Sustainable management of sea urchins

From resource conservation to aquaculture promotion in a circular economy perspective

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1. General Introduction

Sea urchins are among the invertebrates that play a pivotal role in structuring and controlling marine benthic macrophyte communities and can be considered the main grazers in shallow seas around the world.

They, in conditions of uncontrolled proliferations of their population (e.g., due to the lack of predators) can cause severe overgrazing events with complete deforestation of algal communities (Lawrence & Sammarco, 1982; Valentin & Heck, 1999), inducing the formation of bare substrates, dominated by encrusting algae and consequently poor in biodiversity (Kempf, 1962; Vukovic, 1982; Verlaque & Nedelec, 1983; Verlaque, 1984; Palacin et al., 1998a; Sala et al., 1998a; Guidetti & Mori, 2005). Conversely, sea urchin disappearance may also generate negative effects on the ecosystem, as it would promote algal overgrowth, resulting in loss of biodiversity (Trowbridge et al., 2011).

They are intensely harvested for commercial and recreational purposes since the beginning of XVII sec., with dramatic ecological consequences. The world market demand of its gonads has significantly increased since the early 70s (especially in Japan), with both the natural growth of the world population and the increasing interest in this delicacy. In recent years, world production reached 73000 metric tons with an estimated value of 208 million US$ (FAO, 2016a). Sea urchin roe (gonad), which represents the edible part of the urchins, is a valuable excellence and it is appraised for both size and quality criteria (taste, firmness and colour).

More than 20 sea urchin species have been recorded in the Mediterranean Sea, but the most studied one is *Paracentrotus lividus* (Lamarck 1816), since, in addition to being one of the most widespread, it is the one with the greatest commercial interest (Guidetti, 2004). *Paracentrotus lividus* is distributed along the Mediterranean and Atlantic coasts of Western Europe and Morocco, including the Canary Islands and the Azores (Boudouresque & Verlaque, 2013). *P. lividus* is typically a subtidal species and lives from the sea surface to depths of 80 meters, but is most common up to 20 meters depth, on rocky reefs (Tortonese, 1965). Its distribution is strongly influenced by the heterogeneity of the seafloor and the presence of holes, crevices and cracks in the rocks, which provide shelter against attack by predators, usually sparids and wrasses (Benedetti-Cecchi et al., 1995, Guidetti, 2003a).

In Italy, *P. lividus* is a species of commercial interest especially in southern regions (particularly Sardinia and Apulia), with a high demand for its valuable gonads (Furesi et al., 2016). The harvest

of this species has seen a significant increase in recent decades that has led to overfishing of natural populations, causing a consequent decline of *P. lividus* stocks in these areas (Guidetti et al., 2004, Pais et al., 2007; Pieraccini et al., 2016, Farina et al., 2020, Ceccherelli et al., 2022).

However, the decline of *P. lividus* populations has also been reported in areas like the Ligurian Sea, where the sea urchin is not considered a species of commercial interest. So far, the causes of population decline in this area are still unexplained.

In recent years, it has been observed that sea urchins are also very sensitive to indirect anthropogenic impacts: stressors related to climate change (e.g., algal blooms, fluctuating water temperatures, ocean acidification) and the presence of pollutants (e.g., chemicals and emerging compounds) can lead to abnormal larval development, reduced survival of larvae and juveniles, and impaired reproductive success of adults due to effects on gamete quality, threatening survival of entire populations (Ribeiro et al., 2015; Gambardella et al., 2016, 2018; Asnaghi et al., 2020). In the very near future, climate change could accelerate the loss of *Paracentrotus lividus* in the environment with serious consequences, both economic, with the collapse of local harvesting activities, and ecological. In particular, the regression of *P. lividus* populations could favour the proliferation of the competing species *Arbacia lixula* (Guidetti et al., 2004), a species that is more tolerant to rising sea temperature (Guidetti e Dulcic, 2007; Gianguzza et al., 2011; Privitera et al., 2011; Wangensteen et al., 2012) and less prone to the top-down control, as it is not a commercial species and is poorly predated (Guidetti, 2004). The uncontrolled proliferation of *A. lixula* may therefore favour the creation of barrens, with consequent loss of ecosystem services (Benedetti-Cecchi et al. 1998; Micheli et al. 2005; Guidetti and Dulčić 2007; Bonaviri et al. 2009).

Therefore, sea urchin production through aquaculture could be an ecologically sustainable alternative to meet market demand while preserving natural populations and several efforts to develop rearing protocols for gonad improvement have been implemented in recent years (Spirlet et al., 2000; Kelly & Chamberlain, 2010; McCarron et al., 2010; Sartori et al., 2016). Yet, sea urchin aquaculture is still hampered by the slow growth of the species, with critical stages to obtain commercial-sized sea urchins (>5 cm) and about 2-3 years to raise new spawners (Rahman et al., 2014; Castilla-Gavilán et al., 2018).

For these reasons, an industry that grows sea urchins, and particularly *P. lividus*, to market size has not yet been established in the Mediterranean area. The main obstacle to the development of such an industry is the cost and availability of sea urchin diets suitable for use in intensive echinoculture.

To make echinoculture an economically viable activity, feeds and feeding methods must be developed that are cost-effective, maximize somatic growth to reduce time to market, and produce high gonad production with acceptable quality (Lawrence, 1991; Chenoweth, 1994; Pearce et al., 2002; Kennedy et al., 2005).

Several efforts have been made in recent decades to fill this gap. The many studies so far performed, in particular, have provided evidence that sea urchins fed only with macroalgae show good gonad color and taste, but low somatic and gonadal growth, while those fed only with artificially formulated feeds, thus high in protein and lipid content, show significantly higher somatic and gonadal growth, but with color and taste unsuitable for commercialization (Daggett et al., 2005; Shpigel et al., 2005; Unuma et al., 2015; Carrier et al., 2017; Prato et al., 2018).

Moreover, among the many feeds formulated and developed worldwide for sea urchins, only two are commercially available outside of China or Japan. These are the Lawrence/Watts diets (developed by the Texas A&M University feeding laboratories; Lawrence et al., 2001; Hammer et al., 2006; Eddy et al., 2012) and the Nofima diet (formulated by a Norwegian research group; James et al., 2015). Of these two diets, only the Nofima diet is commercially available on a scale to support commercial echinoculture and is presently distributed by Urchinomics (https://www.urchinomics.com).

Sea urchin consumption generates abundant waste due to the high content of inedible parts such as tests, spines and viscera, which consist of about 70 % of the animal's weight. Most of this waste is generally disposed of in landfills and this management is now under examination because it is not environmentally and economically sustainable.

Noteworthly, these wastes can contain a significant amount of nutrients that could have important application in medicine, agriculture, and in particular could be used as enrichment components in the formulation of feed for land farming and especially aquaculture. The recycling and reuse of these by-products would thus represent an emerging sustainable alternative to landfilling.

In the specific case of *P. lividus*, the significant content of calcium and magnesium carbonate of the endoskeletons (Ebert, 2007; Mamelona et al., 2010) has already been used as a soil conditioner in agriculture for acidic or subacidic soils. At the moment, however, the most interesting use from an economic and environmental sustainability point of view seems to be the use of these biocarbonates as an enrichment additive in feeds intended for echinoculture. In fact, these high-

magnesium carbonates powders, obtained by drying and grinding the whole urchin waste, should also be rich in antioxidant compounds, pigments and other biomolecules. Thus, in addition to boosting the somatic growth of reared individuals, they could provide better performance for both the feed's shelf life and their well-being, ensuring a better-quality product.

Therefore, boosting the utilization of these by-products would favor, in the long run, sea urchin aquaculture, safeguarding natural stocks and addressing the goals of the Blue Growth, which is socioeconomic growth based on sustainability and protection of the biodiversity of marine systems and resources (Eikeset et al., 2018). This could happen encouraging the creation of a virtuous circular economy, in which the waste, instead of been disposed, is turned into reusable material, increasing the commercial value of the exploited species.

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Aim of the project

The increase of the harvesting pressure led, recently, to an over-exploitation of sea urchins, including the Mediterranean species *Paracentrotus lividus*. This raised concern for their conservation status and for the possible consequences on coastal ecosystem functioning. Aquaculture represents a possible alternative to harvesting but it still needs to be improved in terms of efficiency and sustainability.

For these reasons, the present research project pursued two main objectives:

1. Characterization of *P. lividus* populations in different sites of the Ligurian Sea, in order to investigate their health status (abundance and size-frequency distribution) and speculate about possible causes of their regression.

   To achieve this objective, the effects that increased seawater temperatures and blooms of toxic microalgae may have on different developmental stages of the sea urchin were also investigated. Such stressors, possibly acting from gametes to adult organisms, could increase the vulnerability of the species and jeopardize the maintenance of the balance of the entire stock.

2. Promotion of sustainable sea urchin aquaculture by formulating new experimental feeds, enriched with high Mg and Ca biocarbonates and antioxidant substances, derived by processing the large amounts of waste materials (mostly constituted by test and spines) from the industries that package sea urchin gonads. Such approach would contribute to develop sea urchin aquaculture, establishing a virtuous system of circular economy, while reducing the impact (and the wastes) of the harvesting of wild specimens. The recycling of the wastes would favor the development of local Small and Medium Enterprises, thanks to a better exploitation of the raw product, capable of increasing the commercial value of the exploited species.

2. Sea urchin population structure along the Ligurian coast

2.1 Introduction

The edible sea urchin *Paracentrotus lividus* (Lamarck) is a key species in the shallow sublittoral of the Mediterranean Sea, regulating the structure and functioning of rocky reefs through its grazing activity (Hereu, 2005). It is a benthic organism inhabiting shallow rocky bottoms and seagrass communities (Boudouresque & Verlaque, 2011), and acting as a key herbivore in coastal marine ecosystems where regulates subtidal communities (Sala et al., 1997; McClanahan & Sala, 1997; Pinnegar et al., 2000; Farina et al., 2020). Several processes (e.g. predation, migration), often exacerbated by anthropogenic environmental pressures (climate change, fishing, pollutants), can play a crucial role in sea urchin population dynamics, affecting theirstructure, distribution and fitness (Scheibling et al., 1999; Guidetti et al., 2004; Gambardella et al., 2013; Migliaccio et al., 2019; Guarnieri et al., 2020).

In some areas of the Mediterranean Sea, *P. lividus* populations have shown large-scale declines over the past 15 years, to the point of collapse due to overharvesting (Pais et al., 2007; Giglioli et al., 2020) and climate change, as recently reported along the coasts of Israel and Lebanon in the eastern Mediterranean (Yeruham et al., 2015). *Paracentrotus lividus* are more sensitive to human activities than previously thought (Zhadan et al., 2017): studies conducted in the last decade reveal that climate change (e.g., microalgal blooms, water temperature fluctuations) and the presence of pollutants lead to abnormal larval development (Ribeiro et al., 2015; Gambardella et al., 2016, 2018). These anomalies could reduce the survival capacity of the larvae adding on the effects that the above stressors exert on the reproductive success of the adults, through effects on gamete quality, endangering the entire population. There is an urgent need to monitor this species also in the Ligurian Sea, where systematic monitoring are not carried out, to detect ecological changes and understand the causes (including anthropogenic impacts) that may affect the recruitment, distribution, and abundance of *P. lividus*, with cascading effects on biodiversity and functioning. In this regard, the aim of this study was to collect data on *P. lividus* population structure at different sites along the Ligurian coast in order to investigate their health status (abundance, size-frequency distribution, reproduction) and possible causes of the regression observed in recent decades.

2.2 Materials and methods

The study was conducted along the Ligurian coast in four locations. Specifically, the sampled sites were, from East to West, Baia di Prelo (Rapallo, GE - 44°20.195" N, 9°13.567" E), Quarto dei Mille (Genoa- 44°23.296" N, 8°59.617" E), Vernazzola (Genoa- 44°23.419" N, 8°58.796" E) and Capo Mortola (Ventimiglia- 43°46.682" N, 7°33.372" E; *Fig. 2.1*). For each site, sampling was performed along three linear transects (25 m each) in the shallow subtidal (2-5 m depth), in a randomly chosen area with natural features typical of *P. lividus* habitat (rocky cliffs or boulders). Each transect was divided into three $5m^2$ sub-transects as replicate units (0-5m, 10-15m, 20-25m), within which sea urchins were counted across 50x50cm quadrats (*Fig. 2.2*; e.g Guala et al 2005). Urchins were collected in special nets to be measured out of water with a calliper, and then assigned to 6 different size classes (class1: 0-10mm; class2: 10.1-20mm; class3: 20.1-30mm; class4: 30.1-40mm; class5: 40.1-50mm; class6: ≥50.1mm) following Bertocci et al. (2014). Density index was calculated for each site, measured in individuals per square meter(ind/m^2).

Figure 2.1: Sampling sites along the Ligurian coast: Baia di Prelo (PRE), Quarto dei Mille (QUA), Vernazzola (VER), Capo Mortola (MOR).

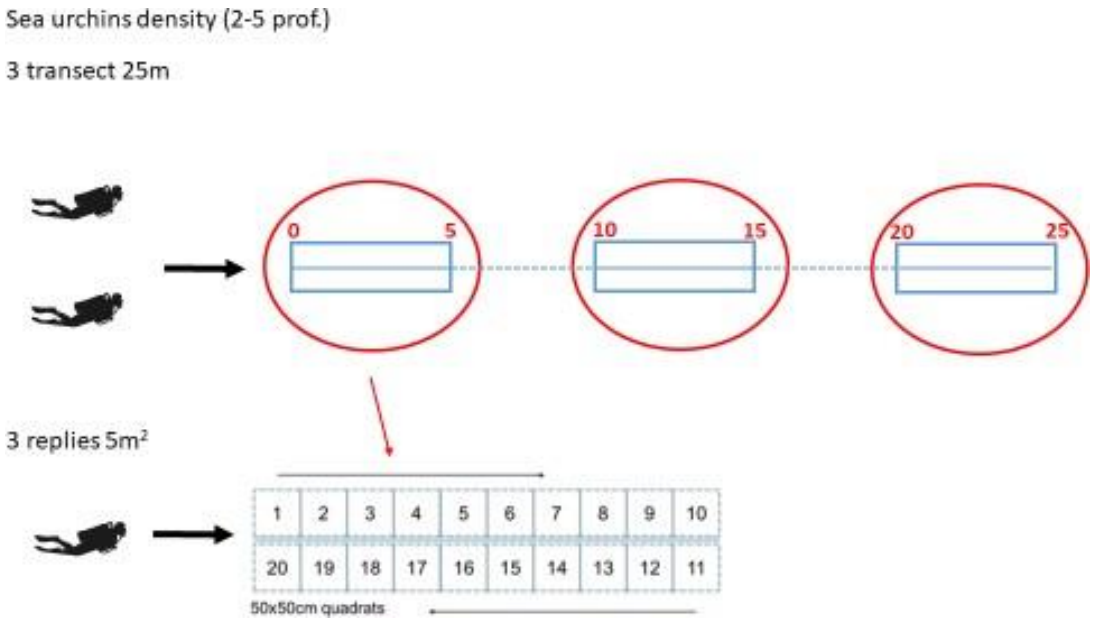

Figure 2.2: Sampling design

2.2.1 Study site

The four sampling sites have very different geomorphological and environmental characteristics.

The "Baia di Prelo" site (*Fig. 2.3*) is located near the town of Rapallo at the base of the Portofino promontory and is characterized by a small, very sheltered bay open to East. The site is located near the Marine Protected Area of Portofino and the seabed is characterized by a *Posidonia oceanica* meadow that is quite heterogeneous in terms of cover and density of shoots (Montefalcone et al., 2007), declared a "Site of Community Interest" (SCI), and interspersed with patches of small stones.

Figure 2.3: "Baia di Prelo" sampling site, bay immediately North of Punta Pagana

The "Quarto dei Mille" site (*Fig. 2.4* and *2.5*) is located within the municipality of Genoa. The sampled area is exposed to West, sheltered to the southeast winds by a small rocky promontory. The seabed consists of medium and small-sized rocks interspersed with areas of fine sand. This site has been extensively monitored with regard to the presence and concentration of the toxic benthic microalga *Ostreopsis ovata*, as it appears to be one of the areas most prone to the occurrence of extreme blooms of this harmful alga, which has been present in Liguria since the early 2000s (first noteworthy bloom occurred in 2005 along Genoa coastline; Ciminiello et al., 2006; Mangialajo et al., 2008a).

Figure 2.4: "Quarto dei Mille" sampling site, cliff West of Monumento dei Mille

Figure 2.5: photo of the seabed at the "Quarto dei MIle" site.

The "Vernazzola" site (*Fig. 2.6* and *2.7*) is also located within the municipality of Genoa, 2 km further West from the previous site near an artificial pebble beach widely frequented during the summer season, facing South-Southeast and sheltered to the West by a small rocky cape. Morphologically, the seabed on which the urchins were sampled is composed by medium and small stones. There is a freshwater discharge near the sampling area, which often releases at sea large amounts of organic matter.

*Figure 2.6: "*Vernazzola" sampling site, bay in the center of the photo

Figure 2.7: photo of the seabed of the site "Vernazzola"

The "Capo Mortola" site (*Fig. 2.8* and *2.9*) is located in the municipality of Ventimiglia, near the French border, and is within the Capo Mortola Area di Tutela Marina. The sampled area consists of a narrow rocky promontory that extends as a shallow seabed for several hundred meters from the coast. The seabed consists of a continuous, fairly sloping reef that progresses into a dense *Posidonia oceanica* meadow. The Cape is very exposed to the prevailing wind (Libeccio, from the Southwest) and is characterized by a strong sea current.

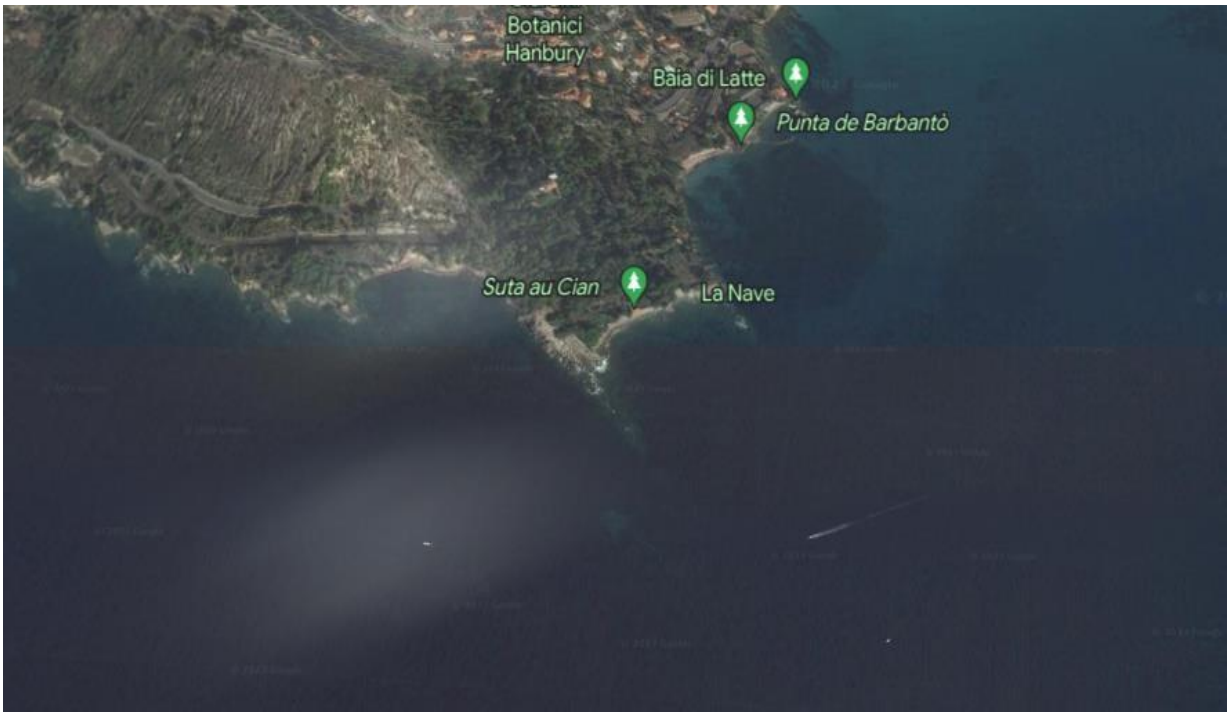

Figure 2.8: "Capo Mortola" sampling site: the promontory southwest of Suta au Cian forest.

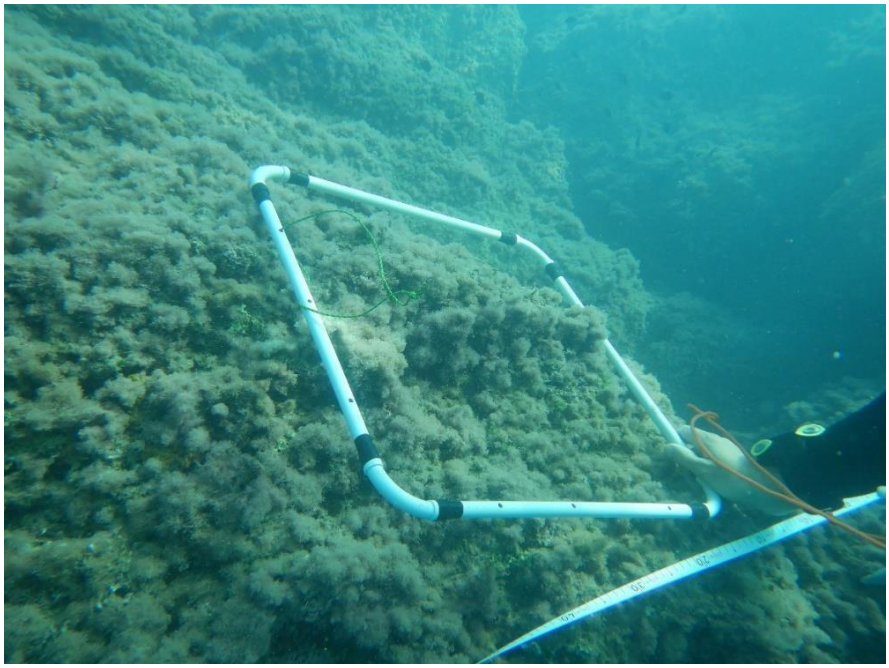

Figure 2.9: photo of the seabed of the site "Capo Mortola"

2.3 Results

A large variability in the density (ind/m^2; *Fig. 2.10*) of sea urchins was observed at the different sites. In particular, the following average densities (± SE) were recorded: in Capo Mortola 0.9 ind/m^2 (± 0.27 SE), in Vernazzola 9.27 ind/m^2 (± 1.93 SE), in Quarto dei Mille 6.13 ind/m^2 (± 1.57 SE) and in Baia di Prelo 5.27 ind/m^2 (± 1.23 SE). In terms of the abundance of individuals in the respective size classes, a large variability in distribution was also observed (Tab. 2.1; *Fig. 2.11-2.14*).

Table 2.1: Abundance of individuals and relative percent in the relevant size classes, per site sampled.

	class1 0-10 mm	class2 10-20 mm	class3 20-30 mm	class4 30-40 mm	class5 40-50 mm	class6 >50 mm	total
Capo Mortola	-	-	3 8.3%	6 16.7%	16 44.4%	11 30.6%	36 100%
Vernazzola	5 1.2%	45 18.8%	89 21.4%	111 26.8%	116 28%	49 11.8%	415 100%
Quarto dei Mille	12 6.3%	61 31.8%	61 31.8%	39 20.3%	16 8.3%	3 1.6%	192 100%
Prelo bay	6 3.8%	30 19%	41 25.9%	38 24.1%	36 22.8%	7 4.4%	158 100%

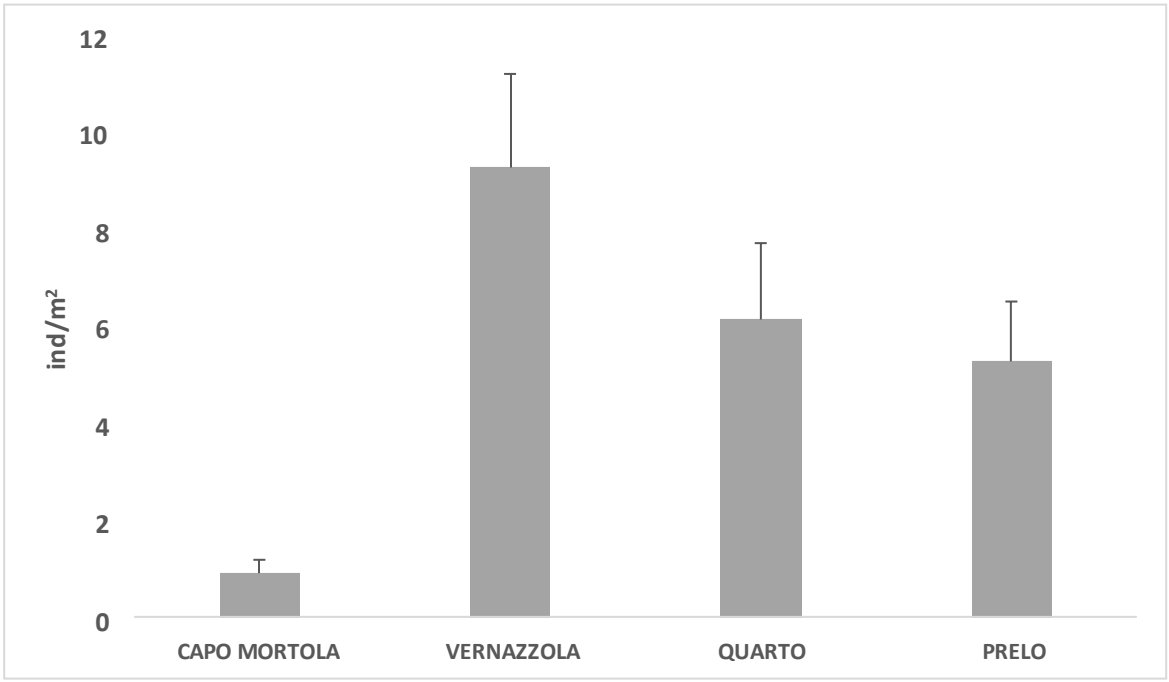

Figure 2.10: Density of *P. lividus* in the four sampled sites (average ind/m² ± SE).

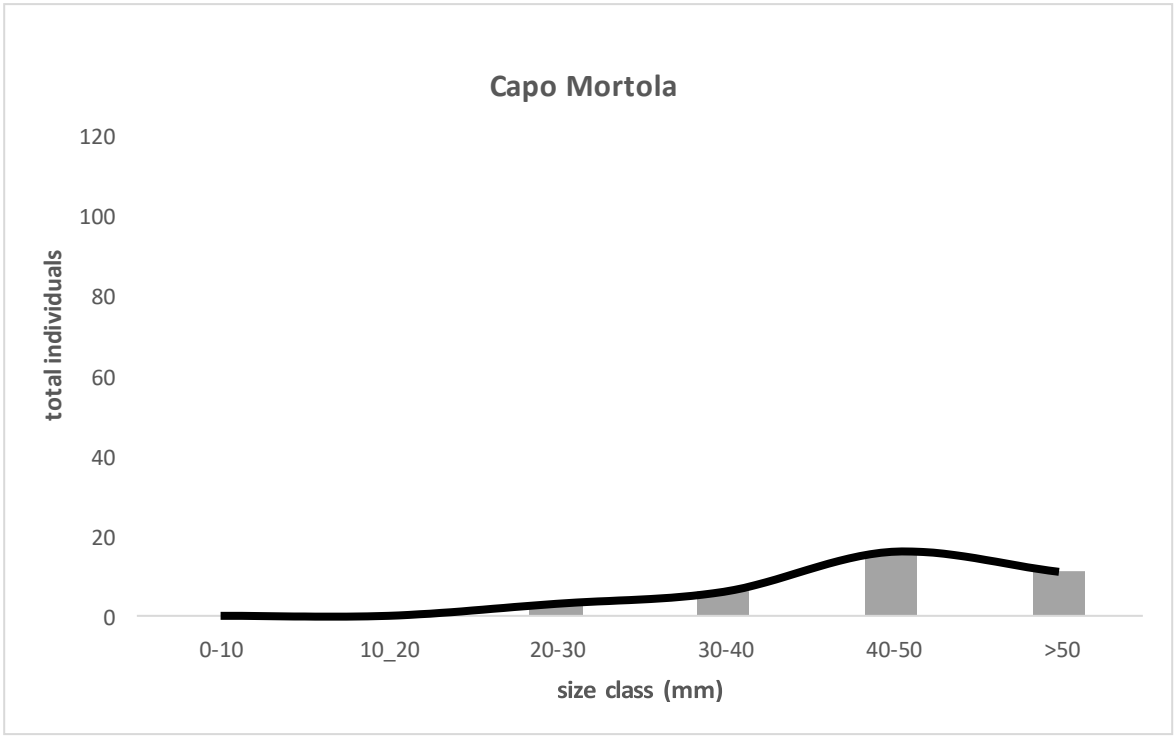

Figure 2.11: structure of *P. lividus* at the "Capo Mortola" site. Abundance of individuals in the respective size classes.

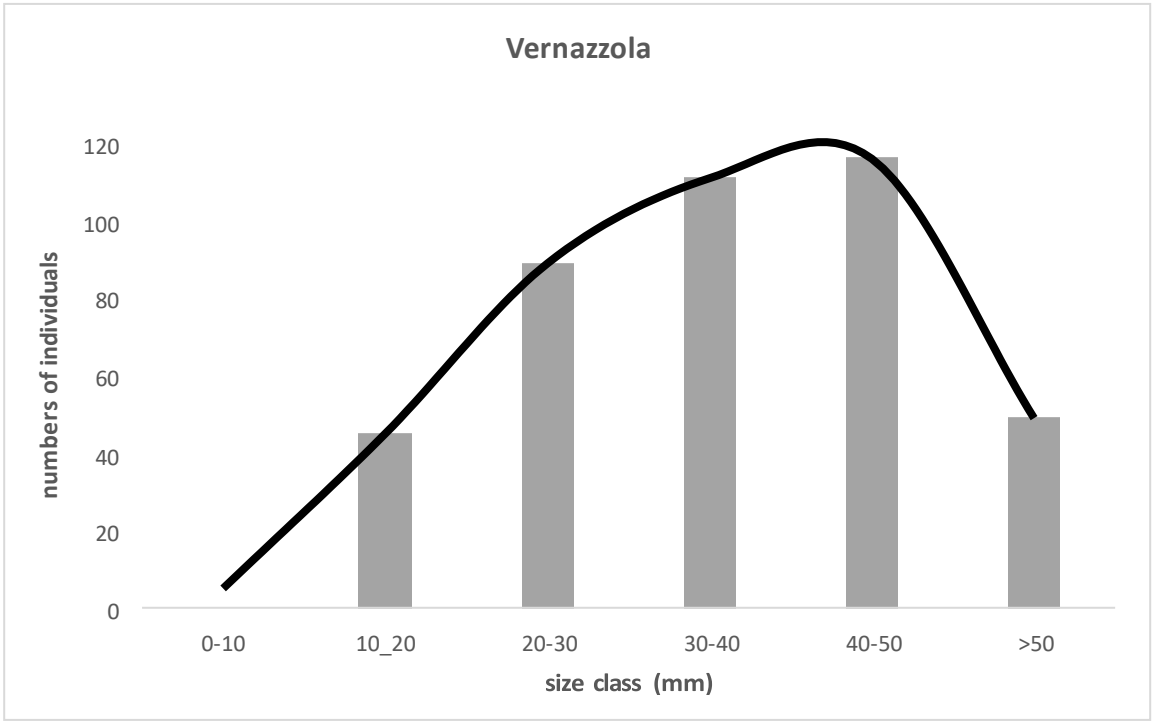

Figure 2.12: Population structure of *P. lividus* at the "Vernazzola" site. Abundance of indivisuals in the respective size classes.

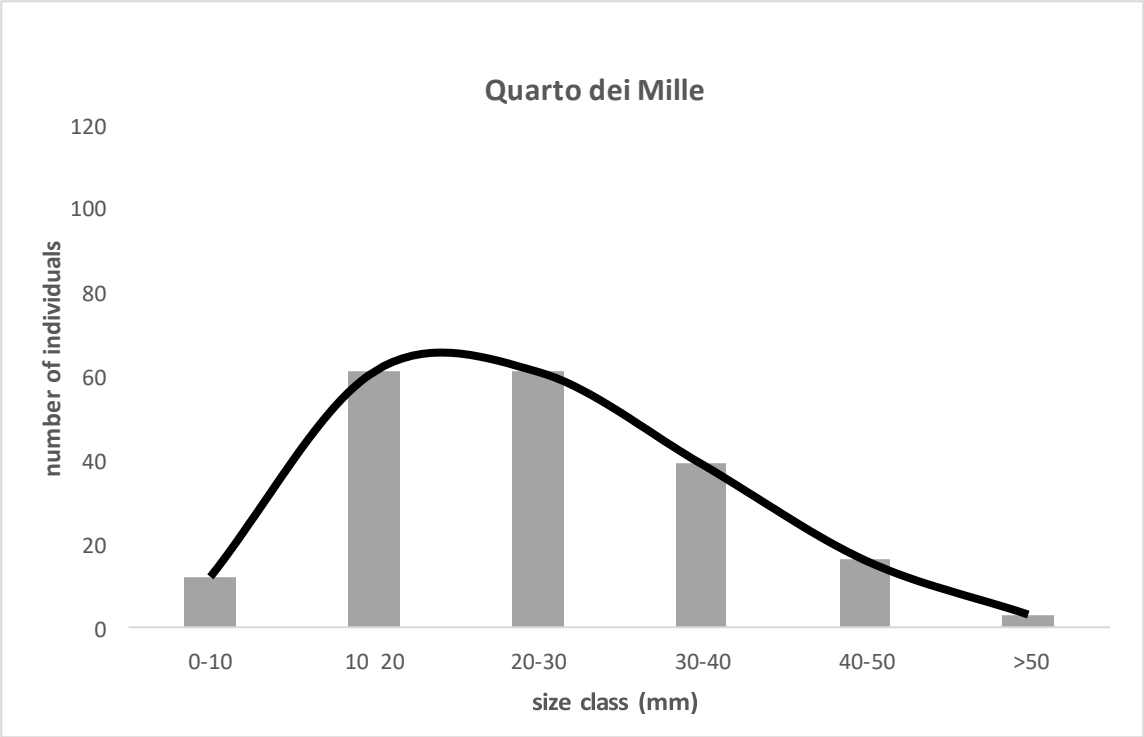

Figure 2.13: Population structure of P. lividus at the "Quarto dei Mille" site. Abundance of indivisuals in the respective size classes.

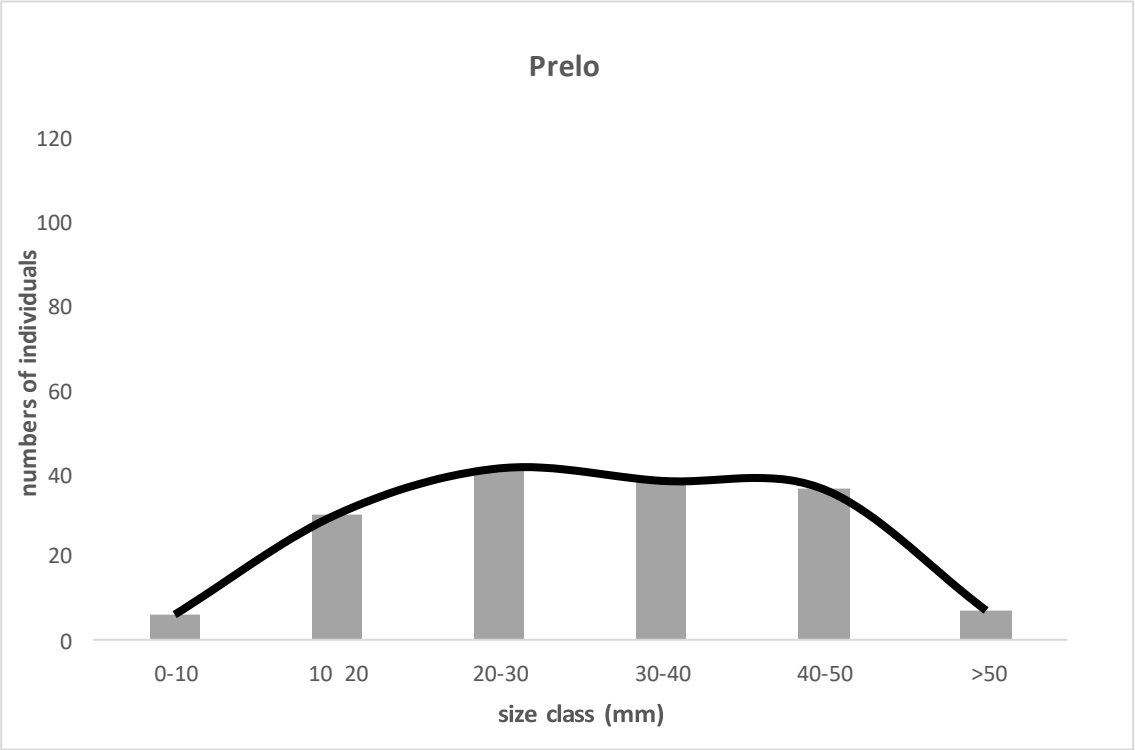

Figure 2.14: Population structure of *P. lividus* at the "Prelo" site. Abundance of indivisuals in the respective size classes.

2.4 Discussion and Conclusions

The density of the sea urchin *Paracentrotus lividus* in the four locations studied is largely variable and several could be the drivers of such differences, both at local (habitat structural complexity, local hydrodynamics, natural predators, human exploitation, toxic microalgal blooms) and larger-scale (regional hydrodynamic processes, heatwave occurrence). Regarding the local factors, one of those that may have the greatest impact on the density of individuals in *P. lividus* populations is the habitat structural complexity. Habitat structural complexity is known to be one of the most important factors regulating the main ecological processes driving sea urchin population dynamic: recruitment and predation (Farina et al., 2020). The present work shows that sites characterized by a seabed with a predominance of small and medium-sized rocks (especially the "Vernazzola" site) offer optimal conditions for the establishment of large *P. lividus* populations, as they prefer substrates rich in holes and shelters where they can hide from predators (Sala and Zabala, 1996; Hereu et al. 2005). In comparison, however, coastal areas characterized by continuous and sloping rocky cliffs, such as the "Capo Mortola" site, do not offer the ideal substrate conditions for their settlement. In addition, this

site, being poorly sheltered by capes or headlands has strong hydrodynamics, another factor that is most likely not favorable to the settlement of new recruits (Lopez et al., 1998; Jacinto et al., 2013).

Anthropization, in the sense of closiness to municipalities and bathers' pressure, does not seem to exert negative effects on the population density of *P. lividus*, because the harvesting of this species in Liguria, contrarily to other Italian regions where this species is largely overexploited (see Puglia: Guidetti et al., 2004; and Sardinia: Pais et al. 2007; Addis et al., 2009; Farina et al., 2020, Ceccherelli et al., 2022), is irrelevant. On the contrary, sea urchins may have indirect benefits from anthropization, a representative example may be that of "Vernazzola," where there is a freshwater sewage discharge, which provides an important supply of organic matter that may promote algal growth and thus consequently increases the amount of food available to urchins.

Relevant differences were also observed in the population structures of the investigated sites. At the Capo Mortola site, the few individuals present were almost all concentrated in the larger size classes. A possible reason for the observed pattern could be related to the life cycle of the species: *P. lividus* has a planktotrophic larva called pluteus, which can survive in the plankton for 20-40 days (Pedrotti, 1993), so the strong current present in this area could not favor the establishment of new recruits. Another reason why the lower size classes are poorly represented can be attributed to the characteristics of the substrate, lacking holes and shelters, which exposes the juvenile stages of *P. lividus* to increased predation by labrids and sparids, the major predators of this sea urchin species.
In the Vernazzola site, on the other hand, all size classes are well represented, and the highest abundance can be seen in the sizes attributable to adults (class4 and class5), synonymous of a stable and healthy population structure.

A population mainly represented by size classes corresponding to juvenile and young adult stages (class2 and class3) is observed at the Quarto dei Mille site. Since individuals with a test diameter of 2 cm are generally considered to belong to the year 2 age class and individuals with a test diameter of 4 cm to be 4 to 5 years old (Boudouresque & Verlaque, 2013), the most abundant individuals present at this site may has from 1 to 3 years old. The lower number of adults (Class4, Class5 and Class6) compared to juveniles is probably due to the loss of one or more cohorts because of an unfavourable event that could have occurred 4 to 5 years before the sampling, during the spawning or settlement period. The event that may have caused this mortality therefore should have occurred between the years 2017 and 2018. Such event could have been represented by a relevant bloom of the toxic microalga *Ostreopsis ovata.* In fact, Privitera et al. (2012) showed that *O. ovata* could

adversely affect the recruitment of *P. lividus* through its impact on the survival of juveniles, whose mortality could be induced by toxins or caused by other factors: such as mechanical impediment due to mucus production (Guerrini et al., 2010).

This hypothesis is corroborated when related to the reproductive cycle of *P. lividus* in the Mediterranean, which, although there is variability from region to region, shows a major peak in spring and a minor one in late fall (Fenaux, 1968; Crapp & Wills, 1975; Régis, 1979; Byrne, 1990; Pedrotti, 1993; Lozano et al., 1995; Fernandez & Boudouresque, 1997; Guettaf et al., 2000; Chiantore et al., 2008). Post-settlement juveniles are commonly found in early summer (Chiantore et al., 2008; Privitera et al., 2008). In the Mediterranean Sea, *O. ovata* blooms have been observed during the summer (July-August, Tyrrhenian Sea and Ligurian Sea; Sansoni et al., 2003; Simoni et al., 2004; Congestri et al., 2006; Mangialajo et al, 2008) or early fall (September-October, Adriatic Sea; Totti et al., 2010; Mangialajo et al., 2011), corresponding to the time of the year when juveniles of *P. lividus* have recently settled.

Particularly in Quarto dei Mille area, during the year 2017, the peak of *O. ovata* bloom was particularly intense, reaching the maximum value of: 81 380 cells × L^{-1} for cells in water, 2 890 528 cells × gFW^{-1} for cells on macroalgae (*Fig. 2.15*). Most importantly, this bloom was observed around the middle of June, earlier than usual, since they usually occur in the second half of July (Mangialajo et al., 2008; 2011; Giussani et al., 2017; for years from 2013 see *Fig. 2.16*). This was almost certainly caused by the surface seawater temperature anomaly that occurred during early summer 2017, which was about 4 °C higher than the average of the past 30 years (Meroni et al., 2018 and *Fig.: 2.17*). Such early and intense *O. ovata* bloom could have affected more seriously than in other years larval survival of *P. lividus*, because a June *O. ovata* bloom is more overlapped with the regular reproductive cycle of the sea urchin. In addition, juveniles' settlement could have been hampered because of the large abundance of toxic microalgae epiphyte on the substrates and the associated large amount of mucilaginous matte (Besada et al., 1982; Reynolds, 2006, 2007; Barone, 2007; Giussani et al., 2015; Totti et al., 2010).

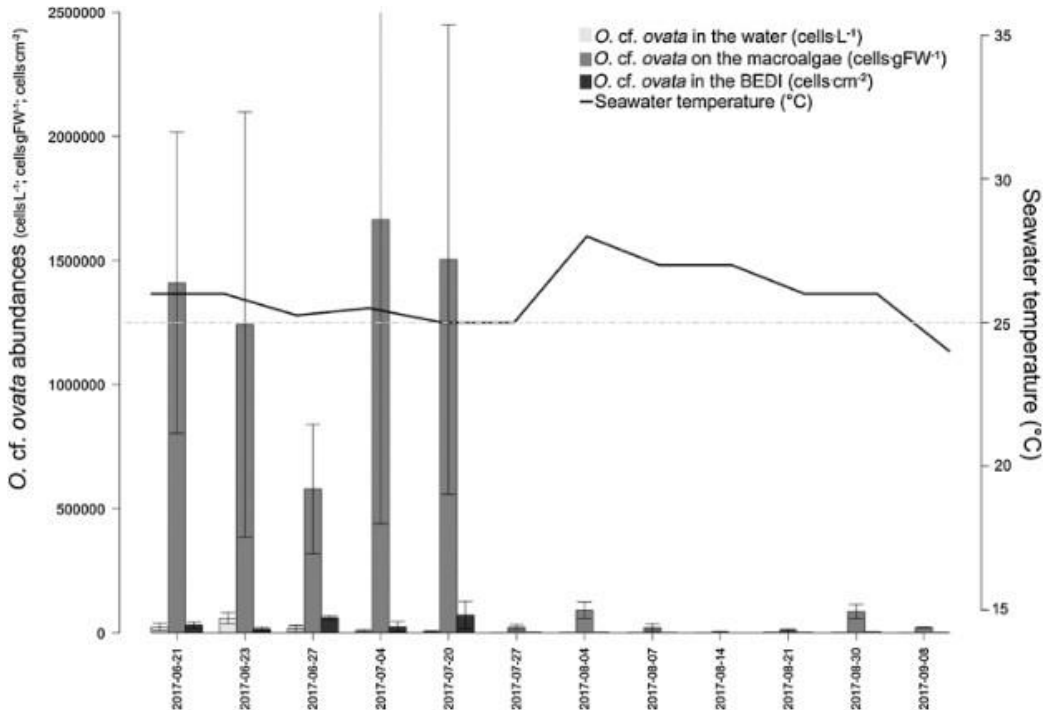

Figure 2.15: *Ostreopsis* cf. *ovata* abundances in Quarto dei Mille during summer 2017 for the three matrices: cells in the water (pale grey), cells on macroalgae (grey) and potentially resuspended cells (dark grey); black line reports seawater temperature. From Meroni et al., 2018.

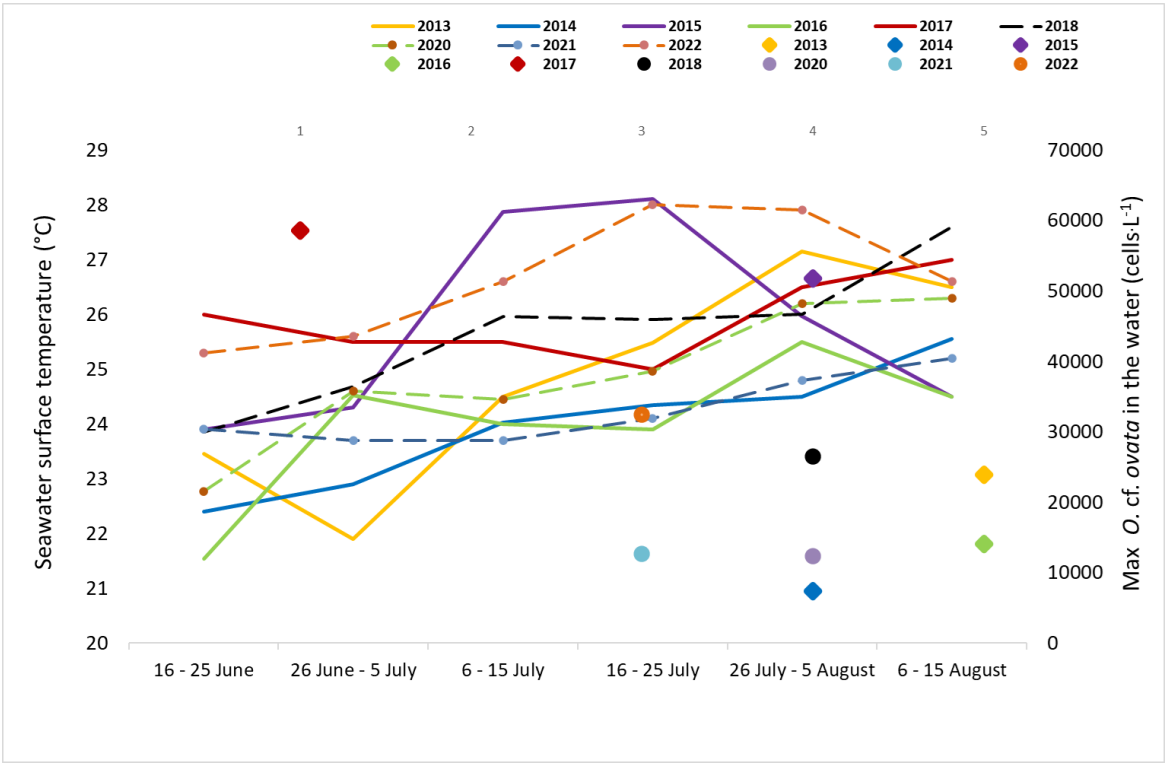

Figure 2.16: Average seawater surface temperature (°C) recorded in Quarto dei Mille (lines) and maximum-recorded *Ostreopsis* cf. *ovata* abundances in the water (cells × L–1) for each year (diamonds). From Meroni et al., 2018, modified.

Figure 2.17: Surface seawater temperature recorded in Quarto dei Mille in 2017 and 2018, and average temperature at Quarto dei Mille over the past 30 years (1992/2022).

Beyond triggering the *O. ovata* bloom, the extreme heat wave event (*Fig. 2.17*) could have, *per se*, hampered the development of the larval and Post-settlment stages. A marine heat wave is a period of unusually high sea surface temperatures and is defined by its duration and intensity (Hobday et al., 2016). There is already large evidence that the recent increase in the frequency of these extreme events in the Mediterranean plays a key role in the collapse of populations of several species of marine organisms (Garrabou et al., 2022).

Although the Vernazzola site is only one nautical mile away from Quarto dei Mille, as previously discussed, it shows a very different population structure. This site could be much less adversely affected by a seawater temperature anomaly, because it is subject to stronger hydrodynamics and because of its southeastern exposure that favors water exchange due to the dominant current present in this area (current from the east to west), as opposed to Quarto dei Mille, that is exposed to the west-southwest. As far as the possible impact of *O. ovata*, it has been largely demonstrated that the occurrence of blooms is a very local phenomenon and is closely dependent on local habitat

characteristics (Pistocchi et al., 2011; Cohu et al., 2013; Meroni et al., 2018; Gemin et al., 2020; Monserrat et al., 2022), leading to a very fragmented distribution of this phenomenon. For this reason, the Vernazzola site being much less confined and presenting a seabed with a continuous degradation towards the open sea, is much less prone to these extreme events of *O. ovata* blooms, compared to Quarto dei Mille, which is much more sheltered and presents a seabed without inclination with the presence of puddle areas.

Finally, regarding the population structure of the Prelo site, a very flattened curve was observed, where both size classes of juveniles (class2 and class3) and adults (class4, class5 and class6) display more or less the same abundance of individuals. As previously reported, a healthy population should have higher numbers of "young adult" and "adult" individuals than the juvenile stages. In this case, the reduced number of these size classes is hypothesized to be due to a strong predation pressure at the site. In fact, being very close to the Portofino Marine Protected Area, the presence of fish predators is significantly higher than at the other sampling sites. It has been amply demonstrated how predation has considerable effects on the population structure of sea urchins, where individuals that are older and therefore larger in diameter are much less capable of hiding in holes and crevices in the seabed, thus being more exposed to attack by predatory fish (Sala et al. 1996; Guidetti & Mori, 2005).

The high variability observed in the different sites may depend therefore on several factors, both biotic and abiotic, which may act individually or in synergy in modelling the various population structures and their respective densities, with largely variable effects even at a very small spatial scale (such as "Vernazzola" and "Quarto dei Mille"). The present study represents the first large scale monitoring of *P. lividus* population along the Ligurian coats, at least in the last couple of decades, and therefore the data are still preliminary and require an increase in the number of sites to be monitored in space and along time in order to achieve a better understanding of the population dynamicsin the region. Studying the population structure and the processes that regulate it assumes considerable importance, as it could provide numerous indications on the state of health and functioning of the local ecosystem.

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3. Effects of Marine Heat Waves (MHWs) on the survival of *Paracentrotus lividus* larvae and possible cascading effects on population structure

3.1 Introduction

Sampling different populations of the sea urchin *Paracentrotus lividus* along the Ligurian coast allowed to highlight different population structures that may be the result of external environmental stressors, one of which is the occurrence of heat wave events characterized by extreme seawater temperatures exceeding the climatological average (Hobday et al., 2016).

In the last decade these abnormal and sudden events have been increasingly recorded (Oliver et al., 2018; Collins et al., 2019; Darmaraki et al., 2019) and it has been widely demonstrated how they can have a strong negative impact on many species in the Mediterranean marine ecosystems, often leading to significant mass mortalities (Garrabou et al., 2022). Most studies of heatwave-related mass mortality events in the Mediterranean have focused on sessile habitat-forming species such as gorgonians and macroalgae (Garrabou et al., 2019; Chimienti et al., 2021; Verdura et al., 2021). Instead, the effects that marine heat waves may exert on other Mediterranean vagile species remain poorly understood, although it has been documented how in some areas of the Mediterranean basin some species, including molluscs and sea urchins, have suffered a collapse in recent decades (Yeruham et al., 2015; Rilov, 2016; Albano et al., 2021).

In the case of the investigated populations of the sea urchin *P. lividus* along the Ligurian coast, we cannot claim mass mortality occurrence, although the present low abundance of some size classes was noted, and which can be attributed to a failure in the recruitment of new individuals in some years. For this reason, the occurrence of extreme heat waves was hypothesized to have affected not directly the adult individuals of *P. lividus* populations, but rather the larval and/or juvenile stages, which may be significantly more vulnerable to thermal stress and less adapted than adults to tolerate wide ranges of seawater temperature (Przeslawski et al., 2015).

To test such hypothesis, a laboratory experiment was set up with the aim of exposing the early developmental stages of *P. lividus* to different, in terms of their duration, sudden increases in seawater temperature, in order to simulate the occurrence of abnormal heat waves, to determine the potential effect in terms of percentage of malformed "plutei", delayed larval development and mortality.

3.2 Materials & Methods

Wild adult specimens of *P. lividus* (test diameter >5 cm) have been collected in Vernazzola, a coastal rocky site in the Genoa city (Ligurian Sea, NW Mediterranean).

Gamete release has been induced through intracoelomic injection of 0.5 ml potassium chloride (KCL 1 M; ***Fig. 3.1***). Eggs from three female specimens were collected into 100 ml Falcon tubes (*Fig. 3.2*), counted using a Sedewick rafter cell under stereoscope and introduced into three 1 l autoclaved bechers with filtered sea water at a concentration of 40 eggs ml^{-1}. Dry sperm from two male specimens was collected directly from the gonoporo using a Pasteur pipette and placed into a 2 ml Eppendorf tube. After a prior dilution (e.g., 10 microliters of sperm in a 10 ml graduated cylinder filled to the mark with FSW) the sperm concentration was estimated using a hemocytometer. Afterwards, dry sperm was added to the bechers with eggs in order to obtain a a proportion sperms:eggs 100:1 and gently mixed. After 20 min, eggs were checked for fertilization.

Then, a volume of zygotes solution corresponding to 1000 eggs/ml has been added to each replicate (4 bechers).

Figure 3.1: Individuals of *P. lividus* after KCl injection, waiting for their egg or sperm release

Figure 3.2: Female individual of *P. livdus* and eggs released in seawater

3.2.1 Thermal anomaly simulation

For this experiment, 20° C was set as the "Cold" (normal) seawater temperature, as it corresponded to the temperature recorded at the location and time when the tested *Paracentrotus lividus* individuals were collected (13/06/2022 in Vernazzola-Genoa- Lat/Long 44°23.419" N, 8°58.796"E), while 24° C was chosen as the "Hot" temperature to simulate the thermal anomaly, since an increase up to 4°C is expected in the Mediterranean Sea in the context of climate change (Cattaneo-Vietti, 2018).

Fertilization was performed at the two different temperatures, (*Fig. 3.3*), in order to simulate the occurrence of the thermal anomaly during this process. The zygotes solution was divided into four 1l bechers, for each temperature, in order to have 2 replicates for each treatment. After one hour, the success rate of fertilization was estimated by counting fertilised eggs (i.e., eggs with the fertilisation membrane fully or partially visible; 3 replicated counts on 1 ml) and then assessing the percentage of fertilised eggs.

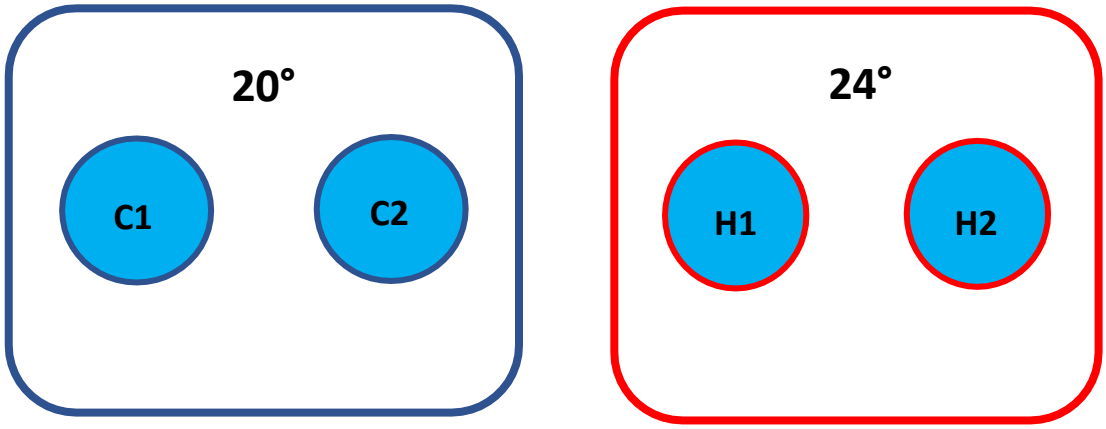

Figure 3.3 Experimental design at fertilization time, "C"= Cold condition of fertilization and "H"= Hot condition of fertilization

The mortality and the percentage of malformed zygotes and plutei or of those with delayed larval development was assessed 24 hours post fertilization (hpf) by a volumetric count (3 replicated counts on 1ml). Then, the bechers containing the individuals fertilized at the lowest temperature (20°C) were divided into 8 smaller bechers (*Fig. 3.4*): 4 of them were maintained at the same temperature (cold condition; C1 treatment; bechers C1a, C1b, C2a, C2b), while the other 4 were exposed to the temperature increase of 3/4°C (hot condition; H1 treatment; bechers H1a, H1b, H2a, H2b) in order to test effects of thermal anomaly occurring at 24 hpf.

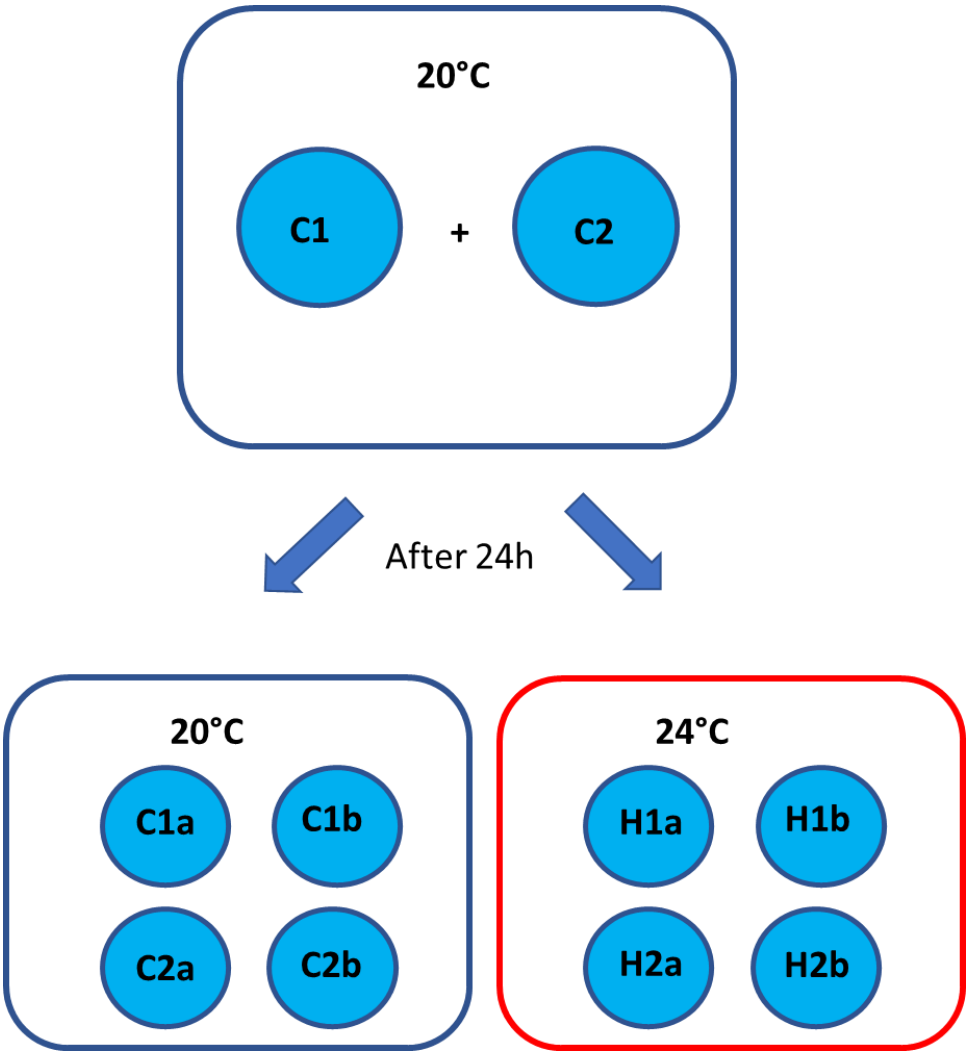

Figure 3.4: Experimental design 24 h after fertilization, "C"= maintained at Cold condition and "H"= subjected to Hot condition (24 °C heat wave) from 24h to 48 h of development

At 48 hpf, the mortality and the percentage of malformed zygotes and plutei or with delayed larval development were evaluated (2 replicated counts on 1ml).

After that, two bechers from the lower temperature condition were subjected to a temperature increase of 3/4°C, while the other two remained at the constant lower temperature (*Fig. 3.5*), in order to test effects of thermal anomaly occurrence at 48 hpf.

As for the bechers that had been subjected to the high temperature condition, two replicates are maintained for additional 24 hours at the highest temperature (so as to simulate a thermal anomaly with a duration of 48h), while the other two were returned again to the lowest temperature (so as to simulate a thermal anomaly of short duration, only from 24hpf to 48hpf (*Fig. 3.5*).

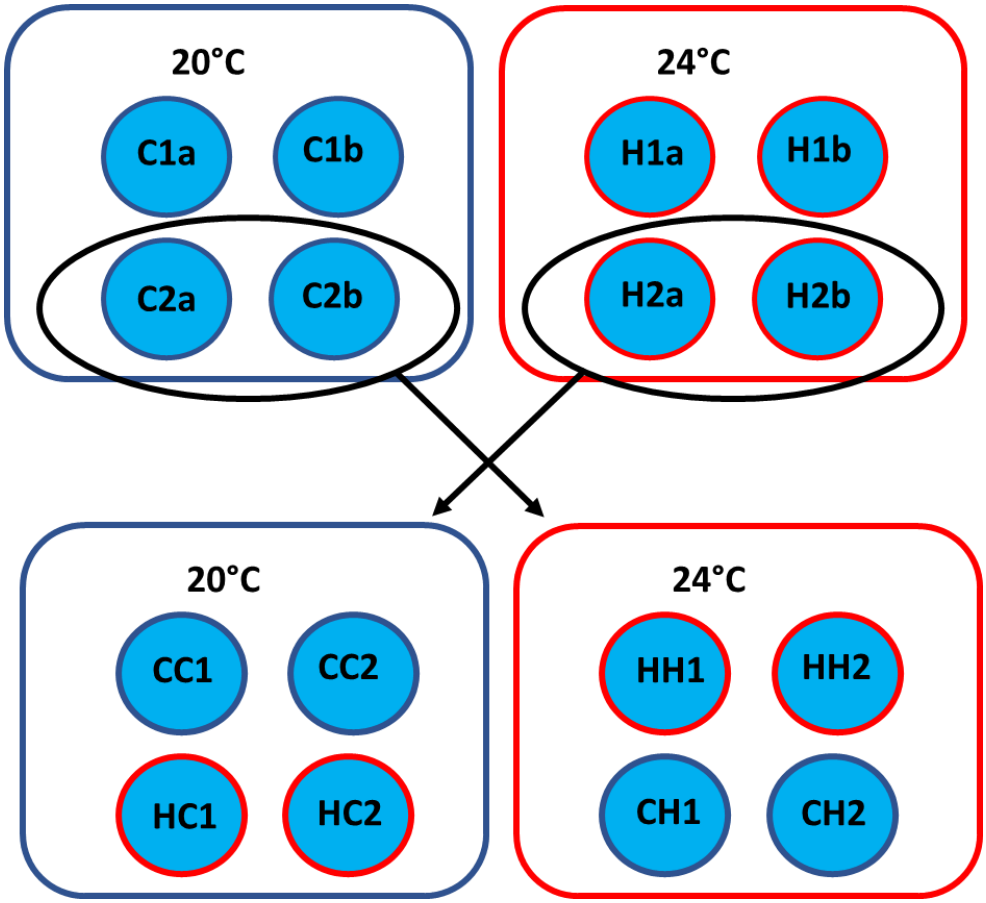

Figure 3.5: Experimental design 48 h after fertilization. "CC"= maintained at Cold condition for all times. "HH"= subjected to Hot condition (heat wave of 24°C) from 24h to 72h of development. "HC"= subjected to Hot condition heat from 24h to 48h of development and then returned to the Cold condition. "CH"= manteined maintained at Cold condition for 48h and after subjected to Hot condition (heat wave of 24°C) from 24h to 48h of development

At 72 hpf the mortality and the percentage of malformed zygotes and plutei or with delayed larval development were assessed for all the 8 beachers (2 replicated counts on 1ml).

3.2.2 Some examples for the identification of malformed individuals:

The following figures (*Fig. 3.7-3.10*) report anomalies in the larval development of *P. lividus*, compared to the normal one (*Fig. 3.6*) in response of different stressors.

Figure 3.6: Correct larval development (photo from PhD thesis Miccichè, 2010)

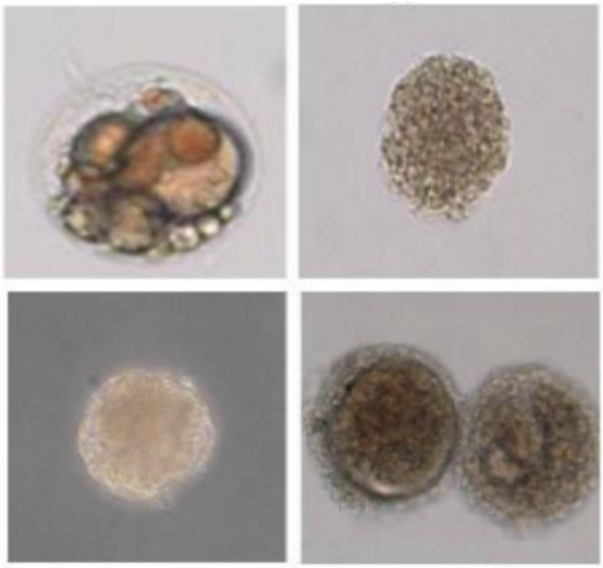

Figure 3.7: Arrest of gastrula development (photo by CNR IAS –Genova, www.ias.cnr.it)

Figure 3.8: Prism with delayed development (photo by CNR IAS –Genova, www.ias.cnr.it)

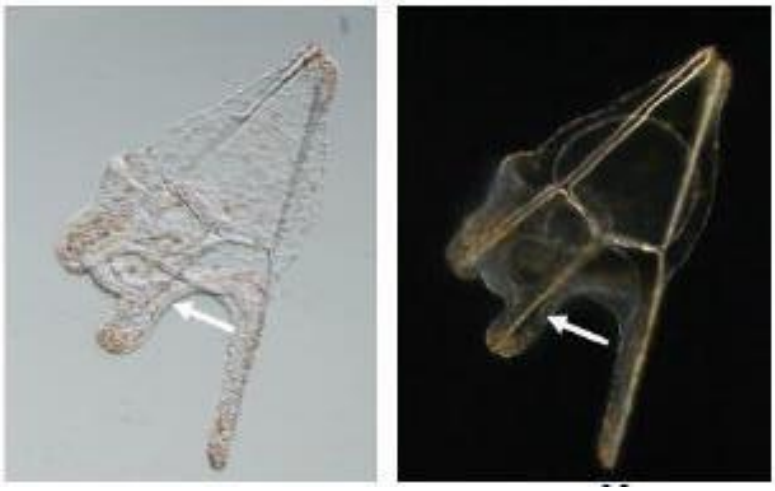

Figure 3.9: Pluteus with fused arms (photo by CNR IAS –Genova, www.ias.cnr.it)

Figure 3.10: pluteus with absent or undeveloped arms (photo by CNR IAS –Genova, www.ias.cnr.it)

3.3 Results

The two different temperature conditions tested did not affect fertilization. In fact, the fertilization rates were definitely high for both temperature treatments: 97% for the "Cold" temperature condition (20° C) and 98% for the "Hot" temperature condition (24° C).

Conversely, after 24hpf (hours post fertilization), a very high mortality of zygotes that developed at the temperature of 24° C was observed. In the two bechers at 24° C, 44.33 ± 39.71 (avg ± SD) ind/ml and 32 ± 5.56 SD (avg ± SD) ind/ml were counted (as mean of multiple microscopic countings), while in the two bechers at 20° C, 434.33 ± 120.67 (avg ± SD) ind/ml and 913.33 ± 186.00 (avg ± SD) ind/ml were counted (*Fig. 3.11*). Given the high mortality of the Hot treatment, H1 and H2 beackers were dropped from the following steps of the experiment.

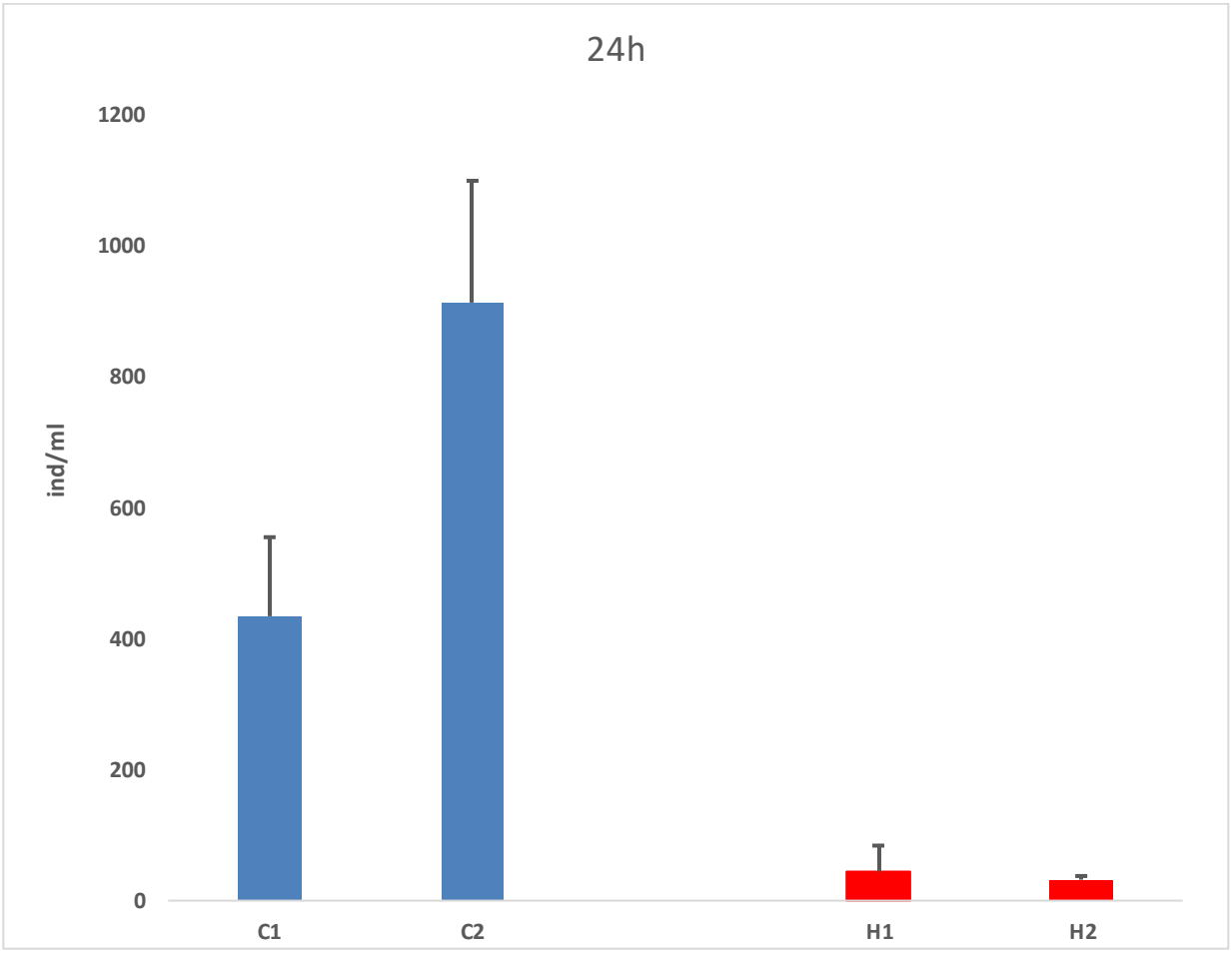

Figure 3.11: Abundance of *P. lividus* zygotes at 24hpf, in the two temperature conditions. "C"= Cold condition (20 °C) of fertilization and "H"= Hot condition (24 °C) of fertilization

At 48 hpf, a difference in total individuals (independent of developmental stage) alive and without the presence of malformations was observed between those that remained at 20° C at all times (C1a, C1b, C2a, C2b) compared to those that were subjected to the 24° C heat wave (H1a, H1b, H2a, H2b). The former were 9 ± 2.8 ind/ml mean ± SD (C1) and 19.5 ± 9.5 ind/ml mean ± SD (C2), while the latter were only 3 ± 1.4 ind/ml mean ± SD (H1) 4.5 and 1.3 ind/ml mean ± SD (H2). Significant differences were also observed in the total number of individuals (healthy individuals + malformed individuals); in the first case it was 24.5 ± 6.2 ind/ml mean ± SD, while in the second it was 11.5 ± 3.1 ind/ml mean ± SD *(Fig. 3.12).*

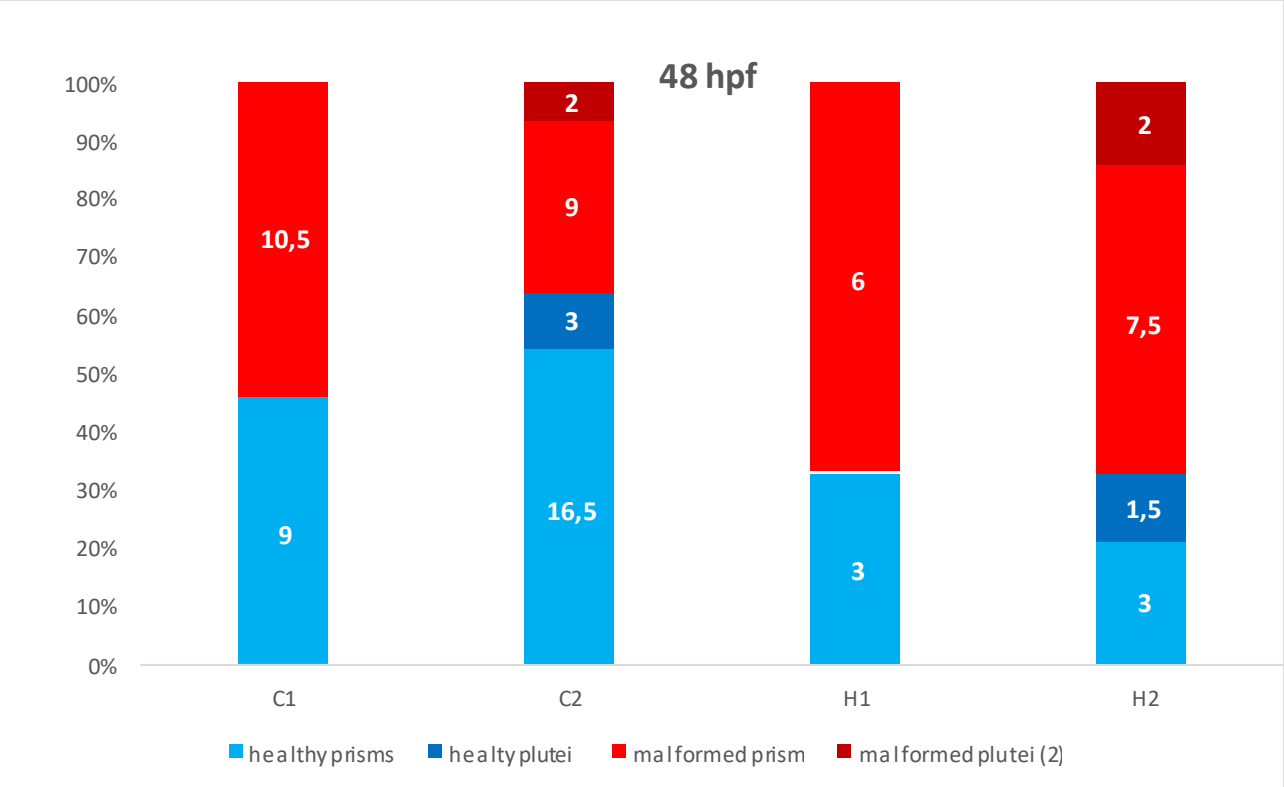

Figure 3.12: Abundance (numbers on the bars) and health status of juveniles of *P. lividus* at 48hpf, under the two temperature treatments. C1 and C2 maintained at 20 °C at all times, H1 and H2 subjected to 24 °C heat wave from 24h to 48 h of development. Plutei (2) refers to two-armed plutei.

At 72 hpf, four different responses were observed for the four conditions (*Fig. 3.13*). In the first case, where juveniles of *P. lividus* were maintained for all 72h at the "Cold" temperature of 20° C ("CC"), a high number of survived individuals was observed: 18.5 ± 4.2 ind/ml mean ± SD, including 5 ± 4.2 healthy and 13.5 ± 7.8 ind/ml mean ± SD malformed plutei. In the second case, where juveniles suffered the heat wave between 24h and 48h ("HC") of development, an average density of 10 ± 1.4 ind/ml mean ± SD was found, in this proportion 4± 1.4 ind/ml mean ± SD healthy and 6 ± 1.4 ind/ml

mean± SD malformed plutei. In the third case, in which juveniles were subjected to the prolonged heat anomaly (i.e., a total of 48h, from 24h to 72h, "HH"), the total number observed was only 6 ± 0 ind/ml mean ± SD, of which only 1.5 ± 0.7 ind/ml mean ± SD healthy plutei. Finally, in the last case, where juveniles were subjected to a later heat wave (only in the last 24h, "CH"), a high number of total individuals was observed: 20.5 ± 4.9 ind/ml mean ± SD ind/ml, but almost all malformed plutei 18.5 ± 5.7 ind/ml mean ± SD. Pluteus have been observed at different stages of development: pluteus (2)= two-armed pluteus; pluteus (4)= four-armed pluteus; pluteus (6)= six-armed pluteus.

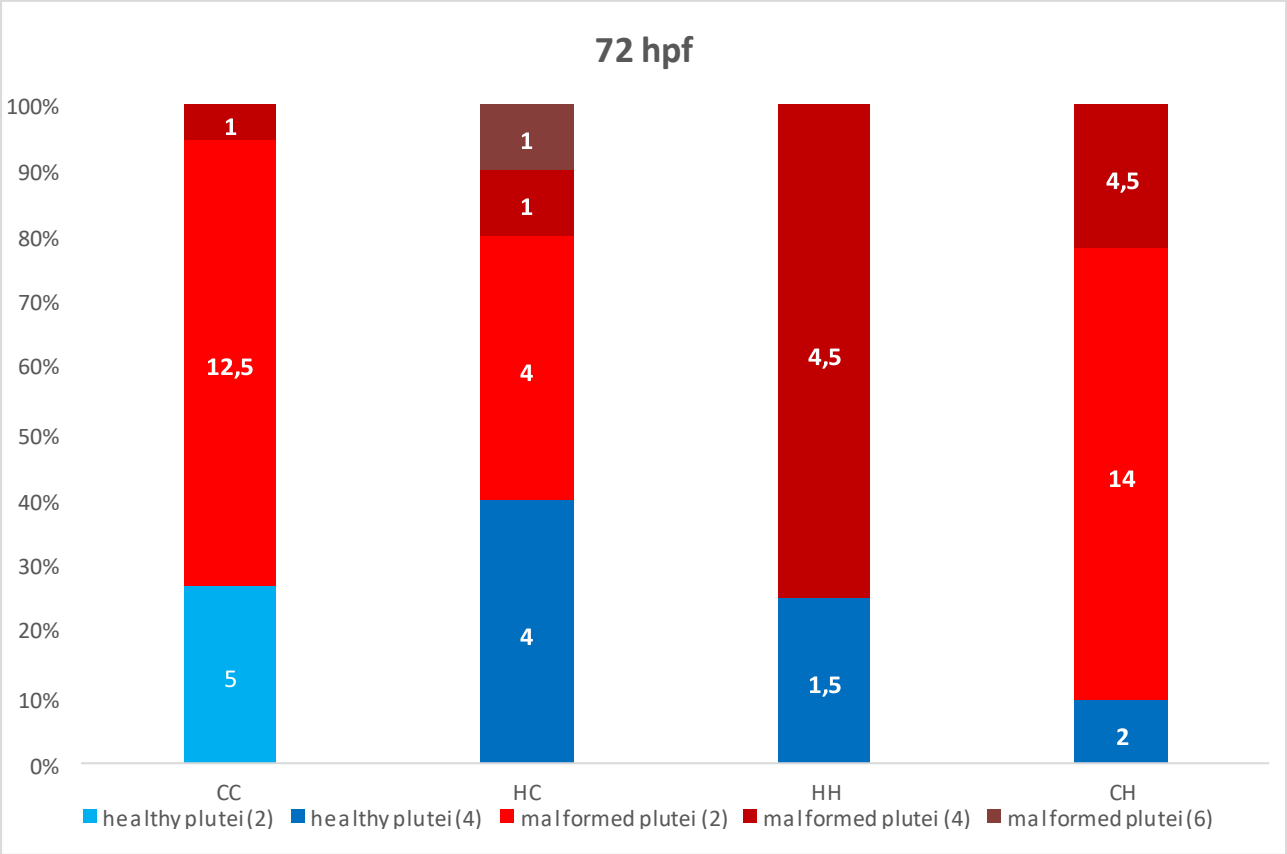

Figure 3.13: Abundance and health status of juveniles of *P. lividus* 72hpf, in the 4 recreated conditions. "CC"= maintained at Cold condition for all times. "HC"= subjected to Hot condition heat from 24h to 48h of development and then returned to the Cold condition. "HH"= subjected to Hot condition (heat wave of 24°C) from 24h to 72h of development. "CH"= maintained at Cold condition for 48h and after subjected to Hot condition (heat wave of 24°C) from 24h to 48h of development.

3.4 Discussion and Conclusions

This experiment provided preliminary information in order to better understand the impact that heat waves can exert on the juvenile stages of *Paracentrotus lividus*, and consequently on population structure. Indeed, our data prove that a sudden and abnormal increase in seawater temperatures can have severe negative effects from the earliest stages of development.

Since the fertilization rate was significantly high and almost the same in both conditions: 97 % for the "Cold" temperature condition (20° C) and 98 % for the "Hot" temperature condition (24° C), it can be affirmed that the temperature, although high, may have no effect at this stage. In contrast, however, 24 hours after fertilization, a drastic decrease of live embryos can be seen in the sample fertilized at 24°C *(Fig. 3.11*). Thus, it is evident that an increase in temperature, in this case 3/4° C higher than that generally found in the Ligurian Sea in late spring and therefore at the time of the highest reproductive peak of *P. lividus* (Fenaux, 1968; Pedrotti, 1993; Lozano et al., 1995; Chiantore et al., 2008), can lead to rapid and high embryonic mortality.

Also a thermal anomaly impact on the zygotes of *Paracentrotus lividus* from 24h to 48h post fertilization (a period when zygotes are in the metamorphosis phase from prisms to two-armed pluteus, Fenaux et al., 1985) would again exert very negative effects. In particular, it was observed that the number of individuals present is greatly reduced compared to the "Cold" conditions, and furthermore, the percentage of healthy individuals is reduced by half from about 60% to 30% *(Fig. 3.12).* Finally, at 72 hours post fertilization, the four different tested conditions provided other important insights. The sample that was maintained throughout the experiment at the constant temperature of 20°C (CC in *Fig. 3.13*), that represented our negative control, displayed a higher abundance of plutei than those that underwent seawater temperature alterations. However, a high percentage of malformed plutei was still observed, this is most likely attributable to the excessive density of individuals in the bechers. In fact, at 72hpf the ideal density for culturing sea urchins should be 1 ind/ml (Fenaux et al., 1985). Comparing the CF and CC treatments (*Fig. 3.13*), it was demonstrated how the negative effect of the occurrence of the thermal anomaly can be directly proportional to its duration. In the case where the induced heat wave was longer (48h *vs* 24h), both the survival of the plutei and the percentage of healthy plutei were reduced. Finally, the last case (of more delayed occurrence of thermal anomaly, CH in *Fig. 3.13*) further confirms the hypothesis that the occurrence of an anomalous and sudden change in seawater temperature adversely affects the development of *Paracentrotus lividus* early stages. As long as plutei were kept at the "Cold"

temperature they were showing a good survival success, evidenced by the high density of individuals in the samples. Conversely, a very high percentage of malformed plutei was observed following the increase in temperature.

Consequently, these results strongly support the hypothesis that the population structures of *P. lividus* along the Ligurian coats may have been largely shaped by the occurrence of these anomalous and increasingly frequent events in the Mediterranean Sea. In fact, such thermal anomaly events may strongly affect the survival of early development phases, hampering the recruitment and causing, therefore, the lack of entire cohorts in affected populations.

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4. The role of calcium carbonate and antioxidants in the Mediterranean sea urchin diet

4.1. Introduction

The present study was conducted to improve knowledge about the role of calcium carbonate and antioxidants contained in natural algae in the diet of sea urchin through a laboratory experiment. Specifically, possible effects of different macroalgal diets tested simultaneously on both species of sea urchin found in the Mediterranean Sea, which coexist naturally in rocky shore communities and *Posidonia oceanica* seagrass beds, were evaluated (Chiantore et al., 2008; Privitera et al., 2008):

- *Paracentrotus lividus* (Lamarck, 1816)

- *Arbacia lixula* (Linnaeus, 1758).

Macroalgae to be used as feed were selected on the basis of their different compositional characteristics, particularly the different amount of calcium carbonate and antioxidant compounds. Considering the role of calcium carbonate in sea urchin diet is very important, as it is the chemical compound that most constitutes the test of sea urchin and, as such, is fundamental for the growth, survival and well-being of the animal (Asnaghi et al., 2019).

The selected macroalgae were:

• *Ellisolandia elongata* (J. Ellis & Solander), a red alga of the Corallinaceae family, whose articulated (geniculate) thallus is mainly made up of calcium carbonate.

• *Padina pavonica* (Linnaeus), a brown alga of the Dictyotaceae family, characterised by abundant depositions of calcium carbonate on the surface of the thallus.

• *Dictyota dichotoma* (Hudson), also a brown alga of the Dictyotaceae family, known to be rich in antioxidants.

The Corallinales are the predominant order of calcified macroalgae found in temperate waters (Williamson et al., 2014). The red *E. elongata* is an articulated calcareous species, whitish-pink to

reddish-lilac, it has branching, pinnate flexible fronds and an erect thallus attached to the rocky substrate by a crustose holdfast. The fronds are made of small calcified segments (intergenicula), separated from uncalcified nodes (genicula), which provide flexibility to the erect algal thallus (Marchini et al., 2019). *P. pavonica* is the only calcareous genus of the brown algae. As a member of the Dictyotales, it is widely distributed in warm, shallow seas. $CaCO_3$ is precipitated in the form of needle-shaped aragonite crystals. *P. pavonica* presents a biologically induced extracellular calcification, which results in whitish precipitations. These carbonate deposits are arranged in concentric bands on both thallus surfaces with interspaces where reproductive structures, such as tetrasporangia, can develop. The side of the blade facing the sea surface with the enrolled margins represents the "upper" surface and is more calcified than the opposite side, the "lower" surface (Iluz et al., 2017). Calcification of the frond amounts to approximately 11% content by dry weight (Benita et al., 2018). The calcium carbonate gives little support to the plants (Miyata, Okazaki & Furuya, 1977).

The content of antioxidants in seaweed is widely studied because of their therapeutic properties. The family Dictyotaceae has been extensively studied for its wide variety of bioactive diterpenes with marked biological activities (Zubia et al., 2009). *D. dichotoma* extract showed high total flavonoid and total alkaloid contents. Flavonoids have been reported to be antioxidants, scavengers of a wide range of ROS and inhibitors of lipid peroxidation, and also to be potential therapeutic agents against a wide variety of diseases (El-Shenody et al., 2019). Antioxidants play a vital role against various diseases like cancer, cardiovascular diseases, inflammation, ageing process, rheumatoid arthritis, diabetes, as well as disease associated with cartilage and Alzheimer's disease (Tariq et al., 2015). Also, the profiles of *P. pavonica*, evaluated using HPLC, reported to contain five phenolic compounds, suggesting using *P. pavonica* as alternative for various diseases including diabetic, cardiovascular problems etc (Sudha & Balasundaram, 2018).

In light of the above, these macroalgae have been used to test the effect of different content of calcium carbonate and antioxidants in sea urchin diet, measuring different morpho-functional parameters indicative of animal health and growth: weight, test diameter without spines, lantern size, robustness, jaw/test ratio, gonadosomatic index, repletion index.

4.2. Materials and methods

Specimens of *Arbacia lixula* and *Paracentrotus lividus* were collected in shallow rocky reefs, along the stretch of coast between Genoa and the Portofino Promontory (GE), NW Mediterranean Sea. Sea urchins were carefully detached from the rocky substrate to avoid damaging spines and injury. They were then quickly transported to the Marine Laboratory of Camogli (CNR-IBF; GE) and allowed to acclimate for some days in aquaria to limit the shock due to sampling.

24 specimens of *P. lividus* and 24 of *A. lixula* were randomly chosen. The sea urchins were divided into 12 tanks (3.7 liters each; 4 individuals per tank), in order to have two replicates for each species, for each diet. The tanks were fed individually by a constant flow of seawater, in order to have independent replicates. The seawater was taken directly from the sea. The turnover of the water was 1 l/minute (around 3 minutes for a total turnover). Prior to the start of the experiment, sea urchins were starved in the experimental setup for 2 weeks.

During the experimental period, temperature was measured continuously with a data logger, while salinity and oxygen concentration were monitored twice a week with a probe. The 3 species of the selected macroalgae (*E. elongata, P. pavonica* and *D. dichotoma*) were collected with a weekly frequency, in the same coastal area where the sea urchin specimens were collected. The algae were stored in dedicated tanks filled with seawater and constantly oxygenated, in order to supply them to the sea urchins in conditions as similar as possible to natural ones. The 9th of July 2020 (T0), we first measured for each specimen the body weight and the size (test diameter without spines, in triplicates) using a calliper (±0.1 mm). Then we fed the sea urchin for the first time. After comparing information from previous aquaculture experiments of these echinoderms (Grosjean et al., 1998; Prato et al.; 2018, Boudouresque & Verlaque, 2020), it was decided to feed each specimen with a daily amount of algae equal to 5% of their wet body weight.

We fed the sea urchins twice a week. Before feeding, we cleaned each tank in order to remove the feces, and we weighed the quantity of the uneaten macroalgae. The food they discarded was removed and replaced with fresh algae. The feeding trial lasted 3 months, during which the health and well-being of the specimens was continuously monitored. We measured the test size (without spines) and the weight of each sea urchin 4 times during the experiment: at the beginning of the experiment (T0) and at three further dates at approximately one-month intervals (T1: 30 th of July, T2: 03 rd of September). T3 (6th of October) represented the end of the experiment. At the end of

the experiment, the sea urchins were moved to the Laboratory of the University of Genova to perform morpho-functional measurements. Besides the total weight and the test diameter, we measured for each specimen:

Algal consumption

Three days after feeding, the remaining portion was retrieved and weighed to estimate the percentage of consumption sing the following formula: Algal consumption = [wet weight seaweed supplied/ wet weight seaweed not eaten] x 100.

Test size and weight

The test diameter and weight of each specimen were measured monthly during the experiment (T0= start of experiment, T1, T2 and T3 one month apart). The sea urchins were left to dry for a few minutes and then weighed, while the diameter was measured without spines.

Gonado-Somatic Index (GSI)

The gonads were extracted and weighed and the GSI was calculated using the formula: GSI = [wet weight of gonads/total wet weight] × 100 as reported by Lawrence & Holland 1965

Repletion Index

For the estimation of a feeding index, the gut contents of the specimens were taken and weighed, and the repletion index (RI) was calculated as proposed by Kempf (1962): RI = [wet weight of gut/total wet weight] × 100.

Test robustness

Test robustness was measured in alive sea urchin specimens using a custom-made device designed to measure the static force required to crush sea urchin tests (used by Asnaghi et al., 2013, 2019). Sea urchins were positioned upside down (in order to mimic fish predator attack) in a glass column. Then, a hollow piston, built to fit and run within the column, was inserted inside the column and progressively filled with lead pellets in order to increase the pressure, until the crushing of the urchin test. The static force required to crush sea urchin tests was measured as the weight (g) of piston and lead added. Test robustness has been normalized dividing the fracture force/surface on which the

force is applied.

Jaw/Test ratio

Jaw/Test ratio (JTR) is the ratio between the length of the jaw pyramids of the Aristotle's lantern and the diameter of the test, expressed in percentage. To measure the length of the demi-pyramids of Aristotle's lantern, it was necessary to immerse the Aristotle's lantern in hydrogen peroxide, which contains 2% of sodium hypochlorite, for 30 min, in order to remove organic matter and facilitate the separation of structural elements (according to Asnaghi et al., 2013).

4.2.1. Statistical analyses

The measured morpho-functional features (i.e. gonadosomatic index, repletion index, test robustness and jaw-test ratio), have been used to perform multivariate analyses. We calculated the triangular matrix of dissimilarity using the Euclidean distance. Potential differences between the two sea urchin species due to algal diet were tested through PERMANOVA, using the factor Species (2 levels), the factor Algal diet (3 levels) and their interaction. Results are visually displayed through the MDS (Multidimensional scaling) plot, a multivariate technique that enables visualizing the level of similarity of individual cases of a dataset.

Additionally, paramentric two-way ANOVAs, following the same design applied for the PERMANOVA test, were performed on the single morpho-functional variables, after checking for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett test). Variables not fulfilling assumptions (GSI and JTR) have been square root transformed. All statistical analyses were performed using R software (R Core Team 2021).

4.3. Results

4.3.1. Algal consumption

The algae consumption for the two species is showed in figure *Fig. 4.1*. and it was generally observed that *Arbacia lixula* left much more uneaten food than *P. lividus*. The preferred algal species of *A. lixula* was *E. elongata*, followed by *P. pavonica*. *D. dichotoma* was the most repelled food item. *P. lividus*, on the other hand, consumed all the food provided, except for occasional events.

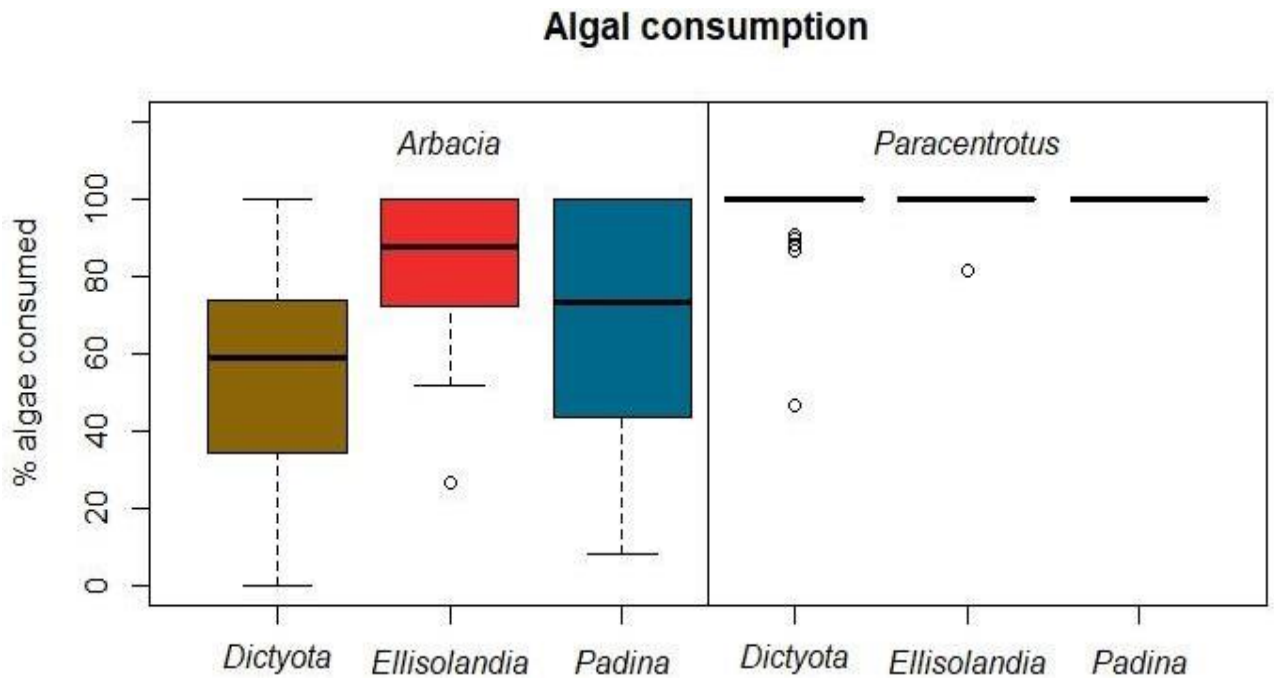
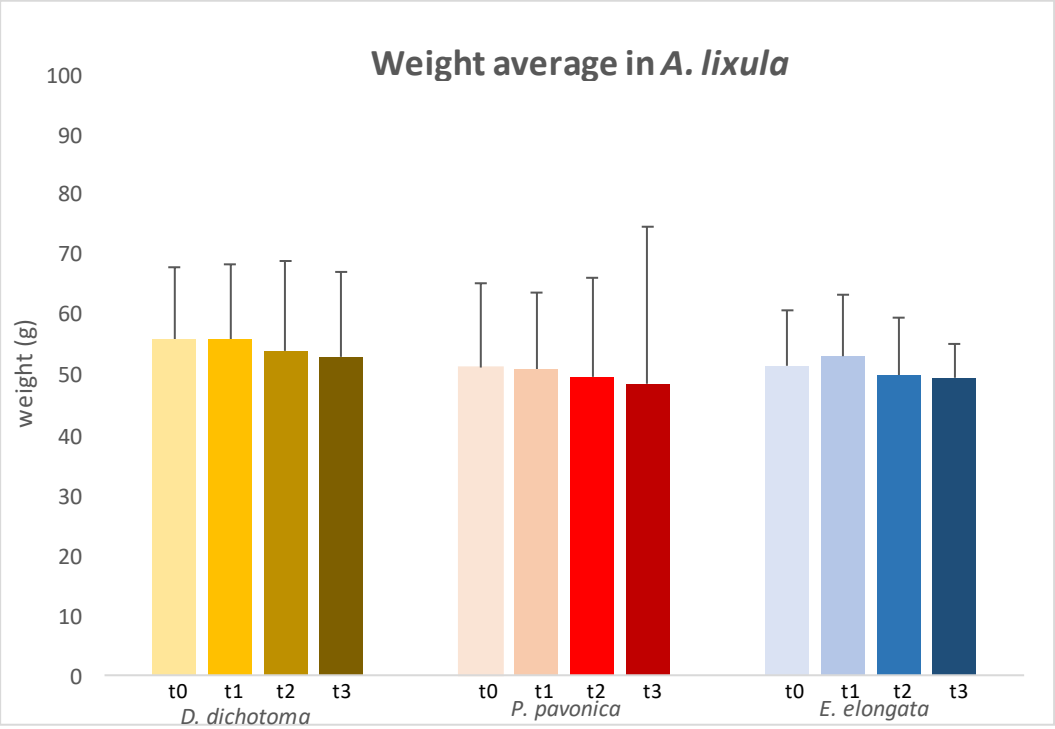


Figure 4.1: Average percentage of macroalgae consumed by the two species during the experiment.

4.3.2. Test size and weight

The diameter and the weight of the specimens did not vary significantly during the experiment (*Fig. 4.2, Fig. 4.3*). This is to be expected since adult specimens were used and the experiment was performed during the summer season, a period when organisms consume more energy and invest less in somatic growth.



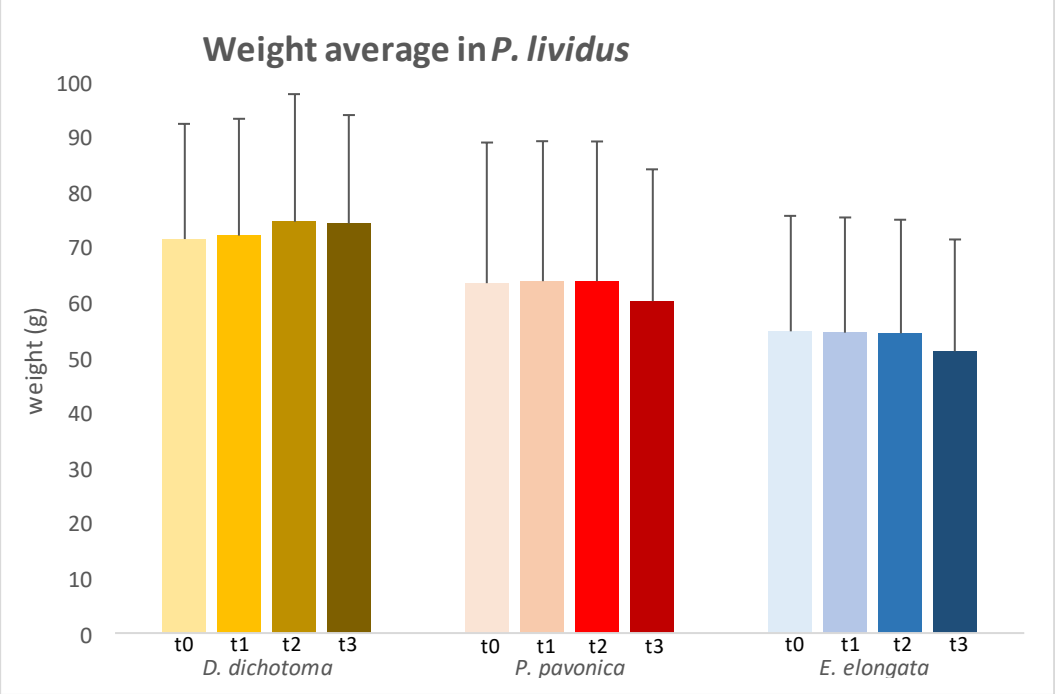


Figure 4.2: Average weight (g) of a) *A. lixula* and b) *P. lividus* during the experiment, measurements taken at intervals of about one month.

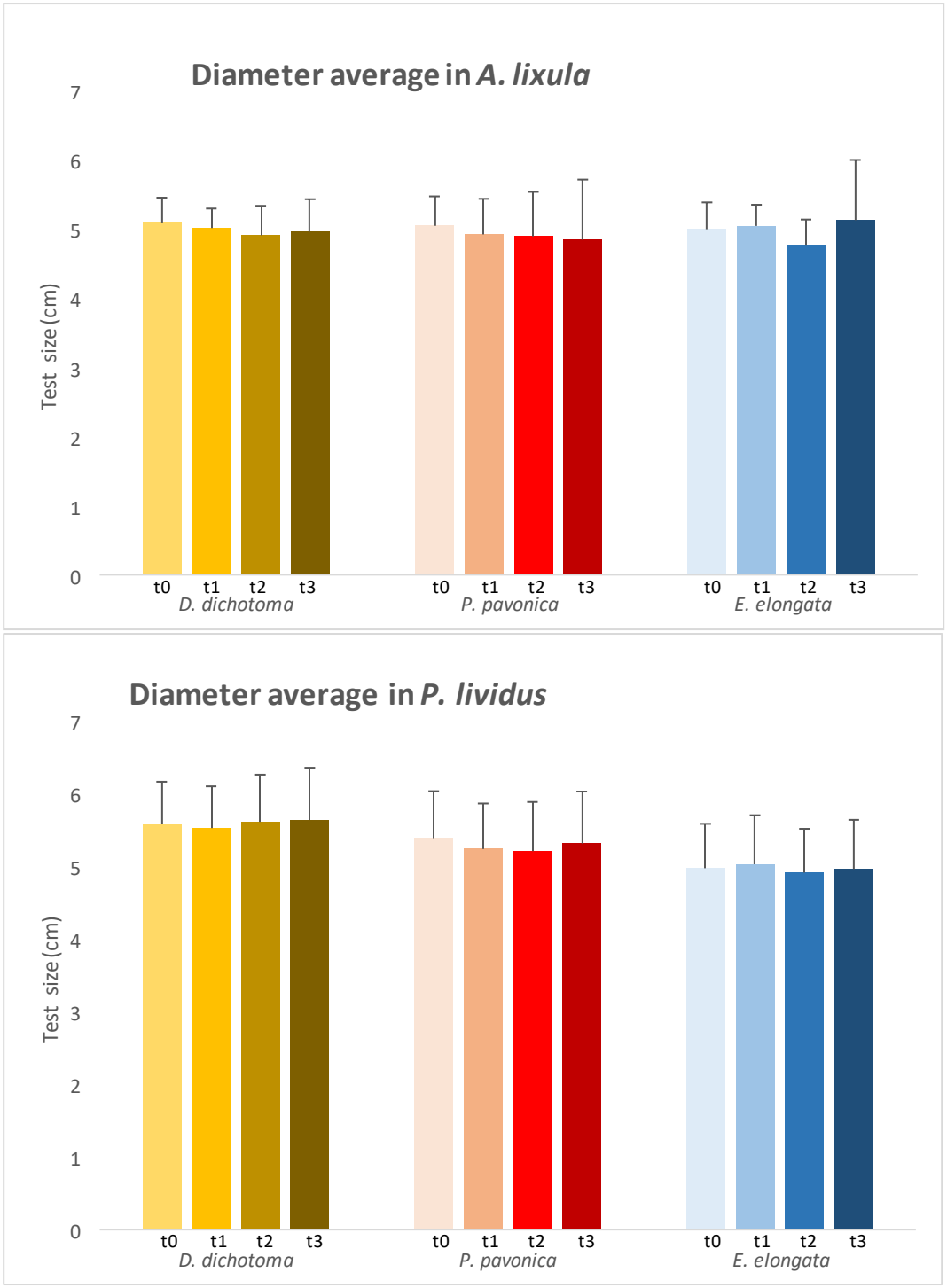


Figure 4.3: Average test size (cm) of *A. lixula* and *P. lividus* during the experiment, measurements taken at intervals of about one month.

4.3.3. Gonado-Somatic Index

The box-plot in *Fig. 4.4* shows the Gonado-Somatic Index calculated for each specimen of the two species after 3 months of breeding. Significant differences were observed between the two species: *P. lividus* showed significantly more developed gonads than *A. lixula* (Tab. 4.1). In addition, the *E. elongata* diet conferred larger gonad development to *P. lividus* than the other species of macroalgae, with a significant difference compared to the *D. dichotoma* diet (Tab. 4.1).

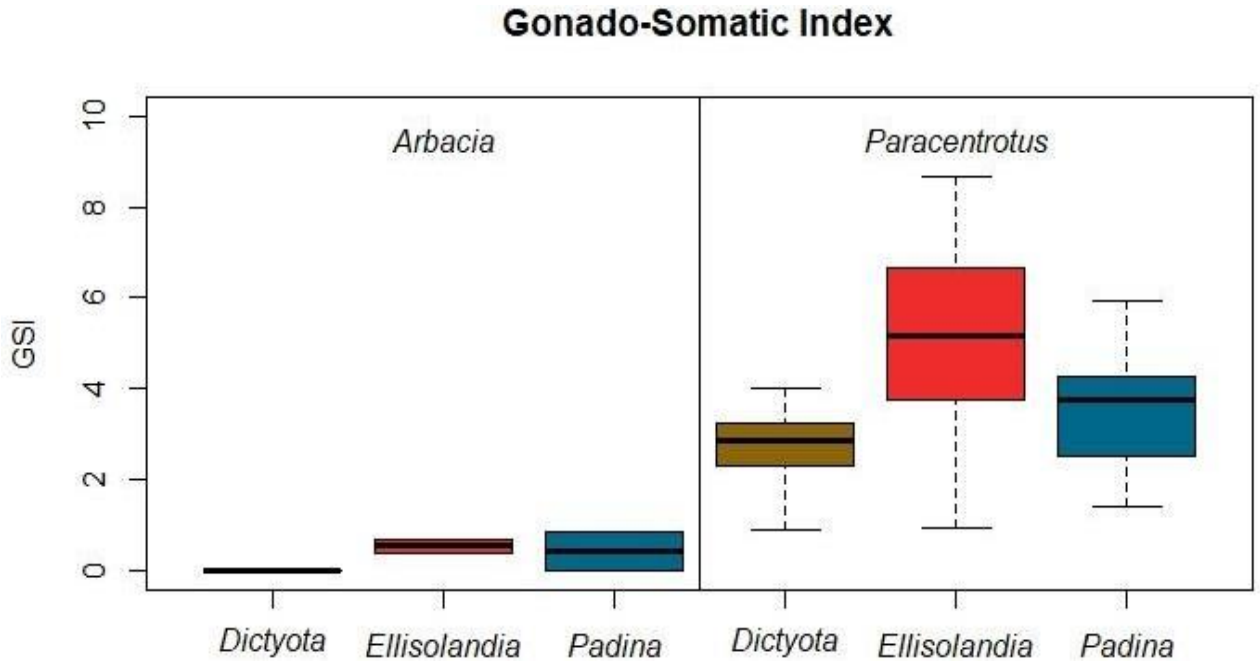


Figure 4.4: Gonad Somatic Index of *A. lixula* and *P. lividus* for the three different macroalgal diets.

Table 4.1: Anova table for the response variable Gonado Somatic Index.

GSI	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Algal_diet	2	2.1082	1.0541	4.9446	0.01685	*
Species	1	10.3136	1.03E+01	48.3785	5.55E-07	***
Algal_diet:Species	2	0.0393	0.0196	0.0922	0.91231	
Residuals	22	4.6901	0.2132			

Tukey post hoc Test: for *P. lividus Ellisolandia* ≠ *Dictyota*, p-value 0.05

4.3.4. Repletion Index

Regarding Repletion Index (RI, *Fig. 4.5*), a significant difference was observed between the two sea urchin species. In addition, *P. lividus* displayed significantly higher RI values for the diet with *P. pavonica* than for the diet with *E. elongata* (*Tab. 4.2*).

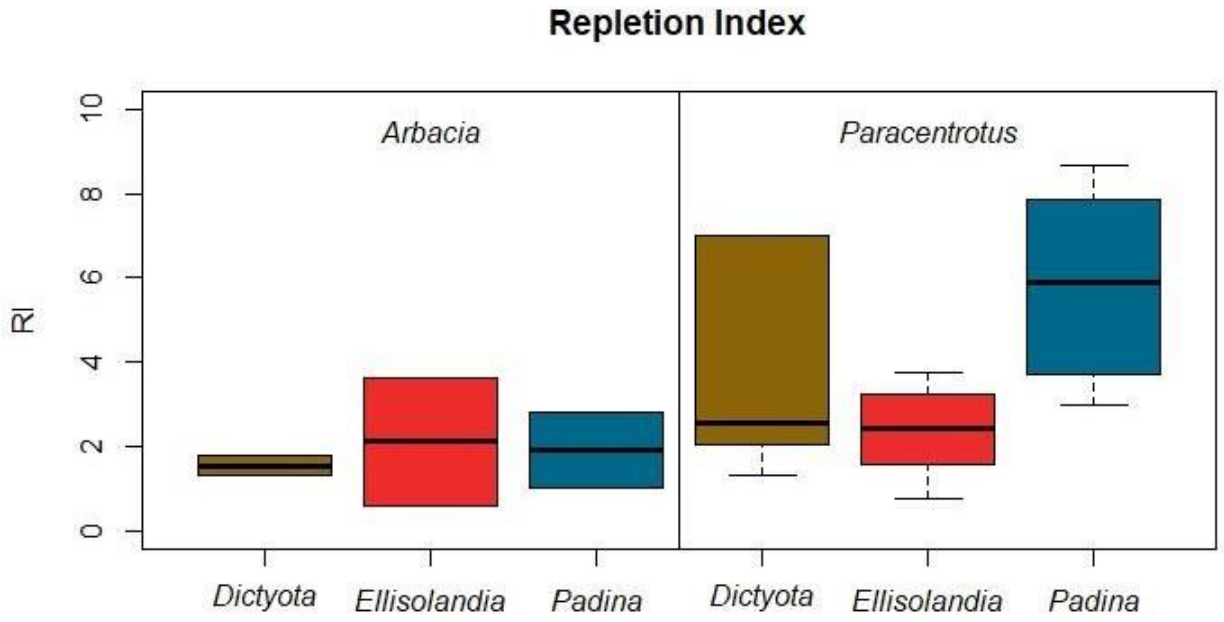


Figure 4.5: Repletion *Index* of *A. lixula* and *P. lividus* for the three different macroalgal diets.

Table 4.2: Anova table for the response variable Replection Index

RI	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Algal_diet	2	37,699	18,8495	4,9979	0,01624	*
Species	1	21,056	21,0562	5,583	0,02739	*
Algal_diet:Species	2	10,556	5,2779	1,3994	0,26786	
Residuals	22	82,973	3,771			

Tukey post hoc Test for *P. lividus: Padina ≠ Ellisolandia*, p-value 0.007

4.3.5. Test robustness

For the robustness of the sea urchin test (*Fig. 4.6*), in contrast to what was expected, *E. elongata* diet (the diet with more calcium carbonate content) did not lead to higher test robustness. *A. lixula* showed similar values with *D. dichotoma* and *E. elongata* diets, higher than the one of urchins fed with *P. pavonica*.

P. lividus displayed higher values with *D. dichotoma* diet. For both species *P. pavonica* diet lead to lower values of test robustness. Yet, none of the algal diet provided significant effect on test robustness (*Tab. 4.3*).

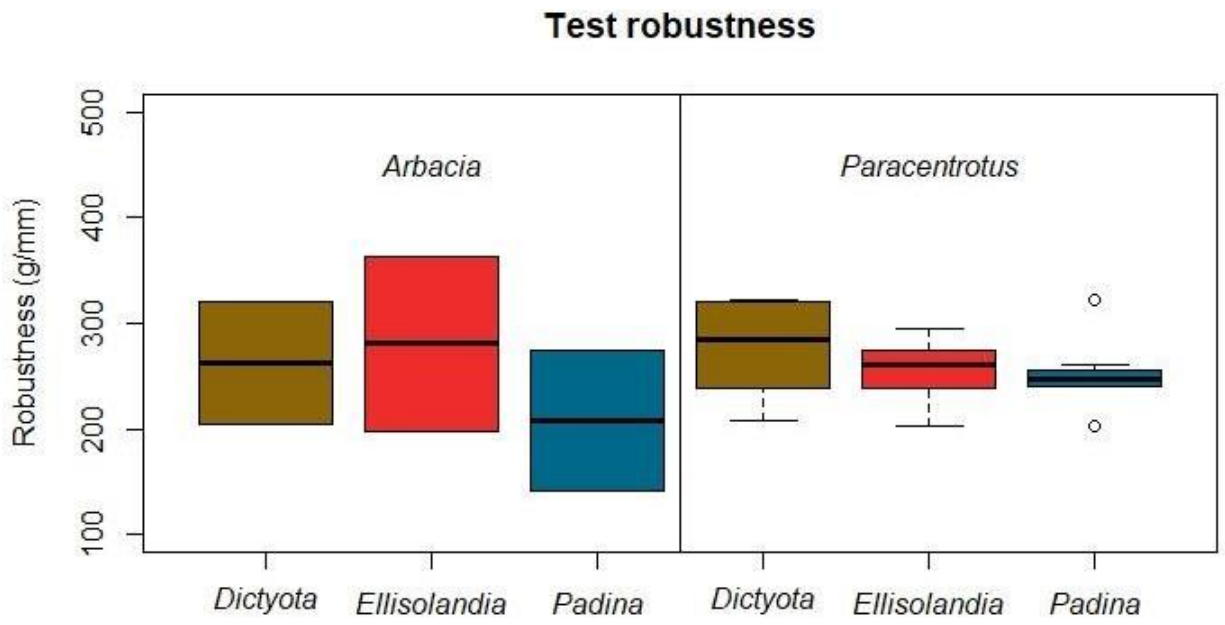


Figure 4.6: Test robustness of *A. lixula* and *P. lividus* for the three different macroalgal diets.

Table 4.3: Anova table for the response variable Test robustness

ROBUSTNESS	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Algal_diet	2	0,004054	0,002027	0,8324	0,4483
Species	1	0,000531	0,000531	0,2181	0,6451
Algal_diet:Species	2	0,003745	0,001872	0,7689	0,4756
Residuals	22	0,053569	0,002435		

4.3.6. Jaw/Test ratio

Regarding the jaw/test ratio (JTR), the values were significantly higher for *A. lixula* (*Tab. 4.4*), especially for specimens fed the diet of *D. dichotoma*, which performed differently between the two sea urchin species (*Fig. 4.7*). In fact, for *P. lividus*, no differences were found between the different algal species.

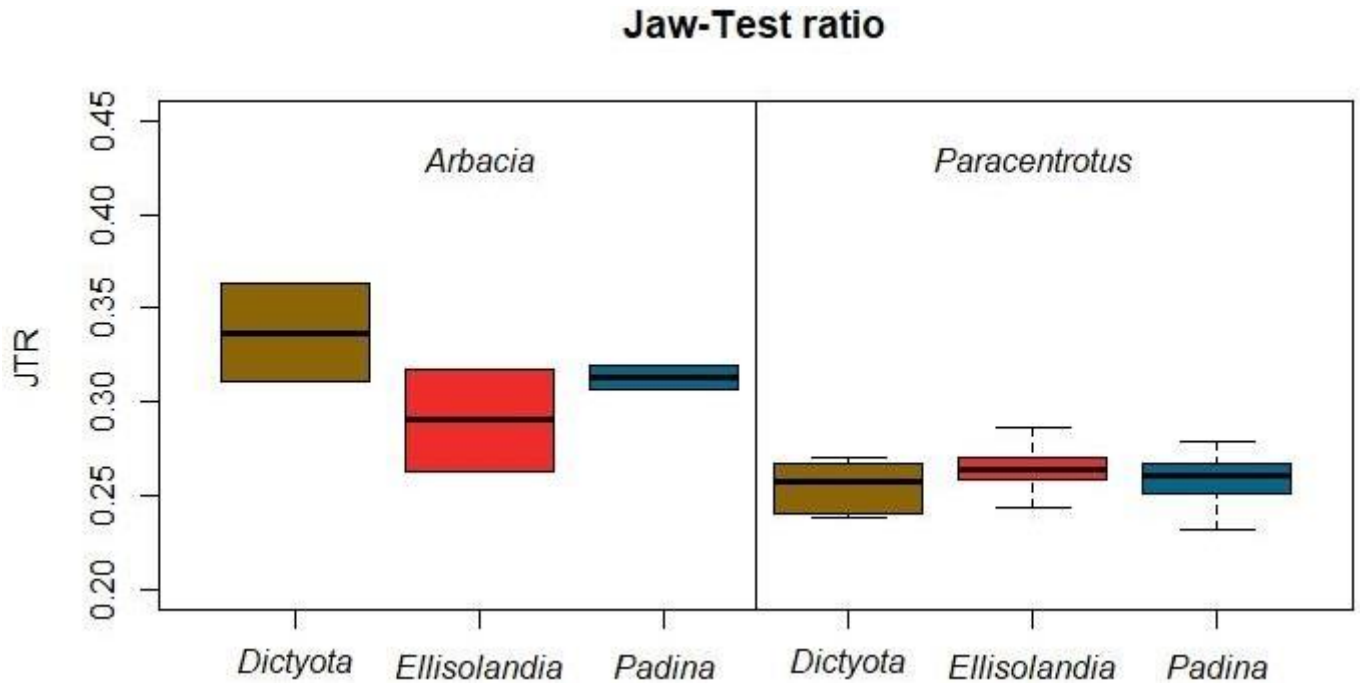


Figure 4.7: Jaw-Test ratio of *A. lixula* and *P. lividus* for the three different macroalgal diets.

Table 4.4: Anova table for the response variable Jaw Test Ratio

JTR	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Algal_diet	2	4.91E-05	2.46E-05	0.1904	0.828	
Species	1	0.00529	0.00529	41.0209	2.39E-06	***
Algal_diet:Species	2	0.000922	0.000461	3.5764	0.04606	.
Residuals	21	0.002708	0.000129			

4.3.7. MDS ordination

The plot in *Fig. 4.8* shows the results of MDS analysis, where all above mentioned parameters were analysed together through a multivariate approach. It is clear that the two species, *Arbacia lixula* and *P. lividus*, are well separated in two groups. Regarding the Algal diets, the plot shows a net division between *E. elongata* and *P. pavonica*, whereas *D. dichotoma* is more dispersed in the plot.

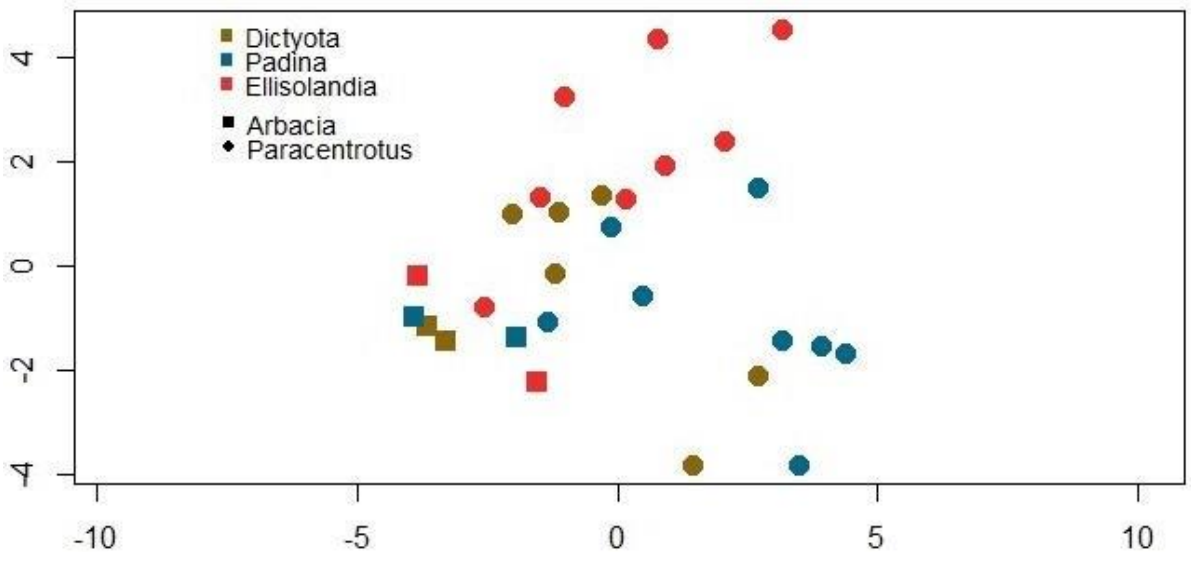


Figure 4.8: MDS plot with all the response variables

The significance of the differences between the two sea urchin species and the effect of the algal diets were highlighted by the PERAMANOVA test in *Tab. 4.5*.

Table 4.5: PERMANOVA table about the effect of Algal diet on the two sea urchin species.

PERMANOVA	Df	SumOfSqs	R2	F	Pr(>F)	
Algal_diet	2	59,018	0,20201	4,5795	0,004	**
Species	1	77,869	0,26653	12,0844	0,001	***
Algal_diet:Species	2	13,504	0,04622	1,0478	0,43	
Residual	22	141,763	0,48523			
Total	27	292,154	1			

PAIRWISE Test: Padina ≠ Ellisolandia p-value 0,039

4.4. Discussion

Sea urchin aquaculture has been extensively investigated in recent years (Grosjean et al., 1998; Privitera et al., 2008; Carboni et al., 2014; Prato et al., 2018; Castilla-Gavilán et al., 2019; DeVries et al., 2019), especially as demand of urchin roe as a food resource has increased and at the same time natural stocks have been drastically reduced (Williamson, 2002; FAO, 2011). Satisfying the human demand for sea urchins is not easy, as echinoculture has some limitations compared to fish aquaculture: the gonads of artificially fed specimens hardly reach an acceptable quality (Shpigel et al., 2005), and also it takes 2-3 years to obtain marketable sized sea urchins (5 cm). Natural stocks of sea urchins, in addition to being threatened by continued human demand, are severely impacted by climate change, particularly ocean acidification (O'Donnell et al., 2009; Asnaghi et al., 2013; Dupont et al., 2013; Kelly et al., 2013). Sea urchins have carbonate structures (skeleton and grazing apparatus) consisting of calcite with a high magnesium content, which is very soluble and particularly sensitive to a decrease in pH. The biomechanical properties of their skeletal structures are of great importance for their individual robustness, as the skeleton provides the means for locomotion, grazing, and protection from predators (Asnaghi et al., 2019). In a future scenario of ocean acidification a decrease of sea urchins' density is expected, due to lower defense from predation, as a direct consequence of pH decrease, and to a reduced availability of calcifying macroalgae, important component of urchins' diet (Asnaghi et al., 2013).

The present study contributed to confirm the importance of calcifying macroalgae in the diet of sea urchins, not in terms of somatic growth but surprisingly in terms of gonadic growth, since they were

the species that ensured a higher Gonado-Somatic Index value and were the most appreciated by both species of sea urchins reared. The macroalgae used were fed fresh to reproduce the natural habits of sea urchins and not to alter their composition in antioxidant compounds and biocarbonate by drying.

Regarding the Gonado-Somatic Index, which as far as echinoculture is concerned is undoubtedly the most important aspect, as the goal is to find the most convenient way to generate sea urchins with large and tasty gonads it was observed that the diet constituted by *E. elongata* performed best in achieving this target, compared to the other macroalgal diets tested.

For both species, we calculated higher GSI values with a calcified macroalgal diet: the GSI was higher for *E. elongata*, intermediate for *P. pavonica*, and much lower for *D. dichotoma*.

However, the GSI was very different between the two species: *A. lixula* showed much lower values for all diets than *P. lividus*. This is due to the fact that the two species have a reproductive cycle with different gonadal maturation periods, *A. lixula* reaches the maximum value in in the late spring and early summer months (Fenaux, 1968; Guettaf et al, 2000) and reaches the minimum values in October-November (Wangensteen et al, 2013), while for *P. lividus*, two distinct peaks have been observed, one in spring and one in late fall (Fenaux, 1968; Crapp and Wills, 1975; Régis, 1979; Byrne, 1990; Pedrotti, 1993; Lozano et al., 1995; Fernandez and Boudouresque, 1997; Guettaf et al., 2000; Chiantore et al., 2008). For this experiment, GSI was measured in the first week of October, so the time of expected maximum gonadal maturation for *P. lividus*, while for *A. lixula* it is the lowest period.

During the three-month rearing experiment, *A. lixula* was found to be the more sensitive species to organic pollution, since many specimens died, probably following a sewage contamination of the incoming seawater, unlike individuals of *P. lividus*, which showed much more resistance to stress. Indeed, it has already been shown that these two species can have different degrees of bioaccumulation of pollutants, with *A. lixula* showing higher sensitivity than *P. lividus* (e.g., Carballeira et al., 2011; 2012).

Higher mortality of *A. lixula* could also reflect a lower plasticity of the species to be maintained in captivity. In fact, *A. lixula* has been shown to be much more selective than *P. lividus*: *A. lixula* specimens fed with *P. pavonica* and even more those fed with *D. dicothoma* have often refused the ration of algae administered, while those fed with *E. elongata* have most often exhausted their dose.

On the contrary, *P. lividus* was much more prone to accept any species of macroalgae and all individuals have always eaten the entire dose administered.

As far as repletion index values, *A. lixula* had similar values for the three diets, slightly higher for the diet of *E. elongata* and lower for that consisting of *D. dichotoma,* instead *P. lividus* had much higher RI values for the diet of *P. pavonica*. The RI values for all diets are much lower for *A. lixula* than for *P. lividus*; this reflects the different algal consumption among the two species during the breeding experiment. As for *P. lividus*, no difference in RI was expected for the different diets, since it consumed almost all the food provided during the three months of the experiment. In contrast, RI values for *P. lividus* were much higher for the diet with *P. pavonica*, so it can be assumed that this macroalgal species remains in the gut for longer, which could mean that it is a macroalgae difficult to digest. To support this hypothses, would be necessary to carry out further analysis of the contents of the gut to get more information about this aspect.

The robustness assessment of the test shows different values than expected, as it had been hypothesized that a diet richer in calcium carbonate (e.g. *E. elongata*) might provide the sea urchin individuals with a thicker and thus stronger theca. In contrast, the robustness of the theca did not seem to be affected by the different macroalgal diets, contrary to what has been shown by Asnaghi et al. (2013), probably because the experiment was conducted with adult specimens that were already developed and therefore invested little energy in somatic growth. Additionally, should be also considered the duration of the experimental period, probably too short to appreciate differences in this morphological aspects of adult specimens.

Finally, no major differences were observed in the Jaw Test Ratio. It defines how developed the grazing apparatus is relative to the size of the specimen. Jaw length (demi-pyramids) changes in relation to test diameter in response to variation in food abundance, so jaw length becomes longer when food is limited (DeVries Maya S. et al., 2019). *A. lixula* is a more selective species, usually feeding on calcareous algae (Privitera et al., 2008, Vielmini at al., 2005), and has larger jaws than *P. lividus.*

In fact, also in this experiment, the values of the jaw/test ratio of *A. lixula* were higher than those of *P. lividus*, mainly in the diet of *D. dichotoma*. This reflects the consumption of algae during the experiment: *A. lixula* rejected much of the food provided, especially the diet of *D. dichotoma*. Thus, the algae used as a diet did not provide enough nutrition to *A. lixula*, which showed a higher JTR,

especially when fed brown algae. In *P. lividus* the JTR results showed no difference between the three diets.

In contrast, Asnaghi et al. (2013) showed that the JTR of *P. lividus* changed with diet, and especially that juveniles of *P. lividus* required a larger grazing apparatus (relative to body size) when fed more calcified algae. They developed a larger grazing apparatus so that they had more strength to chew the calcified thalli of macroalgae such as *E. elongata*.

The present experiment did not show these differences in the jaws of urchins fed the calcified macroalgae diet. The reason could be the same as the robustness results of the test, since the JTR is another parameter that probably needs more time to be appreciated in terms of variability in response to different diets in adult urchins. Therefore, a three-month rearing experiment with adult specimens is not sufficient to study the effect of different diets on jaw length.

4.5. Conclusions

The results obtained from the present study provide useful information on the promotion and development of echinoculture for *P. lividus*, especially in the search for an optimal diet to maximize gonad production. The good performance in terms of gonadal growth observed with the diet of *E. elongata*, a calcified alga rich in calcium carbonate, supports the usefulness of adding biocarbonates in the formulation of new experimental feeds.

This issue could take on further value from a circular economy perspective, since the huge amounts of the waste material from the fishing industry specializing in the production and canning of sea urchin eggs could be used as a source of biocarbonate.

Through the valorization and utilization of these by-products, it would go a long way in promoting sea urchin aquaculture, safeguarding natural stocks. At the same time, however, it would support a circular economy that benefits the development of local Small and Medium Enterprises through better exploitation of the raw product, which can increase the commercial value of the exploited species.

4.6. References

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5. Sea urchin aquaculture promotion in a circular economy perspective

5.1. Introduction

Marine ecosystems represent a huge source of materials of potential biotechnological application, still largely unexplored and which could be widely used for multiple human applications. Among these, the carapaces of crustaceans can certainly be of great interest, as very rich in chitin, from which chitosan is obtained (Madhavan and Nair, 1974; Shahidi and Abuzaytoun, 2005), a polymer used in the medical and pharmaceutical fields. Another very popular biomaterial is collagen, which can be used in numerous applications in the field cosmetic, nutritional, pharmaceutical or biomedical and obtainable from numerous marine species, including sponges (Garrone et al., 1975; 2002; Pallela et al., 2011; Swatschek et al., 2002), jellyfish (Nagai et al., al., 2000; Song et al., 2006), cephalopod molluscs (Kolodziejska et al., 1999; Nagai et al., 2001), fish waste materials, such as skin, bones and fins (Nagai and Suzuki, 2000). Finally, the materials extracted from marine algae, including the Fucoidan, a polysaccharide commonly found in brown algae used as an ingredient in dietary supplement products are under development for many biological and biomedical activities (Aisa et al., 2005; Deux et al., 2002; Fitton, 2011; Park et al., 2011).

In this context, because of their particular richness in biocarbonates and collagen, and from a circular economy and blue growth perspective, sea urchins, a highly valued food resource in Italy and other countries, may be of considerable importance. In fact, sea urchin edible part is limited, as it is reduced to the gonads only, while most of the animal's body mass ends up as waste. In addition, despite so many efforts to initiate aquaculture processes that cover the entire production cycle of this resource (Pedreotti & Fenaux, 1993; Spirlet et al., 1998; McLaughlin & Kelly, 2001; Shpigel et al., 2004; 2005; Woods et al., 2008; Lawrence et al., 2009; Carboni et al., 2012), currently market demand is almost exclusively based on harvesting, and there are numerous cases of depletion of natural stocks due to fishing pressure (Boudouresque and Verlaque, 2007). Aquaculture thus assumes a key role, both in meeting fish market demand and in species conservation.

Global market demand for sea urchins has been estimated by the FAO to be around 60,000- 70,000 tons per year (FAO 2020). Exploited species are numerous and vary according to geographical areas: in Mediterranean regions, the preferred species is the commonly called "purple sea urchin" or "female urchin": *Paracentrotus lividus*.

In recent years, several studies have been conducted regarding optimization of diets for sea urchin aquaculture, with the aim of providing all the necessary nutrients in a single feed. Some studies have shown that artificial feeds can be nutritionally more complete than natural foods and are therefore critical for maximizing gonad production (Lawrence et al., 2011; Prato et al., 2016).

Therefore, it was decided to formulate a new artificial feed, as low-impact and economical as possible, using as an enrichment component (around 25 percent of the total) biocarbonates from the grinding of *P. lividus* thecae discarded by some commercial activities (e.g., small seafood industries or restaurants) that use its gonads.

Since the powder from the grinding of *P. lividus* thecae does not only contain carbonate, but is also rich in other nutrients and antioxidant compounds, it can ensure the reared sea urchin individuals better performance than an common commercial feed (containing $CaCO_3$ of inorganic origin) in terms of somatic and gonadal growth.

This would greatly stimulate echinoculture and create, through the valorization and utilization of these by-products, a virtuous system of circular economy that can promote the development of local Small and Medium Enterprises, through better exploitation of the raw product, which can increase the commercial value of the exploited species and encourage the conservation of natural stocks.

The present study is composed of two main parts: the formuation of the experimental diet (5.2) and the feeding trial on *P. lividus* juveniles (5.3).

5.2. Diet formulation

The recipes to produce the experimental feeds were formulated based on previous experience, in particular following Prato et al. (2016): the different vegetable feeds tested produced good results (see also Chiantore et al., 2016), particularly with regard to gonadal growth and gonad quality, aspect of considerable importance especially at the commercial level. Compared with the recipe used in these works (diet provided by the Scottish Association for Marine Science_SAMS, Oban, UK and finalized in the framework of the FP7 project-RESURCH- FP7- SME-2013-1: GA # 606042), the carbonate component (inorganic $CaCO_3$), was replaced with a powder obtained from grinding and drying the urchin tests. This powder, mostly made by biocarbonates, was provided by the University

of Milan, as part of the BRITEs (Byproduct Recycling: Innovative TEchnology from the Sea) project, funded by the PRIN 2017 (Projects of Significant National Interest, MIUR) program. The BRITEs project, which involves the Universities of Padua, Milan, and Genoa, studies the exploitation of sea urchin waste from the seafood catering/industry to generate valuable by-products.

Furthermore, instead of the macroalgal mix used by Prato et al. (2016), it was decided to use only fresh *Ulva* spp., in order to reduce production time and costs, directly collected from the Gulf of Genoa and later freeze-dried. The feed was produced by the laboratory of the Department of Pharmacy, University of Genoa. In addition to the standard formulation (BRITEs), it was decided to produce another one with twice the amount of fish gelatin (BRITEs 2), to see if more of this ingredient could confer to the feed a higher stability in seawater (*Tab. 5.1*).

Tabele 5.1: Formulation recipes with the percentages of the respective ingredients. SAMS BIND4 is the reference recipe, perfomed by Prato et al. (2016).

	SAMS BIND4	BRITES	BRITES 2
Ingredients	**%**	**%**	**%**
Fish gelatin	**7,5**	**10**	**20**
Wheat Gluten	**10**		
Corn gluten	**15**	**25**	**15**
Soybean meal 48	**2,5**		
Rapeseed meal	**2,5**	**10**	**10**
Wheat meal	**5**		
Pea starch	**5**	**3,5**	**3,5**
Linseed oil	**2,5**	**2,5**	**2,5**
Vit & Min Premix PV01	**1,5**	**1,5**	**1,5**
Soy lecithin - Powder	**2**	**2**	**2**
Binder (zeolite)	**5**		
Macroalgae mix	**24**	**25**	**25**
Antioxidant powder (Paramega)	**0,2**	**0,5**	**0,5**
Monocalcium phosphate	**2,3**		
Ca carbonate	**15**		
Urchin powder		**20**	**20**
Total	**100**	**100**	**100**

The components that make up sea urchin powder obtained from shredded thecae were analyzed. In addition to calcium carbonate, which is in the form of ash, proteins, lipids and carbohydrates were also quantified (Tab. 5.2).

Table 5.2: Percentage compisition of the compounds that make up sea urchin powder obtained by grinding the tests. Data provided from University of Milan laboratory (Prof. Michela Sugni and Dott. Stefania Marzorati).

SAMPLE	Umidity	Mean dry matter	Ashes	Protein (Nx6,25)	Raw lipids	Raw fiber	inazotate extractives (carbohydrates)
	%	%	% dry matter	% dry matter	% dry matter	% dry matter	% dry matter
Urchin powder	0,95	99,05	88,11	5,52	1,71	0,89	3,78

Before using the feeds in sea urchin trials, an experiment was conducted to evaluate the dissolution of the feed in seawater, and consequently its profitability for sea urchins.

5.2.1 Materials and Methods of stability tests

This experiment lasted 72 h, with 5 sampling times at 2, 6, 24, 48, 72 hours, plus one initial dry weight measurement of all feed pellets. 30 portions of feed were randomly chosen, 15 with 10% of Fish gelatine (BRITEs) and 15 with with 20% of Fish gelatine (BRITEs2). Each portion in the shape of a 1g cube (same form in which they were produced), was immersed in a plastic tub filled with filtered seawater (*Fig. 5.1* and *5.2*). After each sampling time, the feed remaining in three replicate tubs per each feed was removed from the water and stored in the freezer at -18°C. Once all samples were removed from the water, they were allowed to dry in an oven at 60°C for 48h to obtain the dry weight. At this point, the weight loss of the two feeds was calculated (the amount that dissolved in seawater). The experiment was conducted at a water temperature of 18°C.

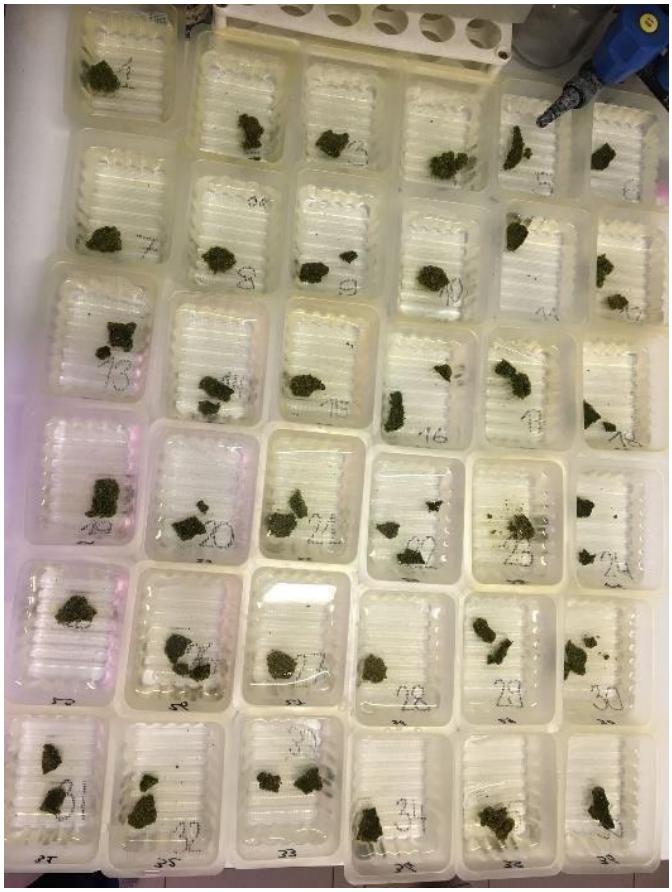


Figure 5.1: Setup of feed stability experiment in seawater.

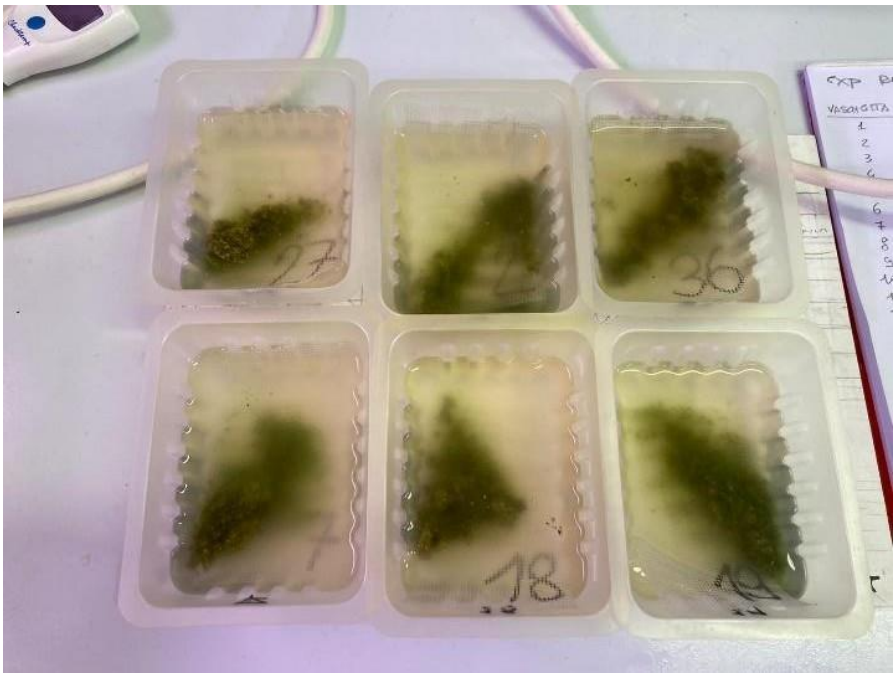


Figure 5.2: Status of the feed after 48h in seawater.

5.2.2 Results on stability of first feed formulations

Both feeds did not perform optimally in the long run; in fact, already after 24h they lost almost 50% of their initial weight. Specifically, the feed with double gelatin content (20% of the total composition) showed better performance in the first hours of water immersion, although still not acceptable to use this formulation to feed urchins (*Fig. 5.3*). This experiment, however, provided us with information to be able to improve, especially in terms of stability, the feed formulation.

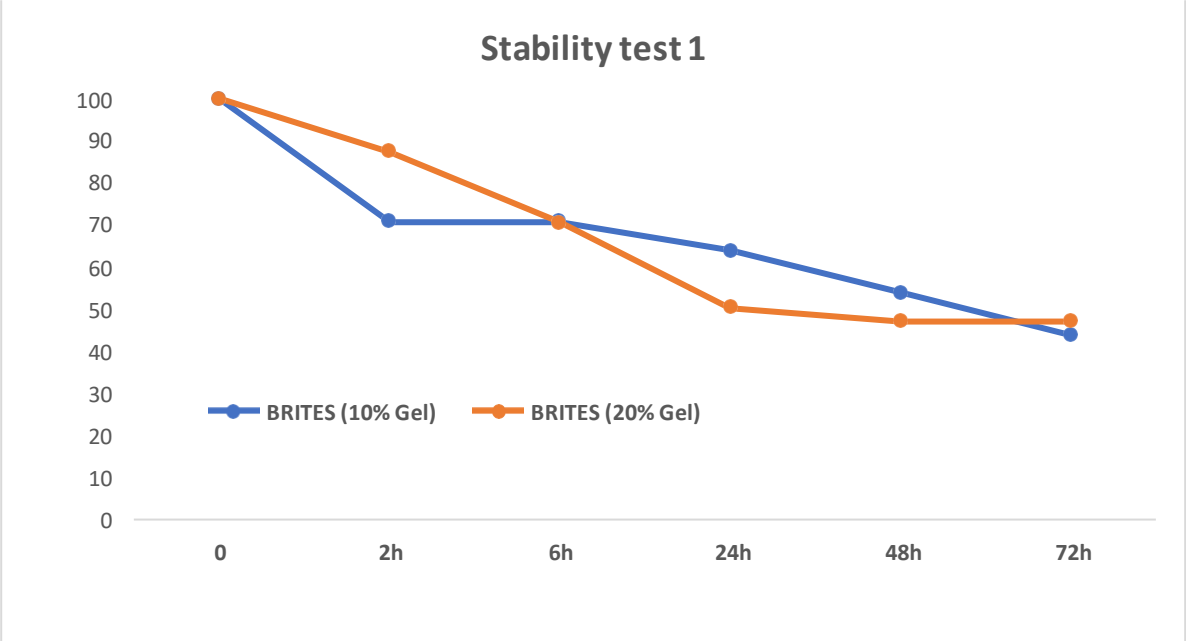


Figure 5.3: Feed stability test in seawater.

5.2.3 Formulation optimization

Following the above results, it was decided to change the formulation of the feed reducing the number of ingredients and increasing the amount of fish gelatin (from 20 to 25%). Sodium alginate was also added to increase the density of the formulation, thus making it more compact and less water soluble. These characteristics not only could provide greater stability to the feed but also make it significantly more palatable to urchins. A harder, more compact feed is definitely more

similar to the food sea urchins are used to in nature, they in fact take up seaweed by grazing it off the surface of rocks.

Therefore, in this second step of the feed formulation testing, two different types of feed were produced (again produced by the laboratory of the Department of Pharmacy, University of Genoa), differing only in terms of the $CaCO_3$ component: one with sea urchin powder (FSU-BRITEs) and one with inorganic calcium carbonate (FSC-BRITEs, CTRL), as a control to investigate the actual benefits of carbonate of organic origin.

Table 5.3: new recipes, obtained by optimizing previous ones.

	FSC (Brites CTRL)	**FSU (Brites)**
Ingredients	**%**	**%**
Fish gelatin	**25**	**25**
Corn gluten	**15**	**15**
Sodium alginate	**5**	**5**
Linseed oil	**2.5**	**2.5**
Soy lecithin - Powder	**2**	**2**
Macroalgae mix	**25**	**25**
Antioxidant powder(Oxivia)	**0.5**	**0.5**
Ca carbonate	**25**	**0**
Urchin powder	**0**	**25**
Total	**100**	**100**

The same experiment that had been conducted on the previous formulations was re-run to evaluate whether the new feed compositions had better stability in seawater (*Fig. 5.4*).

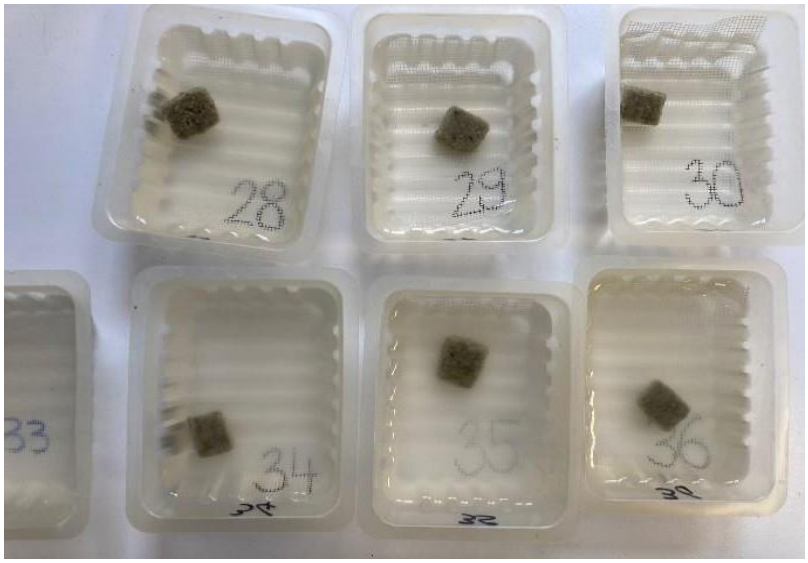


Figure 5.4: status of the feed after 48h in seawater for the new formulation.

5.2.4 Results on stability of second feed formulations and Conclusions:

The results were definitely satisfactory, with both formulations performing optimally in seawater. They both turned out more stable than those in the previous experiment. In fact, weight loss was significantly reduced, both in the short and in the long term (less than 20% after three days). In contrast, between the two formulations (one with organic and one with inorganic carbonate), no particular differences were observed (*Fig. 5.5*).

At this point, both formulations were found to be suitable for direct testing on individuals of *Paracentrotus lividus*.

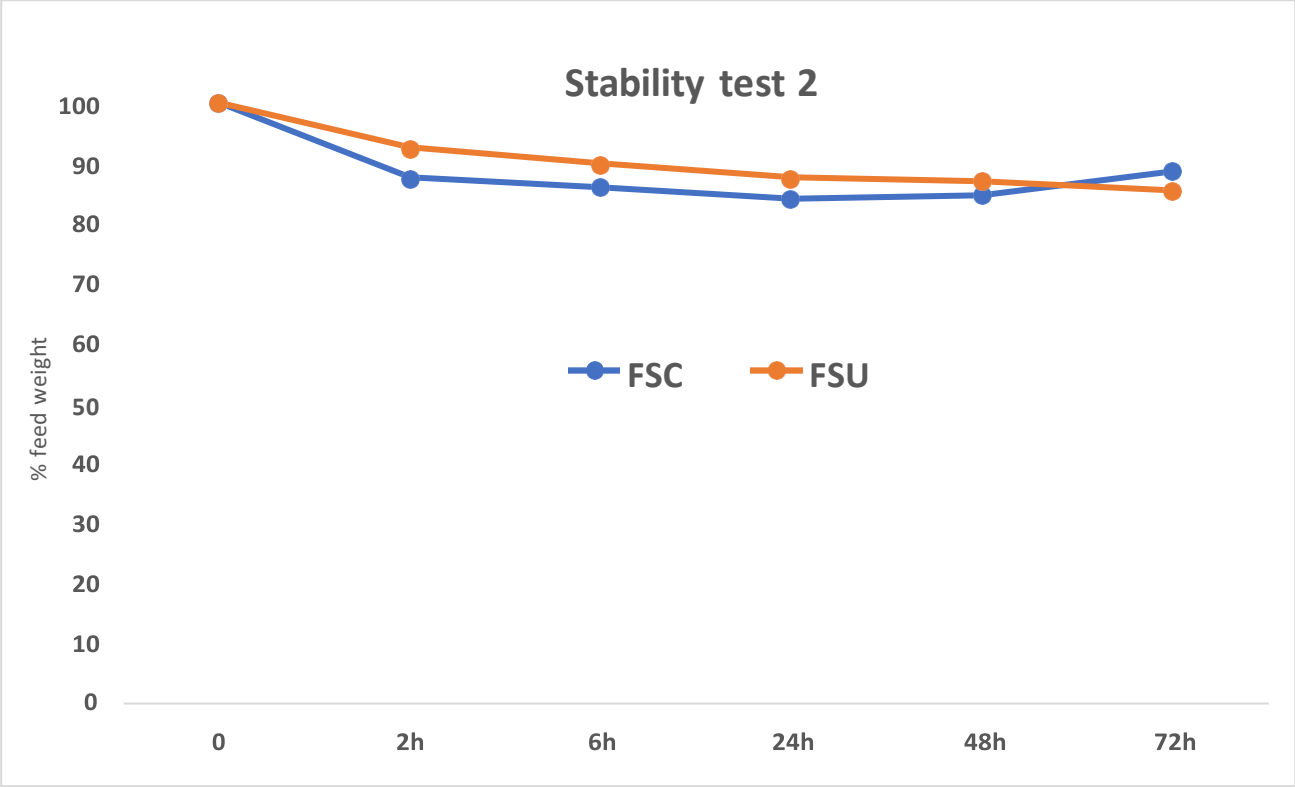


Figure 5.5: Results of feed stability test in seawater for the new recipes.

5.2.5 Antioxidant activity of feeds

In addition, a test ABTS (2,2'-Azinobis-(3-Ethylbenzthiazolin-6-Sulfonic Acid)) was conducted by University of Milan laboratory (Dr. Stefania Marzorati) to verify the antioxidant activities of the two different formulations. The ABTS assay is considered one of the most sensitive techniques to identify antioxidant activity, because the response of antioxidants involves faster reaction kinetics (Chanput et al., 2016).

The results of the ABTS test showed differences in antioxidant activity between the formulation with $CaCO_3$ and with the Biocarbonate from sea urchin powder. The EC50 value, which is the sample concentration required to break down 50% of the ABTS radical, was compared. The lower the EC50 value, the better the antioxidant activity.

The EC50 values related to the feed sample supplemented with inorganic carbonate (corresponding to the gray dots in the figure) are: 0.0062, 0.0061 and 0.0063 mg/ml (three replicates). The EC50 values related to the feed sample additivated with BIOcarbonate (corresponding to the yellow/orange dots in the figure) are: 0.0046, 0.0045 and 0.0044 mg/ml (three replicates).

The EC50 values for the formulation containing BIOcarbonate are lower, so its antioxidant activity is higher than the formulation with inorganic carbonate.

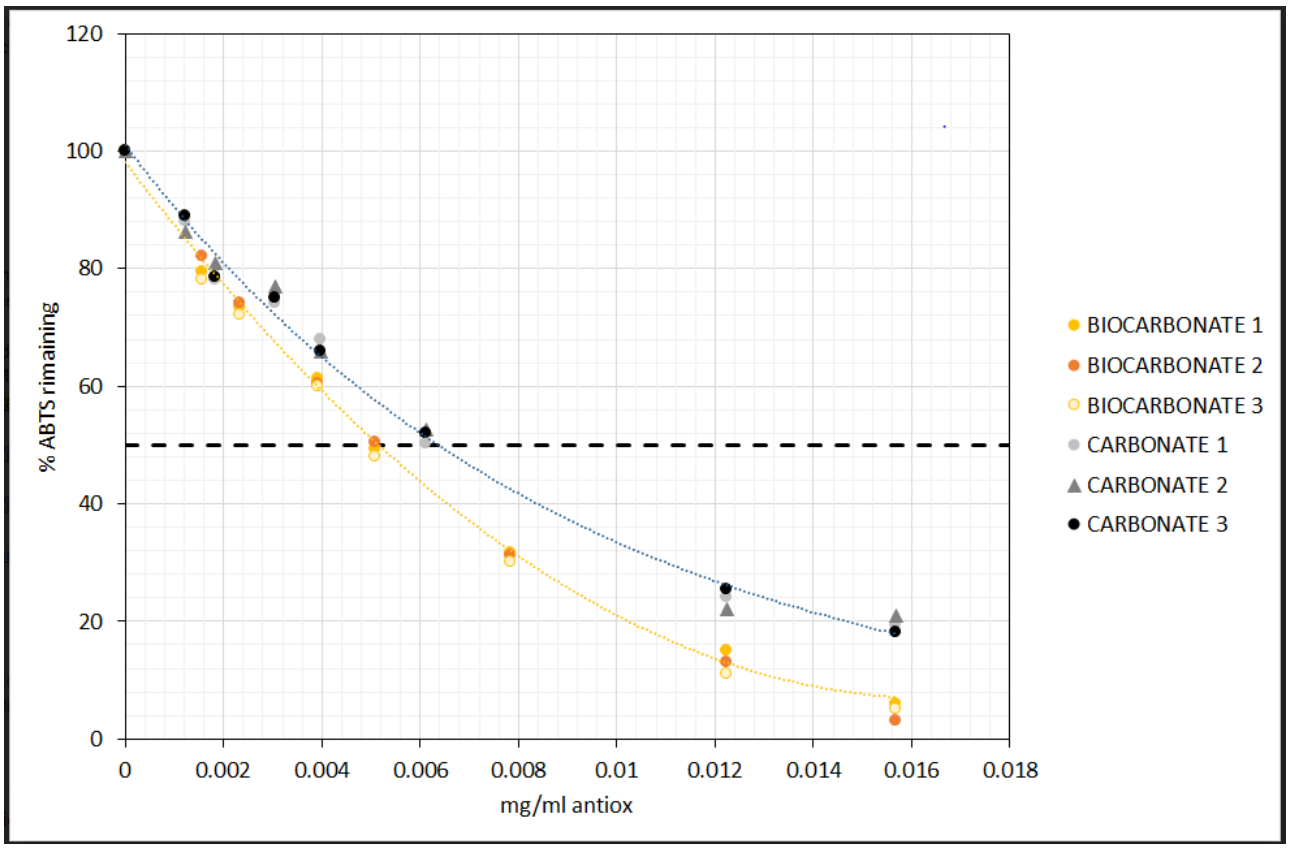


Figure 5.6: Antioxidant activity of the two different experimental formulations, "BIOCARBONATE" = FSU and "CARBONATE" = FSC.

5.3 Feeding trials

To test how efficient these new experimental fedd formulations were in terms of somatic and gonadal growth both were administered to juvenile specimens (class3 and class4, around 30 mm in diameter) of *Paracentrotus lividus* for two months in two different seasons in 2022, summer and fall. As a control, it was decided to use a diet consisting exclusively of fresh macroalgae, 50% *Ulva* sp. and 50% *Ellisolandia elongata*, in order to simulate the normal diet of *P. lividus* in the wild.

5.3.1 Material and methods

5.3.1.1 Urchin collection and experimental conditions

P. lividus specimens were collected for both seasons by SCUBA divers at depths of 3-5 m in the Gulf of Genoa (Ligurian Sea, Lat/Long 44°23.419 "N, 8°58.796 "E). A total of 82 specimens were collected in May (diameter 3.00 ± 0.91 cm and total weight 9.14 ± 2.45 g; mean ± SD), and 82 specimens were collected in October (diameter 2.66 ± 0.32 cm and total weight 9.95 ± 2.80 g; mean ± SD) and subsequently transferred to the CNR-IBF laboratory (Camogli, Genoa). A random sample of 10 *P. lividus* (i.e., WS) was selected and dissected to define baseline conditions. Laboratory setup consisted of 12 tanks of 3.7 litres each, fed individually by a constant flow of seawater, in order to have independent replicates. The seawater was taken directly from the sea, with a water turnover of 0.5 l/minute.

The remaining 72 wild sea urchins were starved for 2 weeks; thereafter they were divided into groups of 6 urchins and randomly assigned to 12 experimental tanks.

5.3.1.2 Experimental diets

Sea urchins were fed with three different diets (Tab. 5.4): FSC diet, FSU diet and a diet of fresh macroalgae (ALGA), in order to have four replicas for each diet. FSC and FSU were pelletized diet provided by the laboratory of the Department of Pharmacy, University of Genoa. The pellets had a cubic shape weighing about 1g each.

Table 5.4: Composition of the sea urchin feed formulation (dry weight %)

	FSC (Brites CTRL)	**FSU (Brites)**
Ingredients	**%**	**%**
Fish gelatin	**25**	**25**
Corn gluten	**15**	**15**
Sodium alginate	**5**	**5**
Linseed oil	**2,5**	**2,5**
Soy lecithin - Powder	**2**	**2**
Macroalgae mix	**25**	**25**
Antioxidant powder(Oxivia)	**0,5**	**0,5**
Ca carbonate	**25**	**0**
Urchin powder	**0**	**25**
Total	**100**	**100**

5.3.1.3 Feeding experiment

Sea urchins were fed with the three different treatments twice a week at a rate of approximately 2.5% of body weight day^{-1} for the specimen fed with sperimental feed (FSU, FSC) and approximately 5% for those fed with fresh macroalgae. The experiment lasted for 8 weeks.

Tanks were cleaned before each feeding event by siphoning off feed residues and faeces.

The test diameter and weight of each specimen were measured monthly during the experiment. The sea urchins were left to dry for a few minutes and then weighed, while the diameter was measured without spines.

5.3.1.4 Total lipid content

Total lipids, in experimental feed and reared *P. lividus* individuals, were extracted and calculated according to the Bligh & Dyer (1956), Marsh & Weinstein, (1966) methods. Total lipid concentration was analyzed for *P. lividus* gonads, for the two experimental diets (FSC and FSU) and for the macroalgal diet (ALGA).

5.3.1.5 Total protein content

Total protein content, in experimental feed and reared *P. lividus* individuals, was obtained according to the "Hartree 1972" colorimetric method, a modification of the "Lowry method," which provides a linear photometric response (Hartree, 1972). Total protein concentration was analyzed for *P. lividus* gonads, for the two experimental diets (FSC and FSU) and for the macroalgal diet (ALGA).

5.3.1.6 Statistical analysis

All the measured parameters (diameter, weight, GSI, protein and lipid contents) are reported as mean and standard error (SE). In order to evaluate the effect of the tested diets (FSC, FSU, ALGA), periods (summer, autumn) and their interaction, on all the response variables considered, i.e. GSI, lipid content and protein content of sea urchin gonads, a crossed two-way ANOVA design was applied, using "diet" and "period" as fixed crossed factors. Additionally, differences in protein and

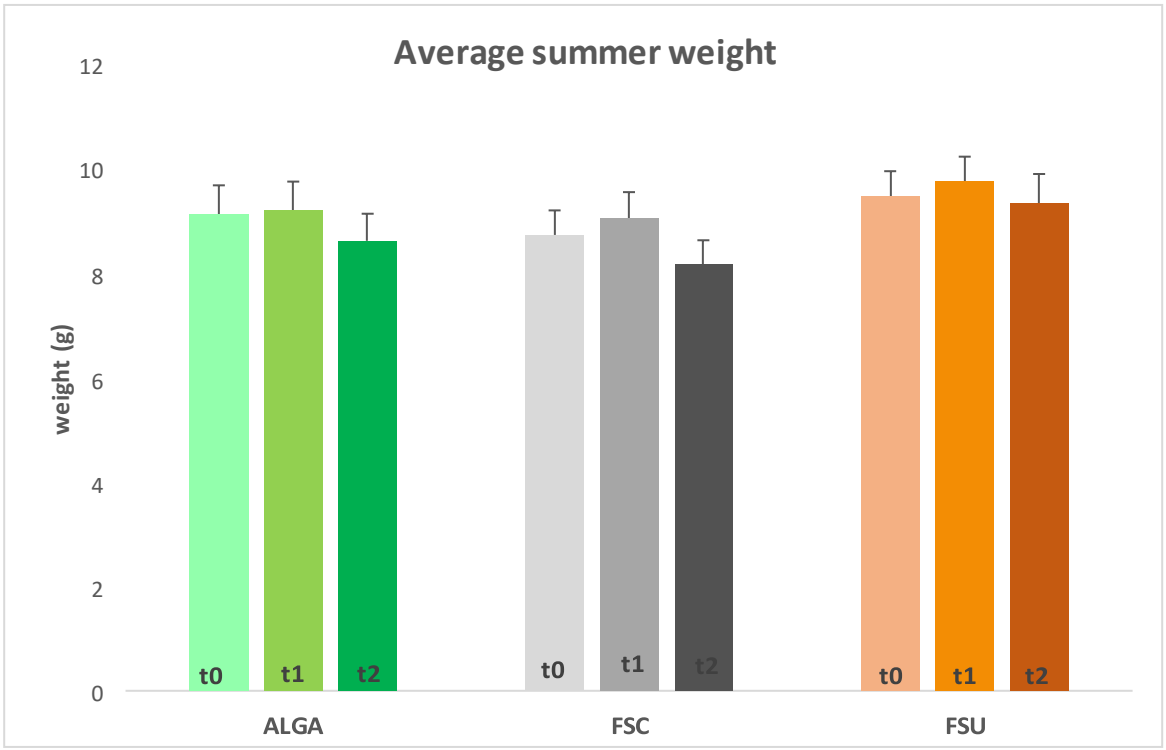
lipid content of the provided diets (FSC, FSU, ALGA 1, ALGA 2, sampled in two different times to take into account natural variability) were tested through a one-way ANOVA using the factor “diet” as fixed. For all the ANOVA analyses, the normality and homogeneity of the variances were verified for all response variables considered using the Kolmogorov-Smirnov test and the Bartlett tests, respectively. All statistical analyses were performed using the R software (R Core Team 2021).

5.3.2 Results

5.3.2.1 Somatic growth: diameter (mm), weight (g)

During the summer period, there was no increase in weight or diameter considering the three diets tested. Instead, a somatic increase in both weight and diameter was observed in the experiment conducted in the autumnal period. Regarding average weight, individuals fed the "ALGA" diet increased from 7.53 ± 0.38 g (mean ± SE) to 9.17±0.49 g (mean ± SE) at the end of the experiment (+ 22%), those fed "FSC" increased from 7.64 ± 0.65 g (mean ± SE) to 8.87 ± 0.78 g (mean ± SE; +26%), and those fed "FSU" increased from 8.67 ± 0.64 g (mean ± SE) to 10.50 ± 0.70 g (mean ± SE; +21%), (*Fig. 5.7*).

While in terms of diameter, individuals fed the "ALGA" diet increased from 2.59 ± 0.05 cm (mean ± SE) to 2.73 ± 0.05 cm (mean ± SE) at the end of the experiment (+6%), those fed "FSC" increased from 2.64 ± 0.07 cm (mean ± SE) to 2.76 ± 0.08 cm (mean ± SE; +4%), those fed "FSU" increased from 2.76 ± 0.07 (mean ± SE) to 2.84 ± 0.07 (mean ± SE; +3%), (*Fig. 5.8*).



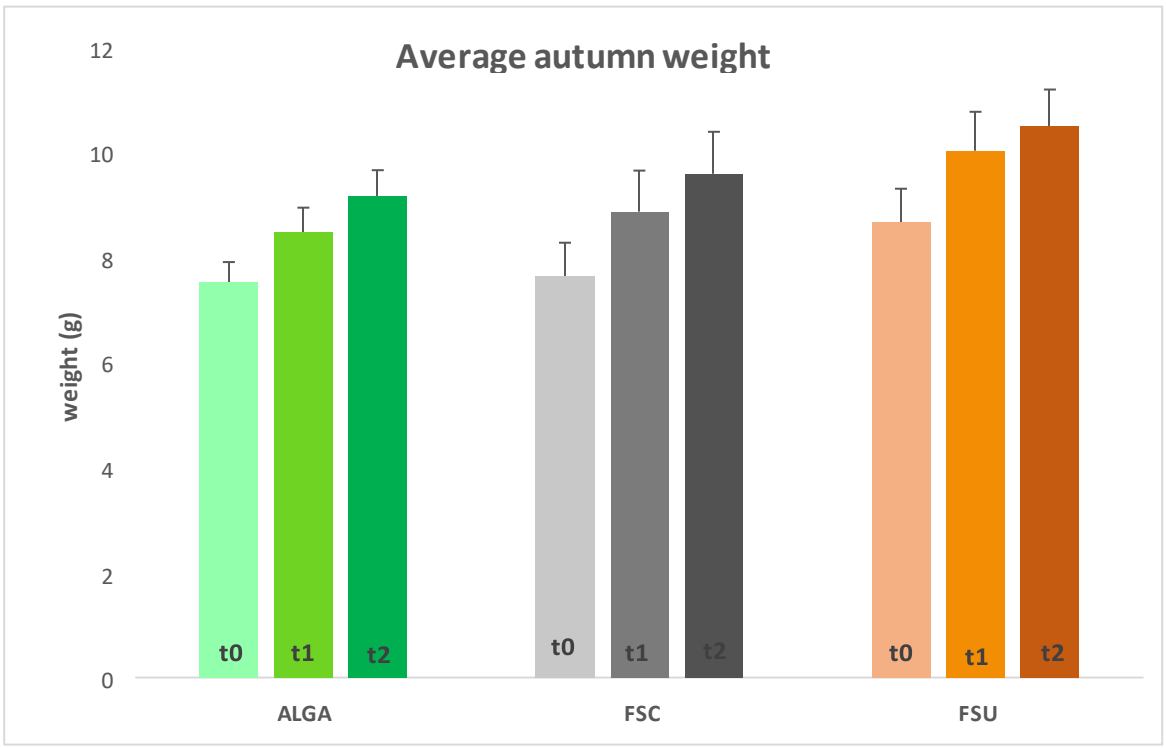


Figure 5.7: Average weight (g) of *P. lividus* during the experiment, measurements taken at intervals of about two weeks

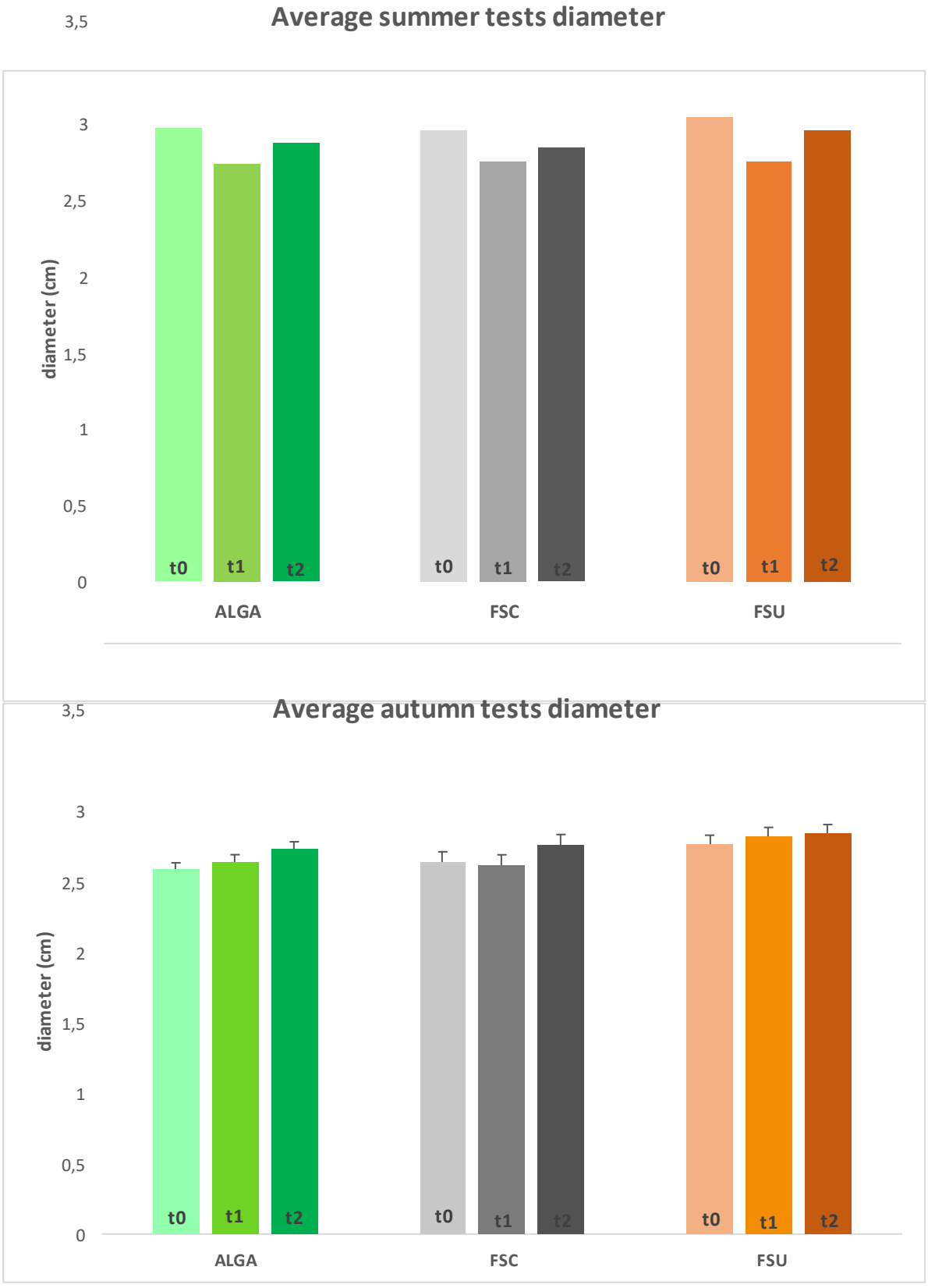


Figure 5.8: Average test diameter (cm) of *P. lividus* during the experiment, measurements taken at intervals of about two weeks

5.3.2.2 Gonadic growth: GSI (%)

In both seasons, the prepared diets (FSC and FSU) produced a significant increase in gonado-somatic index compared to the natural diet (ALGA) (*Tab. 5.5*), in Summer ALGA: 1.90 ± 0.27, mean ± SE; FSC: 4.48 ± 0.48 mean ± SE; FSU: 4.70 ± 0.38 mean ± SE; in Autumn ALGA: 0.93 ± 0.24, mean ± SE; FSC: 1.85 ± 1.70, mean ± SE; FSU: 2.27 ± 0.26 mean ± SE (*Fig. 5.9*). While no significant differences were observed between them, the individuals fed the sea urchin powder enriched (FSU) diet showed higher GSI. Regarding seasonality, a significant difference was observed in the gonado-somatic index value: it was about twice as high in the summer period as in the fall period for all three diets (*Tab. 5.5*).

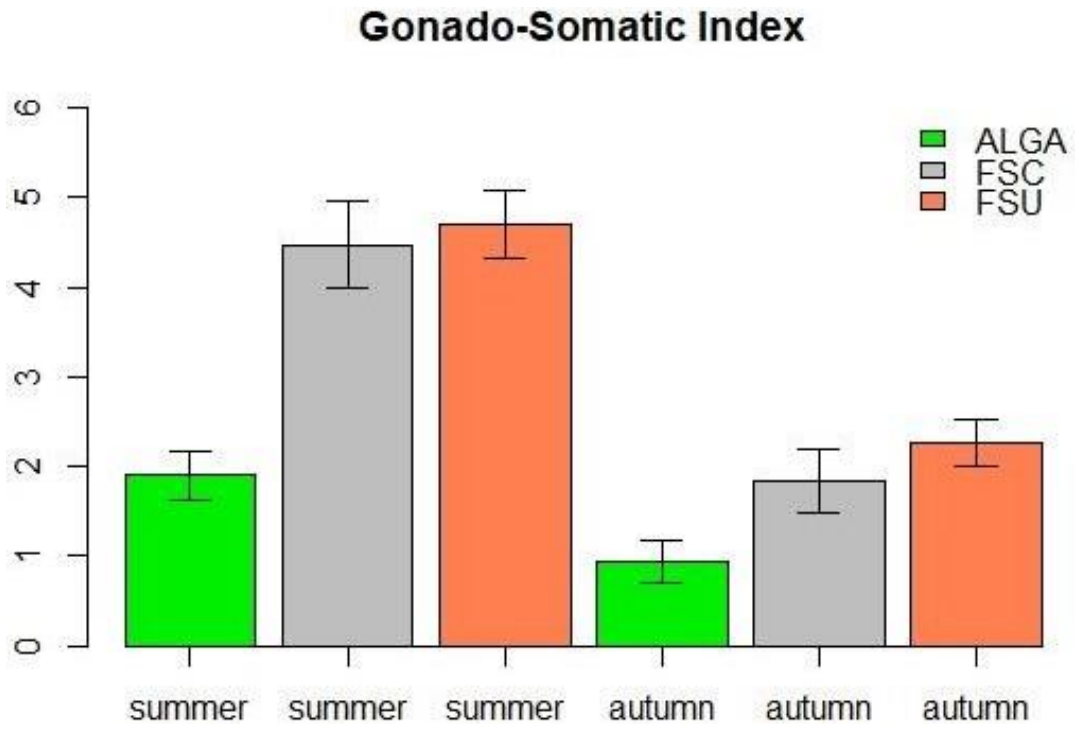


Figure 5.9: Average values with standard error of the GONADO-SOMATIC INDEX (GSI) for the 3 diets in the two breeding periods.

Table 5.5: Anova table for the response variable Gonado Somatic Index (GSI)

GSI (sqrt transf)	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
period	1	11.991	11,9911	45,7478	4,32E-10	***
diet	2	12.873	6,4363	24,5554	9,41E-10	***
period:diet	2	0.947	0,4735	1,8065	0,1684	
Residuals	128	33.550	0,2621			

Tukey post hoc Test: FSC ≠ ALGA p.value 0.03, FSU ≠ ALGA p.value 0.0004.

5.3.2.3 Total lipid concentration in diets

The experimental diets displayed the following lipid concentration: FSC: 81.92 ± 3.43 µg/mg, mean ± SE and FSU: 91.34 ± 9.59 µg/mg, mean ± SE, significantly higher than that of the algal diet, ALGA1: 47.66 ± 2.3 µg/mg, mean ± SE and ALGA2: 47.51 ± 3.06 µg/mg, mean ± SE (*Fig. 5.10*). Among them, lipid concentration did not differ significantly (*Tab. 5.6*).

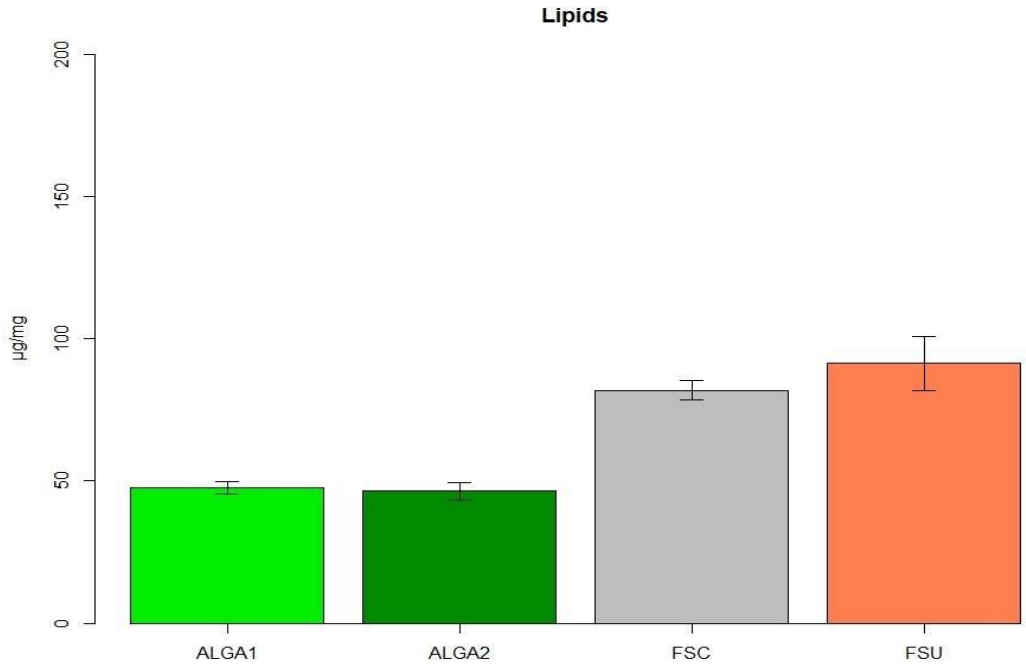


Figure 5.10: Average values of total lipid concentration in diets. Macroalgae data are reported twice because the analyses were performed on two different samples, collected at different periods of the year.

Table 5.6: Anova table for the response variable total lipid concentration in feed

LIPID (µg_mg)	Sum Sq	Df	F value	Pr(>F)
diets	4827.7 3	18,117	**0,000632**	***
Residuals	710.6 8			

Tukey post hoc Test: FSC ≠ ALGA1 p.value 0,009; FSU ≠ ALGA1 p.value 0,002; FSC ≠ ALGA2 p.value0,007; FSU ≠ ALGA2 p.value 0,001

5.3.2.4 Total lipid concentration in gonads

The lipid concentration in the gonads in the summer period was for ALGA: 179.04 ± 18.71 µg/mg, mean ± SE, for FSC diet: 284.82 ± 34.63 µg/mg, mean ± SE and for FSU diet: 363.10 ± 52.46 µg/mg, mean ± SE. While in autumn period for the algal diet: 279.11 ± 31.20 µg/mg, mean ± SE, for FSC diet: 336.68 ± 21.73 µg/mg, mean ± SE and for FSU diet: 254.28 ± 15.10 µg/mg, mean ± SE (*Fig.5.11*).

The macroalgae diet showed significant differences in the two experimental periods, particularly with higher values in autumn. ALGA and FSU formulated diet were significantly different in summer. Finally, in the FSU diet, the concentration of total lipids was significantly higher in summer than in autumn (*Tab. 5.7*).

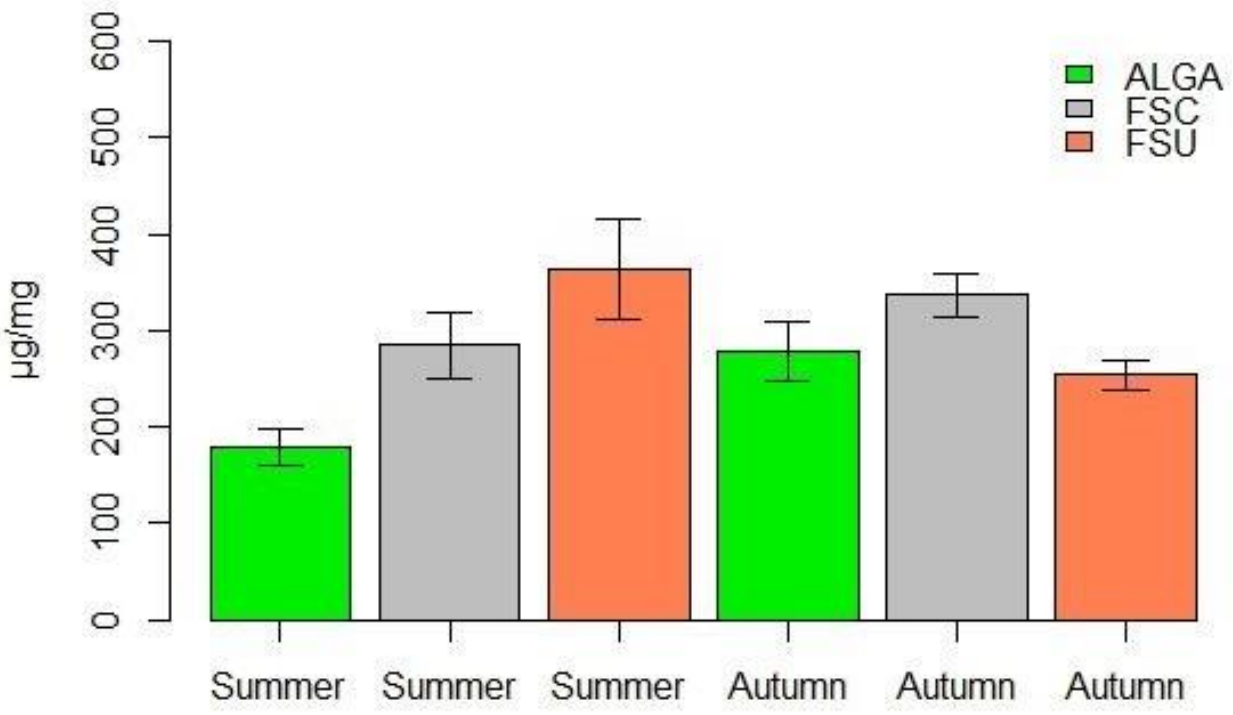


Figure 5.11: Average values of total lipid concentration in *P. lividus* gonads.

Table 4.7: Anova table for the response variable total lipid concentration in gonads.

LIPID (µg_mg)	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
DIET	2	82631	41316	3,668	**0,02965**	*
Period	1	4728	4728	0,4198	0,518792	
DIET:Period	2	155320	77660	6,8947	**0,001679**	**
Residuals	85	957411	11264			

Tukey post hoc Test: in Summer FSU ≠ ALGA p.value 0.005; in Autumn FSU ≠ FSC p.value 0.03.

5.3.2.5 Total protein concentration in diets

The experimental diets have a protein concentration of FSC: 66.82 ± 2.98 µg/mg, mean ± SE and FSU: 91.00 ± 6.73 µg/mg, mean ± SE, significantly higher than that of the algal diet, ALGA1: 28.90 ±

2.44 µg/mg, mean ± SE and ALGA2: 30.43 ± 1.61 µg/mg, mean ± SE (*Fig. 5.12*). Among them, the protein content is significantly higher in diets enriched with sea urchin powder (FSU; *Tab. 5.8*).

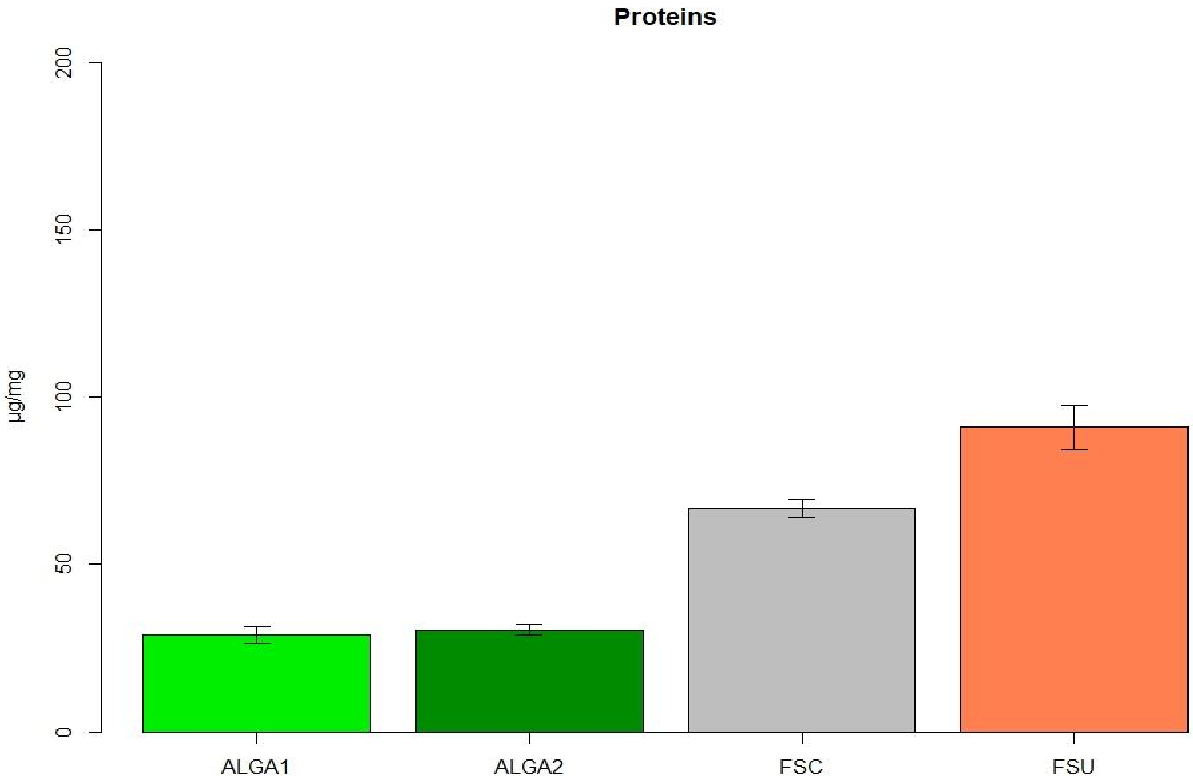


Figura 5.12: Average values of total protein concentration in diets. Macroalgae data are reported twice because the analyses were performed on two different samples collected at different periods of the year.

Table 5.8: Anova table for the response variable total protein concentration in feed.

PROTEIN (µg_mg)	Sum Sq	Df	F value	Pr(>F)
DIET	8157.8 3	59,383	**8,25E-06**	***
Residuals	366.3 8			

Tukey post hoc Test: FSC ≠ ALGA1 p.value <0.001, FSU ≠ ALGA1 p.value <0.001, FSC ≠ ALGA2 p.value <0.001, FSU ≠ ALGA2 p.value <0.001, FSU ≠ FSC p.value 0.01.

5.3.2.6 Total protein content in gonads

The protein concentration in the gonads in the summer period was for ALGA: 199.66 ± 19.36 µg/mg, mean ± SE, for FSC diet: 190.02 ± 11.59 µg/mg, mean ± SE and for FSU diet 167.63 ± 22.85 µg/mg, mean ± SE. While in autumn period for ALGA: 263.44 ± 21.41 µg/mg, mean ± SE, for FSC diet: 201.42 ± 15.56 µg/mg, mean ± SE and for FSU diet 223. ± 10.43 µg/mg, mean ± SE (*Fig. 5.13*).

No significant difference was observed for gonad protein content in the three different diets for both the summer and autumn rearing periods. On the contrary, a significant difference in protein content was found between the two periods, specifically the protein concentration (µg/mg) is higher in the autumn period (*Tab. 5.9*).

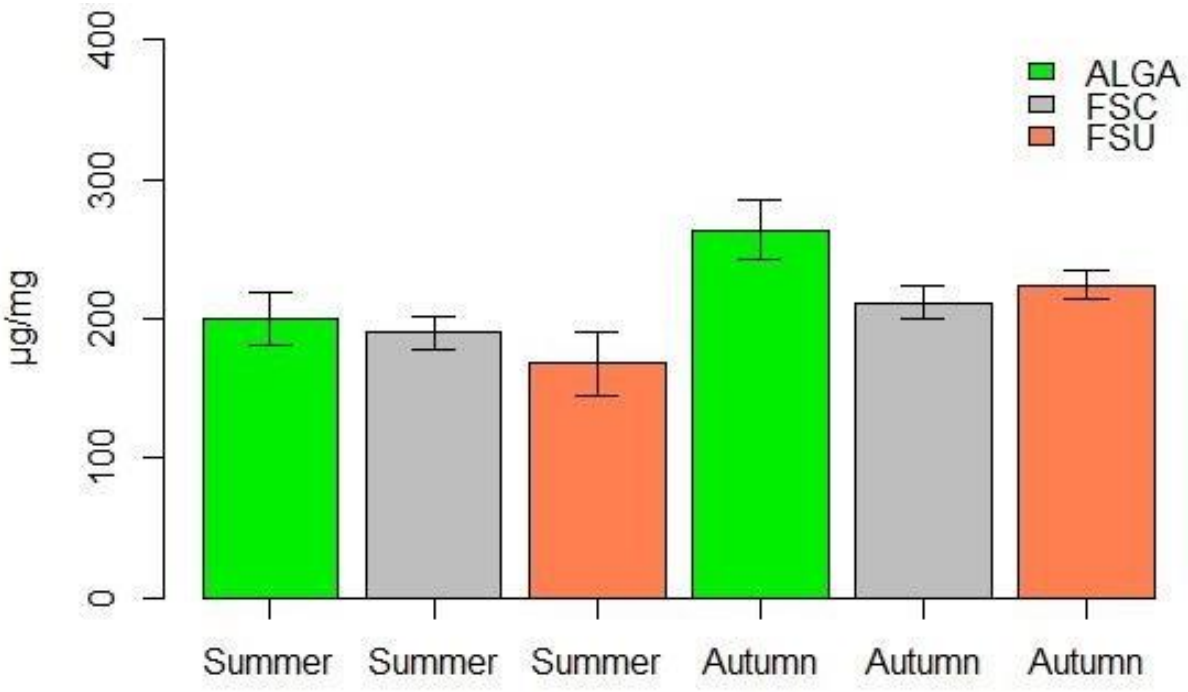


Figure 5.13: Average values of total protein concentration in *P. lividus* gonads.

Tabel 5.9 : Anova table for the response variable total protein concentration in gonads.

PROTEIN (µg_mg)	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
DIET	2	22254	11127	2,788	0,06711	
Period	1	44375	44375	11,119	**0,00126**	**
DIET:Period	2	7093	3547	0,889	0,41494	
Residuals	86	343221	3991			

5.3.3 Discussion

Both experimental feed formulations, either the one containing $CaCO_3$ or the one with the carbonate of organic origin, were found to be palatable and effective for aquaculture of the sea urchin *Paracentrotus lividus.* In particular, they showed significantly higher lipid intake compared to the natural macroalgal diet. This can only be beneficial in echinoculture because lipids are important for sea urchin growth because they are a concentrated source of energy and are important in many physiological functions (Berdanier, 1992; Bruckner, 1992; Chapkin, 1992; Hwang, 1992), including as structural components for the production of cell membranes, a process essential for promoting somatic growth (Takagi et al., 1980; Voogt, 1982). Furthermore, in agreement with other studies, lipids from the diet play a key role in characterizing the fatty acid profile and consequently the quality of sea urchin gonads (Castell et al., 2004; Martinez-Pita et al. 2010a, b; Carboni et al. 2013).

In terms of proteins, the formulated diets also exhibited a significantly higher concentration than the natural diet, another aspect of considerable importance, as data from the literature indicate that an artificial diet containing a moderate protein level produces improved somatic growth rates and gonad production efficiency (Hammer et al, 2012). Conversely, a diet with a relatively low protein content, such as algal-based diets, does not support somatic and gonad growth in sea urchins (Fernandez & Boudouresque, 2000; Cook et al., 2007).

Our results support this hypothesis, as the FSU diet with higher protein content produced the highest GSI. They provided significantly more somatic growth than the control diet (consisting only of fresh macroalgae: *Ulva* sp. and *Elissolandia elongata*). This supports the thesis that artificial feeds can have significant positive effect on gonadal weight gain in sea urchins, compared to urchins fed with natural seaweed (McBride et al., 2004; Shpigel et al., 2005; Prato et al., 2018).

Regarding the protein content in the gonads, a significant difference was observed between the two rearing seasons, with higher values in autumn than in summer. This could be due to the fact that in the fall season, sea urchins are overall in the recovery and depletion stages (i.e., with gonads almost devoid of sex cells; Spirlet et al., 2000; Sánchez-España et al., 2004; Schlosser et al., 2005), which coincides with the start of the gonad maturation process in the Mediterranean Sea (Lozano et al., 1995). In fact, according to the stage of maturation, the gonads contain a different ratio of germ cells to somatic cells (nutritive phagocytes) and are generally characterized by a different gonado-somatic index (GSI) (Byrne, 1990).

Furthermore, the lipid content in the gonads of reared *P. lividus* individuals was found to be significantly higher in the summer season in the experimental diet enriched with sea urchin powder (FSU) than in the macroalgal diet. This aspect holds considerable importance in the perspective of echinoculture, particularly for roe enhancement, since in summer, an ideal period to exploit the gonads (significantly higher GSI), they are also richer in lipids and therefore more valued by the global market.

5.4 Conclusions

This experiment not only demonstrated that prepared diets are more successful in increasing gonad development than the natural feeds tested, but also provided interesting information regarding the shelf life and the persistence in seawater of the feeds used. Both feeds formulated (with inorganic and organic carbonate) exhibited optimal stability in seawater. This characteristic thus makes it possible to avoid the loss of feed and allowing the return on investment to be maximized, especially in relation to the high cost of commercial sea urchin feed.

In particular, this new experimental feed consisting of a limited number of ingredients and enriched with waste material would be an excellent choice for echinoculture both economically and from the point of view of product sustainability.

In addition, the enhanced antioxidant activity provided by the bioactive compounds in sea urchin powder could lead to better performance in the long run, both in terms of feed shelf life and sea urchin welfare.

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6. General conclusions

This study has shown that there is a strong need to monitor *Paracentrotus lividus* populations in the Mediterranean Sea, and in particular along the Ligurian coasts, where regular monitorings have not been implemented, in order to clarify how stressors can directly or indirectly influence population dynamics at individual locations. This takes on added importance since a steady population decline has been observed in many areas of the Mediterranean Sea over the past decade, often caused by excessive harvesting pressure, but in other areas without any certain cause. In addition to this, however, it is now evident that *P. livdus* populations are definitely sensitive to anthropogenic impacts, above all global warming. It, in fact, can act in a direct way in modelling sea urchin populations, e.g. with sudden and extreme heat wave events in seawater temperature that can greatly reduce larval survival and impair the reproductive success of adults. Climate change, however, can also act negatively indirectly on sea urchin populations, for example by favouring toxic microalgal blooms (see *Ostreopsis ovata* blooms), with possible negative effects again on the development and survival of *P. lividus* larval stages, hindering their recruitment. It is therefore urgent to monitor *P. lividus* in the Mediterranean Sea, as any collapse of the various local sub-populations could establish dangerous cascading effects, with negative consequences on the biodiversity and functioning of coastal ecosystems.

In addition to promoting and increasing this monitoring action, it is now clear that it is necessary to invest in the promotion of aquaculture as an alternative to harvesting sea urchins directly from natural stocks for the market or for restocking natural populations.

In this perspective, although many efforts have been made in recent years, a feed has yet to be found that is both cost-effective, has a low environmental footprint, and can provide optimal results in terms of gonad quality and yield. Therefore, this work has focused on the search for a new experimental formulation, consisting of a limited number of ingredients and enriched with material from the waste from processing and packaging activities of the sea urchin gonads. The results obtained are encouraging, as the new formulated feed ensured remarkable performance in terms of somatic and gonadal growth of reared sea urchins. In addition, an attempt was made to promote and support a virtuous system of circular economy, capable of increasing the commercial value of the exploited species thanks to a better exploitation of the raw product, by taking advantage of large amounts of material that would normally be discarded and reduced to waste for disposal, representing additional costs in the supply chain.