) for the same rocky site and sea urchin size class. There was a peak in March, but with GSI values lower than in 2013-2014. During the second year of the present work, the winter season showed the lowest GSI values. Generally, no clear peaks emerged throughout the year as values remained similar. The absence of a clear peak was consistent with another study on *P. lividus* by Sellem and Guillou (2007) who registered a very irregular GSI trend without emergence of an evident peak in the more exposed site called “rock”. This was in contrast with a clear temporal trend in the sheltered site called “meadow”. In our study, the GSI trend was constant from winter 2016 to autumn 2016, in spite of a seasonally different gametogenic stage distribution.

Comparing the two habitats, GSI was significantly higher at the PM site. These results are in agreement with several authors (San Martín 1995; Fernandez and Boudouresque, 1997; Boudouresque and Verlaque, 2007; Sellem and Guillou, 2007; Ouréns *et al.*, 2011) who found that sea urchin gonad indices in seagrass meadows are larger than in rocky habitats. However, they are in contrast with another Sardinian work by Addis *et al.* (2014). These last authors justified their results by pointing out the differences in food availability or in reproductive timing between habitats. We could not ascribe the differences between habitats of GSI to contrasting reproductive timing as the turnover of gametogenic stages was nearly the same at the two sites.

The feeding intensity (assessed through the calculation of the Repletion Index) did not differ between the two sampled sites. However, significant differences were evident comparing seasons. Generally, RI grew just before or during the spring season, in both sites, in connection with the period of maximum maturation of gonads or the spawning events. The relationship between feeding activity and spawning period had already been highlighted for other Mediterranean and Atlantic sea urchin populations (Semroud and Kada 1987; Rico 1989; Semroud 1993; Fernandez and Boudouresque, 1997; Bayed *et al.*, 2005; Murillo-Navarro and Jiménez-Guirado, 2012). It is likely that the increase in feeding activity during the period of maximum gonad growth is to allocate nutrients to the gonads (Sellem and Guillou, 2007).

Even though the reproductive trend we observed can partially explain the trend of RI in both sites, the gonad investment at the RB site did not correspond to the feeding intensity. Although sea urchins sampled at RB seemed to be well-fed during the whole year, and their mean RI was fairly similar to that calculated at the PM site, their mean GSI values were significantly lower in comparison with the sea urchins at PM. These observations suggest that factors other than the amount of food ingested control the intensity of the reproductive cycle and, consequently, the gonad investment. Numerous field and laboratory studies have demonstrated that somatic and gonad growth in sea urchins is directly related to diet quality, which determines the quantity of absorbed nutrients (Briscoe and Sebens, 1988; Caltagirone *et al.*, 1992; Frantzis and Grémare, 1992; Grosjean *et al.*, 1996; Cook *et al.*, 1998; Williams and Harris, 1998; Meidel and Scheibling, 1999; Schlosser *et al.*, 2005). Although no analyses of proximate and caloric content were conducted on the food categories identified in the gut contents at the two sites, the macroalgae ingested at the RB site are not supposed to be of lower quality than *P. oceanica* and its epiphytes at the PM one (Sellem and Guillou, 2007). The different gonad investment cannot be ascribed to differences in temperature or photoperiod, as the two sampled sites were nearby.

Given that the depth of harvesting was the same at each site, the different level of wave energy on sea urchins may explain the different gonad investment, acting as a limiting factor for GSI. It is known that the presence of coastal vegetation (e.g. seagrass meadows) can attenuate wave height as well as wave energy (Infantes *et al.*, 2012; John *et al.*, 2015; John *et al.*, 2016). Although hydrodynamism was not numerically quantified in this study, the sea urchins at the PM site resulted more sheltered than those at the RB one. This is because of the presence of the dense *P. oceanica* canopy which weakens the waves, thus reducing the water flow speed. Data on the effects of hydrodynamic conditions on the reproductive potential of sea urchins in

previous works are contrasting. Lozano *et al.* (1995) and Meidel and Scheibling (1998) indicated that a higher level of hydrodynamism lead to a higher gonad investment in sea urchins. These results were corroborated by James (2006) who observed higher GSI in urchins held in cages subject to increased water movement. On the contrary, Ebert (1968), Gonor (1973), and more recently Guettaf *et al.* (2000), Sellem and Guillou (2007) and Gianguzza *et al.* (2013), pointed out that echinoids had lower gonad indices at wave-exposed sites than at sheltered sites. Over a certain degree of wave exposure, all the energy derived from ingested food must be allocated to the maintenance, repair and test production (Himmelman, 1986), thus subtracting important resources from the reproductive processes. Therefore, we can hypothesize that sea urchins at the PM site had a larger gonad index because the absorbed nutrients were totally employed in the gonad investment.

The gonad color changed over seasons and between sites. Slight differences were detected between the two sampling sites, with the gonads from the RB site showing higher L* and b* values, in correspondence with a high intake of brown seaweeds. Specifically, the L* component was significantly higher in spring than during the rest of the year, with the lowest values in winter. The b* component was higher from spring to autumn. The a* component was the only one which did not differ between sites, although the highest values were observed in summer and autumn and the lowest ones in spring. The changes in the three color components between seasons may be a response to the seasonal variations in diet that were pointed out through analysis of the gut contents, since the quality of food influences the color of the gonads during the reproductive cycle (Tenuzzo *et al.*, 2012). Alternatively, they can be affected by changes in the gametogenic condition of the urchins (e.g. presence/absence of gametogenic cells) during the reproductive cycle, as stated by James and Heath (2008).

Very few works have been published which focus on gonad color in wild sea urchin populations feeding on a natural diet. In a recent study, Addis *et al.* (2014) evaluated the gonad color of two Sardinian *P. lividus* populations for a short time (January 2012), living in two different habitats, i.e. a *P. oceanica* meadow and a rocky bottom covered by a prevalence of *Cystoseira spp.* They did not detect differences in the roe color between habitats. They assumed that the content of pigments in the food available in *P. oceanica* meadows and rocky seabeds were similar, since pigments represent the major factor influencing roe coloration (Shpigel *et al.*, 2006; Symonds *et al.*, 2007). In this work, neither the content of the pigments of *P. oceanica* nor the pigments of the great variety of algal species of the rocky bottom were assessed. While the pigment

profile of *P. oceanica* was previously well-investigated (Dalla Via *et al.*, 1998; Marin-Guirao *et al.*, 2011), that of the seaweeds inhabiting rocky bottoms is fairly difficult to assess, principally because the fluctuation in physical factors throughout the seasons leads to the variation of algal species in terms of richness (Williams, 1993). This also influences their level of proximate constituents (e.g. pigments) and their metabolic responses (photosynthesis and growth rates) (Orduña-Rojas *et al.*, 2002). From the gut content analysis, we can state that the sea urchins at the RB site ingested a larger variety of food items, especially brown and red algae, compared to the urchins at the PM site. The latter mainly ingested *Posidonia* leaves. The consumption of this variety of seaweeds can contribute to a higher quantity or quality of pigments and this has probably produced brighter, yellower gonads at the RB site.

The marketability of sea urchins relies on the quality of the roe. Roe quality, and thus gonad price, depends on several factors among which size (yield) and color. Within wild populations of sea urchins, it is common for the yield and color of roe to be variable (Blount *et al.*, 2017). Gonad yield is subject to seasonal variations due to changes in the environment features, such as hydrodynamic conditions (Sellem and Guillou, 2007), seawater temperature (Byrne, 1990; Bayed *et al.*, 2005; González-Irusta *et al.*, 2009), photoperiod (González-Irusta *et al.*, 2009), algal production (Bayed *et al.*, 2005), food availability (Guidetti *et al.*, 2003; Sánchez-España *et al.*, 2004) and phytoplankton blooms (Lozano *et al.*, 1995; Bayed *et al.*, 2005; González-Irusta *et al.*, 2009). Gonad color also undergoes seasonal variations (Agatsuma, 1998; Robinson *et al.*, 2002), being dependent on the gonad development, feeding activity and food quality (Pearce *et al.*, 2002b; McBride, 2004; Kinoshita *et al.*, 2013). This variability in roe condition thus affects fishing effort, concentrating it in areas where fishermen expect the condition of sea urchin roe to be most consistent (Miller and Nolan, 2008). However, this concentration of fishing effort has led to severe depletion of wild sea urchin populations (Andrew *et al.*, 2002).

This work suggests that it is more profitable to harvest sea urchins in *P. oceanica* meadows especially in the spring, even if color seems better (i.e. results in higher values) in gonads from rocky beds, particularly from spring to autumn, in conjunction with a pretty good gonad size.

These differences in gonad production and quality may have important implications in the management of sea urchin stock, as this information can be helpful for fishery managers to define and improve conservation measures and sustainable harvesting strategies. It can also be used to increase the market value of an important economic product in Sardinia such as sea urchin roe.

Diversifying fishing areas as well as harvesting grounds throughout seasons according to the quality characteristics of gonads should be considered when developing management measures to reduce the overexploitation of wild resources. We suggest, considering *P. oceanica* meadows, which are sheltered areas where sea urchins can invest all their energy in gonad growth, as a possible alternative harvesting ground for sea urchin fisheries. Of course, a thorough understanding of abundance and population structure of sea urchins as well as the size of the meadows themselves is needed before allowing harvesting from the meadows. Where appropriate, alternating the harvesting on hard bottoms and within *P. oceanica* meadows could give a much-needed break to those overfished rocky substrates with a scarcity of commercial-sized urchins.

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4. INFLUENCE OF SEASONAL AND ENVIRONMENTAL PATTERNS ON THE LIPID CONTENT AND FATTY ACID PROFILES IN GONADS OF THE EDIBLE SEA URCHIN *PARACENTROTUS LIVIDUS* FROM SARDINIA.

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Keywords:

Paracentrotus lividus, lipids, Sardinia, sea urchin, multivariate analysis, reproductive cycle

4.1. Abstract

The influence of seasonal and environmental patterns on the lipid fraction of *Paracentrotus lividus* gonads was investigated. For this purpose, sea urchins were collected monthly over a year from two Sardinian coastal areas. Total lipids in gonads follow an annual cyclical trend, described by a sine wave curve, that is more influenced by season than by growing area. The lowest lipid content in gonads corresponds to a high percentage of mature reproductive stages (i.e. winter season), independently of sampling area. A variation in total lipid content follows a change in photoperiod, while it is related to sea surface temperature. Multivariate analysis on fatty acid profiles of gonads, detected by gas chromatography, clusters the collected specimens mainly according to the sampling area, secondly according to the sites within the same sampling area and finally according to season.

4.2. Introduction

Paracentrotus lividus is an echinoderm which prevalently populates intertidal rock habitats in the Mediterranean Sea and Atlantic European coast (Boudouresque and Verlaque, 2007). *P. lividus* is an opportunistic generalist organism able to feed on different food sources. It has an intense grazing activity on *Posidonia oceanica* seagrass meadows (Lozano *et al.*, 1995) and seaweed communities of rocky bottoms (Boudouresque and Verlaque, 2007).

P. lividus gonads represent a valued gourmet delicacy, particularly appreciated in European countries and in Japan, where they are employed as an ingredient of sushi. Their commercial value is generally associated to their size, freshness, color and texture.

Sensory parameters characterizing sea urchin gonads are likely to be simultaneously affected by either environmental, seasonal and physiological factors such as diet, hydrodynamism, climate, photoperiod, reproductive cycle, which in turn affect metabolism and chemical composition (Dincer and Cakly, 2007; Mol *et al.*, 2008; Phillips *et al.*, 2009).

It has been pointed out that dietary lipids have a key role in characterizing the fatty acid profile of sea urchin gonads (Castell *et al.*, 2004; Martinez-Pita *et al.*, 2010a, b; Carboni *et al.*, 2013). However, although a number of experimental evidences of the correlation between dietary fatty acids and gonad fatty acid composition was suggested, a straightforward predictive model cannot be built before the influence of all endogenous and exogenous factors involved is appraised.

Diet, gametogenesis and water temperature may have a marked influence, promoting the indispensable mechanisms of synthesis, selective storage and mobilization of some fatty acids to meet nutritional requirements (Martinez-Pita *et al.*, 2010a, b).

Depending on the maturation stage, gonads contain a different ratio of germinative (ova and spermatozoa) vs. somatic (nutritive phagocytes) cells, and are generally characterized by a different gonado-somatic index (GSI), not strictly correlated to each other (Byrne, 1990; Lozano *et al.*, 1995; Soualili and Guillou, 2009). The observed periodical alternation of gonad structure at cellular level reflects the dual function of gonads as storage tissue and reproductive organ (Hughes *et al.*, 2006). Evidently, the energetic balance of sea urchins is variably influenced by both environmental and physiological factors acting in strict combination. However, an analytical parameter that can unambiguously predict the expected periodical changes occurring in sea urchin gonads is still lacking. Likely, this is due to the complex interplay of environmental and physiological factors in affecting gonad composition. Finding new ways to describe the result of this relationship at both spatial and seasonal level may be of great importance (Hughes *et al.*, 2006).

In order to unravel such complexity, this paper discusses an analytical investigation on the lipid fraction of *P. lividus* gonads sampled monthly from two different areas of the Sardinian coast over one year. It aims, first, to modelling the periodic fluctuation of total lipids of sea urchin gonads; secondly, to describe, by a multivariate analysis of gonad fatty acids composition, the relevance of the different environmental and physiological factors in differentiating the composition of gonad lipids.

4.3. Materials and Methods

4.3.1. Samples collection

Specimens of *P. lividus* of commercial size (test diameter ≥ 50 mm without spines) were collected monthly from June 2013 to May 2014, except November 2013 due to adverse weather conditions, from two Sardinian coastal areas: Tavolara Island and Sinis Peninsula, as shown in Fig. 24. In the following, the two areas will be referred to as Tavolara and Sinis, respectively. Within each area, two different sites have been identified, namely A and B, located approximately 1-3 Km from each other.



Figure 24 - Maps reporting sampling areas along Sardinian coast (Italy).

Tavolara is located along the North-eastern coast of Sardinia inside the homonymous Marine Protected Area. The sampling sites are located on the north of the islands Tavolara and Piana, on hard bottoms of granite. Both sites are exposed to North-eastern winds; they are therefore relatively sheltered from the prevailing winds.

Sinis is located along the central western coast of Sardinia at the North of the Sinis Peninsula. The sampling sites are located on sandstone-carbonate substrates and are exposed to prevailing winds blowing from North-west. Both areas are characterized by photophilic algal communities and differ mainly for the exposure to the prevailing winds and the mineralogical composition of rocks.

At each sampling date, sea urchins for both lipid and histological analyses were collected from each site within each area. Then, part of all specimens collected has been addressed to lipid analyses and the remaining were left for histology.

Lipid content and fatty acid profiles were determined on two groups of fifteen individuals, collected from each site at each sampling date. In total, $N = 30$ individuals per site (i.e. $N = 60$ per area) were allotted to lipid analyses at each sampling date. Sea urchins were immediately transferred to the laboratory alive, then opened, and gonads from each group were removed and pooled together into a Petri dish, obtaining two pools per site. Samples were thus frozen in liquid nitrogen and stored at -80°C until extraction. At each sampling date, histological analyses were done on at least 5 sea urchins of commercial size per site (i.e. $N = 10$ per area).

4.3.2. Lipid extraction

Frozen pools were crushed by using a pre-cooled stainless-steel mortar filled with liquid nitrogen. Then, 10 g of the fine frozen powder obtained were freeze-dried (Cinquepascal s.r.l., Milano, Italy, mod. Lio5P) for 20 h. Lipid extraction was carried out in duplicate on lyophilized powders following the Bligh and Dyer method (1959) modified by Anedda *et al.* (2013), on about 1.25 g of accurately weighed samples. The lipid extracts were finally evaporated to constant weight under a gentle stream of nitrogen gas.

The extracted lipids were stored at -30°C until methylation and GC analysis. Total lipid content will be reported in the following as a percentage of dry gonad tissue (dry weight percentage, % DW). All chemicals and solvents were of analytical grade and were purchased from Sigma Aldrich (St. Louis, MO, USA).

4.3.3. GC analysis of fatty acids

Fatty acids were methylated with a modified version of the method proposed by Antongiovanni *et al.* (2007). Lipid extract (10 mg) was dissolved in 1 mL of a hexane solution of internal standards, containing 1 mg/mL each of methyl valerate, methyl nonanoate, methyl tridecanoate, methyl nonadecanoate. Hence, 40 mL of methanolic KOH 2N were added. The mixture was vortexed for 1 min at room temperature and then the hexane phase was analyzed by GC. Methylations were carried out in duplicate for each lipid extract (resulting in a total of eight methylations per site). Mixtures of fatty acid methyl esters (FAMES) were analyzed using a modified version of the method proposed by Santercole *et al.* (2012), using one temperature program with a 175°C plateau.

Each (FAMES) was identified using pure FAME reference materials, a custom reference mixture and Menhaden fish oil analytical standard (Sigma Aldrich, St. Louis, MO, USA). All chemicals and solvents were of analytical grade and were purchased from Sigma Aldrich (St. Louis, MO, USA).

4.3.4. Histological study

A section of one of the five gonads was fixed in 10% formalin, dehydrated, embedded in paraffin, sectioned at 7 µm by a rotary microtome and stained with Hematoxylin (Sigma Aldrich, St. Louis, MO, USA) / Eosin (Carlo Erba Reactifs, Val De Reuil Cedex, France). Reproductive stage was assigned to each sample using the nomenclature of Byrne (1990): stage I (recovery), stage II (growing), stage III (premature), stage IV (mature), stage V (partly

spawned), stage VI (spent). Gonads in recovery stage (I) contain nutritive phagocytes forming a meshwork across the acinus, and few germ cells (oocytes and spermatocytes) along the acinal wall that, during the growing stage (II), begin to increase in size. In premature gonads (III), nutritive phagocytes are displaced from the centre by the first mature gametes accumulating in the lumen of the acinus (mature ova and spermatozoa). Mature gonads (IV) are packed with mature gametes whereas the nutritive phagocytes are limited to the periphery. In partly spawned stage (V), gonads are characterized by thin acinal wall and spaces in the acinal lumen due to the spawning of mature gametes. Spent gonads (VI) appear empty except for relict gametes; a pale meshwork of nutritive phagocytes around the periphery starts to sequester reserves for the next reproductive cycle, including material phagocytosed from relict gametes.

4.3.5. Photoperiod and sea surface temperature (SST)

Photoperiod was determined from the US Navy Observatory data (<http://aa.usno.navy.mil>), using the following GPS coordinates: Tavolara E 009 40 N 40 53 and Sinis E 008 25 N 40 03. The data associated to the two areas were then averaged.

SST data were obtained from the “Mediterranean Sea - High Resolution and Ultra High Resolution L3S Sea Surface Temperature” product (<http://marine.copernicus.eu/web/69-myoccean-interactive-catalogue.php>). Mean daily temperature registered by satellites was extrapolated from the MyOcean catalogue, choosing an intermediate point between the two stations within the sampling areas.

4.3.6. Fitting of experimental data

Fitting of experimental data was carried out using algorithms included in the Statistic Toolbox™ of Matlab R2014a version (The Mathworks Inc., Natick, MA, USA). Total lipid content of gonads, photoperiod and SST were preliminarily modelled using a total of 156 equations, comprising several polynomial (35), rational (40), co-sinusoidal (32), Gaussian (25), and differential (24) functions. The best fitting equation, characterized by the higher ranking value, was selected among the abovementioned 156 functions. Optimization of the selected equation parameters was performed following the non-linear regression procedure (NLIN) of the Statistical Analysis System (SAS Institute. 1987. SAS user's guide: Statistics. SAS Institute, Cary, N.C.).

4.3.7. Monovariate and multivariate statistical analyses (MVA)

All experimental values are reported as their mean and standard deviation (SD). Statistical differences among mean values of total lipids and GC FAMES between samples from Tavolara and Sinis were estimated using Student's t-test with a P value of less than 0.05 for the rejection of the null hypothesis. One-way analysis of variance (ANOVA), followed by Tukey's test, was used to estimate seasonal differences in total lipid content ($P < 0.05$). All monovariate statistical analyses were carried out by using GraphPad Prism 5.03 (GraphPad Software Inc., La Jolla, CA, USA).

MVA was carried out for the entire GC FAMES dataset (44 samples, each with 42 variables), after data centering and pareto scaling (Wold *et al.*, 1993) and using mean percentage values for each sampling area and site in each sampling date. Both unsupervised approaches (principal component analysis, PCA) (Jolliffe, 2002), and supervised orthogonal partial least square discriminant analyses (OPLS-DA) (Trygg *et al.*, 2007) were used. From the OPLS-DA modelling, the GC FAMES that contribute significantly to the observed discriminations were identified by evaluating variable predicted importance (VIP) scores, with a cut-off value of 1. Goodness of all MVA models was evaluated by the cumulative R^2 (cum) and the predictive Q^2 (cum) parameters, calculated according to the cross-validation method (Eastment and Krzanowski, 1982; Martens and Naes, 1989). All multivariate analyses were carried out by using the software SIMCA-P v.13 (Umetrics Inc., Kinnelon, NJ, USA).

4.4. Results

4.4.1. Histological analyses

Fig. 25 shows the trend of reproductive stages observed in Tavolara and Sinis by pooling, for each area, the data from both sites. From June to October, samples from Sinis were mostly in the recovery stage (Stage I). Premature individuals (Stage III) appeared in December when gametogenesis started. Premature and mature (Stage IV) individuals were well represented for the whole winter season. The main spawning event occurred in April and was followed by a high percentage of spent individuals (Fig. 25).

In Tavolara, a somewhat more heterogeneous development was highlighted. Urchins were still spawning in June and July; the recovery stage was well represented from July to October, although low percentages of premature and mature individuals were still observed in the same period. These individuals turned into spent stage in December. Similarly, to what observed in

Sinis, gametogenesis began in December, but continued only up to March, and fully mature individuals were found in the last two winter months (February, March). The main release of gametes occurred in March; in April, all the individuals already spawned and were mainly in the spent stage (Fig. 25).

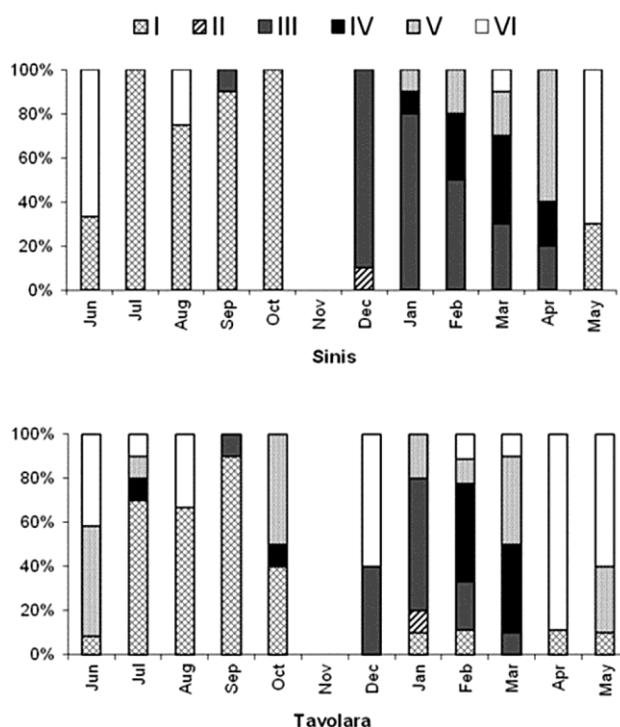


Figure 25 - Histograms of reproductive stages observed by histology in *P. lividus* gonads over one year in Sinis and Tavolara (data from both sites were pooled). Reproductive stages I-VI were assigned according to Byrne (1990).

4.4.2. Total lipid content of gonads

The analysis of total lipid content in gonads of *P. lividus* shows that mean values are not statistically different between Tavolara and Sinis over one year, except for the samples collected in May, when a small difference among areas ($P = 0,0493$) was detected (Table 8). Even more so, no significant difference was found between the two sites of each area.

Considering that lipid content did not appear to be significantly influenced by the area, further investigations were directed to the analysis of average lipid contents associated to each month, regardless the area of collection. In particular, data reported in Fig. 26 correspond to grand mean values between mean lipid content in Tavolara and mean lipid content in Sinis for each month. As well as for photoperiod and SST, an annual cyclic trend was observed for the total lipid content (%DW) of *P. lividus* gonads (Fig. 26). The trend of total lipid content throughout

the year is characterized by a maximum peak during the warm season, a minimum in the cold season, while the inflection point is observed between October and November. In particular, in each area, the maximum and minimum total lipid values were reached in July and in January, respectively (Table 8). A significant variation in lipid content ($P < 0.05$) can be found when summer and winter values are compared, as shown by letters reported in Fig. 26. Moreover, it is worth noting that the variability in the lipid content of gonads in the period from December to April was lower than that evidenced from June to October and in May, as reflected in the error bars. Clearly, a more relevant variability is observed during the summer than in winter (Fig. 26). Furthermore, comparison between the seasonal variation of lipid content and the gametogenic cycle suggests that lipid uptake in sea urchin gonads is favored during the advanced stages of gametogenesis, since premature (III) and mature (IV) stages are mainly present in the cold season (i.e. from December to March-April in both areas) (Fig. 25).

Table 8 - Seasonal evolution of total lipid content in gonads of *P. lividus* over one year in the two areas of Tavolara and Sinis. Reported values represent monthly mean values of A and B sites within each area (% DW (SD)). (N = 8).

Month	Tavolara	Sinis
June	18.4 (0.70)	18.27 (1.07)
July	19.26 (1.71)	18.92 (0.76)
August	19.16 (1.63)	18.32 (0.91)
September	16.96 (1.59)	17.49 (0.88)
October	17.82 (1.50)	18.42 (1.00)
December	16.98 (1.12)	16.55 (0.49)
January	15.52 (1.16)	15.63 (0.65)
February	15.54 (1.27)	15.69 (0.93)
March	16.25 (1.01)	15.72 (0.47)
April	16.33 (1.06)	16.39 (0.46)
May	17.78 (1.05)	19.16 (1.47)*

Note: * indicates statistical difference within the same month ($P < 0.05$).

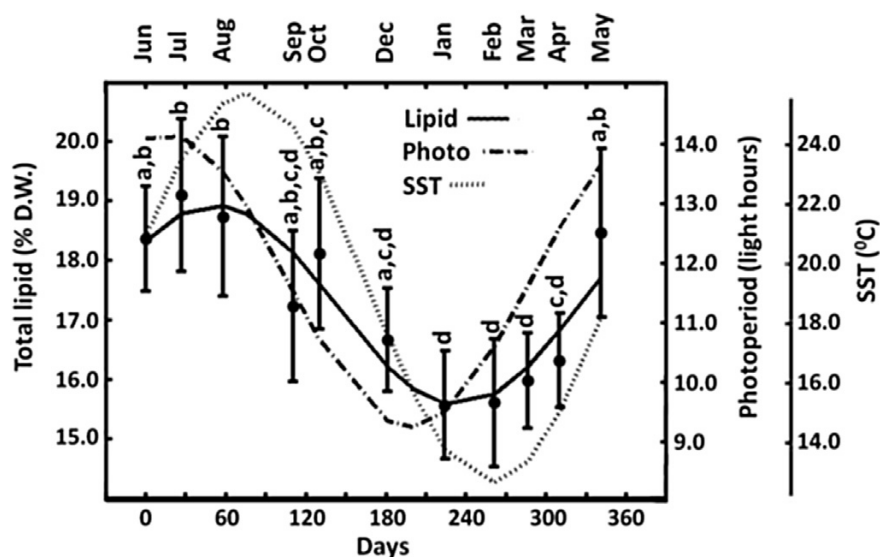


Figure 26 - Seasonal variation (monthly grand mean values between mean lipid content in Tavolara and mean lipid content in Sinis) of total lipids content in gonads of *P. lividus* compared with fitted photoperiod and SST. Black circles and solid line represent experimental data and fitted residuals (according to Eq. (1)) of total lipids. Means sharing same letters stand for not significant differences ($P > 0.05$).

By comparing the seasonal evolution of total lipids, photoperiod and SST, it is possible to observe that a change in the total lipid content of gonads follows a change in the photoperiod, while it precedes the corresponding change in SST. To better analyze such changes, experimental data were fitted by proper mathematical functions. The following sine wave equation was selected to fit experimental data:

$$y = y_0 + A \sin\left(\pi \frac{x + \phi}{w/2}\right) \quad (1)$$

where y_0 is the vertical shift, A is the phase amplitude, ϕ is the phase shift and w is the period, which refers to an average solar year duration, fixed at 365,242 days (Table 9). Since ϕ gives a measure of the curves displacement-time, it can be used to compare curves since they all share the same w . Therefore, it can be observed that ϕ of photoperiod exceeds of 36 days the ϕ of total lipids, while ϕ of sea surface temperature is 25 days shorter than the ϕ of total lipids. Fit goodness index, reflected in the R^2 value, shows high correlation ($R^2 > 0.8$) with the sine wave modelling for total lipids, photoperiod and SST experimental data.

Table 9 - Total lipids of gonads, photoperiod and SST models. Summary of the best estimated parameters: y_0 vertical shift, A amplitude shift and ϕ phase shift, with standard errors (SE) and fit goodness index, R^2 , with related root mean square errors (RMSE), calculated according to the Eq. (1) with a fixed wavelength w .

Models	y_0	A	ϕ	w	R^2
Photoperiod (light hours)	12.195 (0.015)	2.826 (0.021)	76.861 (0.439)	365.242	0.999 (0.071)
SST (°C)	18.623 (0.094)	18.623 (0.094)	15.542 (1.241)	365.242	0.929 (1.160)
Total lipids (%DW) of gonads	17.272 (0.167)	1.683 (0.239)	40.620 (7.980)	365.242	0.861 (0.545)

4.4.3. Gonad fatty acids composition

Fatty acid composition of *P. lividus* gonads is reported as mean values of the samples collected from June 2013 to May 2014 in each sampling area (Table 10).

Polyunsaturated fatty acids (PUFAs) are the dominant FA class, representing approximately 40% of the total. EPA (Eicosapentaenoic acid, C20:5 n-3) and ARA (Arachidonic acid, C20:4 n-6) represent the main components of the n-3 and n-6 PUFAs families respectively. Saturated fatty acids (SFAs), corresponding to approximately 30% of total FAMES, represent the second largest class, with C16:0 being the main FA, followed by C14:0 and C18:0. Finally, monounsaturated fatty acids (MUFAs) sum to slightly over 20% of all FAMES. Tavolara and Sinis areas showed significant statistical differences associated to the fatty acid composition of gonads. Concerning the main PUFAs, the percentage of ARA did not show any significant difference between Tavolara and Sinis, whereas EPA content is significantly different in the two sampling areas ($P < 0.01$). Among the SFAs, only C16:0 showed highly significant different values ($P < 0.001$) between Tavolara and Sinis. Regarding MUFAs, all the C18:1, C20:1 and C22:1 isomers, together with C16:1 5c and C16:1 9c showed a strongly different statistic associated to the sampling area ($P < 0.001$), whereas less relevant differences were found in C24:1 15c and C14:1 9c ($P < 0.01$).

Table 10 - Mean percentages values (SD) of GC FAMES in gonads of *P. lividus* collected from June 2013 to May 2014. N = 44 per each area.

Fatty acid	Tavolara	Sinis
C14:0	8.37 (0.63)	8.23 (0.86)
C15:0	0.92 (0.13)	1.02 (0.10)**
C16:0	15.14 (0.66)	16.46 (0.87)***
C17:0	0.25 (0.05)	0.25 (0.05)
C18:0	2.45 (0.20)	2.49 (0.12)
C20:0	0.35 (0.04)	0.43 (0.04)***
C21:0	2.49 (0.23)	3.39 (0.26)***
C14:1 9c	0.33 (0.20)	0.22 (0.05)*
C16:1 5c + C16:1 6t	0.64 (0.12)	0.37 (0.06)***
C16:1 7c	0.23 (0.02)	0.24 (0.02)
C16:1 9c	4.64 (0.60)	2.98 (0.58)***
C16:1 11c	1.07 (0.24)	1.00 (0.24)
C18:1 9t	0.26 (0.03)	0.22 (0.03)***
C18:1 9c	1.45 (0.18)	2.05 (0.28)***
C18:1 11c	3.85 (0.20)	2.85 (0.50)***
C18:1 15c	0.17 (0.05)	0.13 (0.02)***
C20:1 5c	3.14 (0.31)	3.62 (0.35)***
C20:1 7c + C20:1 8c + C20:1 9c	0.81 (0.16)	0.63 (0.11)***
C20:1 11c	2.45 (0.39)	3.76 (0.67)***
C20:1 13c	1.00 (0.12)	0.84 (0.15)***
C22:1 11c+C22:1 13c	3.48 (0.26)	4.93 (0.61)***
C18:3 n-3 (ALA)	1.73 (0.53)	2.96 (0.77)***
C18:4 n-3	0.17 (0.03)	0.17 (0.03)
C20:4 n-3	0.72 (0.12)	0.66 (0.14)
C20:5 n-3 (EPA)	13.64 (1.79)	12.28 (1.48)**
C21:5 n-3	0.13 (0.03)	0.03 (0.02)***
C22:5 n-3	0.73 (0.10)	0.58 (0.07)***

C22:6 n-3 (DHA)	1.94 (0.53)	0.89 (0.11)***
C16:2 n-4	0.46 (0.13)	0.17 (0.05)***
C16:3 n-4	0.23 (0.05)	0.09 (0.05)***
C20:2 n-4	0.58 (0.08)	0.68 (0.09)***
C18:2 n-6 (t)	0.13 (0.07)	0.09 (0.05)*
C18:2 n-6	1.53 (0.36)	1.96 (0.35)***
C18:3 n-6	0.52 (0.08)	0.38 (0.03)***
C20:2 n-6	1.87 (0.27)	2.68 (0.43)***
C20:3 n-6	0.41 (0.05)	0.54 (0.03)***
C20:4 n-6 (ARA)	12.23 (1.98)	11.23 (1.52)
C22:4 n-6	0.62 (0.19)	0.53 (0.09)*
C22:5 n-6	0.67 (0.10)	0.40 (0.13)***
NMI C20:2	3.20 (0.43)	2.60 (0.56)***
Phe	0.31 (0.12)	0.18 (0.03)***
SFA	29.97 (1.12)	32.27 (1.59)***
MUFA	23.53 (0.86)	23.84 (1.04)
PUFA	41.51 (1.39)	38.91 (2.35)***
n-3	19.06 (1.56)	17.56 (1.48)**
n-6	17.98 (1.96)	17.81 (1.74)

Note: *, **, *** indicate significant difference in each GC FAME between the two areas, with $P < 0.05$, $P < 0.01$ and $P < 0.001$.

NMI – non-methylene interrupted; Phe - methyl phytenate

4.4.4. Multivariate analysis of GC FAME data

MVA was carried out on GC FAME profiles collected from both Tavolara and Sinis sampling areas over the year. PCA scores plot (Fig. 27a) shows no outliers and evidences clustering of the samples according to sampling area (i.e. Tavolara and Sinis), with R2X (cum) and Q2X (cum) values of 0.657 and 0.582, respectively. Both these parameters appear to be above the threshold limit values for a good biological model ($Q2X \text{ (cum)} > 0.5$).

Supervised OPLS-DA can also be implemented on the same GC FAMES data, using a priori classification of the samples based on the sampling area (Fig. 27b). Results are in agreement with the clustering observed by unsupervised PCA of the same data, but a clearer distinction is observed between the two clusters associated to the area (Sinis or Tavolara) where sea urchins were collected. Indeed, OPLS-DA classification according to the sampling area shows an excellent model goodness, with R^2Y (cum) and Q^2 (cum) > 0.9 and R^2X (cum) < 0.7 .

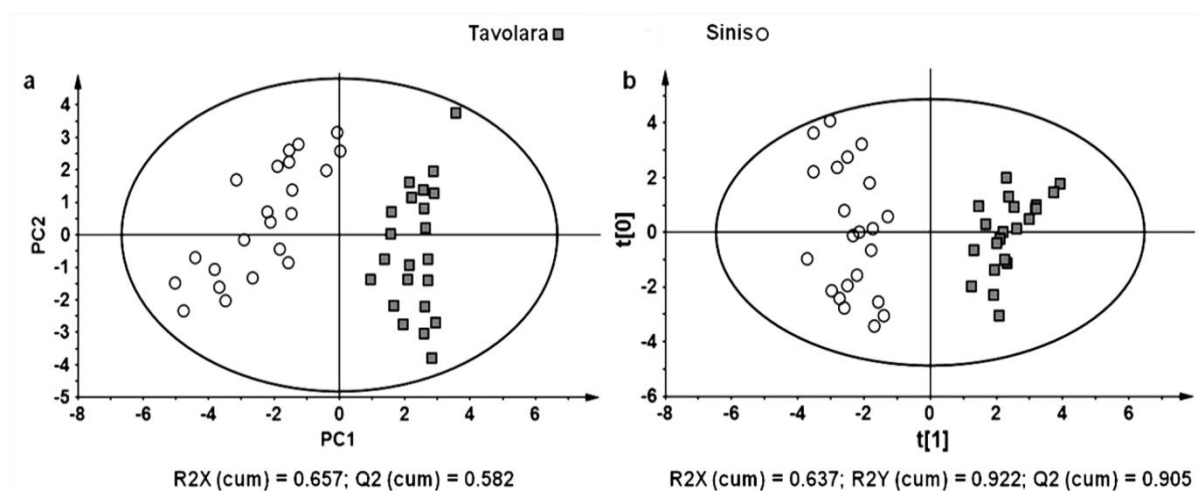


Figure 27 - a) PCA scores plot based on the GC FAMES profiles of *P. lividus* gonads of all specimens collected over one year period; **b)** OPLS-DA scores plot based on GC FAMES profiles according to the two areas of origin. R^2X (cum) and Q^2 (cum) for PCA and R^2X (cum), R^2Y (cum) and Q^2 (cum) for OPLS-DA are reported at the bottom of each graph. The black ellipses drawn represent T2 Hotelling's plots with 95% confidence.

The most discriminant fatty acids for such clusterization are described by the Variable Importance on Projection (VIP) scores (Table 11 - column AREA). As it clearly appears, MUFAs C16:1 9c and C22:1 11c + C22:1 13c (VIP > 2) possess the highest VIP value in discriminating FA profiles of *P. lividus* gonads according to the sampling area. In the order from the most relevant to the least relevant (according to VIP values), they are followed by other MUFAs, SFAs and PUFAs, namely C20:1 11c, C16:0, DHA (docosahexaenoic acid, C22:6 n-3), ALA (α -linolenic acid, C18:3 n-3), C18:1 11c and C20:2 n-6 ($2 > \text{VIP} > 1.5$).

Table 11 - GC FAMES predicted VIP scores derived from the OPLS-DA classifications according to area (Tavolara and Sinis), sites (A and B within each area) and season (warm or cold season). Only variables with VIP score > 1 are reported.

Fatty acid	AREA	SITE		SEASON	
		Tavolara	Sinis	Tavolara	Sinis
C16:1 9c	2.259		1.760	2.120	
C22:1 11c + C22:1 13c	2.135		2.254		
C20:1 11c	1.950		1.497		
C16:0	1.800	1.201	1.059		1.919
C22:6 n-3 (DHA)	1.800	2.205			
C18:3 n-3 (ALA)	1.781		2.259	1.184	
C18:1 11c	1.732		2.516		
C20:2 n-6	1.510			1.444	1.888
C20:5 n-3 (EPA)	1.412	4.162	2.516	1.352	2.822
C18:1 9c	1.330	1.185	1.034		1.214
NMI C20:2	1.073		1.603	1.895	1.458
C20:1 5c	1.033			1.150	1.544
C20:4 n-6 (ARA)	1.021	1.899	2.424	3.958	2.780
C16:1 11c		1.621			1.170
C22:4 n-6		1.455			
C18:2 n-6		1.226		1.471	1.505
Phe		1.074			
C20:1 7c + C20:1 8c + C20:1 9c		1.025			
C14:0			1.629	1.039	2.093
C16:2 n-4				1.017	
C20:1 13c			1.144		
C22:5 n-6			1.132		
C20:4 n-3			1.033		

Supervised OPLS-DA has been implemented on the same GC FAMES data, using a priori classification of the samples according to the sampling site (A and B) within each area (Fig.

28a and b). Due to the fewer samples considered in site-discrimination OPLS-DA (Fig. 28a and b) with respect to those relative to area discrimination (Fig. 27b), the former model is characterized by a slightly lower, though well validated, discrimination goodness. This is reflected in the cross-validation parameters R²X (cum) and Q²X (cum) (<0.9) (Fig. 28a and b).

The OPLS-DA score plots related to site-associated compositional differences, and the corresponding VIP ordered GC FAMES list (SITE column in Table 11), individuate discriminant GC FAMES responsible of the observed grouping of samples, and show that they are different depending on whether Tavolara or Sinis are considered. This implies that the growing site, in a given area, may result in a specific gonad composition. As far as the area of Tavolara is concerned, the main discriminant GC FAME is the n-3 PUFA EPA, with larger VIP value (4.162), followed by n-3 DHA (VIP>2). On the other hand, for Sinis several GC FAMES having $2.25 < \text{VIP} < 2.5$ were found, such as C18:1 11c and C22:1 11c+13c among MUFAs, and ARA and ALA among PUFAs.

A third OPLS-DA model was implemented according to a priori classification criteria based on grouping collected specimens in two clusters: one containing samples collected in both areas from May to October, corresponding to the warm season, and the other containing samples collected from December to April, corresponding to the cold season (Fig. 28c and d).

Compared to the previous supervised modellings (Figs. 27b, 28a and b), the OPLS-DA classification according to seasonal variation has the lowest goodness parameters (R²X (cum), R²Y (cum) and Q²X (cum) (<0.7)) in both Tavolara and Sinis. Notably, ARA and EPA are the only GC FAMES having relevant VIP value in all OPLS-DA based classifications (column SEASON in Table 11). In particular, VIP value of ARA in seasonal differentiation (3.958) was much higher than in area (1.021) and site discrimination models (1.899 and 2.424 in Tavolara and Sinis, respectively).

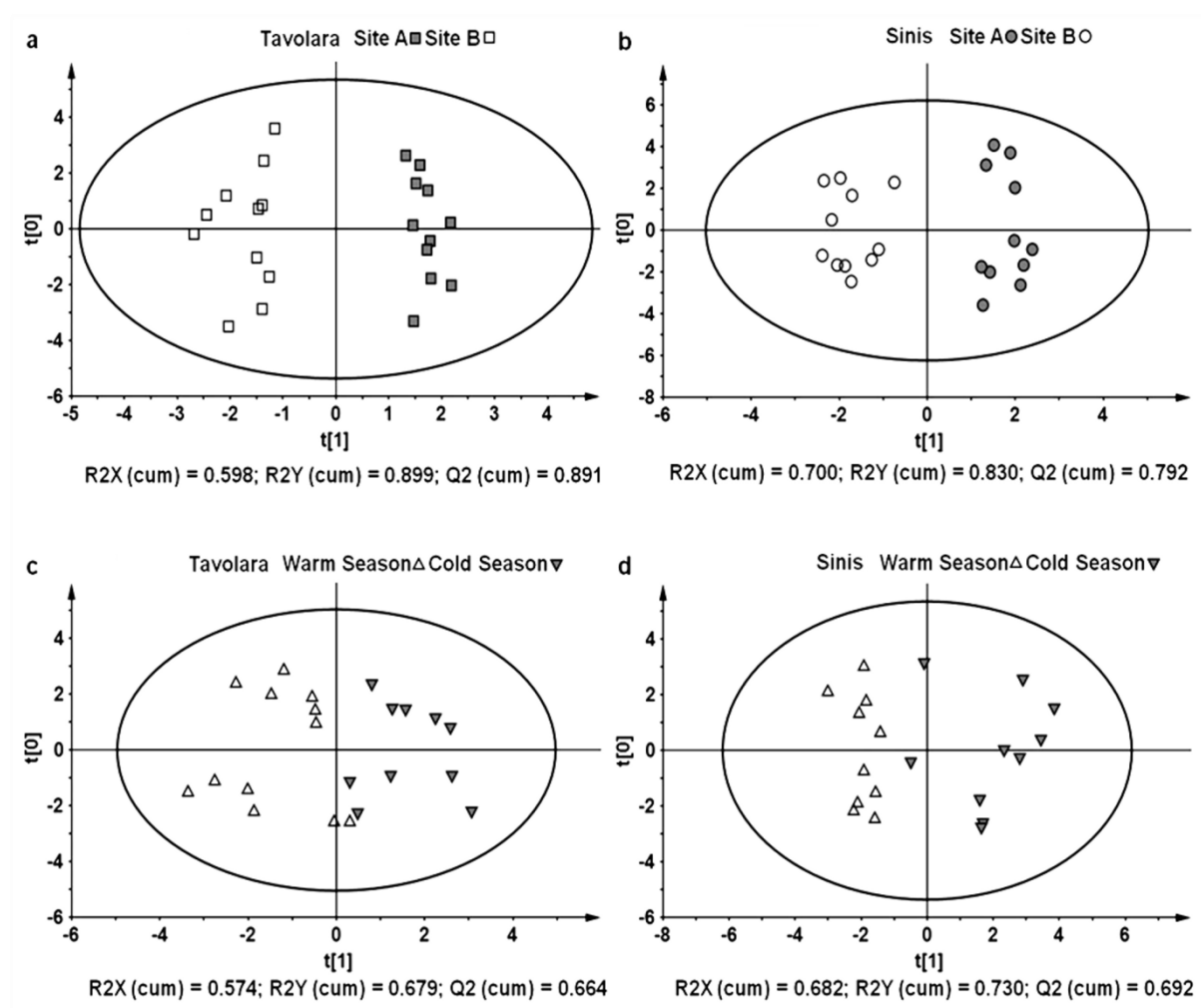


Figure 28 - OPLS-DA scores based on the GC FAMES profiles for Tavolara and Sinis areas, according to site (a, b) and seasonal (c, d) discrimination. R^2X (cum), R^2Y (cum) and Q^2X (cum) parameters are reported below. The black ellipses represent the T^2 Hotelling with 95% confidence.

4.5. Discussion

Total lipid content (%DW) found in gonads of Sardinian *P. lividus* is similar to that found, for the same species, in other environments (Dincer and Cakli, 2007; Fernandez, 1998; Mol *et al.*, 2008; Montero-Torreiro and Garcia-Martinez, 2003; Serrazanetti *et al.*, 1995). In agreement with Fernandez (1997), who suggested that the lipid content does not seem to be influenced by different growing areas in natural condition, we found no significant difference in total lipid content between Tavolara and Sinis samples collected concurrently (Table 8). Indeed, the annual variation in total lipids of the gonads shows that this biochemical parameter is less influenced by growing area rather than by season (Fig. 27). Such observation might suggest that

physiological and seasonal factors affect lipid absorption and storage mechanisms more than diet or other environmental parameters.

Despite the large body of evidence in scientific literature to support lipid evolution in sea urchin gonads, this relevant issue is still debated. Previous works have already evaluated the time-evolution of the total lipid content in *P. lividus* gonads, but data reported so far have not been systematically collected and are sometimes contrasting. Arafa *et al.* (2012) studied *P. lividus* specimens sampled from the Gulf of Tunis, and found a maximum in gonads total lipids content in August/September and a minimum in April, in general agreement with our findings. Dincer and Cakli (2007) also found a minimum value in April for total lipids in gonads of *P. lividus* collected in Turkey monthly from February to July. Conversely, Montero-Torreiro and Garcia-Martinez (2003), in a study carried on in the Galicia region (Spain), showed that lipid content remained almost constant throughout most of the year, except for two maxima evidenced in February and September; it is worth noting that this latter study was carried out in climatic conditions that are extremely different from those in the Mediterranean area. Fernandez (1998) showed, by studying *P. lividus* specimens collected in a Corsican lagoon (France), a maximum in total lipids of gonads in February. Mol *et al.* (2008), by sampling *P. lividus* gonads bimonthly from Turkey, did not spot relevant fluctuations in fat content over one year period. In this regard, Martinez-Pita *et al.* (2010b) suggested that the higher fat content during the growing stages and its decrease at maturity imply that fat is used as energy source by sea urchins during gametogenesis. Indeed, also in our observation, the lowest lipid content in gonads corresponded to high percentages of reproductive stages III (premature) and IV (mature) (Byrne, 1990, Fig. 25), irrespective of sampling area. The larger influence of season than sampling area on gonad lipid content has been observed also in other echinoderms (Hughes *et al.*, 2006). Similarly, in this work, we have found very similar seasonal trends in the two different areas (Table 8). Our histological study confirms this similarity between Tavolara and Sinis samples, showing that the premature and mature stages are present, for both areas, mainly in the cold season. The low error bars for lipid content obtained from December to April (Fig. 26) can be explained by considering that, in the cold season, the gonads are acting as a reproductive organ more than as a storage tissue (Hughes *et al.* 2006). A reduction in compositional variability, as reflected in the error bars in Fig. 26, may indeed be related to gametogenesis, and in particular to the appearance of maturation stages III and IV according to the classification suggested by Byrne (1990). With increasing maturity stages, gonads are replenished with spermatozoa and oocytes, which in turn confer peculiar characteristics to the lipid composition of gonad tissue.

It is by now established that biochemical parameters in gonads are influenced by specific reproductive cycle stages, in turn triggered by seasonal changes. In this regard, photoperiod and temperature are generally considered to be, among all exogenous environmental factors, the most probable proximate mediators of reproduction in echinoderms (Carboni *et al.*, 2013). On the other hand, the influence of temperature in gametogenesis and spawning still remains uncertain in *P. lividus* (Mercier and Hamel, 2009). Fig. 26 gives a clear picture of the variation of total lipids in gonads of *P. lividus* as a function of photoperiod and water temperature. For the first time to our knowledge, the periodical variation in total lipid content of gonads is described by a mathematical model (eq. (1)). Very few methodical studies, if any, have been published comparing biochemical composition of gonads with photoperiod and water temperature in a systematic approach. In our observation, total lipids in gonads exhibited annual periodical cycle that can be described by an oscillating wave function similar to that describing the behavior of both photoperiod and sea surface temperature. It was found that while a change in photoperiod anticipates the corresponding change in gonad total lipids content, a change in sea surface temperature follows it (Fig. 26). Our findings suggest that the influence of photoperiod is more important than water temperature in triggering and regulating fat utilization and storage in *P. lividus* gonads. It is suggested that such metabolic changes might be then correlated to an adaptative process of the sea urchin to seasonal changes, mainly regulated by day-length. The mathematical approach presented here (eq. (1)) led us to estimate in numerical terms (Table 9) the influence of the aforementioned factors on total lipid content. In particular, in the specific conditions of our study, a change in day-length led to a variation of total lipid content in ~40 days. This result might explain why, in sea urchins reared in controlled conditions, a 45 days treatment (Spirlet *et al.*, 2000) may not be long enough to allow to evaluate the influence of photoperiod on gametogenesis, whereas a 90 days treatment does reveal the influence of photoperiod on the gametogenic cycle (Shpigel *et al.*, 2004). Furthermore, as mentioned above, these results confirm that gonads should not be only referred to as a reproductive organ, but as a very important nutrient storage tissue (Russell, 1998; Hughes *et al.*, 2006). In this sense, the evolution of lipid content is likely due to a combination of several factors, and one of these may be the amount of fat introduced with the diet. Even if we did not specifically investigate the lipid content of algae in Tavolara and Sinis, it is reasonable to speculate on the possible role of the lipid content of algae on the aforementioned fat enrichment of gonads. In this regard, several authors found a positive correlation between the lipid content of algae and photoperiod, and concluded that a higher lipid content has to be related to increased exposition of some algae species to light and to an increase in water temperatures (Olofsson *et*

al., 2012; Weldy and Huesemann, 2007). In particular, El Maghraby and Fakhry (2015) found a high lipid content in spring for some algae, including *Padina pavonica*, a strongly preferred species for *P. lividus* (Boudouresque and Verlaque, 2007), that is very common in both Tavolara and Sinis sea bed (Guala, personal observation). Further studies on the effects of lipid content of algae on that of urchins' gonads are certainly desirable. It is also generally agreed that in marine ecosystems lipid composition of animal tissues is greatly affected by the source of dietary lipids (Martinez-Pita *et al.*, 2010a, b).

A statistically significant influence of growing area on total lipid content of sea urchin gonads has not been evidenced in this work, except in May (Table 8). Nevertheless, we suggest that environment imprints a marked effect on gonadal composition at a finer level, acting on fatty acids profiles. As a consequence, while total lipid content is not significantly affected, MVA on fatty acids profiles demonstrates that the qualitative profiles of such lipids are markedly influenced by the sampling area (Table 10 and Fig. 27). Such conclusion is certainly of primary importance when nutritional, sensorial and technological qualities of sea urchins' gonads are to be evaluated or need to be predicted.

The fatty acid composition of *P. lividus* gonads found in the present work (Table 10) is in general agreement with literature (Serrazanetti *et al.*, 1995; Mol *et al.* 2008; Martinez-Pita *et al.*, 2010b; Arafa *et al.*, 2012; Pereira *et al.*, 2013; Angioni and Addis, 2014; Prato *et al.*, 2017;). 14:0, 16:0, EPA and ARA are the most represented fatty acids in the lipid extract of *P. lividus* sea urchin gonads.

However, it is worth noting that the average gonadal contents of EPA measured in the present work (Table 10) are surprisingly high if compared with other specimens of *P. lividus* recently sampled in different areas of Sardinia (Angioni and Addis, 2014), but more similar to those reported by Serrazanetti *et al.* (1995), Mol *et al.* (2008) and Arafa *et al.* (2012).

The fatty acids profiles allow discrimination of sea urchin gonads with respect to the two different growing areas considered (Sinis and Tavolara). Indeed, MVA of experimental GC data strongly suggests a discriminant capacity of GC FAMES profiles in differentiating the sampled sea urchins in the series area > site > season. This means that the discriminant effect imparted by the area where urchins grew to the fatty acid profile of their gonads is greater than those imparted by different sites within each area or by seasonal fluctuations. In particular, gonadic MUFAs are the main GC FAMES responsible for the observed clustering between Sinis and Tavolara (Table 11). Discrimination between different sites within the same area (approximately 1-3 Km far from each other) evidenced that gonad MUFAs are the main

discriminative FA for Sinis but not the most relevant for Tavolara, in which such discrimination is primarily due to EPA and DHA contents (Table 11). These findings are in agreement with Martinez-Pita *et al.* (2010a), who reported an influence of environmental factors on gonad fatty acid composition in *P. lividus* harvested from two different sites. In their work, Martinez-Pita *et al.* (2010a) mostly attributed such difference to 14:0, some MUFA, such as C18:1 11c, C20:1 9c and C20:1 11c, to the two main long chain PUFAs, EPA and DHA, and to another essential fatty acid, ARA. To interpret such site-associated compositional differences, they suggested that water temperature could have only a marginal role, while diet composition is expected to impart more marked lipid fingerprint to the gonads. Since Tavolara and Sinis have different physical characteristics (i.e. mineralogical composition of rocks and exposition), that potentially reflect different seaweed composition, also in our group of samples the different trophic availability could explain the different fatty acid profile among the sampling areas.

The influence of marine ecosystem (area and site) on the fatty acid profiles of sea urchin gonads appear to be so strong that it is able to mask the influence of season, which has been previously explained (Hughes *et al.*, 2006; Martinez-Pita *et al.*, 2010b). In fact, although the MVA carried out to compare warm and cold season in each area (Fig. 28c and d) confirm the presence of an effect of season, the discriminative power of growing area and site is more relevant than that imposed by seasonal changes. The FAs responsible for seasonal clusterization are the long chain PUFAs ARA and EPA. The role of EPA on the metabolism of *P. lividus* (Gago *et al.*, 2009; Martinez-Pita *et al.*, 2010a, b; Carboni *et al.*, 2013; Angioni and Addis, 2014) and other echinoids (Hughes *et al.*, 2006; Mol *et al.*, 2008) has been the subject of several studies, overall reaching the general conclusion that this essential FA is selectively absorbed in gonads during gametogenesis if present in the diet up to reach a certain nutritional requirement. Another explanation considers the involvement of EPA in homeoviscous adaptation of phospholipid membranes to water temperature changes (Martinez-Pita *et al.*, 2010b), a phenomenon well known also in other poikilotherm marine organisms (Anedda *et al.*, 2013; Melis and Anedda, 2014). Also ARA is expected to be related to seasonal events, and its annual profile is likely linked to gonad development and positively correlated with gonadosomatic index (Martinez-Pita *et al.*, 2010b). Some authors (Gonzales-Duran *et al.*, 2008) associated the presence of ARA to endogenous conversion of precursors such as 18:2 n-6, while others (Martinez-Pita *et al.*, 2010b) related an increase in ARA content in gonads to eicosanoid synthesis occurred to face the stress associated to spawning or during high water temperatures. For what stated, among ARA and EPA are certainly biochemical markers of seasonal variations in the lipid content of

sea urchin gonads, and their seasonal-induced variation is higher than that dictated by other exogenous factors affecting their concentration. It is worth to note that ARA and EPA were also found to be putative endogenous markers of site discrimination (Table 11).

4.6. Conclusion

The present study focused on the evaluation of total lipids content and fatty acids profile of *P. lividus* gonads in different growing areas along Sardinian coast and in different seasons during a year.

Total lipid content follows an annual cyclical trend, for the first time described by a mathematical model, and it is more influenced by season rather than by growing area. In addition, the lowest lipid content in gonads corresponds to high percentages of premature and mature reproductive stages, independently of sampling area. The seasonal change in total lipid content follows a change in photoperiod, while it is related to sea surface temperature. On the other hand, it was found that FA profile is more influenced by growing area rather than by season. In particular, FA in gonads of *P. lividus* clusters the collected specimens mainly according to the sampling areas, secondly according to the sites within the same area and, finally, according to season. Multivariate statistical analysis of GC FAME data allows to spot the main compositional differences associated to growing area, site and season. MVA results indicate that fatty acid profile of gonads is principally affected by sampling area, followed in discriminative importance by site- and season-associated differences. Biochemical markers of area distinction are some MUFA such as 16:1 and 22:1. Essential fatty acids (EPA and ARA) have a higher discriminant value in differentiating sea urchins collected in different seasons. Site-related differences of the fatty acids profiles are mainly associated to EPA content, and this is particularly evident in Tavolara (VIP value = 4.162).

The results of this work confirm that gonad content of some fatty acids, in particular EPA and ARA, is markedly modulated by both seasonal and environmental factors.

It is suggested that dietary fatty acids may have the highest impact on gonad fatty acid composition so as to link the lipid quality of sea urchin gonads to growing area. Further investigation on the complex dependency between dietary (macroalgae populations) and gonad composition is ongoing in our laboratory. In this sense, appropriate multivariate models built on the lipid quality of sea urchin gonads can be considered promising tools for addressing traceability, certainly safeguarding and adding high value to some selected products.

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Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.marenvres.2015.12.001>.

5. PROTEOMIC CHANGES OCCURRING ALONG GONAD MATURATION IN THE EDIBLE SEA URCHIN *PARACENTROTUS LIVIDUS*.

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Keywords:

Roe, maturation marker, reproductive cycle, Sardinia, shotgun proteomics

5.1. Abstract

The reproductive stage of *Paracentrotus lividus* strongly influences product quality that, in turn, impacts significantly on the market price. Large, compact and sweet gonads are preferred, and sensory attributes are positively related to the ratio of nutritive phagocytes to gametes. Gonads at advanced maturation stages, although larger, have less desirable attributes, being more watery and bitter especially in females. Therefore, the best compromise among size, texture, and taste needs to be reached. In this study, wild *P. lividus* were collected along coastal Sardinia, and gonads in the recovery, pre-mature, mature, and spent stages were analyzed by gel-based and by shotgun proteomics. A detailed characterization of the proteome changes occurring in gonads of both sexes along maturation was achieved, and significant modifications were seen in numerous proteins involved in nutrient accumulation in nutritive phagocytes, as well as in gamete biology and maturation. Adding to an improved understanding of the *P. lividus* reproductive cycle in its natural environment, the results described in this work may form the basis for defining novel protein markers and procedures for an easier sexing and staging, and for monitoring sea urchin gonad maturation cycles in aquaculture plants. Mass spectrometry data are deposited in ProteomeXchange (PXD004200).

Significance: The sensory quality of *P. lividus* gonads is strongly influenced by the reproductive cycle, with significant changes in flavor, texture, and size. A better knowledge of the protein profiles, patterns, and markers associated with gonad sex and maturation stage can have useful implications for understanding and monitoring these changes. One of these is the ability to identify protein profiles specifically associated with a given stage and, in perspective, to identify maturation and sex markers. The comprehensive proteomic evaluation achieved in this work was made possible by the application of combined gel-based and shotgun approaches. As a result, this study generated the largest proteomic dataset available in the literature for *P. lividus*, as well as a general picture of protein abundance changes occurring along maturation.

5.2. Introduction

The sea urchin *Paracentrotus lividus* (Lamarck, 1816) is found abundantly throughout the Mediterranean Sea and in the eastern Atlantic Ocean, from Scotland and Ireland to Southern Morocco and the Canary Islands. *P. lividus* lives mainly in territories where winter water temperatures range from 10 to 15°C, and summer temperatures from 18 to 25°C (Boudouresque and Verlaque, 2007; Ceccherelli *et al.*, 2011). *P. lividus* is typically a subtidal species, inhabiting from the mean low water to 10–20 m, in intertidal rock pools (Crook *et al.*, 2000)

and in seagrass meadows of *P. oceanica* and *Zostera marina* (Boudouresque and Verlaque, 2007). In spite of its popular name “purple sea urchin”, *P. lividus* color is highly variable from black-purple, purple, red-brown, to dark-brown.

P. lividus gonads, also known as roe or uni, are a highly valued seafood product and are considered a delicacy in many parts of the world for their flavor and taste. In recent years, their increasing demand has resulted in commercial overexploitation of the wild population. For this reason, this species is becoming an important candidate for the emerging aquaculture industry in Europe (Phillips *et al.*, 2009; Angioni and Addis, 2014), although mainly with a focus on an increase in gonad yield (Liyana-Pathirana *et al.*, 2002) and less emphasis placed on quality parameters such as appearance, color, texture, flavor and nutritional value, despite the strong influence of these factors on market price (Pearce *et al.*, 2002a; Yokota *et al.*, 2002; Agatsuma *et al.*, 2005; Phillips *et al.*, 2009; Prato *et al.*, 2017).

The reproductive apparatus is constituted by five gonads that are usually bright red-orange in females and yellow-orange in males. Gonads of both sexes contain two main cell types: germinal cells (GCs) (oogonia and ova in the ovary and spermatogonia and spermatozoa in the testis) and somatic cells defined as nutritive phagocytes (NPs). NPs have the function of storing the nutrients (proteins, lipids and carbohydrates) for germinative cells during gametogenesis, and to eliminate residual eggs and sperms at the end of the reproductive cycle (Yokota *et al.*, 2002; Walker *et al.*, 2005), in order to mobilize and recover nutrients for the next cycle. *P. lividus* gonad sensory quality is strongly influenced by sex (Lawrence *et al.*, 1997), diet, and availability of food resources (Byrne, 1990; Lozano *et al.*, 1995; Lawrence *et al.*, 1997; Yokota, 2002; Shpigel *et al.*, 2006; Siikavuopio *et al.*, 2007; Arafa *et al.*, 2012; Angioni and Addis, 2014), season/reproductive period (Yokota *et al.*, 2002), geographic location and by environmental factors such as photoperiod and water temperature. The attributes of a high quality sea urchin gonad are size, color, texture, and flavor, being the largest, bright orange, compact, and sweet gonads the most appreciated. This implicates that gonads should be developed but not too mature, since a high gamete to NP ratio makes gonads watery and bitter (Unuma, 2002; Phillips *et al.*, 2009).

In order to assess the influence of seasonal and environmental patterns on sea urchin gonad biochemistry, our research group (Siliani *et al.*, 2016) has recently shown a periodical gonad lipid content fluctuation in *P. lividus* which seems more influenced by seasonal factors (photoperiod and water temperature) than harvesting area. On the other hand, statistical

evidence was given of the modifications introduced by the area of collection on the lipid quality (fatty acid composition), the season having much less impact on the observed fat compositional profiles, suggesting a role of dietary lipid uptake in modulating roe composition.

During the reproductive cycle, *P. lividus* gonads undergo important changes classified in different developmental stages. One of the most exhaustive classification system of *P. lividus* was described by Byrne in 1990 (Byrne, 1990). She examined the reproduction of *P. lividus* determining the annual cycle from contrasting habitats (intertidally from an exposed rocky shore and subtidally from a protected bay) on the west coast of Ireland, documenting gametogenesis through histological examination of the gonads. Then, she classified the annual reproductive cycle of *P. lividus* in 6 developmental stages: 1, recovery; 2, growing; 3, premature; 4, mature; 5, partly spawned; 6, spent.

At the beginning of the cycle (stage 1, recovery), the gonadal tissue is vacuolated for the presence of numerous nutritive phagocytes scattered around the matrix. During gonad maturation, the ratio of NPs to GCs varies drastically (Byrne, 1990). Immature oocytes and spermatocytes developing in the acinal walls gradually move towards the acinal lumen, replacing phagocytes (confined to the acinal walls) and becoming mature eggs and sperms. After spawning, gonads are devoid of both gametes and phagocytes, and display thin acinal walls.

A useful parameter that supports histological analysis in the reproductive cycle is the gonadosomatic index (GSI), calculated as a ratio of the gonad mass to the whole-body wet mass. The GSI trend varies during the reproductive cycle and, generally, high values of GSI are observed before the spawning phase. This index is commonly used in the study of the echinoid reproductive cycle (Moore, 1934; Fernandez and Boudouresque, 2000; Gago *et al.*, 2003; Shpigel *et al.*, 2005; Arafa *et al.*, 2006; Barbaglio *et al.*, 2007; Harrington *et al.*, 2007), although its general usefulness is somewhat debated due to the dimensionless nature. The development of novel, rapid, and possibly quantitative systems for assessing sex and maturation stage would be useful to speed up and provide reliability to the process. Therefore, a detailed description of the proteomic makeup of *P. lividus* gonads, and of how it changes along sexual maturation, might contribute to the identification of potential markers of gametogenesis crucial to monitor the reproductive process, paving the way to more conscious harvesting and farming activities.

Adding to their importance as food, sea urchins are considered a research model system for modern molecular, evolutionary, and cell biology studies. Nevertheless, up to now, only a few studies have investigated the proteome of echinoderm gonads, and a complete dataset of proteins expressed at different developmental stages is not yet available. In addition, the genome of the purple sea urchin *Strongylocentrotus purpuratus* is the only sea urchin annotated reference genome available to date (Sodergren *et al.*, 2006). In 2008, Sewell *et al.* (2008) used a combined shotgun and gel-based proteomic approach to describe the major proteins in mature ovaries of the New Zealand sea urchin *Evechinus chloroticus* to determine changes in protein profiles of gonads from sea urchins fed different experimental diets.

Proteomic studies on other aspects of sea urchin biology are more numerous, including the studies on the maternal to zygotic transition, on unfertilized eggs through first cell division, and as a model in biology studies (Guo *et al.*, 2015; Wan *et al.*, 2015; Phanse *et al.*, 2016). Mann *et al.* (Mann *et al.*, 2010) examined the proteome content of the spicules (calcitic skeletal elements). Dheilly *et al.* (2011) investigated the response of coelomocytes to bacterial infection with different proteomic approaches (Dheilly *et al.*, 2011; Dheilly *et al.*, 2012; Dheilly *et al.*, 2013), and Fasoli *et al.* (2012), in 2012, characterized the sea urchin coelomic fluid via previous capture and enhancement of low abundance proteins with combinatorial peptide ligand libraries (CPLL, ProteoMiner).

With the aim of improving our knowledge of the changes occurring in gonads along maturation, in this study we investigated the gonadic proteomic profile of wild *P. lividus* sea urchins collected in the Mediterranean Sea along coastal Sardinia by applying gel-based and shotgun proteomic approaches.

5.3. Materials and methods

5.3.1. Protein extraction and quantification

This study was carried out on specimens of *P. lividus* of commercial size (test diameter ≥ 50 mm without spines) collected in the context of a previously described study (Siliani *et al.*, 2016). Sex and stage characterization were carried out by histology. One of the five gonads extracted from each sea urchin was stored in 10% neutral buffered formalin and then dehydrated, embedded in paraffin, sectioned at 7 μ m by a rotary microtome (Leica 2125 RM, Leica Biosystems Nussloch GmbH, Germany) and stained with hematoxylin (Sigma Aldrich, St. Louis, MO) / eosin (Carlo Erba Reactifs, Val De Reuil Cedex, France); the sections were

analyzed under the binocular microscope (Leica DM RB, Leica Microsystems Wetzlar GmbH, Germany) and samples were classified as females or males belonging to stages from 1 to 6 according to Byrne (1990). Samples in stages 2 and 5 were not included in the study due to their insufficient number, probably due to heterogeneity in the histologic appearance in the first case and to difficulties in finding samples at the exact moment of spawning in the second case. Protein extraction was performed as described previously (Ghisaura *et al.*, 2014), with minor modifications. Briefly, a small portion of tissue was minced with a sterile scalpel and 100 mg were placed in 2 mL Eppendorf safe-lock tubes (Eppendorf, Hamburg, Germany), immersed at 2% w/v in lysis buffer (7 M urea, 2 M Thiourea, 2% CHAPS, GE Healthcare, Little Chalfont, UK) plus protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO). Each sample was then processed and subjected to three cycles of 5 min at 30 oscillations/s in a TissueLyser mechanical homogenizer (Qiagen, Hilden, Germany) followed by freezing/thawing. All extracts were then subjected to sonication on ice using an ultrasonic homogenizer (three 30 s bursts with a titanium microtip) (BioLogics, Inc. Manassas, Virginia) and then clarified for 15 min at 14,000 rpm at 4°C, quantified with the Pierce 660 nm Protein Assay Kit (Thermo Scientific - Rockford, IL), tested for quality and quantity by SDS-PAGE, and stored at – 80°C until analysis.

5.3.2. Gel-based proteomics

Twenty-five micrograms of each protein extract were separated by SDS PAGE in 8% polyacrylamide gels according to Laemmli (Laemmli, 1970). Gels were stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions and digitalized with an ImageScanner III (GE Healthcare, Little Chalfont, UK). Pattern intensities were compared using Quantity One 1-D analysis software (Bio-Rad, Hercules, CA) (Salvatore, 2014). For densitometry, two gels containing all gonad samples of the same sex were processed in parallel, including electrophoretic and staining procedures. After destaining and image digitalization, whole lane intensities were measured, and the average intensity value was calculated for each lane. The overlay of the means for each sample class was then plotted. For calculating relative protein abundances, band areas were estimated using Origin-Pro 8 SR0 software (OriginLab Corporation, One Roundhouse Plaza, Northampton, MA) and band area ratios were calculated.

For Western immunoblotting experiments, proteins were transferred to nitrocellulose after SDS-PAGE and probed with anti-actin and anti-tubulin antibodies according to standard procedures, as described previously (Addis *et al.*, 2009; Addis *et al.*, 2011). For calculating

relative band abundances, tubulin and actin band intensities were estimated with Quantity One, and tubulin was normalized on actin using band intensity ratios.

For protein identification in electrophoretic gels, the bands of interest were excised from one lane and then destained, reduced, carbamidomethylated, and trypsin digested as described previously (Ghisaura *et al.*, 2014). Briefly, samples were reduced in 50 mM NH_4HCO_3 buffer with 10 mM DTT at 56°C, and then carbamidomethylated in 50 mM NH_4HCO_3 buffer with 55 mM iodoacetamide at room temperature in the dark. Tryptic digestion was performed at 37°C overnight using an average amount of 80 ng of trypsin per gel slice. LC–MS/MS analyses of tryptic digests were performed on a XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA). Samples were concentrated and desalted on an enrichment column with formic acid, and peptides were fractionated on a C18 reverse-phase column directly coupled to a nanospray source. Data Analysis software (6300 Series Ion Trap LCMS), provided by the manufacturer, was used to analyze raw MS and MS/MS spectra and to generate peak lists, as MGF files.

5.3.3. Shotgun proteomics

The same samples analyzed by GeLC-MS/MS were also investigated by shotgun proteomics. Protein extracts were diluted to 200 μL with UA solution (8 M urea in 100 mM Tris-HCl, pH 8.8), loaded into Microcon Ultracel YM-10 filtration devices (Millipore, now Merck Millipore, Billerica, MA, USA), and then processed according to the “FASP II” protocol (Wiśniewski *et al.*, 2009), with minor modifications (Tanca *et al.*, 2013). Briefly, samples were subjected to repetitive washings by filter centrifugations with buffers, reducing and alkylating agents, followed by over-night on-filter digestion with trypsin, final collection of peptides in acetonitrile and formic acid, drying and reconstitution of the peptide mixture in 0.2% formic acid to a final concentration of 2 mg/mL. Peptide mixture concentration was estimated with the Pierce™ BCA protein assay kit (Thermo Scientific - Rockford, IL).

LC–MS/MS analyses were performed on a Q-TOF hybrid mass spectrometer equipped with a nano lock Z-spray source and coupled online with a NanoAcquity chromatography system (Waters) (Pagnozzi *et al.*, 2014). The peptide mixture was concentrated and washed on an enrichment column, and fractionated on a C18 reverse-phase column directly coupled to a nanospray source, using a 250 min gradient. The mass spectrometer was set up in a data-dependent MS/MS mode where a full-scan spectrum was followed by tandem mass spectra, selecting peptide ions as the three most intense peaks of the previous scan. ProteinLynx

software (Version 2.2.5), was used for analysis of raw MS and MS/MS spectra and the production of the peak lists, as pkl files.

5.3.4. Protein identification and differential proteomic analysis by label-free quantitation

Both the peak lists from Q-TOF analysis, converted into a MGF file, and the MGF files from Ion Trap mass spectrometer, were analyzed by Proteome Discoverer software (version 1.4.0.288; Thermo Scientific), with a workflow consisting of the following nodes (and respective parameters): Spectrum Selector for spectra pre-processing (precursor mass range: 350–5000 Da; S/N Threshold: 1.5), Sequest-HT as search engine (Protein Database: Echinoidea sequences from UniProtKB/ SwissProt 15,530,586 residues, 32,894 sequences, release 2014_4; Enzyme: Trypsin; Max. missed cleavage sites: 2; Peptide length range 5–50 amino acids; Max. Delta Cn: 0.05; Precursor mass tolerance: 50 ppm for Q-TOF analysis, and 300 ppm for Ion Trap analysis; Fragment mass tolerance 0.4 Da for Q-TOF analysis, and 0.6 Da for Ion Trap; Static modification: cysteine carbamidomethylation; Dynamic modification: methionine oxidation), and Percolator for peptide validation (FDR < 1% based on peptide q-value). Protein annotations concerning protein families and gene ontology were retrieved from UniProtKB (<http://www.uniprot.org>). The uncharacterized sequences obtained from Uniprot database were blasted on another non-redundant database: NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to find the homologous proteins.

For shotgun results, the Normalized Spectral Abundance Factor (NSAF) was used in order to estimate the extent of differential protein abundance of functional categories among sample groups; it was calculated according to Zybaylov *et al.* (2006) as follows: $NSAF = SAF_i / \sum_{i=1}^N SAF_i$, where subscript *i* denotes a protein identity and *N* is the total number of proteins, while SAF is a protein spectral abundance factor that is defined as the protein spectral counts divided by its length. Then, the NSAF log ratio (R_{NSAF}) was calculated according to the following formula: $R_{NSAF} = \log_2(\sum NSAF1 / \sum NSAF2)$, expressing the log₂ ratio of the abundance of a protein category between sample groups 1 and 2, whereas $\sum NSAF1$ and $\sum NSAF2$ are the summed NSAF values for all the proteins belonging to a given category in sample groups 1 and 2, respectively. Functional categories showing $R_{NSAF} > 0.5$ or < -0.5 were considered as differentially abundant between groups. Statistical significance of differential protein abundance was determined by applying Student's t-test (two-sample comparison, $p < 0.05$) to logarithmized (normally distributed) NSAF values.

The MS proteomics data have been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (Vizcaino *et al.*, 2014; Vizcaino *et al.*, 2016) with the dataset identifier PXD004200, and are reported in Ghisaura *et al.* (2016) [Data in Brief].

5.4. Results

5.4.1. Gametogenic cycle

The annual gametogenic cycle was extensively described in Siliani *et al.* (2016). *P. lividus* was found mainly in recovery stage (stage 1) during summer and autumn months (from June to October). Gametogenesis began at the end of autumn (December) with the appearance of few individuals in growing (stage 2) and several in premature stage (stage 3), and lasted until April, with lower abundance. Fully mature stage (stage 4) was found mainly from January to April, with a higher frequency between February and March. Spawning occurred mostly in spring, although sea urchins in stage 5 were observed throughout the whole winter and occasionally in summer and in October. Spent individuals, at the end of the annual gametogenic cycle (stage 6), were found in December and then continuously from February to August, generally after a spawning phase. Fig. 29 depicts the histological appearance of the maturation stages.

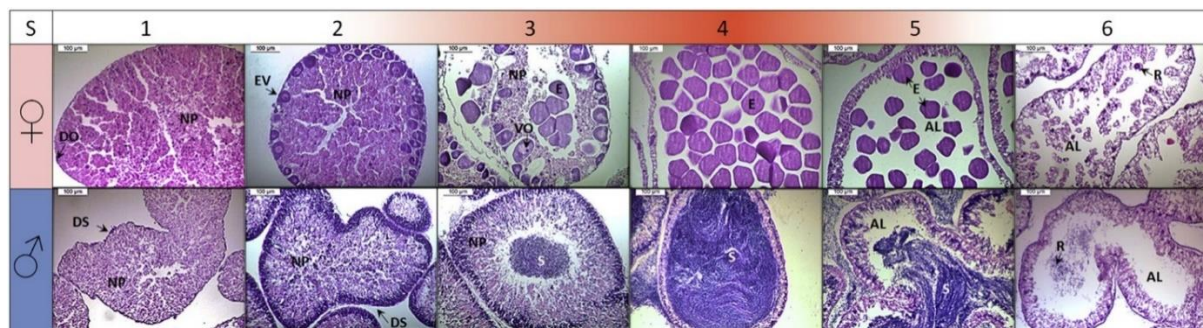


Figure 29 - Developmental stages (S) of females (top) and males (bottom) of the sea urchin *P. lividus*: 1, recovery; 2, growing; 3, premature; 4, mature; 5, partly spawned; 6, spent. In the figures: NP, nutritive phagocytes; DO, developing oocytes; EV, early vitellogenic oocytes; VO, vitellogenic oocytes; E, mature eggs; AL, acinal lumina; R, relict gametes; DS, developing spermatocytes; S, mature sperms.

5.4.2. Comparison of total protein profiles by differential gel-based proteomics

To identify the differences in protein abundance occurring in *P. lividus* gonads along maturation, total proteins from female and male gonads at stages 1, 3, 4, and 6 were separated by SDS PAGE (Fig. 30). Stages 2 and 5 were not examined because of the very low number of

gonads found in these stages, probably due to their transitional nature and to the difficulties in correctly identifying gonads at these stages by histology. According to densitometric analysis (Fig. 31), inter-stage differences were more pronounced than inter-sex differences, that is, there was more difference among stages of the same sex than among females and males of the same stage. The main differential bands (1, 2, 3, 4, 6 in Fig. 30) were subjected to mass spectrometry identification, together with one common band as a reference (band 5 in Fig. 30). Since stages 4 and 6 were the most differential according to gel densitometry, the protein identification procedure was carried out on gels from these stages.

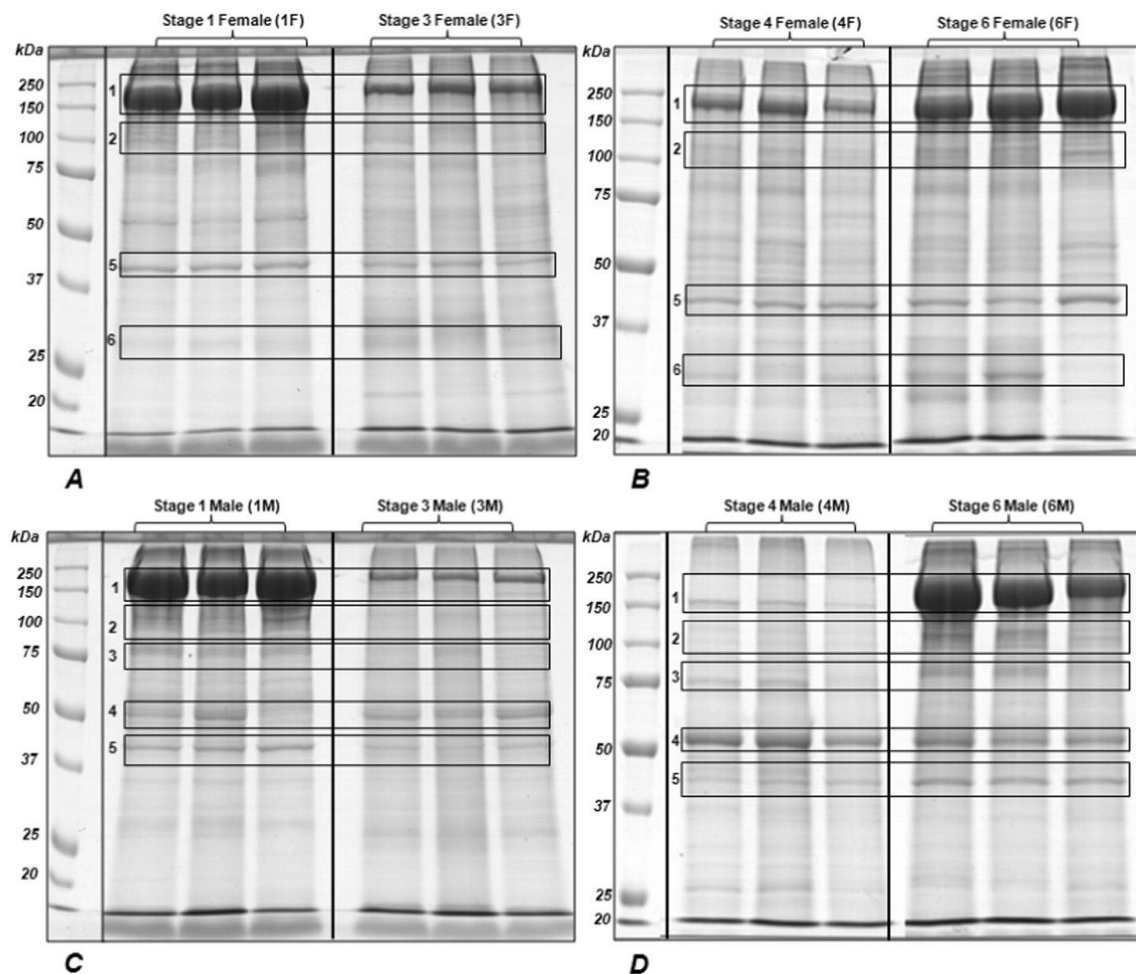


Figure 30 - SDS-PAGE profiles of female and male gonads in the 4 stages examined (A: stage 1 and 3 females; B: stage 4 and 6 females; C: stage 1 and 3 males; D: stage 4 and 6 males). Differential bands are boxed and numbered. Band 5 was similar in all samples and it was used as reference. Supplementary Table S1 reports the proteins identified in each band.

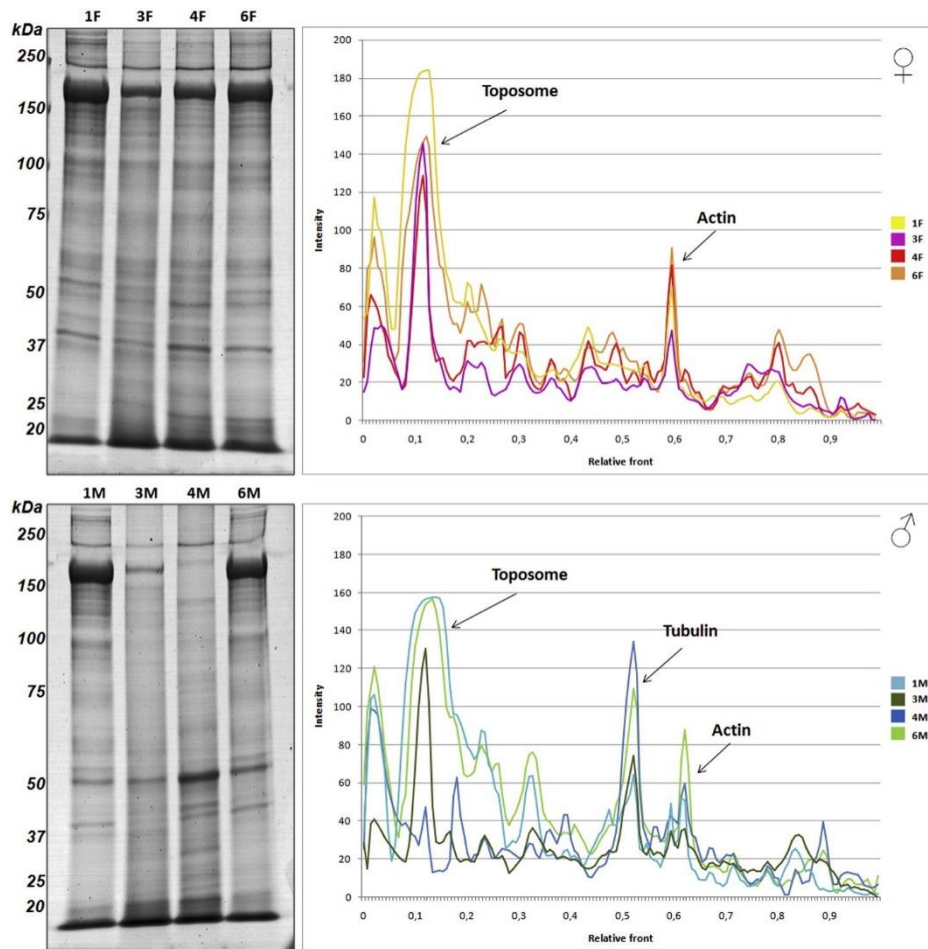


Figure 31 - Densitometric analysis of samples and comparisons according to gonadal stage and sex. Top, females; bottom, males. The left side of the image reports the SDS-PAGE patterns of pooled samples belonging to each maturation stage. The right side of the image reports the overlay of the averaged sample class densitograms. The arrows point to the main differential protein band areas.

The most differential band (1) contained mainly toposome, also reported as major yolk protein (MYP), and the second most differential band (4) contained mainly tubulin. In order to provide a relative measure of these changes, the band area ratios were calculated for toposome and tubulin in the different sexes and stages. To account for presence of other proteins in the same band area, the ratio was also calculated against actin as an internal normalization reference. The results concerning the four stages for both sexes are reported in Table 12.

The first and more evident result in both sexes was the dramatic drop occurring in toposome abundance in gonadal stages 1 and 6 when compared to stages 3 and 4. This massive decrease in toposome abundance along gonadal maturation was more prominent in males, especially in stage 4, at which it disappeared almost completely. The decrease in toposome abundance along

maturation was seen also in females, but with a lower magnitude. When measured against actin, the toposome/actin ratio in females was 12.8 in stage 1, vs 3.3 in stage 4, while in males it went from an impressive 19 in stage 1 to 1.7 in stage 4. Concerning tubulin, males at stage 4 showed the highest amounts when compared to the other stages. In the case of females, there were no evident tubulin bands at the same molecular weight, and no significant differences were seen among females at different stages in this gel area.

Table 12 - Band area ratios for toposome (MYP), actin, and tubulin, in the four stages for both sexes, according to gel densitometry.

Proteins	1F ¹ /3F	3F/4F	4F/6F	6F/1F	1F	3F	4F	6F
Toposome/Toposome	2.3	1.0	0.6	0.7	-	-	-	-
Toposome/Actin	-	-	-	-	12.8	5.7	3.3	7
Tubulin/Actin	-	-	-	-	-	-	-	-
	1M ² /3M	3M/4M	4M/6M	6M/1M	1M	3M	4M	6M
Toposome/Toposome	3.8	2.1	0.12	0.8	-	-	-	-
Toposome/Actin	-	-	-	-	19.0	7.1	1.7	15.6
Tubulin/Actin	-	-	-	-	1.38	2.4	3.8	2.5

¹F, females

²M, males

5.4.3. Shotgun proteomics for investigating protein abundance changes: maturation stage

Shotgun proteomic analysis was then carried out on all samples by combining FASP with Q-TOF-MS/MS and protein quantitation by a label-free approach, with the aim of obtaining a wider picture of the proteome changes occurring along maturation and of easing evaluation of their relative changes. The details on protein identifications obtained in all samples, the list of all proteins identified and the differential proteins ($-0.5 > \text{RNSAF} > 0.5$, $p \text{ value} < 0.05$) observed in the four stages in female and male gonads are reported in Ghisaura *et al.* (2016) [Data in brief].

Table 13 and Fig. 32 provide a general overview of the proteins showing the most significant changes in abundance among the developmental stages investigated in females and males.

Table 13 - Differential proteins identified in the four maturation stages in females and males; Differential proteins with a Log Ratio NSAF>0.5 or <-0.5 and with a Log Ratio NSAF>1.5 or <-1.5 are illustrated.

# Diff. proteins	1F ¹ /3F	3F/4F	4F/6F	6F/1F
-0.5 >R _{NSAF} >0.5	46/357	33/355	33/242	19/257
R _{NSAF} >1.5	2	1	1	4
R _{NSAF} <-1.5	13	3	6	3
	1M ² /3M	3M/4M	4M/6M	6M/1M
-0.5 >R _{NSAF} >0.5	43/447	48/468	59/304	21/270
R _{NSAF} >1.5	1	7	12	3
R _{NSAF} F<-1.5	8	3	2	6

¹F, females²M, males

Based on these observations, toposome and tubulin were among the proteins undergoing the most significant changes. Therefore, their relative abundance ratios were calculated in the different sexes and stages based on the respective NSAF values (Fig. 33). Results were in line with the gel-based data (Fig. 31 and Table 12): in females, the toposome underwent a decrease from stage 1 (19.4%) to stage 3 (4.4%), and increased again in stage 4 (8.7%) and then in stage 6 (17.6%). In males, the toposome dropped dramatically from stage 1 (14.1%) to stage 4, where it was almost absent, and started increasing again in stage 6 (8.8%). Concerning tubulin (Fig. 33, panel B), similar amounts were seen in both sexes in stage 1 (13% for females and 15% for males). The highest amounts of this protein were seen in stage 4 males (27%). In female gonads, tubulin was present in similar and lower amounts in all stages.

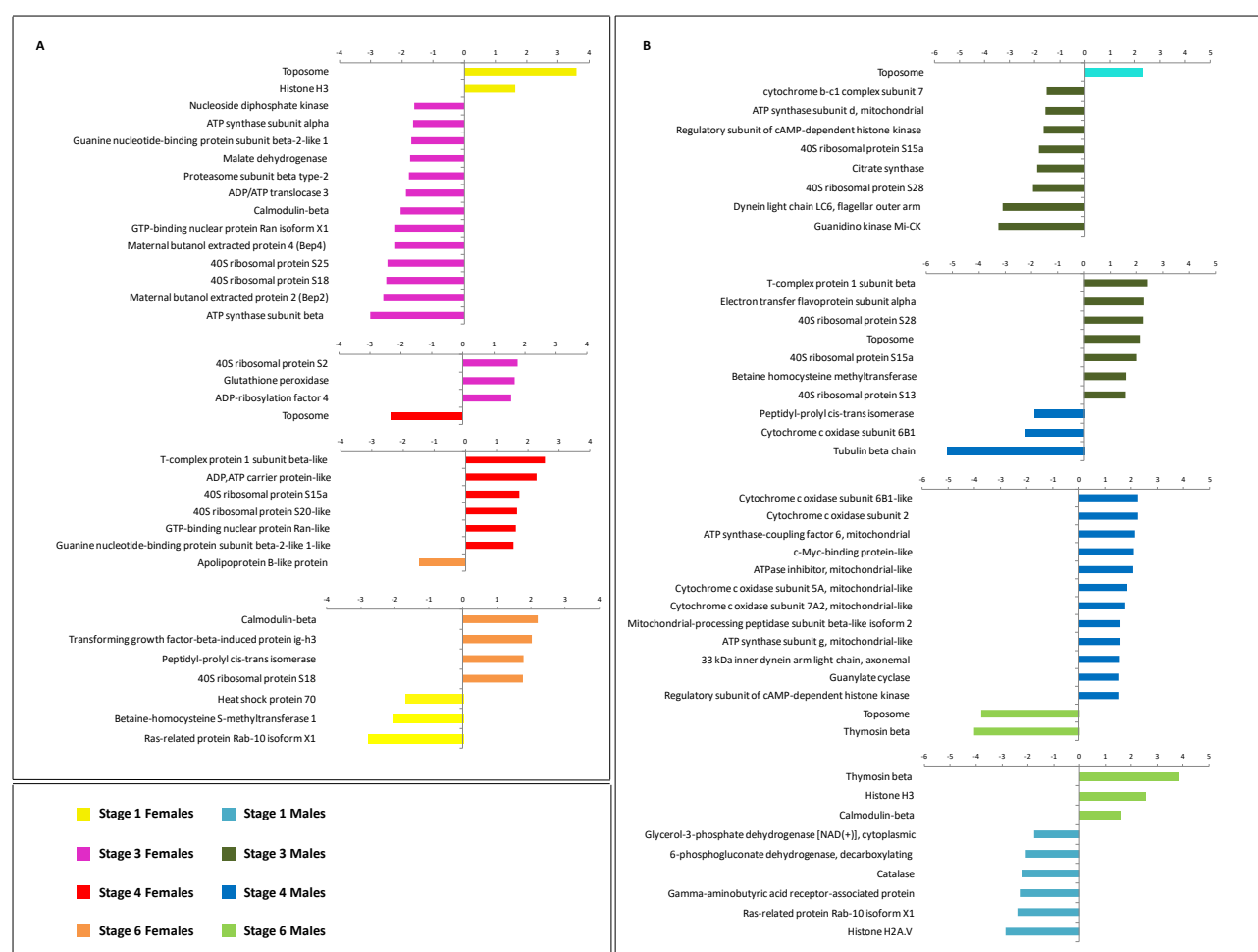


Figure 32 - Bar graph of main differential proteins in the comparison of the four stages in females (panel A) and males (panel B). Only protein changes with a Log Ratio NSAF > 1.5 or < -1.5 and P value < 0.05 are visualized. For the complete dataset please refer to Ghisaura *et al.* (2016) (Data in Brief).

The changes in abundance concerning toposome were in line with the works of Unuma and coworkers (Unuma, 2002; Unuma *et al.*, 2007; Unuma *et al.*, 2003). Adding to this, in order to provide a validation to the proteomics data with an alternative approach, we evaluated tubulin abundance by Western immunoblotting and densitometry in all experimental samples, upon normalization with actin (Supplementary Fig. S2). The results agree with the observations emerging from gel-based and shotgun proteomic experiments: tubulin undergoes minimal fluctuations in female gonads, while it significantly increases in male gonads at advanced maturation stages.

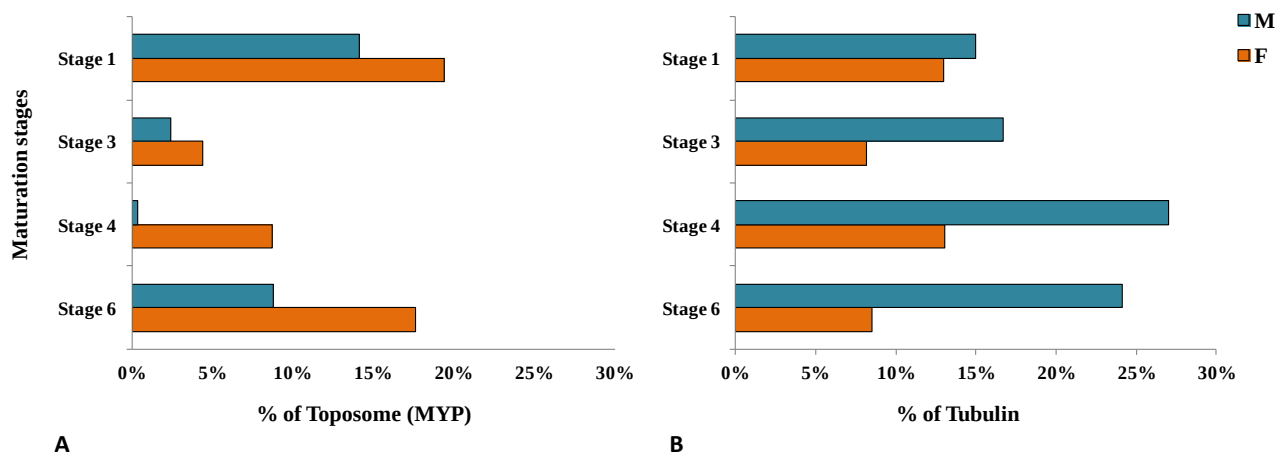


Figure 33 - Histograms illustrating the relative percentage of toposome (panel A) and tubulin (panel B) in all maturation stages for both sexes. F, females; M, males.

5.4.4. Shotgun proteomics for investigating protein abundance changes: sex

5.4.4.1. Mature gonads

In stage 4 gonads, an impressive 37.99% (130/392) of proteins showed statistically significant differences in abundance between females and males (Ghisaura *et al.*, 2016). Of these, 65 were higher in females, and were mainly represented by proteins present in eggs, such as metallothionein, maternal butanol extracted protein, toposome, and ribosomal proteins. A balanced 65 proteins were significantly higher in males, mainly spermatozoa constituents such as tubulins, other flagellar proteins, mitochondrial proteins, and histones. Fig. 34, panel A, provides an overview of the 57 proteins showing the highest changes in abundance in mature gonads.

5.4.4.2. Post mature gonads

In gonads of stage 6, only 32/214 proteins showed statistically significant differences in abundance between females and males (Ghisaura *et al.*, 2016, Data in Brief). Among these, only 13 proteins showed the most intense changes in females (ribosomal proteins, dehydrogenase enzymes, toposome, myosin heavy chain), versus males, mainly represented by tubulin and histones (Fig. 34B).



Figure 34 - Bar graphs of main differential proteins in the comparison of stage 4 (panel A) and stage 6 (panel B) in both sexes. Proteins with a Log Ratio NSAF >1.5 and <-1.5 and P value <0.05 are reported. For the complete dataset please refer to Supplementary file S2.

5.5. Discussion

The sensory quality of *P. lividus* gonads is strongly influenced by the reproductive cycle, with significant changes in flavor, texture, and size. A better knowledge of the protein profiles, patterns, and markers associated with gonad sex and maturation stage can have useful implications for understanding and monitoring these changes. One of these is the ability to identify protein profiles specifically associated with a given stage and, in perspective, to identify maturation and sex markers.

By applying combined gel-based and shotgun approaches, this study generated the largest proteomic dataset available in the literature for *P. lividus*, and provided a general picture of the protein abundance changes occurring along maturation. Our work was carried out on three individuals per sex and stage; therefore, potential individual variation still needs to be taken into account. Some observations may require further refinement by analysis of a larger number of individuals, especially considering that environmental differences, including water temperature or food availability, might have an influence on gonadal maturation or the protein reserves stored in NPs.

Upon gel-based proteomic analysis, female and male gonads showed significant differences in electrophoretic patterns, both between them and among the four developmental stages. Some major proteins changed drastically during the four stages and behaved differently in the two sexes; toposome and tubulin were the most evident, in terms of abundances and trends. Toposome is a glycoprotein of 170 kDa contained mainly in immature gonads (Unuma *et al.*, 1998) and in eggs, where it was originally identified as the most abundant component of yolk granules and termed the major yolk protein or major yolk glycoprotein (MYP) (Unuma *et al.*, 2003). Toposome, which is considered to act as a protein reserve for oogenesis, spermatogenesis, and early development (Scott *et al.*, 1990; Unuma *et al.*, 2003; Walker *et al.*, 2007), is predominantly synthesized in the gut of the adult sea urchin as a 195-kDa protein precursor (Shyu *et al.*, 1986; Brooks and Vessel, 2003) and then secreted into the coelomic fluid, absorbed by the NPs, and stored in large granules before gametogenesis (Ozaki *et al.*, 1986; Unuma *et al.*, 1998; Unuma *et al.*, 2003). It was found that toposome can reach so high values as to dominate the protein pool of nutritive phagocytes (Harrington and Easton, 1982). Moreover, it was suggested that the high energy cost for toposome synthesis can be associated to an upper limit in growth of nutritive phagocytes, and therefore gonad size (Marsh and Watts, 2001). In our study, toposome was observed to be highly expressed in stages 1 and 6 when compared to stages 3 and 4 in both females and males. However, its abundance trends were

different: females showed a decrease in abundance from stage 1 to stage 3, followed by a slight increase at stage 4, which continued in stage 6, while males showed a dramatic decrease from stage 1, to stage 3 and 4, at which the protein virtually disappeared. Then, a strong increase was again seen in stage 6. These observations are in line with those by Unuma (Unuma *et al.*, 2003), reporting that toposome accumulates in NPs in both sexes before gametogenesis. In fact, at this stage the gonads start increasing in size by accumulating nutrients into the NPs that fill the gonadal lumina in both sexes. During gametogenesis, NPs start to gradually supply nutrients to the developing germ cells and decrease in size, while the gonadal lumina fills with eggs and sperm (Walker *et al.*, 2001; Unuma *et al.*, 2003; Siliani *et al.*, 2016). The toposome starts to decrease until it disappears completely in mature testes (Unuma *et al.*, 1998), while in mature ovaries ripe eggs still contain toposome (Ozaki *et al.*, 1986), and toposome synthesis probably continues in the course of gametogenesis (Shyu *et al.*, 1986; Unuma *et al.*, 2003). In our work, both the immature ovary and testis contained similar abundant amounts of toposome (about 9% of wet gonad mass), constituting about 80% of total protein, while as gametogenesis proceeded, the toposome content decreased rapidly in males, and more gradually in females. In the fully mature gonad (stage 4), toposome was almost absent in males, and it was less than half of the initial amount in females. Another protein showing intense changes in abundance and differences between males and females was tubulin. Tubulin is the major component of cytoskeletal microtubules, key structural elements of cilia and flagella. Tubulin was highly abundant in males at all investigated stages, showing an intense increase in stage 4. This was in contrast with females, for which the protein was less abundant and showed less dramatic changes. This observation is likely due to the high abundance of tubulin in the flagella of sea urchin sperms, which are constituted by 9 + 2 doublets microtubules, in which the nine doublets surround a central pair (Darszon *et al.*, 2011).

Shotgun proteomics followed by label-free quantitation and differential abundance analysis revealed changes in a high number of other proteins among the four stages, both in female and in male gonads. Figs. 31 and 33 provide a picture of the protein variations occurring along the four analyzed stages in female and male gonads, outlining a progressive increase in the number of differential proteins along maturation, and a decrease in spent stages. The detailed list of all proteins undergoing changes in abundance is reported in Ghisaura *et al.* (2016) [Data in Brief]. Along female gonad development, also in agreement with the aforementioned electrophoresis results, a decrease is seen in toposome and molecular transport and signaling proteins, such as Apolipoprotein B-100 like (Gotthardt *et al.*, 2000; Sewell *et al.*, 2008; Dheilly *et al.*, 2013),

with a parallel increase in proteins associated with eggs including ribosomal proteins, implicated in protein synthesis, and maternal butanol extracted protein, abundant in the fully mature ovary and in the egg (Wessel *et al.*, 2000; Unuma *et al.*, 2003), as well as proteins such as T complex proteins (TCPs), associated with the mitotic spindle and centromeres (Dheilly *et al.*, 2013). Along male gonad development, toposome decreased even more significantly, as discussed above, according to the observations emerged with the gel-based proteomic approach, while mitochondrial proteins increased, including citrate synthase, ATP synthase subunit d, cytochrome *b-c1* complex, and guanidine kinase (Mi-CK), commonly named mitochondrial creatine kinase (Wyss *et al.*, 1995) and principally implicated in energy metabolism of sperms. Flagellar proteins, including dynein light chain (Darszon *et al.*, 2011) and histones, did also increase, as well as cytochrome c oxidase, identified in different subunits: subunit 5A, 5B, 6B and 6C, 7A. Another highly abundant protein in mature male gonads was ATP synthase and ATP synthase associated proteins, also in this case reflecting the significant mitochondrial component of sperms. Other proteins associated to flagellar motility of spermatozoa such as dynein arm light chain and dynein heavy chain were strongly increased in mature male gonads (Brokaw, 2009; Darszon *et al.*, 2011).

Accordingly, when comparing the protein abundance profiles in mature female and male gonads, the differences in protein profiles were numerous and significant, reflecting the massive presence of eggs and sperms, respectively; on the other hand, in immature gonads of both sexes the protein content was highly similar, being mainly constituted by NPs with toposome as the most abundant protein. In immature gonads, the only significant differences were seen in the basic components of spermatozoa, including tubulin and histones. It is known that gonad quality is linked to a high NP to GC ratio (Unuma, 2002); since toposome is linked to NP content while dynein/metallothionein/ribosomal proteins/tubulin/histones are linked to GC content, this may provide an indirect measurement of quality, together with the GSI. Finding the right relationship of toposome or other protein abundances to GSI might indicate which is the best quality roe, large but not too mature.

Currently, the identification of sex and maturation stage of gonads is carried out by histology. Gonads are fixed in formalin, and then analyzed individually at the microscope after slicing and staining. This requires an expert operator and a long time frame for obtaining information that may be useful in shorter times from gonad collection. Wet squash preparations may be a faster alternative, but this technique may still have limitations in correctly classifying some reproductive stages of male sea urchin gonads (Kelly, 2001). According to the observations

made in this study, some of the most intensely changed proteins, such as toposome, tubulin, or histones, may open the way to the development of novel systems, such as rapid electrophoretic tests or field immunoassays, enabling a less cumbersome, more precise and quantitative definition of the maturation stage, thus improving our ability to monitor ecology, quality, and developmental aspects of the sea urchin. Such rapid, dedicated tests may find potential applications for improving the definition of regulatory time frames for wild sea urchin catches, as well as for monitoring development cycles and quality in sea urchin aquaculture plants. In addition, the availability of a novel, detailed dataset of protein abundance changes occurring in gonads may contribute to improve our understanding of sea urchin biology and reproduction.

Acknowledgements

The MS proteomic data in this paper have been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (Vizcaino *et al.*, 2014): dataset identifier PXD004200. This work was funded by the Sardinia Regional Government by means of Sardegna Ricerche (art. 26 L.R. 37/98).

Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2016.05.035>.

6. THE INFLUENCE OF MACROALGAL DIETS ON GONAD YIELD AND COLOR OF ADULT *PARACENTROTUS LIVIDUS*.

Not submitted

6.1. Abstract

In recent years, several studies have been conducted to test different feeds with the aim of improving the sea urchin aquaculture in terms of both the production efficiency and color of gonads. In this study, the effects of *Ulva lactuca* and *Gracilaria gracilis* on gonad condition (yield and color) were evaluated in adult *Paracentrotus lividus*.

Gonad growth and development were evaluated through changes in gonadosomatic indices (GSI) and histological analysis, respectively. Color was assessed by colorimetric analysis. Wet feed intake, absorption efficiency and food conversion ratio (FCR) were also considered.

GSI and gonad development were not affected by diet, but were different from the initial sample. After treatment, gonad color was similar to that of the initial sample with only yellowness differing between the two diets. The yellow index was better (higher) in those urchins fed *U. lactuca*. Wet feed intake and absorption efficiency were higher in urchins fed *G. gracilis*. However, in spite of a similar growth between diets, FCR was lower in urchins fed *U. lactuca*, due to the smaller amount of food ingested.

Although similar results were found between the two diets, the smaller amount of *U. lactuca* ingested makes this alga a more suitable and cost-effective feed in sea urchin aquaculture, also due to its fast growth and the ease of enrichment of its nitrogen content to obtain a highly proteic diet.

6.2. Introduction

Sea urchin gonads represent a delicacy in many parts of the world (Dincer and Cakli, 2007; Arafa *et al.*, 2012a). Therefore, in several countries a continuous harvesting of sea urchins has led to the depletion of wild stocks (Andrew *et al.*, 2002). This makes aquaculture an essential tool for satisfying the increasing demand for gonads (Siikavuopio *et al.*, 2007a).

The marketability of sea urchin roe, as well as its economic value, depends on gonad yield (as a percentage of the total body wet weight) and quality (in particular, color) which are both affected by food availability and diet composition (Gutierrez *et al.*, 2017).

Several studies have been conducted to test different feedings with the aim of efficiently improving gonad production and color. Many authors found that manufactured feeds, which are generally hyperproteic, can enhance the gonad growth more efficiently than macroalgae (Pearce *et al.*, 2002a; Pearce *et al.*, 2004b; Shpigel *et al.*, 2005; Phillips *et al.*, 2010a; Lawrence *et al.*, 2011; Cyrus *et al.*, 2014; Prato *et al.*, 2017). In fact, seaweeds have a highly variable composition, which depends on the species, time of collection and habitat, as well as on external conditions such as water temperature, light intensity and nutrient concentration in the water (Mišurcová, 2012; Makkar *et al.*, 2016). Consequently, macroalgae-based feeds are generally less growth-efficient than artificial ones.

On the other hand, manufactured diets cause a paler gonad color than seaweeds (Lawrence *et al.*, 1997; McBride *et al.*, 1997; Barker *et al.*, 1998; Walker and Lesser, 1998; McBride *et al.*, 2004; Pearce *et al.*, 2004b; Shpigel *et al.*, 2005). Therefore, some authors recently investigated the possibility of producing valuable gonads of a desirable size and color using a combination of algal and prepared diets (Shpigel *et al.*, 2005) or of supplementing artificial diets with a certain amount of dried macroalgae (Cyrus *et al.*, 2014).

The massive production of macroalgae for animal feeding is currently not economically feasible. However, the recent set-up of IMTA (Integrated Multi-Trophic Aquaculture) plants in several countries is considered a promising possible way to reduce production costs. Specifically, the IMTA system allows for the rearing of various aquatic species of different trophic or nutritional levels in the same plant (Chopin, 2006; Neori *et al.*, 2007), thus promoting the re-use of resources such as water, feed, energy and space for the production of other commercial species (Chopin *et al.*, 2001). Regarding species which can be reared in IMTA systems, Barrington *et al.* (2009) identified *Paracentrotus lividus* for Echinoderms and *Ulva spp.* and *Gracilaria spp.* for seaweeds among the genera of interest with the highest potential in marine temperate waters.

Due to its roe content, *P. lividus* is a promising candidate for the emerging aquaculture industry of some European countries (Tomšić *et al.*, 2010; Carboni *et al.*, 2012). *Ulva spp.* are among the few species of green macroalgae to be cultivated. They are characterized by a rapid reproduction and fast growth in suitable environmental conditions (Littler and Littler, 1980),

and are therefore commonly used in aquaculture as fresh feed for herbivores (Robertson-Andersson, 2003). The genus *Gracilaria* has been demonstrated to be the most attractive candidate for sea urchin culture due to its ability to achieve high yields and produce commercially valuable products (Zhou *et al.*, 2006). Both genera are particularly abundant in the Sardinian lagoons, thus representing a cheap, sustainable source of nutrients for *P. lividus*.

In this study, we evaluated the effects of *Ulva lactuca* and *Gracilaria gracilis* on gonad growth and color of adult *P. lividus*. Somatic growth, gonad development, feed intake, absorption efficiency and food conversion ratio were also considered.

6.3. Material and methods

6.3.1. Sample collection, experimental design and measured parameters

Adult individuals of *P. lividus*, with a diameter ranging from 40 to 45 mm, were collected in January 2017 by scuba divers from a rocky bottom at Su Pallosu Bay (40°03' N; 008°25' E), at a mean depth of five meters. The bay is located on the central-western Sardinian coast. In this size class, the greatest amount of energy is allocated to reproduction (Fabbrocini *et al.*, 2012). Sea urchins were wrapped in cloth soaked in salt water, stocked in iceboxes and immediately transported to the laboratory. Once in the laboratory, individuals were stocked in plastic, truncated-cone containers with a volume of 2.9 L and a surface of 0.085 m², with one individual per container. The containers were perforated throughout the entire surface, to allow the seawater to flow inside them.

As for previous works (Siikavuopio *et al.*, 2007b; Cárcamo, 2015), all the containers floated in the same 600 L rectangular tank (385 cm L x 18 cm W x 85 cm H), which contained filtered seawater (1 µm) at 37.4 ± 0.1 ‰ salinity (mean $\pm$ SE), temperature of 18.1 ± 0.1 °C, pH of 8.4 ± 0.1 and dissolved oxygen of 90.4 ± 0.8 %. The tank was supplied with a recirculating system (flow of 5 L/min) and equipped with a biological and a mechanical filter (10 µm). A 50% seawater exchange was done once a week. Gentle aeration and natural photoperiod were applied.

Prior to the experiment, individuals were starved and held for 2 weeks in the experimental laboratory system to acclimatize and standardize their nutritional condition (Vadas, 1977; Minor and Scheibling, 1997). During this period, no mortality was observed.

The experimentation began in February 2017. At the beginning (T0), the state of the sea urchins was assessed. Twelve individuals were randomly selected, blotted dry with a paper towel to remove excess water, and weighed (total wet weight, TWW). Test diameters (TD) were

measured using a caliper and the mean test diameter was calculated. Individuals were then dissected and the gonads were removed and weighed (wet weight). The gonadosomatic index (GSI) was calculated through the formula [gonad wet weight / total wet weight] x 100 as reported by Lawrence *et al.* (1965).

One gonad section from each individual was prepared for histological analysis. Each gonad sample was preserved in neutral buffered formalin to determine the reproductive state. According to Byrne (1990), six stages were recognized: (I) recovering stage with many large nutritive phagocytes and only few small primary oocytes or spermatocytes, (II) growing stage with plenty of nutritive phagocytes and larger oocytes or a thicker layer of spermatocytes, (III) premature stage with the first eggs or spermatozoa accumulating in the lumen, (IV) mature stage with no or few nutritive phagocytes surrounding a large number of mature eggs or spermatozoa, (V) partly spawned stage containing few nutritive phagocytes and loosely packed gametes, and (VI) spent stage with gonads without gametes.

Gonad color was measured according the colorimetric method specified in Addis *et al.* (2014). Analyses were carried out by a digital colorimeter (Chroma meter CR-400; Konica Minolta, Tokyo, Japan) at standard luminant D65, which specifies the color according the Commission Internationale de l'Eclairage (CIE) L*a*b* color space (CIELAB) (CIE, 2008).

Color was broken down into three coordinate values: L* (lightness), a* (redness), and b*(yellowness). Three replicate measurements of each component were taken for each gonad sample.

Sea urchins used for the experiment (N=12 for each treatment) were fed *ad libitum* with fresh thalli of *Ulva lactuca* (U diet) and *Gracilaria gracilis* (G diet) for three months. *U. lactuca* was harvested in the Santa Gilla lagoon (39°13'N, 9°04'E), whereas *G. gracilis* was collected in the Mistras lagoon (39°90'N, 8°46'E). Thalli were collected between the end of July and the beginning of August 2017, carefully washed with fresh water to remove epiphytes and promptly frozen at -80°C. Prior to the administering of algae, thalli were defrosted and weighed (wet weight).

Uneaten feed, when present in the containers, was removed and weighed (wet weight). Five samples of each feed were dried in an oven at 60°C for 3 days and weighed (dry weight) (Cárcamo, 2015). The average ratio dry weight/wet weight (0.11 and 0.14, for *U. lactuca* and *G. gracilis*, respectively) was used to calculate the dry weight of feed intake (McCarron *et al.*, 2009). Feces were siphoned weekly, dried at 60°C for 3 days and then weighed (dry weight) (Cárcamo, 2015).

At the end of the experiment, the wet feed intake (gr/urchin/day) was calculated as the difference between the total amount of wet feed provided and the total amount of wet feed uneaten, divided for the days of experimentation. The absorption efficiency was calculated by $[(\text{average dry feed intake per individual} - \text{average dry feces siphoned per individual}) / \text{average dry feed intake per individual} \times 100]$ (Hammer *et al.*, 2004). The food conversion ratio (FCR) for each treatment was calculated as $[\text{total dry feed intake (gr dry weight/urchin)} / \text{total wet body weight gain (gr wet weight/urchin)}]$ (Hammer *et al.*, 2004).

All animals that survived until the end of the experiment were weighed, test diameter was measured and gonads were weighed as well. One gonad section from each individual was histologically examined. Gonad color of each individuals was measured following the method described above for the initial sample.

6.3.2. Statistical analysis

Statistical comparisons for somatic growth, GSI, color, wet feed intake, absorption efficiency and FCR were performed using Statistica 6.1 StatSoft, Inc. (2004). Prior to analysis, all data were tested for normality (Shapiro Wilk's W test) and homogeneity of variance (Levene's test). If data were normal and homoscedastic, one-way ANOVA was performed. When data violated the ANOVA assumptions, nonparametric tests (Kruskal-Wallis, Mann-Whitney U tests) were preferred. Tukey's honestly-significant difference (HSD) test was used to evaluate all pair-wise treatment comparisons ($p < 0.05$).

6.4. Results

6.4.1. Survival

At the end of the experiment, one dead individual was counted for the U diet, while two dead urchins were recorded for the G diet.

6.4.2. Somatic growth

By the end of the trial, there was no significant difference in TD between the two diets ($p > 0.05$), without any increase in size with respect to the initial sample (T0) (Table 14).

No significant difference in TWW was registered between diets neither from the initial sample ($p > 0.05$), although they were slightly lighter than urchins at T0 (Table 14).

6.4.3. Gonad index

GSI increased from a mean value of 0.87 ± 0.27 in the initial sample to 2.34 ± 0.51 in the sea urchins fed the U diet and 2.88 ± 0.73 in the G diet (Table 14). Values did not differ between the two diets, and both diets produced significantly higher GSI ($p < 0.01$) than in the initial sample (Table 14).

Table 14 - Mean test diameter (TD), total wet weight (TWW), gonadosomatic index (GSI), CIE L* (lightness), CIE a* (redness) and CIE b* (yellowness) observed in the initial sample and at the end of the feeding trial. Superscript letters indicate the results of a Tukey's multicomparison post hoc test showing significant pair-wise differences between treatments. Different superscripts indicate significant differences for data in the same column.

Treatment	TD (mm)	TWW (g)	GSI	CIE L*	CIE a*	CIE b*
Initial sample (T0)	44.17 ± 0.32^a	33.99 ± 1.49	0.87 ± 0.27^b	42.34 ± 1.58	19.7 ± 1.15	31.29 ± 1.67^{ab}
<i>Ulva lactuca</i> (U)	43.27 ± 0.38^{ab}	36.49 ± 0.88	2.34 ± 0.51^a	46.75 ± 1.33	18.31 ± 1.50	34.41 ± 1.42^a
<i>Gracilaria gracilis</i> (G)	42.30 ± 0.33^b	36.52 ± 1.28	2.88 ± 0.73^a	45.97 ± 2.12	14.83 ± 1.92	26.87 ± 1.87^b

6.4.4. Reproductive conditions

Urchin gonads from the initial sample were mainly in spent (67%) and partly spawned (17%) stages. The rest of individuals (18%) were found to be in the recovery or premature stages (Fig. 35). At the end of the experiment, sea urchins fed the U diet were mostly in the growing stage (47%), with some individuals in the recovery (27%) and premature (27%) stages (Fig. 35). Individuals fed the G diet were approximately in the same reproductive condition of those fed the U diet but distributed differently (recovery 10%, growing 60%, premature 20%), plus 10% mature individuals (Fig. 35).

6.4.5. Quantitative color assessment (CIE L*a*b)

The two treatments produced a lighter roe color (CIE L*, Fig. 36), although it was no significantly different from the gonad color of the initial sample ($p > 0.05$; Table 14).

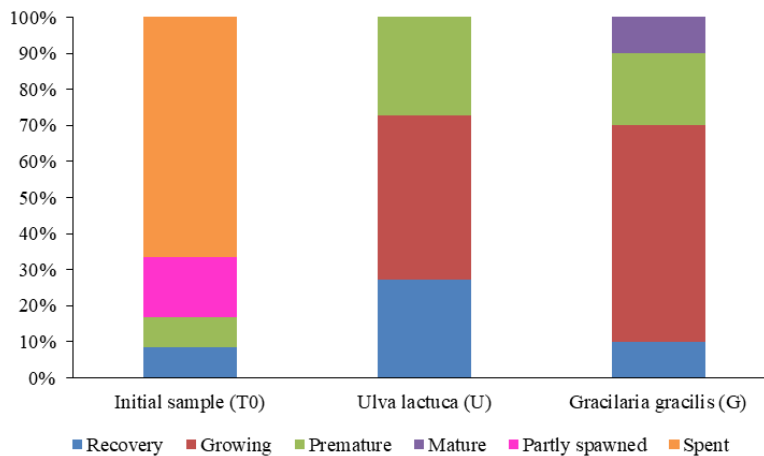


Figure 35 - Reproductive conditions of gonads from the initial sample and the experimental treatments at the end of the experiment.

At the end of the trial, the two diets did not produce a redder gonad color (CIE a^*) than in the initial sample (Fig. 36). The urchins fed the U diet showed redder gonads (18.31 ± 1.50) than those fed the G diet (14.83 ± 1.92) (Fig. 36 and Tab. 14), nevertheless a^* values were not significantly different between treatments ($p > 0.05$, Table 14). The U diet produced significantly yellower gonads (CIE b^* , 34.41 ± 1.42 , $p < 0.05$) than the G one (26.87 ± 1.87) (Fig. 36). Neither diets showed any differences when compared to the initial sample (31.29 ± 1.67 , Table 14).

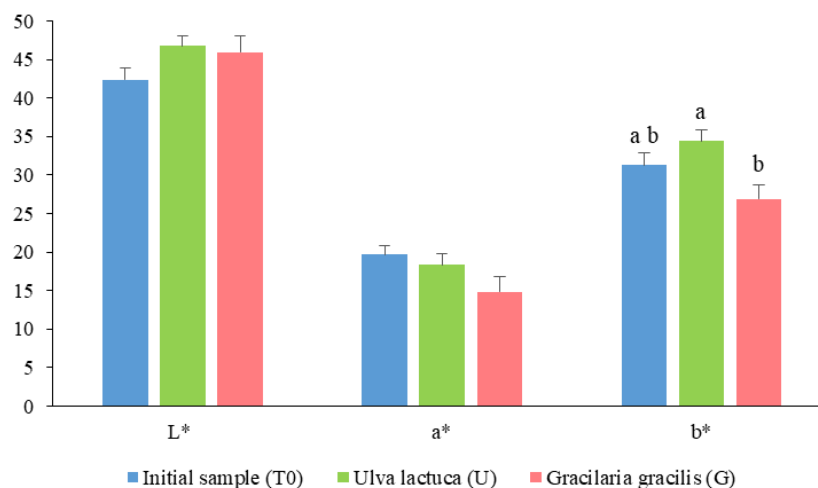


Figure 36 - Mean lightness (L^*), redness (a^*) and yellowness (b^*) of gonads from the initial sample and the experimental treatments. Different letters above each bar indicate the results of a Tukey's multicomparison post hoc test showing significant pair-wise differences between treatments. Differing letters indicate significant differences.

6.4.6. Feed intake, absorption efficiency and food conversion ratio

Individuals fed the G diet resulted in a significantly higher amount of wet feed intake in comparison with the U diet ($p < 0.001$): the daily grams per urchin were 1.5-fold higher (Table 15). The absorption efficiency of the G diet was over twice that of the U diet ($p < 0.01$, Table 15). The food conversion ratio resulted in a significantly higher value (nearly 3-fold higher) for the urchins fed the G diet than the U diet ($p < 0.05$, Table 15).

Table 15 - Wet feed intake, absorption efficiency and food conversion ratio (FCR) observed at the end of the feeding trial, for both treatments. Superscript letters indicate the results of a Tukey's multicomparison post hoc test showing significant pair-wise differences between treatments. Different superscripts indicate significant differences for data in the same column.

Treatment	Wet feed intake (g/urchin/day)	Absorption efficiency	FCR
<i>Ulva lactuca</i> (U)	0.88±0.01 ^b	11.52±3.05 ^b	9.15±1.71 ^b
<i>Gracilaria gracilis</i> (G)	1.35±0.03 ^a	26.10±2.61 ^a	26.75±6.47 ^a

6.5. Discussion

Although the two diets showed differences in food consumption, absorption efficiency and food conversion ratio, they produced similar somatic, gonad growth and development of gonad color.

On a wet-weight basis, sea urchins ingested significantly more *G. gracilis* than *U. lactuca*. These results are in contrast with those obtained by Cárcamo (2015). This author, in a feeding experiment which consisted in supplying several monospecific macroalgal diets to individuals of *Loxechinus albus*, found that the ingestion of wet *Ulva* sp. was one of the lowest, although it did not differ from that of *Gracilaria chilensis*. It is well-known that sea urchins, when offered different natural diets, may display a preference for some seaweed species over others (Steinberg and van Altena, 1992; Dworjanyn *et al.*, 2007). This preference could depend on several factors: the nutritive value of the food, its physical properties such as texture, shape, and consistency, or the presence/absence of attractant/deterrent compounds (Nagi and Kaneko, 1975; Vadas, 1977; Sakata *et al.*, 1989; Hay and Steinberg, 1992; Hay, 1996; Wright *et al.*, 2005; Cárcamo, 2015). This is considered an adaptive behavior because sea urchins often grow faster when fed with highly preferred seaweeds (Dworjanyn *et al.*, 2007).

Absorption efficiency was strongly affected by diet, being higher in the urchins fed the G diet. The low absorption observed for the U treatment was related to i) the small difference between the amounts of algae ingested and the fecal material produced (i.e. they digested a smaller amount of food in comparison with those fed *G. gracilis*), and ii) the low dry feed intake. On the contrary, the high dry feed intake of the urchins fed *G. gracilis* contributed to a higher absorption, despite the fact that the feces produced were quantitatively more consistent. Generally, seaweeds are absorbed with a significantly lower efficiency than other diets, due to a certain amount of non-digestible material in the algae (mainly complex polysaccharide components) as shown by a high production of feces (Otero-Villanueva *et al.*, 2004). The difference in absorption efficiency observed between our two algal treatments may be ascribed to the fact that *U. lactuca* is less digestible than *G. gracilis*.

Feed conversion ratio is an important meter in evaluating the capacity of individuals to convert feed into body mass (assimilation) (Heflin *et al.*, 2013). Several studies have highlighted that the consumption of relatively protein-rich feed is coupled with an increased feed conversion ratio (FCR) (McBride *et al.*, 1998; Fernandez and Boudouresque, 2000; Hammer *et al.*, 2004). On the contrary, Hammer *et al.* (2012) found that high dietary protein content corresponded to a lower FCR (and higher gonad production efficiency). The same results were obtained by Heflin *et al.* (2012) who reported comparatively low FCR when *Lytechinus variegatus* was fed with artificial feeds with high protein levels. In Cárcamo (2015), the lowest and best FCRs were obtained with *G. chilensis* followed by *Ulva* sp. There were no statistical differences between treatments even if the former was higher in organic matter and protein content. It seems that *Gracilaria* species have higher proteic content than the *Ulva* species. In fact, the protein content in *Ulva* spp. is in the range of 15-20% (dry weight) (Pycke and Faasse, 2015), although lower (4.2%) or higher (27.2%) values have been reported (Portugal *et al.*, 1983; Ortiz *et al.*, 2006). The protein levels in *Gracilaria* spp. ranges from 5.6% to 30% (Briggs and Smith, 1993; McDermid and Stuercke, 2003; Marrion *et al.*, 2005; Renaud and Luong-Van, 2006; Wen *et al.*, 2006; Marhino-Soriano *et al.*, 2007) although Francavilla *et al.* (2013) reported the highest protein concentrations (31-45% d.w.) for *G. gracilis* from the Lesina lagoon. It is known that the crude protein content of seaweeds can vary according to season and environmental conditions (Fleurence, 1999).

In our study, the urchins fed with *U. lactuca* had the lowest, i.e. best, FCR, while those fed with *G. gracilis* showed the worst FCR due to the two-fold larger amount of dry food ingested.

Typically, individuals exhibiting a lower FCR grow faster than those with a high one (Heflin *et al.*, 2013). Despite the fact that somatic growth was not the focal point of our work, it is worth saying that both the test size and the total weight of the urchins were similar between treatments. In addition, no differences were observed when comparing the measures obtained at the end of the trial with those from the initial samples. Shpigel *et al.* (2005) did not notice any somatic growth during a 12-week experiment during which adult *P. lividus* (40.18 ± 3.08 mm and 29.62 ± 5.79 g, test diameter and whole wet weight respectively) were fed a mix of *U. lactuca* and *G. conferta*. In echinoids, the fastest somatic growth is generally observed in the first year (10-20 mm test diameter). Subsequently, sea urchins continue growing for several years but growth slows as animals increase in size until it may eventually stop or become immeasurably low (Emlet, 2002).

Sea urchin gonads act as the main organ of nutrient storage (Gonor, 1973; Siikavuopio *et al.*, 2006). The amount of nutrient intake, and subsequent gonad growth, depends on food quantity and/or quality and the rate of consumption, digestion, and absorption (Lawrence, 1975; Lares and McClintock, 1991; Lawrence *et al.*, 2003). At the end of the experiment, gonads were significantly larger than those of the initial sample independent of diet. However, the two algae produced similar gonad yields despite the different amount of food ingested and the different absorption efficiencies between diets. Similar results were obtained by Schlosser *et al.* (2005) in a two-months feeding test where adult *P. lividus* (31-34 mm test diameter) were fed *U. lactuca* and *G. conferta*. At the end of the test, gonad index was not affected by algal diet, even if the quantity of the *G. conferta* ingested daily by urchins was higher on average than that of the *U. lactuca*.

The gametogenic state progressed from having a majority of urchins in the initial sample in partly spawned or spent stages (i.e. at the end of their reproductive cycle), to a more advanced condition of gonads after three months for both diets. Gonads were mostly in the growing stage or even in further ones, e.g. the premature one with the first mature gametes and the mature stage. Our results show that food quality and quantity promote gametogenesis, although Pearse and Cameron (1991) stated that food quantity and quality can affect gonad size, but do not initiate gametogenesis. In this work, the two algae reached a “critical level” of nutrients which triggered the onset of gametogenesis in sea urchin gonads (Pearse, 1969).

Depending on the local market preference, acceptable gonad color can range from a bright yellow to a dark orange or almost red. Lighter, pale-colored or dark-brown gonads are not as

desirable in the market (Robinson *et al.*, 2002). Dietary carotenoids are the major influencing factor in gonad coloration (Griffiths and Perrott, 1976; Agatsuma *et al.*, 2005). β -carotene, in particular, appears to be the pigment responsible for the yellow-orange coloration in most echinoid species (Griffiths and Perrott, 1976; Matsuno and Tsushima, 2001; Robinson *et al.*, 2002; Pearce *et al.*, 2004a). Algal diets have been successfully used in improving the coloration of the gonads of several echinoid species (Robinson *et al.*, 2002; Vadas *et al.*, 2000; Shpigel *et al.*, 2005), however the macroalgae selected for our experiment were not very effective in enhancing gonad color. Sea urchins fed on the two diets achieved lower CIEL*a*b* values than those reported by other authors for the same species fed with kelp (McCarron *et al.*, 2009; McCarron *et al.*, 2010). Neither the U nor G diets promoted any significant improvements in color compared to the initial post-starvation conditions (T0, $p > 0.05$). Green seaweed species (such as *Ulva* sp.) include β -carotene, lutein, violaxanthin, neoxanthin and zeaxanthin, while red seaweed species (such as *Gracilaria* sp.) contain mainly α - and β -carotene, lutein and zeaxanthin (Holdt and Kraan, 2011). The mix of these pigments resulted in no significant differences between treatments, except for yellowness (b^*), which was significantly higher in the urchins fed *U. lactuca* ($p < 0.05$).

In conclusion, *U. lactuca*- and *G. gracilis*-based diets were moderately effective in terms of gonad growth, even though with an increase in gonad size lower than the one obtained by administering formulated diets (Prato *et al.*, 2017). However, they were not efficient in improving gonad color. The total amount of *U. lactuca* consumed during the trial was more than 1.5-fold lower than the total quantity of *G. gracilis* ingested. Therefore, from an economic point of view, it would be most profitable to feed animals with *U. lactuca*. This green alga is a perfect aquaculture candidate due to its ability to produce large biomass rapidly (Bolton *et al.*, 2008). Consequently, it is one of the most cultured species together with marine animals (Cohen and Neori 1991; Jimenez del Rio *et al.*, 1996; Neori *et al.*, 1996; Neori *et al.*, 1998; Neori and Shpigel 1999; Neori *et al.*, 2000; Schuenhoff *et al.* 2003). This is also due to its excellent bioremediation capabilities when cultivated under high N regimes (Nielsen *et al.*, 2012). Growing *Ulva* spp. thalli in enriched nitrogen waters (e.g. aquaculture wastewater) allows for the production of a highly proteic feed (Shpigel *et al.*, 1999). Since the N content of green algae is positively correlated to protein content (Lourenco *et al.*, 2002), the use of “enriched” *Ulva* sp. as high-protein source in invertebrate or fish feed could be a cost-effective way to enhance the production of commercial species such as sea urchins.

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7. GENERAL DISCUSSION

In many countries such as Japan and the US, sea urchin gonads represent a gourmet delicacy and are often eaten as sushi. Due to the importance placed on product presentation in the market, the quality of the roe (i.e. size, color and chemical composition) affects its appearance, and influences the price of the product (Whitaker *et al.*, 1997; Unuma, 2002; McBride *et al.*, 2004). Also in southern Italy, especially in Sardinia, *Paracentrotus lividus* harvesting has an important socio-economic role (Pais *et al.*, 2007; Addis *et al.*, 2012).

The high variability of quality may concentrate the fishing effort in areas where conditions are likely to be favorable, thus compromising the survival of wild stocks. Identifying the factors implied in these changes and their effect on gonads may be helpful to improve the overall management of the fishing activities.

With this perspective, this PhD thesis aimed to enhance the existing knowledge of some biological aspects of this sea urchin species, to i) improve current management practices for fishery in Sardinia and ii) encourage the developing of echinoculture in Europe, in the wake of Japan (Unuma *et al.*, 2015) and China (Liu and Chang, 2015).

The following paragraphs will briefly summarize the main findings.

7.1. Gonad production

In chapter 2, we suggested that the continuous catches of large sea urchins ($TD \geq 50$ mm) lead to the reduction of individuals' body-size, which in turn influences the size of gonads (Mita *et al.*, 2007). The reduction of diameter due to high fishing pressure has been already described in several Atlanto-Mediterranean areas (Guidetti *et al.*, 2004; Gianguzza *et al.*, 2006; Pais *et al.*, 2007; Ceccherelli *et al.*, 2009; Bertocci *et al.*, 2014). Precisely the smaller diameter seems to have caused the reduction of gonad yield of the fertile individuals in Su Pallosu Bay (subject to high fishing pressure), which in turn may have led to the decrease in the number of reproductive events. Actually, this hypothesis seems to be corroborated by a previous work conducted in a nearby area (Baroli *et al.*, 2006) intensely affected by fishing (Marra *et al.*, 2016). This reduction of the number of reproductive events could lead, in future times, to both economic and ecological consequences.

Contradictory results have been obtained from field works on the effects of habitat on *P. lividus* gonadosomatic index. For instance, Guettaf (1997) found no differences in gonad yield in populations from different habitats. On the contrary, other authors (San Martín 1995; Ouréns *et al.*, 2011; Boudouresque and Verlaque, 2007; Fernandez and Boudouresque, 1997; Sellem and Guillou, 2007; Addis *et al.*, 2014) demonstrated the influence of habitat features on GSI by pointing out the differences in food availability or environmental conditions between habitats. In this work, as explained in chapter 3, the habitat in which sea urchins live seems to affect gonad production. Neither food availability (similar Repletion Index between habitats) nor environmental features (same photoperiod and temperature due to the closeness of the sampling sites) were the responsible for the difference gonad production observed. A plausible explanation could be that the *P. oceanica* meadow fosters sea urchins to address all the energies derived from food to gonad investment, thanks to the efficiency of its canopies in weakening the waves and reducing water flow speed (Infantes *et al.*, 2012; John *et al.*, 2015; John *et al.*, 2016). On the contrary, in rocky bottoms, sea urchins are more exposed to waves than in meadows, and gonad production is lower.

Gonad production varies according to season and is strictly linked to the sea urchin's developmental stage (Chen *et al.*, 2013). Gonads go through a change of their cellular structure, with the ratio of germinative and somatic cells varying during the reproductive cycle (Unuma, 2002). As we observed by matching the results of the histological analysis with those relative to gonad size (GSI) for two sampling years (chapter 3), gonad yield begins to grow in conjunction with the start of gametogenesis (which in turn is triggered by decreasing photoperiod and temperature) to reach its highest values when gonads are just about to spawn (Chen *et al.*, 2013).

Gonad production is also affected by dietary factors. When urchins are fed ad libitum with desirable foods, roe yield increases (Lawrence *et al.*, 2003; Hammer *et al.*, 2004). This reflects the dual function of gonads as a reproductive organ and storage tissue (Hughes *et al.*, 2006). Dietary proteins have been identified as the main contributing factor for the gonad enhancement (de Jong-Westman *et al.*, 1995; McBride *et al.*, 1998; Pearce *et al.*, 2002a,b; Hammer *et al.*, 2004; Pearce *et al.*, 2004; Cook and Kelly, 2007). In chapter 6, since no differences in gonad growth were found in urchins fed ad libitum with *U. lactuca* and *G. gracilis*, it has been assumed that the two macroalgae have the same proteic content. However, it is worth to note that the crude protein content of algae varies seasonally and according to environmental features

(Fleurence, 1999), and that several works (among all, Francavilla *et al.*, 2013) highlighted a higher protein amount in *Gracilaria* species. Further analysis on the proximate composition of algae is ongoing in our laboratory and will permit to unravel this issue.

7.2. Gonad color

Gonad color varies seasonally and marginally, according to the growing habitat. In chapter 3, we compared gonads from two different habitats, i.e. a rocky bottom covered by photophilic macroalgae and a dense *P. oceanica* meadow standing on a sandy bottom. When examining the three color components separately, only L* (lightness) and b* (yellowness) seem to be habitat-related, being higher in urchins from the rocky bottom. In particular, the best L* is found in spring, followed by summer and autumn, a* is higher in summer and autumn while b* shows similar values all year round, except in winter when it decreases significantly. The dissimilarities between habitats could be related to the different amounts and compositions of pigments contained in seaweeds and in *P. oceanica* leaves. Although the pigment content of *P. oceanica* has been deeply investigated (Dalla Via *et al.*, 1998; Marin-Guirao *et al.*, 2011), that of algae in rocky sites is difficult to assess, since algal composition changes during the year as well as the contribution of carotenoids for each algal species (Addis *et al.*, 2014). Further analyses on pigments in macrophytes collected in the two habitats are certainly desired.

It is known that the ingestion of food containing a certain amount of pigments may influence gonad color in sea urchins. Generally, macroalgae-based feeds are more effective than artificial feeds in enhancing gonad color (Lawrence *et al.*, 1997; McBride *et al.*, 1997; Barker *et al.*, 1998; Walker and Lesser, 1998; McBride *et al.*, 2004; Pearce *et al.*, 2004b; Shpigel *et al.*, 2005). However, in the experiment described in chapter 6, neither *U. lactuca* nor *G. gracilis* allowed us to obtain gonads with an improved color, when compared to an initial sample of starved urchins. In fact, L*, a* and b* did not grow with respect to the initial conditions, and this was true despite they were administered ad libitum over a three-month period. Although a small difference was found in the yellowness of gonads, with higher values for individuals fed *U. lactuca* than for those fed *G. gracilis*, sea urchins fed on the two diets achieved lower CIEL*a*b* values than those reported in the literature for the same species fed with kelp (McCarron *et al.*, 2009; McCarron *et al.*, 2010). This may indicate a more effective pigment composition of brown algae and it should be considered for future feeding experiments.

7.3. Gonad chemical composition

Chemical composition is affected by either environmental, seasonal or physiological factors (Dincer and Cakly, 2007; Mol *et al.*, 2008; Phillips *et al.*, 2009), which in turn affect sensory parameters characterizing sea urchin gonads.

As stated in chapter 4, both physiological and seasonal factors affect lipid absorption and storage mechanisms more than diet or other environmental parameters related to the growing area. This is in agreement with Fernandez (1997). In general, total lipid content found in gonads of Sardinian *P. lividus* is similar to that found in other locations for the same species (Serrazanetti *et al.*, 1995; Fernandez, 1998; Montero-Torreiro and Garcia-Martinez, 2003; Dincer and Cakli, 2007; Mol *et al.*, 2008). Data concerning the time-evolution of the total lipid content in sea urchin gonads are often contrasting (Fernandez, 1998; Montero-Torreiro and Garcia-Martinez, 2003; Dincer and Cakli, 2007; Mol *et al.*, 2008; Arafa *et al.* 2012). We found the lowest lipid content in gonads corresponding to high percentages of reproductive stages III (premature) and IV (mature), as observed by Martinez-Pita *et al.* (2010b) and in general agreement with Arafa *et al.* (2012). This implies that fat is used by sea urchins as an energy source during gametogenesis. In addition, since a change in the photoperiod anticipates a variation in gonad lipid content, this is a more efficient exogenous factor than temperature in triggering and regulating fat utilization and storage in *P. lividus* gonads. Anyway, it is probable that the evolution of lipid content during the year may partly depend on the quantity of fat introduced with the algal diet. In fact, in marine ecosystems, the lipid composition of animal tissues is greatly affected by dietary lipids (Martinez-Pita *et al.*, 2010a, b), and the lipid content in algae is positively correlated to light and water temperature (Olofsson *et al.*, 2012; Weldy and Huesemann, 2007). Further investigation on the relation between dietary macroalgae and gonad lipid composition is desirable.

The fatty acid composition of gonads found in our work is mainly represented by 14:0, 16:0, EPA and ARA, in general agreement with literature (Serrazanetti *et al.*, 1995; Mol *et al.* 2008; Martinez-Pita *et al.*, 2010b; Arafa *et al.*, 2012; Pereira *et al.*, 2013; Angioni and Addis, 2014). Gonadal content of EPA resulted higher than in other specimens of *P. lividus* harvested in other areas of Sardinia (Angioni and Addis, 2014), but similar to those reported by Serrazanetti *et al.* (1995), Mol *et al.* (2008) and Arafa *et al.* (2012). The growing area strongly influences the gonad fatty acid profile. This result is certainly of primary importance when nutritional and sensorial qualities of sea urchin gonads have to be evaluated or predicted. Our findings are in

general agreement with Martinez-Pita *et al.* (2010a), who mainly discriminated different sampling sites by 14:0, some MUFAs (C18:1 11c, C20:1 9c and C20:1 11c), the two main long chain PUFAs, EPA and DHA, and another essential fatty acid, ARA, explaining such site-associated compositional differences as imparted by diet composition. Specifically, in our work MUFAs are the main discriminative fatty acids; the differences between growing areas may be linked to the dissimilar physical characteristics that potentially reflect a different seaweed composition. Only minimal seasonal influences have been observed on the fatty acids of gonads, with ARA and EPA, normally involved in reproductive or temperature adaptation processes (Gago *et al.*, 2009; Martinez-Pita *et al.*, 2010a, b; Carboni *et al.*, 2013; Angioni and Addis, 2014), as the main discriminative FAs.

Gender and developmental stage affect the gonad protein profile (chapter 5). The protein content is similar in immature gonads of both sexes, mainly constituted by nutritive phagocytes, with toposome as the most abundant protein (Harrington and Easton, 1982; Unuma *et al.*, 2003). On the other hand, the differences in protein profiles in mature female and male gonads are numerous and significant. In females, the decrease of toposome is followed by an increase in proteins associated with eggs, such as ribosomal proteins, maternal butanol extracted protein, metallothioneins and T complex proteins (Wessel *et al.*, 2000; Unuma *et al.*, 2003; Dheilly *et al.*, 2013). In males, the decrease of toposome is concomitant with an increase in mitochondrial proteins implicated in energy metabolism of sperms (e.g. citrate synthase, ATP synthase subunit d, cytochrome *b-c1* complex, and mitochondrial creatine kinase) as well as flagellar proteins (e.g. tubulin, dynein and histones) (Wyss *et al.*, 1995; Brokaw, 2009; Darszon *et al.*, 2011).

7.4. Conclusions, suggestions for fishery and aquaculture, and further studies

With this PhD thesis, I aimed to broaden the existing knowledge on *P. lividus* from Sardinia, focusing attention on those factors (extrinsic and intrinsic) that may be involved in the changes in gonad yield, color and chemical composition.

7.4.1. Wild resources

Although sea urchin harvesting in Sardinia is already regulated by regional rules, wild stocks are threatened (Pais *et al.*, 2007; Ceccherelli *et al.*, 2011; Pais *et al.*, 2012). Continuous harvesting activities cause the reduction of gonad yield of commercial-sized individuals, and may have consequences on the survival of populations in the case of the absence (due to low recruitment rates and/or overfishing) of the fertile sub-commercial sized urchins. Therefore, the

regional authority designated to manage the sea urchin resources should prevent, by stringent measures, the catch of the intermediate size class that effectively contributes actively to the maintenance of population in overfished areas.

Another strategy for making fishing more sustainable and profitable could be to alternate harvesting areas or habitats. In this way, those highly-fished rocky substrates with a scarcity of commercial-sized urchins could be relieved of fishing pressure and fishermen could harvest profitable gonads without stopping fishing activities. Regarding this, it seems that *P. oceanica* meadows represent a valid alternative, at least in terms of gonad yield, although a precise assessment of the size of meadows and the abundance of sea urchins of commercial size is required in each harvesting area. Figure 37 summarizes graphically the match of the seasonal trend of GSI with that of each color coordinate, observed in the years 2016-2017 in the two sites (rocky bottom and seagrass meadow) chosen at Su Pallosu Bay. While keeping in mind that yield and color vary seasonally and are habitat-dependent, favorable conditions of gonads (acceptable in size and appearance) have been described from spring to autumn (Fig. 37 a,b). It is particularly evident that the coldest season (i.e. winter) is the least suitable time for harvesting profitable gonads, since GSI and color coordinates have the lowest values (Fig. 37 a,b).

However, since gonad conditions are extremely variable, it is difficult to harvest sea urchin individuals with an acceptable match for both gonad yield and color. Fishermen would need to immediately open each sea urchin to verify the real gonad conditions. This would imply the harvest of huge quantities of individuals in order to obtain adequate quantities of marketable product. This would not be economically nor ecologically feasible.

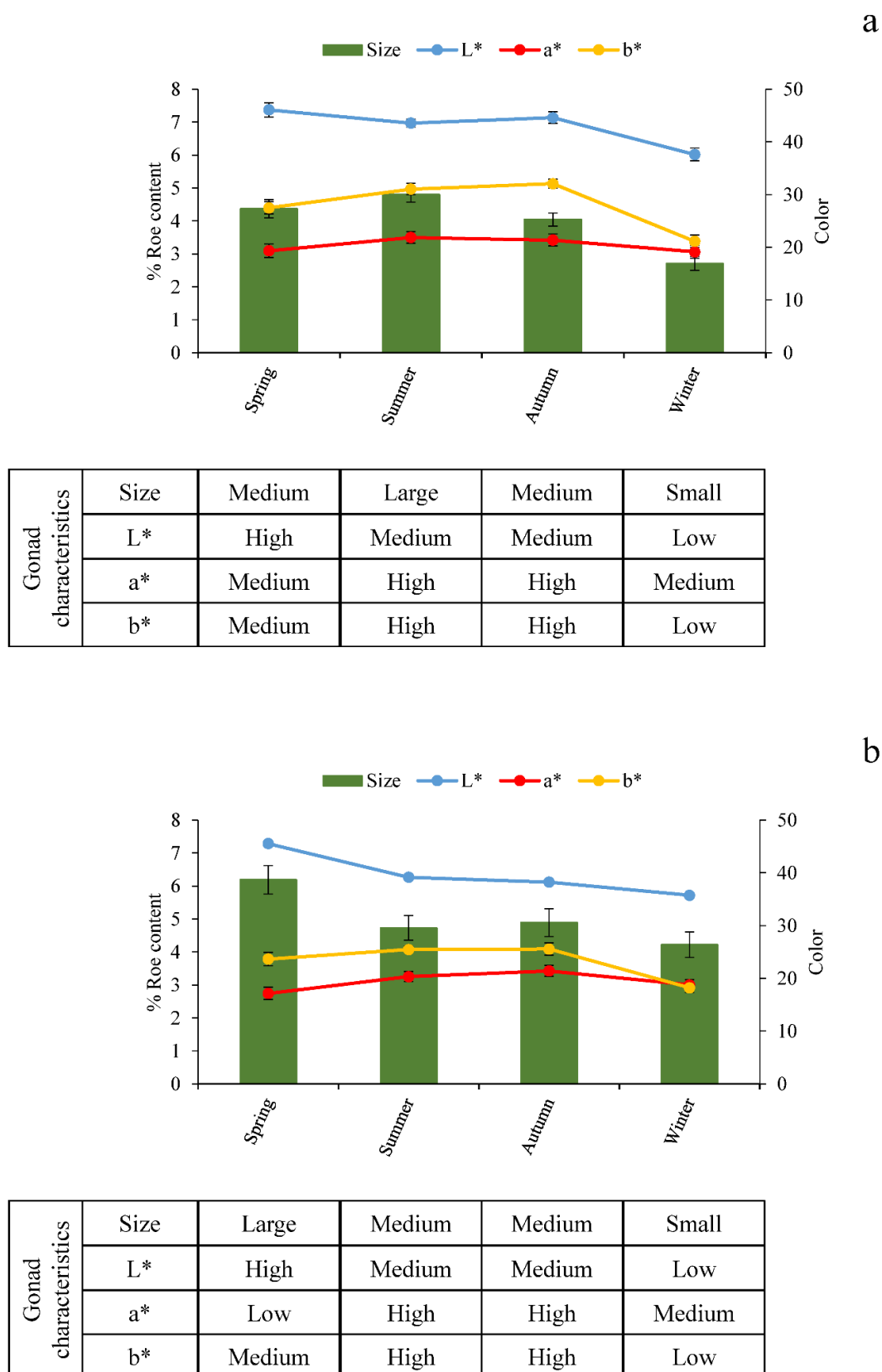
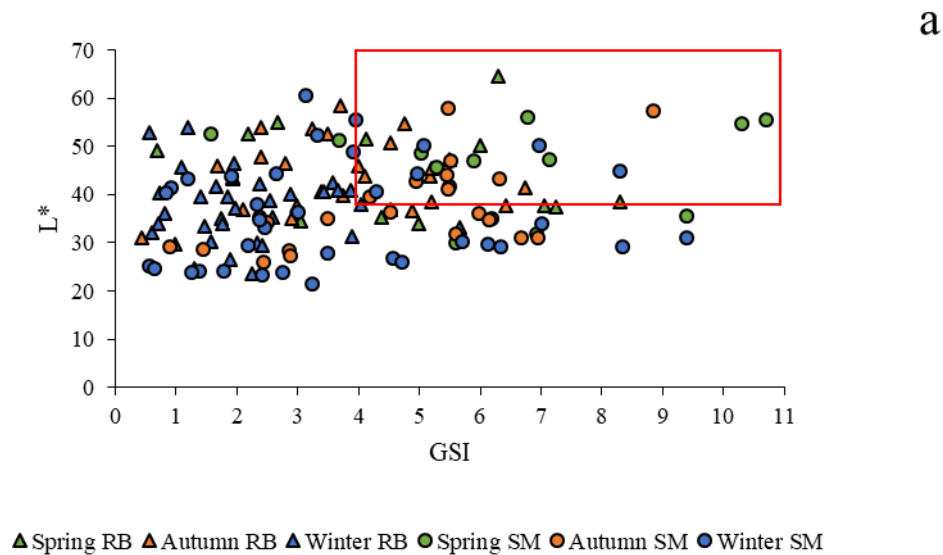


Figure 37 - Graphic representation of the match of some gonad sensory characteristics, in sea urchins from a) rocky bottom and b) *P. oceanica* meadow.

Taking into account the fishing period approved by Sardinian laws, I tried to pinpoint in which season and habitat it would be easier to collect valuable gonads by harvesting the fewest urchins. Here, I correlated the three color coordinates (L^* , a^* and b^*) in turn to the gonadosomatic index (Fig. 38a, b, c).

No standards for the objective characterization of quality of *P. lividus* gonads exist. Both in rocky bottom and seagrass meadow habitats, it is more probable to harvest gonads with $GSI > 4$ and at least one color coordinate which is high in value during the autumn months which are included in the legal fishing season (i.e. November and December) (Fig. 38). The same result is reached when considering the match between GSI , a^* and b^* simultaneously. These are the main parameters responsible for the reddish-yellow gonad color.



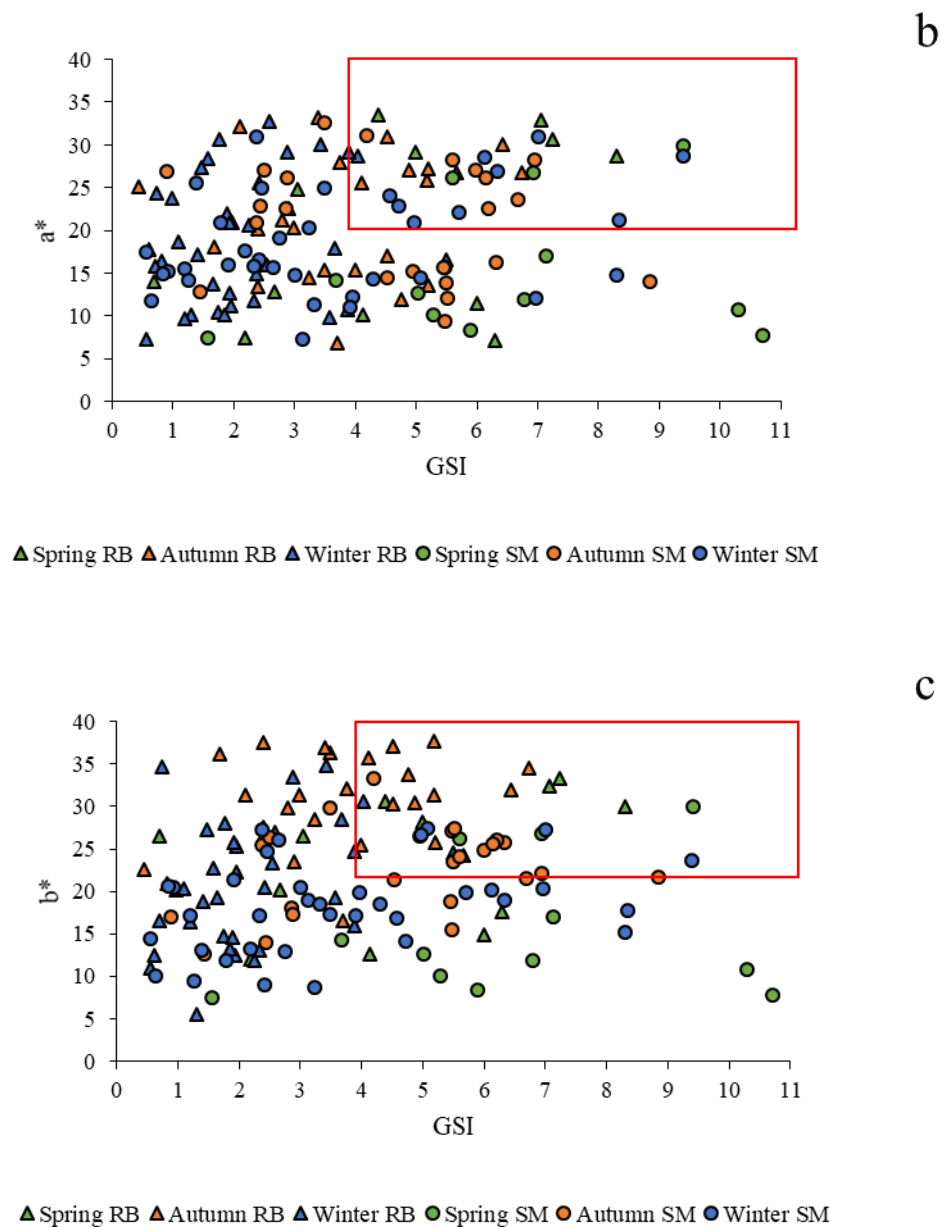


Figure 38 - Graphical representation of the relationship between GSI values and the three color coordinates L* (a), a* (b) and b* (c). Red rectangles enclose gonads with acceptable GSI and color features. Green = spring; orange = autumn; blue = winter; triangles = rocky bottom (RB), circles = seagrass meadow (SM).

Autumn is likely to be a profitable season in which to harvest gonads not only with a certain size and color, but also at the most consumers' most-favored gametogenic stage. In this period, *P. lividus* gametogenesis starts, and immature individuals are found with gonads at the developmental stages I (recovery), II (growing) and III (premature). In most countries where sea urchins are consumed, these immature gonads are generally preferred (Walker *et al.*, 2001; Unuma, 2002) since they are characterized by a high nutritive phagocyte (NPs)-to-germ cell

(GCs) ratio (Unuma, 2002). On the contrary, a high germ-cell-to-nutritive-phagocyte ratio would make gonads watery and bitter and therefore qualitatively poor (Unuma, 2002; Phillips *et al.*, 2009). Generally, immature gonads are highly proteic since they accumulate a large amount of proteins (especially toposome) in nutritive phagocytes available for the gametogenic processes.

The high proteic content in autumn is accompanied by the gradual decrease of the total lipid content in gonads due to the utilization of fat reserves during gametogenesis, thus making roe a balanced and nutritious food product. In this period, moreover, gonads begin to accumulate some long-chain PUFAs such as EPA and ARA. From a human health point of view, polyunsaturated fatty acids (PUFAs) provide a variety of health benefits against cardiovascular diseases, including well-established hypotriglyceridemic and anti-inflammatory effects (Garaffo *et al.*, 2011).

7.4.2. Echinoculture

In recent years, with the increasing demand for sea urchin gonads and the concomitant decline of natural stocks in many European countries, interest has grown in the development of echinoculture as a way to meet market demand and preserve natural stocks. It is obvious that enhancing growth and quality of the gonads from wild or cultured urchins is vital for increasing the competitiveness and profitability of future echinoculture industries (Prato *et al.*, 2017).

Important information for the growing echinoculture field has emerged from this PhD thesis.

The reproductive cycle can affect the sensory quality of sea urchin gonads. Therefore, it is essential for farmers to know the exact moment of the cycle in which harvesting the most profitable gonads. Currently, the identification of the gonad maturation stage is carried out by histology. This technique requires an expert operator and is time consuming. The dedicated proteomic methods described in this thesis probably cost as much as the histological ones, but may allow to obtain information on sex and maturation stage in a shorter time from gonad collection. A detailed description of the gonad protein profiles and patterns through proteomics, and how they change along maturation, could help to understand and monitor the changes in the organoleptic characteristics of roe. Therefore, these methods could potentially be applied to monitor development cycles and quality in sea urchin aquaculture plants, paving the way to more conscious farming activities.

In this work, toposome, or other proteins linked to the development of mature gametes (dynein, tubulin, metallothionein, ribosomal proteins, etc.) have been identified as markers of gender

and maturation stage. Toposome is linked to the NP content being the most abundant in immature gonads, while the others are linked to the GC content filling mature roe. Finding the right relationship between these proteins and gonad yield may provide an indirect measure of gonad quality in reared sea urchins, indicating which is the best quality roe, large but not too mature.

Gonad lipid content varies throughout the reproductive cycle, and these changes are likely to be triggered by the photoperiod. This is particularly true for sea urchins living in the wild. This information may be applied in aquaculture plants where, for instance, farmers could obtain gonads with a desired lipid content adjusting the daylight to dark ratio.

Monitoring the reproductive cycle may be useful for harvesting gonads with a high yield, particularly in the period of maximum maturity just before gamete deposition.

Gonad production, in addition, can be improved by feeding animals *ad libitum*. Macroalgae can be used as feed since they improve yield, but a higher proteic feed should be identified to obtain larger and more profitable gonads. It has been reported in the literature that macroalgae are able to improve gonad color better than manufactured diets. However, in this thesis I did not identify any seaweed capable of significantly enhancing the appearance of gonads. In any case, the green seaweed *U. lactuca* performs better than the red *G. gracilis*, because less than half the quantity of the former is needed to obtain the same results as the latter.

7.4.3. Knowledge gaps and future studies

This thesis emphasizes the urgent need to improve current fishing activities as well as accelerate the development of echinoculture in the European area.

Some knowledge gaps have emerged and future studies are desirable.

In the study on the reproductive output of two wild sea urchin populations subjected to different harvesting pressure, the dissimilarity in the number of spawning events between the two zones has been justified by the number of commercial-sized individuals, which changed according to the pressure. This observation should be confirmed in future studies conducted in other areas with harvesting pressures similar to those comprised in this work, considering not only the food availability but also the feeding intensity, an indirect measure of the nutritional state of urchins which can affect reproduction (Minor and Scheibling, 1997; Meidel and Scheibling, 1999).

This thesis indicated the *P. oceanica* meadows as sheltered habitats where gonads grow more than on exposed rocky bottoms, as observed by several authors (San Martín 1995; Fernandez

and Boudouresque, 1997; Guettaf, 1997; Boudouresque and Verlaque, 2007; Sellem and Guillou, 2007; Ouréns *et al.*, 2011) but in disagreement with Addis *et al.* (2014) in the same geographical area (Sardinia). Therefore, the influence of the growing habitat on sea urchin gonad size is still ambiguous and it may vary on a very small scale. In addition, the two-year study described here took into account only two sampling sites in the same bay, with no replicates. A study like this should be replicated in other Sardinian hard bottoms and meadows, possibly taking into account environmental features such as the food availability and the hydrodynamic conditions, and different spatial scales.

The complex dependency between dietary and gonad lipid composition in samples from the wild has not been investigated in this thesis, and could be carried out in areas covered by different macroalgae populations, since it is likely that diet composition affects the gonad lipid content more than water temperature (Martinez-Pita *et al.*, 2010a).

In-depth analysis on gonad proteins should be conducted on a higher number of samples, taking into account potential individual variability, also considering the environmental differences of the harvest sites, such as water temperature or food availability, which might have an influence on gonad development or protein content (Hammer *et al.*, 2006; Arafa *et al.*, 2012).

Finally, the study on an appropriate feeding that enables a higher gonad quality should be implemented. Chemical composition of the two algae tested in the experiment still needs to be analyzed. With those analyses, a clearer understanding of the results obtained may be possible. Currently, neither the natural diets nor the manufactured feeds, when used alone, allow the production of high quality gonads. A new frontier may be the use of manufactured feeds additivated with feeding stimulants and source of pigments (Dworjanyn *et al.*, 2007; Cyrus *et al.*, 2014). Small quantities of macroalgae or microalgae included in nutritious feeds may increase their palatability, concurrently enhancing gonad yield and color.

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