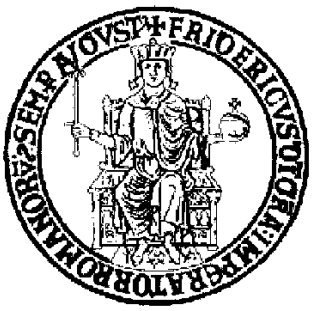
Università degli Studi di Napoli *Federico II*



Scuola di Dottorato in Scienze Agrarie e Agro-Alimentari
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Indirizzo Acquacoltura
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Echinoculture: rearing of *Paracentrotus lividus* in recirculating aquaculture system. Experimentations of artificial diets for sexual maturation.

Echinocoltura: allevamento di *Paracentrotus lividus* a circuito chiuso. Sperimentazioni di diete artificiali per la maturazione sessuale.

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Preface

Fisheries and aquaculture produced in 2010, 148 million tonnes of fish (for a total of 217.5 billion US $), and 128 million of these were consumed as food; preliminary data for the 2011 show an increase in production to 154 million tonnes, but if the share of fish remained stable from 2001 on values of 90 million tonnes, aquaculture has continued to grow strongly at an annual rate of 6.3% from 34.6 million tonnes in 2001 to 59.9 million tons in 2010 (FAO, 2012).
Over the past five years, with the growth of fish production and improvement of distribution channels, even the world fish food demand has grown, with an estimated average growth rate of 3.2% per year from 1961 to 2009. As a result even the per capita fish consumption has increased from an average of 9.9 kg (live weight) in 1960 to 18.4 kg in 2009, and preliminary estimates for 2010 indicate a further increase in fish consumption to 18.6 kg per capita (FAO, 2012). Every European citizen consumes even more, with 22.1 kilograms of fish annually (25.4 Kg Italy per capita per annum) and this values are expected to grow (FAO, 2008) although the catch in European waters has drastically declined since 1993 to an average of 2 percent per year with a total reduction of approximately 25 % (NEF, 2012). Considering that the global population will continue to grow until it reach 9 billion people by 2050 we can conclude that the pressure on fish stocks will cause the collapse of natural resources.
In such a context, even natural stocks of echinoderms, have suffered over the years a marked reduction in production. The fishery of echinoid, has reached its zenith in 1995 with a production of 113,654 tons, an amount three times higher than that recorded in 1970 (William, 2002), to decline to about 100,000 tons a year of 2009 (FAO, 2009). From a simple data analysis, the share of sea urchins caught would seem to have been, at least in appearance, only a small decline over the years, however, if we exclude the quantity fished annually in Chile (an area where the quantities caught annually recorded a sharp increase in those years), in all other regions, the proportion sea urchins suffered a strong decline. It is obvious that this apparent masking of the overfishing conditions on natural stocks, due to strong production of Chile and related to the continued expansion of the fishing area towards south of this country, is a situation that cannot continue for a long time (Andrew *et al*., 2002). This scenario is further exacerbated by the slow growth rates of these organisms; to understand the growth rate of *Paracentrotus lividus*, one of the most widespread species in the Mediterranean Sea, it is necessary to reflect on these data; 2 cm individuals are generally considered to average 2 years old; an individual employs on average 4-5 years to reach 4 cm in diameter (Turon *et al*., 1995; Fernandez 1996; Grosjean *et al*., 1996; Gago *et al*., 2003; Grosjean *et al*., 2003; Sellem *et al*., 2003). It follows that populations of sea urchins, particularly *P. lividus,* are doomed to collapse without the adoption of specific management strategies that allow the stock recovery and the mitigation of impacts on natural populations. At this point it becomes difficult to think on a future without aquaculture project for any species of fish, echinoderms included.
Aquaculture is the worldwide fastest growing industry in the context of food production. The productivity of this sector, although not comparable to the growth recorded in the ‘80 and ‘90 (with an increase of average production of 11% per year from 1984) (AA.VV, 2001), recorded in 2010 its highest peak of production with 60 million tons (echinoderms included) worth US $ 119 billion (FAO, 2012) and currently provides more than 1.2 million tons of fish a year to European markets (NEF, 2012). Aquaculture, however, cannot be considered as the solution to every problems, in fact, with aquaculture are often linked environmental issues. The environmental impact varies greatly depending on the type of animal bred and used system, but there are some critical points that are common to all cases. The biggest problem is that the reared species are feed with derived fishmeal, whose production affects significantly marine stocks. Cases, where to "produce" an animal of 1 kg, are sufficient 1 kg of transformed fish are few; usually the ratio is higher; with salmon, for example, goes up to 1:5 and in some cases can reach up to 1:22.

Moreover we have to consider the rearing conditions, the high density in rearing system often lead to an easily spread of disease. This situation contributes, not a little, to the pollution of surrounding water both for the animals excreta and the remains of those dead, both for antibiotics, animal feed and other products (such as hormones to stimulate growth) administered to farmed organisms.
Should not be neglected also the escape of animals from breeding systems, a situation nearly impossible to avoid and at the same time dangerous because it leads to the competition between reared and wild organisms for natural resource (over-exploitation of resource) and also contributing to genetic impoverishment of wild stocks. Not forgetting, finally, the modification of natural habitats caused by farming systems, as happened to mangrove forests in Southeast Asia, replaced by intensive farming of shrimp.
These issues are partly solved by recirculating aquaculture systems (RAS) where residues and feces are well conveyed can be subjected to physical (settling), mechanics (filtration) and biological (surface impoundment) treatments and allow the total or partial reuse of waters in rearing system, guaranteeing a sustainable use of hydrological resources. This theme has always been for the "Centro interdipartimentale di ricerca per la gestione delle risorse idrobiologiche e per l'acquacoltura CRIAcq " at the University of Naple, Federico II, since its inception in 2000, one of the goals of its mission and has been pursued through basic and applied research in the field of aquaculture, for the exploitation of native species, and hydrobiologic resource management through the study and design of innovative technological solutions aimed at minimizing the effects arising from production processes.
Aquaculture must necessarily perform in the near future a central role in the policies of “restoration” of population of sea urchin as well strongly threatened by excessive fishing. For *Paracentrotus lividus*, the breeding for restocking, is certainly desirable even under further consideration: starting from '80 this species was recognized worldwide among the most reliable as bioindicator (ICES, 1997), and its gametes used for biological assays for monitoring marine pollution. If these condition led *P. lividus* to be considered a biological model, in other words a species widely used by researchers to study biological phenomena, on the other hand has produced on this species, although to a lesser extent than commercial fishing, a further "fishing pressure". From this, comes the need to develop rearing techniques for this species for the production of gametes for scientific use, to get individuals to be used in restocking natural stocks and at the same time to cope the growing market demand for gonads, highly valued as seafood that otherwise the natural populations are unable to meet. Restocking aquaculture requires appropriate technologies, not just fill the sea with urchins, to do so in a sustainable manner will require responsible behaviour and appropriate scientific and technological tools. We must reflect on a central theme: put a species in a rearing system is not the same thing as sending it in an environment. In this second case the dynamics are complex and it is not possible to predict all possible consequences such as those related to the alteration of the genetic structure of natural populations. In the spirit of sustainable development, without taking rigid positions which could reveal wrong, it would be desirable to make restocking aquaculture a tool to retrieve simultaneously aquatic environments and provide new economic opportunities.

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Chapter 1
Introduction

1.1 Overview of the biology and the ecology of *Paracentrotus lividus*

Paracentrotus lividus (Lamarck) belongs to the Echinodermata phylum (class Echinoidea, Diademantoida order). The name assigned to the group, of Greek origin, refers to the fact that these animals are frequently covered with spine.

1.1.1 Morphology and structural organization

Echinoderms are deuterostome and possess a well-developed coelom. The cavities are lined by peritoneum and the coelomic fluid plays an important role in circulatory system. Sea urchin are stenoaline marine organisms that have low mobility. *P. lividus* have developed a body protection system; a sort of shell (dermal skeleton), consisting in calcareous plates welded, so stiff and forming together a reliquary containing the viscera (fig. 1.1.1 A). The body is spherical and slightly flattened, covered in spines, lined with skin, torn at the tip of each spine. Spines are not very long, but acute and strong and evenly located throughout the body. Their color varied from green to violet, to reddish up to brown, and this depends on various spines' chromophore contained in spines in various proportions. As widely documented in the literature the spines color is not related with the size or the depth of the habitat (Koehler, 1883; Mortensen, 1943; Cherbonnier, 1956; Tortonese, 1965; Gamble, 1966-1967).
Sea urchins have pentamerous structure. Each sector consists of two zones, radial and interradial: along the radial areas there are very particular organelles, called tube feet, which have locomotive and tactile function and in some cases even prehensile tail (for this reason these areas are also called ambulacrale areas). The interambulacral zones are devoid of tube feet. On ambulacral and interambulacral areas there are primary tubercles on which are implanted the spines. Even in the interambulacral area there are well-developed secondary tubercles.

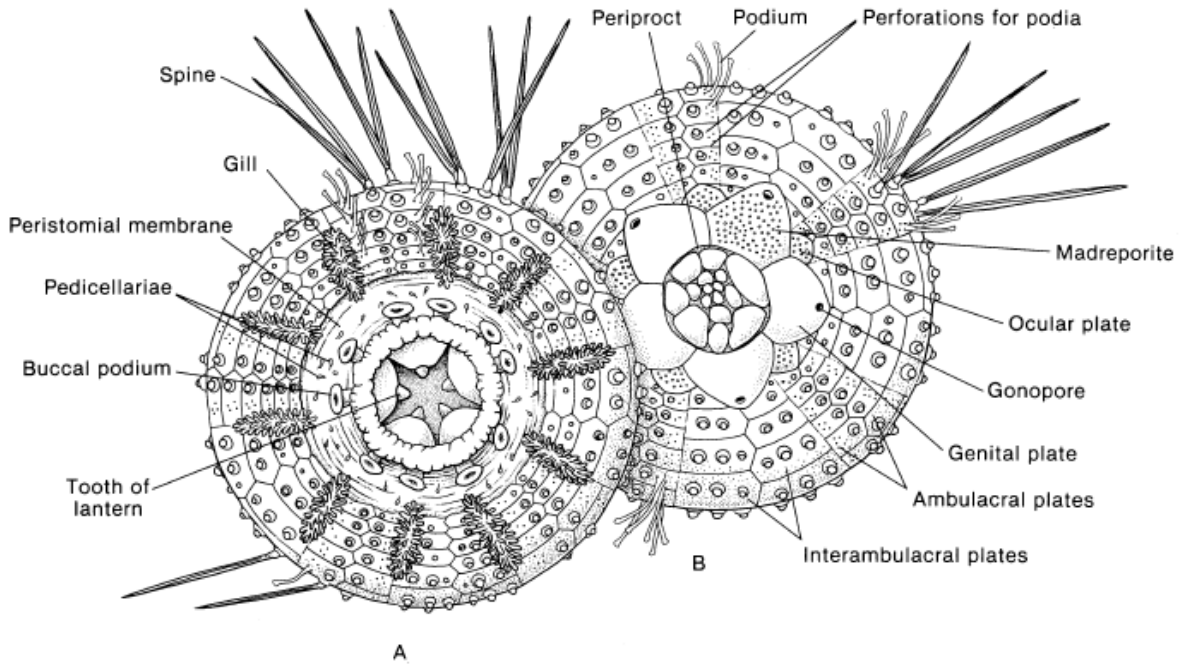


Fig. 1.1.1 Anatomy of regular sea urchin. A. Oral view B. Aboral view .

The mouth and the anus of these animals are located on two opposite poles of the body. Oral area always facing downwards, resting the substrate. In the center of oral zone is placed a space called

peristome covered by peristomal membrane and coated with small plates. In the central part of peristome is placed the mouth. Mouth is composed by an ossicles system constituting a structure called Aristotle's Lantern. The mouth opens into a long and simple intestine which flows in an anus. On the opposite side of the oral zone is located the aboral area where is located the anal region, consisting of a round shaped area (periproct) covered with many platelets, in the midst of which opens the anus (fig. 1.1.1 B). In the aboral area, surrounding the periproct, it is possible to observe 5 genital plates, with a small hole directly connected to a gonad. Other 5 ambulacral plates, smaller than the previous one, are present beside the genital plates

An aquifer system (originally derived from the coelom which belongs solely to echinoderms) and a non-centralized nervous system are present. Both are composed of a ring around the mouth from which depart radial channels which radiating ambulacral areas. The radial channels of aquifer system run along the entire ambulacral zones, from which originates the tube feet, going outward through small holes left in the dermal skeleton.

There are no specialized respiratory systems. Around the mouth there are 5 pairs of coelomic expansion called "gills" and also the aquifer system plays an important role in respiratory exchanges, especially with tube feet which increase the exchange surface.

The gonads are 5, covered by peritoneum. Gonad are located in interambulacral areas and are directly connected with genital plates

1.1.2 Distribution and Habitat

Paracentrotus lividus (Lamarck 1816) is a fairly large sea urchin; test diameter (without spines) can reach, in biggest individuals 7 cm (Bonnet, 1925; Boudouresque *et al*., 1989; Lozano, 1995) and it is one of the main herbivores of the Mediterranean coastline. The geographic distribution of the species includes the Atlantic coastline from Ireland to Morocco, including the Canary Islands and the Azores, and the coasts around the Mediterranean Sea (San Martin, 1995; Hayward and Ryland, 1990). Lives generally in infralittoral area, occours mainly on horizontal or slightly inclined rock (Palacin *et al*., 1997), but is also present on vertical walls and less stable substrates, such as *Posidonia oceanica, Zostera marina* meadows. Its surprising absence in *Cymodocea nodosa* meadows, though this seagrass is an important element in the diet of this sea urchin species, is probably due to two factors: the inadequacy, for the locomotion, of the sand flats where *Cymodocea* is present and the high predators pressure in these environments (Traer 1980). Although it is difficult to observe *P. lividus* on sandy and detrital bottoms, on this type of substrates sea urchins tend to cluster on isolated stones, large shells or various residues (Zavodnik, 1980).

Individuals living in areas, particularly exposed to the wave motion, have developed the ability to dig in the substrate (such as sandstone, limestone, basalt, granite) creating cup-shaped cavities where they live. This behavior is also a protective adaptation against predators.

In coastal lagoons (Thau and Urbinu lagoons in the Mediterranean; Archachon Bay, Atlantic Ocean, France) *Paracentrotus lividus* can even live either on muddy substrates or on coarse sand (Allain, 1975; San Martìn, 1987; Fernandez *et al*., 2003). In these lagoons, as well as in the tide pools, the size of individuals, is always far smaller than those observed in open sea. Although present in coastal lagoons in the Mediterranean and Atlantic "rías", *P. lividus* is sensitive to high and low salinity. Long-term exposure to salinity less than 15-20 ‰ and over 39-40 ‰ cause the death of the organism (Allain, 1975; Pastor, 1971; Le Gall, 1989). In the autumn of 1993, a stormwater (450 mm in 48 h) in the lagoon of Urbinu (Corsica) resulted in the collapse of salinity to 7 ‰ causing a mass mortality in the population of *P. lividus* (Fernandez *et al*., 2003).

P. lividus appears to be relatively insensitive to organic pollution, indeed these compounds will enhance the growth (Tortonese, 1965; Allain, 1975; Zavodnik, 1987; Delmas, 1992). Dense populations of sea urchins are found in the polluted Bay of Brest (Brittany), close to the urban discharge in Rabat (Morocco) and in the heavily polluted Berre lagoon near Marseille. Laboratory experiments have shown the sensitivity of *P. lividus* to ammonia (Lawrence *et al*., 2003), even if in

concentrations found only in aquaculture system rather than in natural environments. In addition, *P. lividus* is able to tolerate high concentrations of heavy metals, and even accumulate them, although they can affect the growth rate of the organisms (Augier *et al*., 1989; Delmas, 1992; San Martin, 1995). In contrast, at least in tide pools, oil spills can cause the mass mortality. In consequence of the "ERIKA" tanker incident, took 3 years so that *P. lividus* density returned to normal levels (Barille-Boyer *et al*., 2004) in tide pools. In spite of the low sensitivity of adults towards contaminants, the sperm toxicity tests, involving gametes of mature individuals, has a great value as bioindicator and has been included in the list of the International Council for the Exploitation of the Sea (ICES, 1997) as one of the most reliable tests for pollution monitoring and assessment of environmental quality.

Small individuals (< 1-2 cm) particularly exposed to predation, constantly living in holes, crevices, under pebbles and boulders, within the "matte" of *Posidonia oceanica* and sometimes under a thick blanket of multicellular photosynthetic organisms (MPOs) (Kempf, 1962; Gamble, 1966-1967; Kitching and Thain, 1983; Verlaque, 1984, 1987a; Azzolina and Willsie, 1987; Azzolina, 1988; San Martin 1995). Larger individuals, may or may not, depending on their size and based on the presence of predators, return to their "lair" once daily grazing activities (Sala, 1996; Palacín *et al.*, 1997) has finished.

The density of *P. lividus* generally results from a few to a dozen individuals per square meter, however very high density (>50-100 individuals for square meter) usually occur in shallow water environments, on rocky substrates with low slope, in intertidal pools and in polluted environments (Kempf, 1962; Pastor, 1971; Crapp and Willis, 1975; Harmelin *et al*., 1981; Delmas and Régis, 1986; Delmas, 1992). Density values, higher than 1600 individuals per m^2, although the basis of this phenomenon remain unclear, may be a defensive strategy against predators, a food behavior or reproductive strategy (Mastaller, 1974; Keegan and Könnecker, 1980).

Despite having been found up to a depth of 80 m (Cherbonnier, 1956; Tortonese, 1965), *P. lividus* colonizes predominantly surface bottoms, with abundances decreasing with increasing depth (Bulleri *et al*., 1999).

Paracentrotus lividus generally lives in subtidal area between the limit of low tide and 10-20 m depth (Gamble, 1965; Tortonese, 1965; Allain, 1975; Règis, 1978; Harmelin *et al*., 1980; Crook *et al.*, 2000). It is particularly aboundant in areas where the water temperature in winter varies between 10 and 15° C and in summer ranges between 18 and 25° C. The northern and southern limit of the natural range of *P. lividus* is bounded by isotherm of 8° C in winter and that of 28° C in summer.

In the English Channel, temperatures lower than 4 C° or greater than 29° C are lethal to *P. lividus*; however in Mediterranean lagoons, sea urchins can survive at temperatures above 30° C, which suggests a certain physiological diversity between populations of different environments

In the Mediterranean, a sea characterized by low amplitude tide, when sea level rapidly drops during high atmospheric pressure days, emerged *P. lividus* quickly go to death. Normally, rigid winter couldn’t cause lethal effects, and the low temperatures are not a limiting factor for the larvae of this species.

1.1.3 Eating habits

Most knowledge about food preferences of *P. lividus* were acquired by means aquarium experiment. Another important source of information about the diet of *P. lividus* is derived from the gut contents and habitat analysis (Ivlev index) (Ivlev, 1961).

The analysis of gut contents of sea urchin indicate that *P. lividus* is basically a "herbivore" (Mortensen, 1943; Kitching and Ebling, 1961; Kempf, 1962; Ebling et al., 1966; Neil and Larkum, 1966; Neill and Pastor, 1973; Verlaque and Nédélec, 1983b; Verlaque, 1987a, 1987b).

Among the preferred species of *P. lividus* we can mention *Rissoella verrucolosa* (rhodobionta), *Cymodocea nodosa* (magnoliophyta), *Cystoseira amentacea*, *Padina pavonica* and *Undaria*

pinnatifida (Brown algae), contrary *Asparagopsis armata*, *Gelidium spinosum*, *Anadyomene stellata*, *Caulerpa taxifolia*, and *Flabellia petiolata* are strongly avoided (Traer, 1980; Cuomo *et al*., 1982; Nédélec, 1982; Kitching and Thain, 1983; Verlaque and Nédélec, 1983a, b; Verlaque, 1984, 1987b; Zupi and Fresi, 1984; Knoepffler-Péguy *et al.*, 1987; Shepherd, 1987; Verlaque, 1987a; Frantzis *et al*., 1988; Odile *et al*., 1988; Fernandez, 1989; Rico, 1989; Boudouresque *et al*., 1993; Knopffler-Péguy and Nattero, 1996; LeMée *et al*., 1996; Aubin, 2004). *P. lividus* consumes all the parts of the seagrass *P. oceanic*; leaves "lives" with and without epiphytic, dead leaves, rhizomes and roots. The behavior of *P. lividus* in avoiding algal species is often linked to the presence of toxic or repellents metabolites. *Caulerpa taxifolia*, containes large quantities of terpenes (Guerriero *et al*., 1992; Lemée *et al*., 1996) while the Rhodobionta *Asparogopsis armata* synthesize brominated compounds (Codomier *et al*., 1977). However, the presence of these toxic metabolites does not always justify the feeding preferences of *P. lividus*. The brown algae *Cystoseira compressa* and *Stypocaulon scoparium* contain 23% and 2% (in relation to total dry weight) polyphenols, respectively, despite this fact, are consumed by *P. lividus* in equal measure where both are present (Frantzis and Gremare, 1992). Even the presence of calcium carbonate in the algae cell walls *(L. incrustans e Amphiroa rigida)* is a reason of avoidance although some tiny articulated corallines (*Jania rubens*), are normally consumed by *P. lividus* (Boudouresque and Verlaque, 2007).

The food selection is greatly conditioned by the relative abundance of seaweed; the choice of "preferred" macrophytes in plenty of food is very high, but quickly falls, until it disappears, when the number of individuals of sea urchin and the pressure exerted on the algal community grows rapidly. An important source of food for *P. lividus* is represented by algae, seagrass, or fragments of these transported by current flow. In the Mediterranean sea, the leaves of *P. oceanica*, can constitute up to 40% of the gut contents of sea urchin, located hundreds of meters from seagrass meadow (Verlaque and Nédélec, 1983b; Maggiore *et al*., 1987; Verlaque 1987a).

The food selection is however conditioned not only by the size and the ease which this can be manipulated, but also by its nitrogen content. Consumption of leaves of *P. oceanica* grow rapidly when their nitrogen content increases; fact that normally happens in polluted environments (Ruiz-Fernandez, 2000). In contrast, seaweeds which are not among the “preferred species” have a high nitrogen content and thus low C/N ratios (*Asparagopsis armata* and *Halurus flosculosa*). *Padina pavonica* despite being among the most consumed species has very low values of aminoacids. Finally, there is no clear correlation between consumed algae and their calorific value. The morphology of spines of *P. lividus* seems to be influenced by the availability of nutrients in the habitat. In areas with high organic pollution caused by domestic sewage, the spines of *P. lividus* tend to elongate and become thinner. The elongation of the spines, and their greater porosity of the internal structure, is considered a morphofunctional adaptation for a more active and efficient uptake of organic material (Delmas and Régis, 1985; Régis, 1986). The increase of "food capture surface" may partly explain the high density of this species in environments with high organic load, the presence of individuals “trapped” in burrows and sea urchin populations that live in rocky pools without algal coverage. In fact, it is highly unlikely that seaweed, in an environment like that, are the sole food source for sea urchins, considering that other herbivores such as limpets compete for the same resource (Mastaller, 1974; Crapp and Willis, 1975). Although seaweed and seagrass are the main elements in the diet of *P. lividus*, this species have a generalist and opportunistic behaviour in food consumption, which makes it able to exploit any food source. In conditions of limited food availability *P. lividus* is able to "shift" from a “preferred” but insufficient food source to another, less appreciated, but plentiful seaweed (switching behaviour). Photosynthetic unicellular organisms, sponges, hydroids, copepods, etc can be found in gut contents (Mortensen, 1943; Tortonese, 1965; Pastor, 1971; Neill and Pastor, 1973; Régis, 1978; Délmas and Régis, 1986; Fernandez, 1990; Mazzella *et al*., 1992).

As for algae, even for sponges there are more “preferred” species, as *Dysidea avara* and less favourite species, as *Crambe crambe* respectively (Uriz et al., 1996). According to Harmelin *et al*. (1981) *P. lividus* can also eat dead fish found on the bottom, while in aquarium, sea urchins can be

fed with mussels (Powis de Tenbossche, 1978; Haya and Régis, 1995). If in the environment are present only inedible algae, such as *C. taxifolia*, *P. lividus* ingests large amounts of sand (Lemée *et al*., 1996). Even acts of cannibalism were recorded, as witnessed by sea urchin residues found in the intestine of individuals in populations with high densities. In aquarium the same phenomenon can occur at the expense of organisms of 2-3 cm in diameter by larger individuals (Pastor, 1971).
Paracentrotus lividus, both in its natural habitat and in the aquarium, tends to cover the aboral region with shells, algae, small stones, plastic parts etc (Kempf, 1962; Dambach and Hentschel, 1970; Pastor, 1971; Martinelli, 1981; Rico, 1989; Benedetti-Cecchi and Cinelli, 1995). This behavior, particularly frequent in summer, although it has been found both in the presence and absence of light, has been considered by many authors a mechanism to protect itself from light (Mortensen, 1927, 1943; Sharp and Gray, 1962; Barnes and Crook, 2001b; Crook and Barnes, 2001; Crook, 2003), against UV rays (Verling *et al.*, 2002) and predators (Mortensen, 1927; Pastor, 1971). The fact that in small individuals, this behavior is more pronounced than in large individuals (Crook *et al.*, 1999; Barnes and Crook, 2001b) would seem to confirm this last hypothesis. For Richner and Milinski (2000) the covering behavior serves to protect the apical opening of aquifer system, which allowing ambulation of *P. lividus,* by the occlusion caused by sand and other suspended particles . This behavior also seems to play an important role in sea urchins nutrition, allowing them to take and carry on algae of which they feed.

1.1.4 Predation

The main natural predators of *P. lividus* are Mediterranean seabream *Diplodus sargus*, *Diplodus. vulgaris*, the wrasses *Labrus merula* and *Coris julis*, the crustacean decapod *Maja crispata* and gastropod *Trunculariopsis trunculus*. *Diplodus sargus* is able to feed on individuals with test diameter up to 5 cm while Coris julis generally feeds on individuals with diameter less than 1 cm (Tertschnig, 1989; Sala, 1996, 1997; Heureu *et al*., 2005). The starfish *Marthasterias glacialis* can eat sea urchins with test diameter up to 63 mm. In coastal areas where *D. sargus* and *D. vulgaris* populations are subjected to an intense fishing effort, predation of sea urchins is due to the 57% for other species of fish and the remaining 43% is by *T. trunculus*. In contrast, in marine protected areas, fishes are responsible for 100% of predation of *P. lividus* (Sala and Zabala 1996).
In the Atlantic, the situation is slightly different, in fact, the predators main role is played by starfish and crustaceans. The crabs *Cancer pagurus*, *Necora puber*, *Maja brachydactyla* and *Carcinus maenas* are able to feed on any individuals of any size class. Adult of *Cancer pagurus* can get to consume two sea urchins per day while *Homarus gammarus*, can get to eat individuals of *P. lividus* with test diameter greater than 6 cm. (Muntz *et al*., 1965; Ebling *et al.*, 1966; Kitching and Ebling, 1967, Neil and Pastor, 1973; Kitching and Thain, 1983; Bernádez *et al.*, 2000).

1.1.5 Reproduction and Growth

Somatic growth of *Paracentrotus lividus* can be influenced by water temperature, the type of food available, and gonadal development (Fernandez, 1996), although seasonal variations of growth rate seem to be mainly related to water temperature. Le Gall *et al*., (1990) reports that, in the population of sea urchins in the English Channel, growth is absent between 4 and 7° C. Growth, increase proportionally with increasing temperature between 7 and 18° C, although the optimum condition for growth is obtained between 18 and 22 °C. Over 22 °C, growth slows, to a halt completely when temperature exceed 28 °C. In the Mediterranean Sea, the highest growth occurs when the water temperature is between 12 and 18° C (spring) while most hardly occurs in autumn and almost never in winter (Azzolina, 1988; Fernandez and Caltagirone, 1994; Turon *et al*., 1995, Shpigel *et al*., 2004). To understand the speed of growth rate of *P. lividus* we must reflect on these data: individuals of 2 cm in diameter are approximately 2 years old urchins; an individual spend on average 4-5 years to reach 4 cm in diameter (Turon *et al.*, 1995; Fernandez, 1996b; Grosjean *et al*.,

1996; Sellem *et al*., 2000; Gago *et al*., 2003; Grosjean *et al*., 2003).
Generally, the high gonadosomatic index values were observed in individuals with size ranging from 40 to70 mm rather than individuals belonging to class size 20-40 mm (Martínez *et al.*, 2003; Sánchez-España *et al.*, 2004)
In the Mediterranean and in Atlantic Ocean, studies on the gonadal growth of *P. lividus* reported the presence of two growth peaks whose temporal localization, even in populations of neighbouring areas, can vary considerably (Lozano *et al*., 1995; Guettaf, 1997; Spirlet *et al*., 1998; Sánchez-España *et al*., 2004). Both field studies and in vitro studies seem to confirm that the somatic and the gonadal growth occur when food availability is high (Lawrence *et al*., 1992; Gago *et al.*, 2003) and the organic matter ingested is high (Frantzis and Grémare, 1992)
Temperatures between 18 and 22° C and short photoperiod, seems to enhance gonadal development (Shpigel *et al.*, 2004). Neverthless some conflicting data were obtained from *in situ* studies; in fact very large gonads were observed in well-feed subtidal populations both in open sea and in the lagoon environments (Byrne, 1990; Fernandez, 1990, 1996; San Martín, 1995; Fernandez and Boudouresque, 1997).
High gonadal indices were found in low-density populations (low competition for the food source) (San Martín and Guettaf, 1995) while poor correlations have been found between the gonadosomatic index and the repletion index (Régis, 1978; Semroud and Kada, 1987). On the contrary, in Spain (Catalonia), high gonadal indices were found in organisms of shallow-water, with high density population, where the substrate is populated by a few algal species rather than in stable environments of deep waters characterized by low population density (Lozano *et al.*, 1995). According to these authors, these results suggested a greater investment by *P. lividus* in reproductive strategy in unfavorable conditions for the availability of food. Although, gonadal growth, could be supported by high supply of algal fragments or food of high nutritional value transported by current flow .
P. lividus has separate sexes and there is no sexual dimorphism, though for this species, hermaphroditism cases have been observed (Drzewina and Bohn, 1924; Neefs, 1937; Byrne, 1990). In vitro, sexual maturity is reached in individuals of size ranging between 13 and 20 mm and/or after 5 months (Fenaux L in Azzolina, 1987; Cellario and Fenaux, 1990), however in the natural population, sexual maturity can be reached in the longer times. Even limiting conditions, such as the availability of food and unfavourable environmental conditions, can lead to a decrease in size for mature organisms (Lozano *et al*., 1995).
P. lividus has an annual reproductive cycle. According to some authors this species presents a single spawning event (Byrne, 1990; Dominique, 1973; Lozano *et al.*, 1995), while others support the hypothesis that in a year may occur two reproductive events (Crapp and Willis, 1975; Fenaux, 1968; Regis, 1979). The reproductive cycle of *P. lividus* has been studied in detail by several authors and is known, as the cycle of many echinoids, is influenced by various environmental factors such as temperature (Byrne, 1990, Lozano *et al*., 1995), photoperiod (Byrne, 1990; Lozano *et al.,* 1995, Sphigel, 2004), hydrodynamics conditions (Guettaf, 2000), and trophic availability (Regis, 1979; Fenaux, 1968, Lozano *et al*., 1995, Guettaf, 2000).
According to Fenaux (1968), although the production of gametes takes place up to a temperature of 8° C, spontaneous emissions are not possible under 13.5 °C. Thus, the reproductive period at our latitudes takes places, from autumn to spring, until the temperatures do not exceed 20-22 °C.
Along the french Mediterranean coasts, two main reproductive moments were observed, one between May and June and the other in September and October (Fenaux, 1968). In accordance with what is reported in the literature for the population of *P. lividus* (Byrne, 1990; Dominique, 1973; Lozano *et al*., 1995) animals living along the Italian coast have a single spawning period much longer, which generally runs from October to June (Giambartolomei, 1990)
Generally, during the spawning events, male and female of *P. lividus* aggregated and simultaneously release their gametes (Cherbonnier, 1954). These episodes do not always involve all individuals of a population. However, the homogenized suspension of sperm and eggs that is created, can be a

trigger and encourage the release of gametes by other sea urchins located in remote places (Kečkeš, 1966). Both in case of double or single spawning periods during the year, the water temperature seems to play a key role in determining the start of the event. Where two spawning events occurred, the first occurs when the temperature reaches 14-16° C and the second episode when the temperature returns to these values (Fenaux, 1968; Byrne, 1990; Pedrotti, 1993; Bayed *et al*., 2005). The first release can also be triggered by the lengthening of photoperiod (about 15 h of daylight) rather than the temperature, while the end of spawning events seems to be controlled by temperature (Spirlet *et al*., 1998, 2000). The presence of one or two spawning periods can be observed even within the same region between locations and different habitats (Guettaf 1997) However, according to Lozano *et al.* (1995) the natural emission of gametes would occur only during spring and early summer, although the presence of larvae in Fall, and individuals post-metamorphosis (1 mm in diameter) in October would seem to reveal the presence of a spawning events in late summer. However, considering all the variables that affect the release of gametes as water temperature, photoperiod, habitat and individual variability, indipendently of the single or double emission, the spawning can occur almost year-round, although in small quantities. This behavior could be a strategy to facilitate the dispersal of larvae and ensure greater reproductive success of the species (Boudouresque and Verlaque, 2007).
Eggs of *P. lividus* are generally isolecithal and possess relatively low quantities of yolk. The egg activation involve a series of signal transduction steps after sperm binds to a receptor protein on the egg surface which determines the raising of the fertilization membrane. The initial process of cell division of the fertilized egg is called segmentation. Sea urchins exhibit radial, holoblastic cleavage which culminates in the formation of a large blastula. For cell invagination of vegetative pole of the blastula is formed the gastrula. During the last stages of gastrulation and coelom development, the embryo takes on a bilateral symmetry and afterwards becomes a lecithotrophic or planktotrophic larva (pluteus) which, after being transported passively by the current, undergoes a metamorphosis taking adult form and benthal behavior.

1.1.6 Gametogenesis in *Paracentrotus lividus*

The pattern of gametogenic cycle in sea urchins is classified by the activities of the two major cells population that compose the germinal epithelium: the germinal cells and the nutritive phagocytes (NP); These two cell types during gametogenesis show an inversely proportional trend (Walker *et al.*, 2005).
According to Byrne (1990) it is possible to identify, in *Paracentrotus lividus* oogenesis, six stages
Stage I: recovery stage
In the ovary are present primary oocyte of variou size (from 5 to 30 μm in diameter) and cluster of oocyte along the ascinal wall. Ovary may contain unspawned ova and residual oocytes within residual NP incubation chambers. The NP forms a mesh-like structure across the ascinus, giving the ovary a vacuolated aspect (fig. 1.6.1.1A).
Stage II: growing stage
Primary oocyte, attached to the ascinal wall and surrounded by NP, increase in size with the beginning of vitellogenesis (ranging from 10 to 50 μm in diameter) (fig. 1.6.1.1 B).
Stage III: premature stage
With the continuation of vitellogenesis, inside the ovary, oocytes are present at all stages of development. Size can vary from 10 to 90 μm. The phagocytes are now displaced, by the presence of larger ova accumulated in the lumen of the ovary, from their central position. Primary oocytes once reached the maximum size begin their maturation process. Oocytes change their shape from almost spherical form to polyhedral form, and the nucleus is no longer visible. (fig. 1.6.1.1 C).
Stage IV: mature stage
In mature stage, ova (90 μm) are closely-packed in the ovaries. Few oocytes (10 to 60 μm) are present along the ascinal wall and NP are absent (fig. 1.6.1.1 D).

Stage V: partly spawned stage

Ova do not appear closely packed as in the previous stage. Inside the ovary there are many spaces, left empty by spawned ova. Sometimes ova can be present within the oviduct (Fig. 1.6.1.1 E).

However, the ovaries, in this stage, may have appearance extremely different from each other: in some cases there may be oocytes at all stages of development (as in stage III), in other cases, as described for stage IV, there may be a large number of ova. From this, it is clear that, with the onset of spawning period, both individuals with stage III and stage IV can undergo to spawning events.

It is evident, that in those ovaries in stage III, where spawning have happened there will be a small number of ova with primary oocytes ready to replace the spawned ones. In this condition, the vitellogenesis continues even during the initial phases of spawning, as confirmed by the presence of oocytes surrounded by nutritive phagocytes. In contrast, in ovaries progressing from stage IV to stage V may have a large amount of ova ready to be spawned. If not absent, primary oocytes are ready to replace the spawned ova.

Stage VI: spent stage

The ovaries have thin-ascinal wall and contain unspawned ova. The number and type of oocyte present is extremely variable; however ova and oocytes present in this stage within the ovary will face to resorption in order to recover the resources necessary to the next oogenic cycle (fig. 1.6.1.1 F).

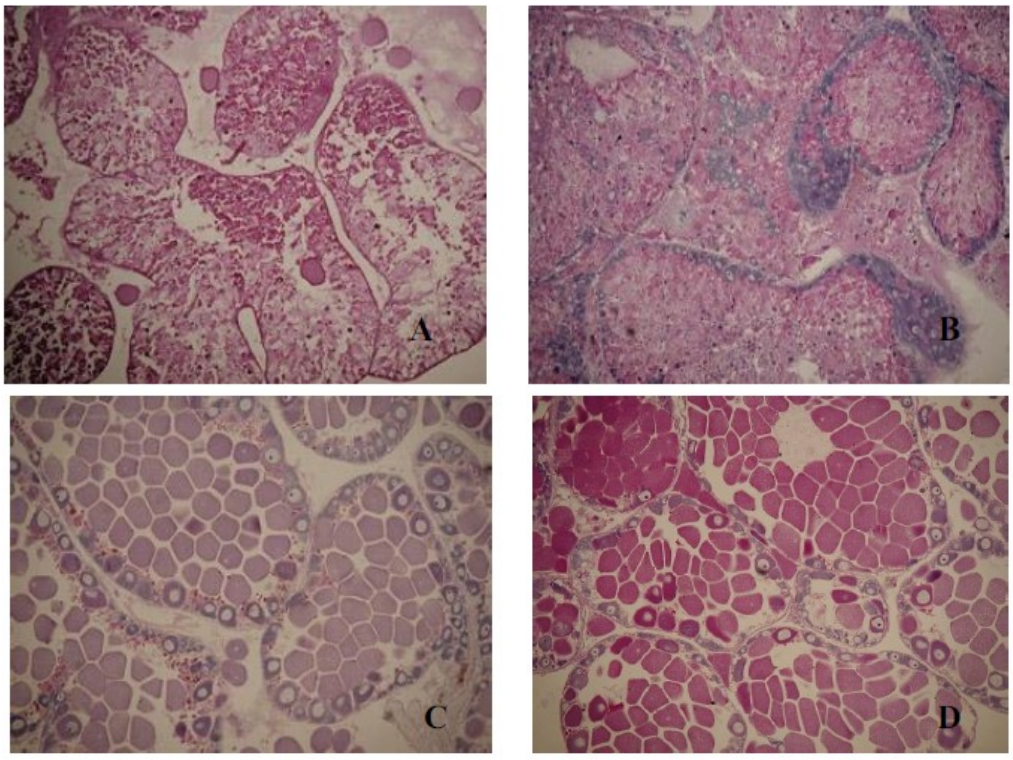


Fig. 1.6.1.1. Histology of ovaries : A) recovery stage (stage I) ; B) growing stage (stage II); C) premature stage (stage III); D) mature stage (stage IV)(Visconti *et al.*, 2008).

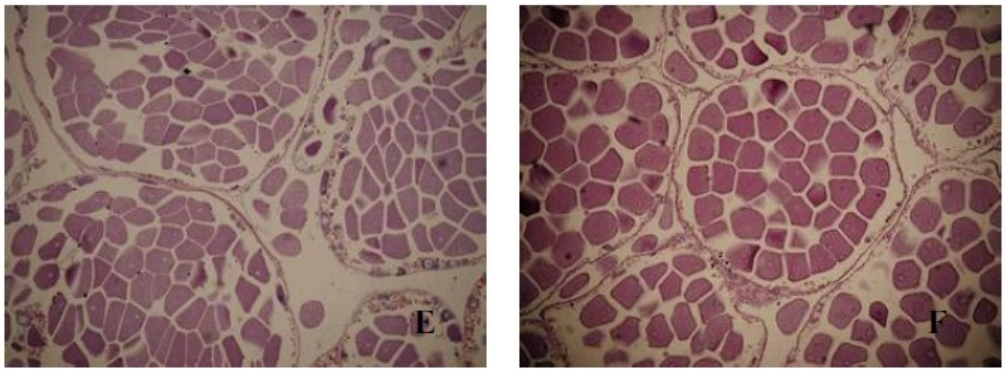


Fig. 1.6.1.1. continued. E) partly spawned stage (stage V); F) spent stage (stage VI (Visconti *et al.*, 2008).

As described for the ovaries, also for testis six different stages can be identified:

Stage I: recovery stage

The testis ascinal wall is characterized by the presence of a large amount of NP. Relict spermatozoa may be present while, a thin layer of primary spermatocytes and spermatogonia, lined the ascinal wall (fig. 1.6.1.2 A).

Stage II: growing stage

Immersed in the mesh of NP one begin to see columns of developing spermatocytes that project toward the center of the lumen of testis (fig. 1.6.1.2 B).

Stage III: premature stage

The spermtozoa accumulate in the lumen of testis while NP are displaced from the centre of testis and are localized along the ascinal wall. (fig. 1.6.1.2 C).

Stage IV: mature stage

Large amounts of spermatozoa in mature stage are accumulated in the lumen of testis while a thin layer of phagocytes lined the ascinal wall (fig. 1.6.1.2 D).

Stage V: partly spawned stage

In stage V testis have a similar appearance to mature stage, however the spermatozoa are less concentrated and there are empty spaces in the lumen generated by spawned gametes (fig. 1.6.1.2 E).

Stage VI: spent stage

Testis are usually empty except for the presence of relict spermatozoa, the phagocytes form a thin layer along the ascinal wall (fig. 1.6.1.2 F).

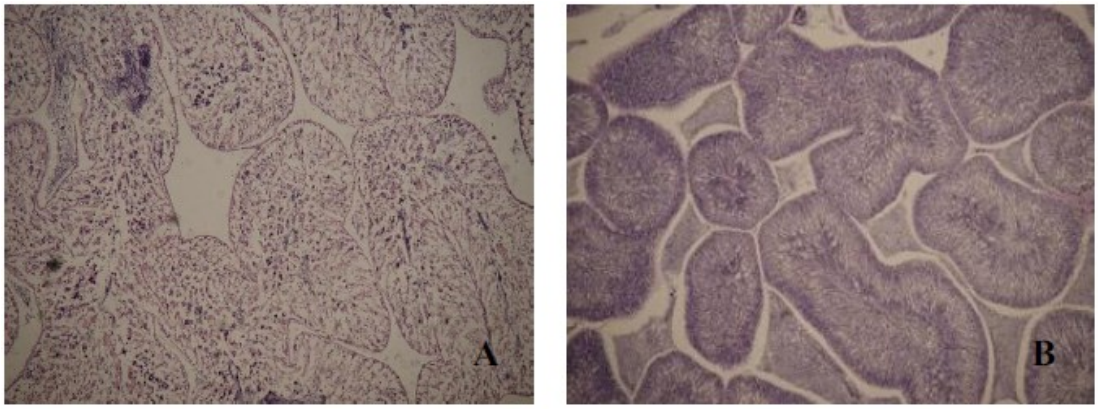


Fig. 1.1.6.2. Histology of testis: A) recovery stage (stage I) ; B) growing stage (stage II); (Visconti *et al.*, 2008).

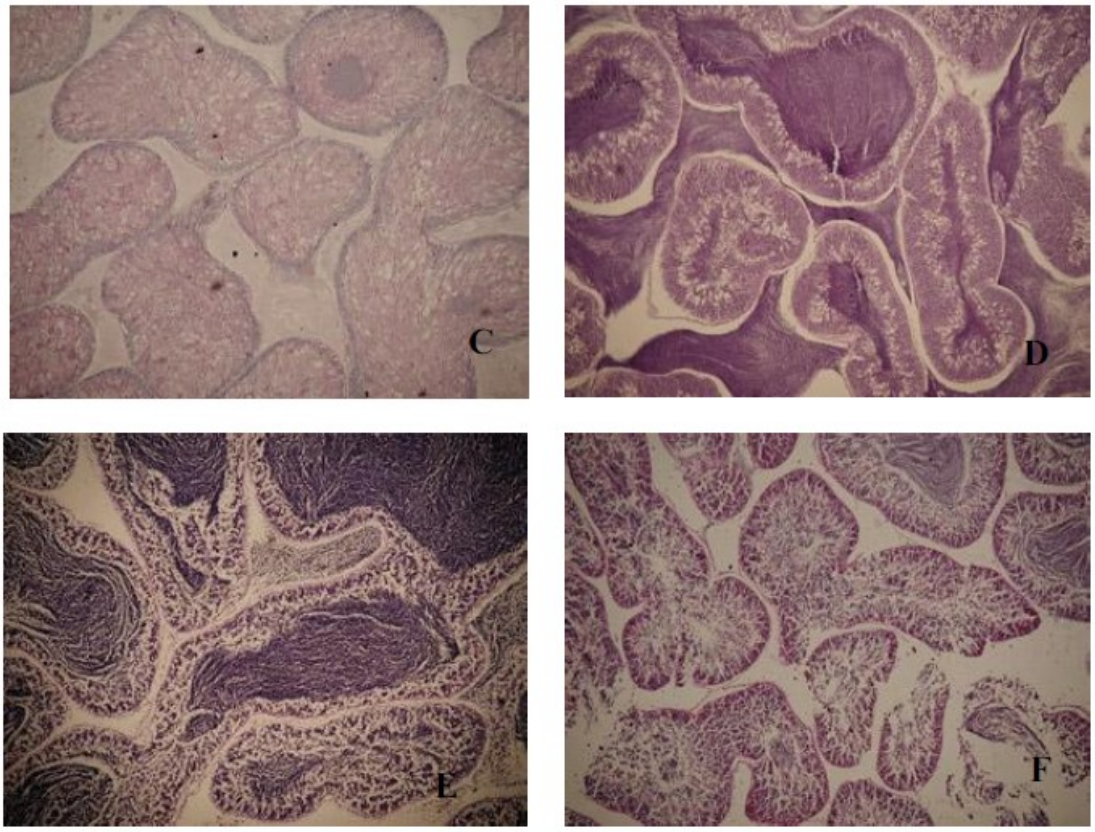


Fig. 1.1.6.2. continued. C) premature stage (stage III); D) mature stage (stage IV); E) partly spawned stage (stage V); F) spent stage (Visconti *et al.*, 2008). (Visconti *et al.*, 2008)

1.1.7 Induction of gonadal growth

The reproductive cycle of echinoids has been extensively studied and documented since the early '30 (Moore, 1934; Boolootian, 1966; Jangoux and Lawrence, 1982; Pearse and Cameron, 1991). Generally, to describe the gonadal growth, is used the gonadal index (GI), which is the ratio between the gonads fresh weight and the total weight of sea urchin. The advantage of using the gonadosomatic index in evaluating the seasonal changes of the gonads weight, has produced numerous studies in the literature. However, being a dimensionless value, the GI does not appear to be a suitable tool to retrieve information relating with the size of the specimens .

From the point of view of the market, the most appreciated gonads are those in which the phagocytes, shortly before the beginning of the maturation process of gametes, have reached their maximum size. Indeed at this stage, where phagocytes have accumulated the necessary substances to be used in the maturation of gametes, gonads have high levels of protein, carbohydrates and lipids.

The development of phagocytes is closely related to the assimilation of nutrients from the diet and then, being able to understand what are the biochemical requirements necessary for the growth and development of nutritive phagocytes could facilitate the selection of optimal diets to use in aquaculture.

Numerous studies have been conducted on *Loxechinus albus* (Lawrence *et al*., 1997), *Strongylocentrotus droebachiensis* (Klinger, 1997), *Evechinus chloroticus* (Barker *et al*., 1998) *Strongylocentrotus franciscanus* (McBride *et al*., 1997) and *Paracentrotus lividus* (Fernandez and Pergent, 1998), regarding the effectiveness of artificial diets in improving the gonadal growth and it is not surprising that diets with high protein content (20-25% dry weight) lead to a high increase in gonadal mass. However, from analysis of ingestion rates and the relative increase in gonadal weight,

registered for these studies, it can be deduced that, although the ingestion of protein with diet can be high, there is a limit to the ability of assimilation of proteins by phagocytes during their early growth phase (Marsh and Watts, 2007). An important aspect to consider, in determining the suitability of diet in promoting gonadal growth is to evaluate, from the biochemical point of view, the relationship between the components of a diet and the corresponding produced gonadal growth in sea urchins. Indeed, very often we faced with the following paradox: increase the quality of the diet (protein and lipid) increases the energy required to assimilate these nutrients (Marsh and Watts, 2007).

As amply demonstrated by the numerous published studies, although gonadal growth is closely related to the availability, and the quality of the food, other physical parameters, such as light regime and temperature can positively affect the gonadal growth.

With respect to the photoperiod, in literature there are numerous studies concerning the effects of light regime on gonadal growth of sea urchins (Table 1.1.7.1).

Table 1.1.7.1. Photoperiod tested in published sea urchin trials (McCarron *et al*., 2009)

Species	Experimental photoperiod	Reference
Strongylocentrotus droebachiensis	Short day	Minor and Scheibling (1997)
Strongylocentrotus droebachiensis	Seasonal photoperiod	Walker and Lesser (1998)
Strongylocentrotus droebachiensis	15.5 h L:8.5 h D	Pearce *et al*., (2004)
Strongylocentrotus franciscanus	Darkness;Continuous light	Beyer *et al*., (1998)
Paracentrotus lividus	Continuous light; Darkness; 12 h L:12 h D	Grosjean and Jangoux (1994)
Paracentrotus lividus	12 h L:12 h D juveniles; 17 h L:7 h D market size	Grosjean, Spirlet, Gosselin, Vaitilingon and Jangoux (1998)
Paracentrotus lividus	12 h L:12 h D	Spirlet Grosjean and Jangoux (1998a)
Paracentrotus lividus	8 h L: 16 h D/16 h L:8 h D	Shpigel *et al*., (2004)
Paracentrotus lividus	Darkness; Continuous light	Fernandez and Pergent (1998)
Psammechinus miliaris	16 h L:8 h D	Kelly (2001)
Psammechinus miliaris	10 h L:14 h D	Pantazis *et al*., (2000)

Photoperiod may have contrasting effects on gonadal growth, depending on the species investigated (Pearse *et al*., 1986; Pearse and Cameron, 1991; Walker and Lesser, 1998; Kelly, 2001; Shpigel *et al*., 2004). Whit a "summer" light regime some species may have a high gonadal growth (Walker and Lesser, 1998), in other cases, only by reducing daylenght, the gonadal growth improve considerably (Yamamoto *et al*., 1998). Numerous studies have "included" photoperiod within their experimental design or evaluated directly the effects of photoperiod on somatic and gonadal growth (Minor and Scheibling, 1997; Walker and Lesser, 1998; Pantazis *et al*., 2000; Shpigel *et al*., 2004; Siikavuopio *et al*., 2007). However, considering the contrasting data available in the literature, sometimes remain unclear to understand how the photoperiod can play a key role in promoting the growth of sea urchins. Le Gall (1990), to promote somatic growth of juvenile *P. lividus* suggests breeding in the absence of light, while Grosjean and Jangoux (1994) examined the effect of three light regimes (constant light, no light, 12 h L: 12 h D) on feed consumption in *P. lividus* showed that higher consumption rates were recorded for the sea urchins kept in the dark, with the lowest consumption rates recorded for animals reared on a photoperiod of 12 h L: 12 h D. Beyer *et al*., (1998) evaluating the effects of continuous light and darkness on *Strongylocentrotus franciscanus* has shown that the growth rate was significantly higher for organisms kept in the dark rather than

those reared in the presence of continuous light. In contrast to Fernandez and Pergent (1998), which obtained best results, rearing *P. lividus* under continuous light .
The tendency of many sea urchins to pick up objects, such as shells, seaweed, etc., and place them on the aboral surface through the tube feet is already known by many authors (Millott, 1975, Lawrence, 1976; Verling *et al*., 2002); and has been described for many species of echinoids such as *P. lividus*, *Evechinus chloroticus* and *Strongylocentrotus droebachiensis* (Millott, 1954, Dix, 1970, Crook *et al*., 1999; Crook *et al*., 2000, Adams, 2001; Verling *et al*., 2002),
However, this behaviour could predict that, in natural conditions sea urchins prefer an environment with poor or absent light. Regardless the conflicting data in the literature, we can certainly assert that, when food availability is unlimited, the temperature is the most important factor that affect the growth rate in sea urchins (Spirlet *et al*., 2000, Shpigel *et al*., 2004). Spirlet *et al*., (2000) examined the effect of temperature on gonadal growth in *P. lividus* showed that, a combination of 24 °C and 9 h daylight improve the gonadal growth, unlike treatments with lowest water temperature and longer photoperiod. In partial disagreement with what has just been said, Shpigel *et al*., (2004) showed that the growth of the gonads is influenced by the water temperature only when it exceeds 26 ° C. McCarron *et al*., (2009), in contrast with data reported by Fernandez and Pergent (1998), testing at constant temperature of 17 ° C, the effects 16 H L: 8 H D and 0 H L: D 24h photoperiods on *P. lividus*, showed that the complete absence of light improve the somatic growth, ingestion rates and gonadal growth.

1.2 Sea urchin market and fisheries

Sea urchin fisheries has a long tradition and historically developed on the Atlantic coasts of Europe, in the Mediterranean, North Asia (Japan and Korea), in New Zealand, and Chile. The request of gonads on the market since the early 70s, has increased significantly (especially in Japan), both for the natural growth of the world population and the increasing interest in this food. The continuous growth in demand for sea urchins in the Japanese market and the consequent inability to meet demand with local resources has been a push, ever since the mid 70's, to develop and find new fishing areas in the whole Pacific Ocean (William, 2002).
Over the past 40 years, the sea urchin fishery is significantly changed; in 1970 the more fishy area was the Northwest Pacific (Japan and Korea), with a production of approximately 30,000 tons per year destined almost exclusively to internal market for daily consume. As early as the mid ‘70 new relevant fishing areas of sea urchin have developed in French Polynesia, on the East and West Coast of Canada and the USA and on the West Coast of South America (Chile). The peak of production in sea urchin fisheries, was reached in 1995 with a landing of 113,654 tonnes, a value three times higher than those caught in 1970 (William, 2002), up to the 100,000 tons per year of our days (FAO 2009). From a simple data analysis, the capture of echinoderms appears to have suffered, at least in appearance, only a small decline over the years, but if we exclude the quantity of sea urchin fished annually in Chile (area where the quantities caught annually, recorded a sharp increase in those years), in all other regions, the share of fished sea urchins, decreased significantly. It is evident that, the apparent masking, of the condition of over-exploitation of natural stocks due to strong Chilean production, linked to the ever-expanding of fishing area to the south of this country, is a condition that will not last long (Andrew *et al*., 2002).
To date, the most important markets for sea urchin gonads are represented by Japan and the USA. Gonads (uni in Japanese) are sold in various forms, such as fresh produce (65%), dried, salted, frozen or already cooked (35%) (Saito, 1992; Hagen, 1996a). Among the various ways in which they are consumed, sea urchin gonads are particularly appreciated in the preparation of the decorations of sushi. The main species sold on the Japanese market are *Strongylocentrotus intermedius* (A. Agassiz), *Strongylocentrotus nudus* (A. Agassiz), *Heterocentrotus pulcherrimus* (A. Agassiz), *Pseudocentrotus depressus* (A. Agassiz), *Anthocidaris crassispina* (A. Agassiz) and

Tripneustes gratilla (L.) (Fuji 1967, Fuji & Kamura, 1970; Fernandez, 1996, Hagen, 1996a).
In 2002 in Japan were imported 18,525 tons of gonads for a total value of 247 million U.S. dollars, a value 10 times higher than those recorded in 1975. This increase is partly due to growing product demand and the consequent rise in prices for the gonads of sea urchin. To get a rough idea of the different quantities traded on the Japanese and the American market, it is sufficient to compare the data on the imports in 1999: in Japan in 1999 were imported gonads, fresh or frozen, for a total value of 216 million U.S. dollars, in the same year, the U.S. imported products were $ 19 million (FAO, 2002).
Most of the sea urchin gonads are sold in Japan, by auction to the Central Market of Tokyo and the price is determined primarily by the quality of the product but also by the local production and total quantity imported. The months in which are recorded the highest prices in Japan, are January and September; months during which there is less availability of the product. On the wholesale, the best price is made with whole gonads bright yellow or orange, compact in appearance and packaged in traditional wooden trays, unlike what occurs in New Zealand where the creamy appearance typical of the mature stage is the preferred condition
The average price of gonads in the Japanese market ranging from 18.6 €/kg for local products to 7.9 €/kg of imported products (Hagen, 1996a) for a total annual business estimated at 657 million.
Of the 29 countries engaged in the fishery of sea urchin, in 2001, Chilean production was 54% of the total catch, the United States contributed to 14%, while Japan, which from 1950 to 1984 was the leading country in fishing echinoderms, in 2001 occupied a market share close to 13% (Sonu, 2003). The species most intensively exploited in the world are *Loxechinus albus* and *Strongylocentrotus spp*. (Table 1.2.1). Indeed, between 1991 and 2001 *Loxechinus albus* accounted for between 24 and 55% of the total landed while, *Strongylocentrotus spp*, in the same period represented a share ranging from 38 to 68% of total sea urchin caught (Sonu, 2003).

Table 1.2.1. World sea urchin landings of genus *Strongylocentrotus* and major species of sea urchins, 1950-2001 (metric tons).

	Echinoderm Species				
Year	***Loxechinus albus***	***Strongylocentrotus spp***	***Altre specie***	***Paracentrotus lividus***	***Echinus esculentus***
1950	1.600	6.415	<0.5	*	.
1951	2.300	6.325	<0.5	.	.
1952	1.700	7.033	<0.5	.	.
1953	1.400	7.213	<0.5	.	.
1954	2.600	6.525	-	100	.
1955	3.700	9.326	<0.5	100	.
1956	3.900	14.254	<0.5	100	.
1957	3.600	9.346	-**	300	.
1958	4.000	12.429	-	100	.
1959	3.700	14.149	-	200	.
1960	2.100	16.050	<0.5	100	.
1961	2.200	16.034	100	100	.
1962	2.700	16.733	100	100	.
1963	2.800	19.638	300	100	.
1964	3.300	20.755	300	300	.
1965	2.500	20.757	200	300	.
1966	2.600	23.265	400	300	.

Table 1.2.1 continued: World sea urchin landings of genus *Strongylocentrotus* and major species of sea urchins, 1950-2001 (metric tons).

	Echinoderm Species				
Year	***Loxechinus albus***	***Strongylocentrotus spp***	***Other Species***	***Paracentrotus lividus***	***Echinus esculentus***
1967	2.900	23.750	700	200	.
1968	3.700	27.038	600	200	.
1969	3.800	27 . 537	1.000	200	.
1970	3.200	27.194	3.400	200	.
1971	4.200	25.231	2.000	100	.
1972	4.200	21.800	1.900	<0.5	.
1973	2.500	23.129	2.700	<0.5	.
1974	1.206	21.878	3.405	502	300
1975	2.105	19.705	2.420	417	242
1976	9.809	20.502	3.655	335	209
1977	8.517	26.104	4.265	233	352
1978	6.925	28.875	3.196	308	170
1979	13.206	28.843	2.866	312	114
1980	13.649	18.563	3.565	373	132
1981	15.502	17.573	5.309	422	108
1982	12.159	26.982	5.721	445	102
1983	11.826	25.610	5.715	200	97
1984	16.154	31.464	5.470	229	48
1985	30.577	32.291	6.539	454	113
1986	25.408	44.735	8.113	278	77
1987	24.574	52.900	5.624	213	49
1988	22.953	60.087	4.719	248	52
1989	25.527	58.267	4.451	285	64
1990	15.648	58.316	9.232	301	62
1991	21.382	60.885	6.745	218	95
1992	29.197	64 . 608	5.845	401	104
1993	31.300	57 . 204	7.622	257	89
1994	39.705	55.838	8.094	159	739
1995	54 . 609	53.354	7.433	78	1.443
1996	51.437	45.583	7.054	63	933
1997	45.560	45.724	6.521	48	425
1998	44.843	39.344	4.040	59	25
1999	55.654	39.656	5.702	84	1
2000	54.096	36.744	6.936	198	13
2001	46.794	33.097	7.009	101	1

*Source: FAO 2003. * = data not available; -**= more than zero but less than 0.5 metric tons*

As for Europe, the main market for sea urchin gonads is represented by France, although the quantities treated are far lower than those of Japanese and American market. In the 60s and 70s in France were caught approximately 1000 tons per year of live sea urchins. In the following years there has been a sharp decline in the catch, up to the 250-300 tonnes per year (Allain, 1972a; Ledireac ' h, 1987; Le Gall, 1987, 1990). Local production, however, unable to meet the demands of the domestic market, has been implemented over the years by imports from Spain, Ireland and Greece to reach a total amount (local and imported) of 500 to 600 tons per year between 1988 and - 1990 (Fernandez, 1996). The main species treated in the French market is the *Paracentrotus lividus* (Lamarck) but also *Psammechinus miliaris* (Gmelin) and *Sphaerechinus granularis* (Lamarck) are sold. Most sea urchins are eaten fresh when the gonads, between December and March, have reached their maximum size (Ledireac'h, 1987).

Since the time of ancient Greece, the sea urchin *P. lividus* was considered a delicacy; gonads are

reddish-orange for the considerable presence of carotenoids and are marketed fresh, frozen and pasteurized. In some regions of Italy (especially in Puglia, Sicily and Sardinia), this product is appreciated so much to determine a growing demand. It is especially during the autumn-winter season, that the gonads of *P. lividus* reach their maximum size and their color more intense, enough to deserve, in some locations as Alghero, the appellation "red gold".
From the bromatologic point of view, this food shows a considerable amount of water (about 80%) and a high protein content (12%) compared to a small aliquot of lipid (2-3%) (Dincer and Cakli, 2007; Mol *et al*., 2008), characterized also by considerable presence of polyunsaturated fatty acids (Martinez-Pita *et al*., 2010). Some precious elements such as iron and phosphorus give this food excellent nutritional qualities. In general, the gonads of *P. lividus* have a low calorie (approximately 150kcal for 100 grams) and are mostly eaten raw. The retail price of each sea urchin lies on average between 0.15 and 0.25 euro, but it is possible to find, in supermarkets, sea urchin gonads packed in small jars 50-70 grams, with an approximate price of around € 15.

1.2.1 Italian Legislation on sea urchin fisheries

The sea urchin fishery, currently, is regulated by Ministerial Decree of January 12, 1995. This legislation comprises five articles whose main points are listed below:

- ✓ Fishing for sea urchin is allowed in professional divers and sportsmen, who can perform it only by immersion and manually, using as only tools for collection the rake (art. 1);
- ✓ Professional anglers may not catch daily more than 1000 specimens; unlike the daily limit for sport anglers is fixed at 50 sea urchins (art. 2);
- ✓ The minimum size of capture of sea urchin is equal to 7 cm in diameter including spine;
- ✓ Professional and sports fishing of sea urchin is forbidden in May and June.

As regards farming, adopting the more generic term, used in legislation, of shellfish farming, is regulated by d.lgs. 530/1992 e s.m.i. and regulations EC 852 and 853 of the April 29, 2004. The latter lay down the health rules for the production and placing on the market of bivalve molluscs, marine gastropods, echinoderms and tunicates. The d.lgs. 530/1992 laying down the health rules for the production and the placing on the market of echinoderms for immediate human consumption or further processing before human consumption.

1.3 The echinoculture

To date, a species is not a subject of interest for aquaculture until the survival of the natural stock of that species and consequently, the earnings and the lifestyle of the fishermen, is not strongly affected by the excessive fishing effort (Robinson, 2003).
Similarly, the growing demand of gonads in recent decades has led to overexploitation of natural populations of echinoids (Keesing and Hall, 1998, Andrew *et al*., 2002) and has begun to grow interest in the aquaculture activities that employ the sea urchin.
Several approaches have been tried, the "seeding" of juveniles from aquaculture facilities (Yokota, 2002b), induction of gonadal growth of sea urchins belonging to natural populations (Fernandez and Caltagirone, 1994, Klinger *et al*., 1997; Lawrence *et al*., 1997; Kelly *et al*., 1998; Robinson and Colborne, 1998; Spirlet *et al*., 2000; Olave *et al*., 2001; Pearce *et al*., 2002 a, b, c, Mortensen *et al*., 2003, James, 2006; Cook and Kelly, 2007; Pantazis, 2009) until the establishment of so-called "closed systems" of echinocoltura where they follow all the stages of the life cycle of the sea urchin, from fertilization of gametes until obtaining adults *P. lividus* of marketable size (Le Gall, 1990; Grosjean *et al*., 1998; Devin, 2002).
For the latter two approaches, there is a strong need to establish what are the feed and feeding regimes that determine the production of gonads of high quality with acceptable cost. The use of

feed formulations is a common element in aquaculture for a several reasons including the easy availability, the constancy of their composition and quality, stability in water and ease of use. These factors are essential for the creation of aquaculture facilities on a large scale of sea urchins (Caltagirone et al., 1992, Lawrence *et al*., 2001). Some feed formulations are available today, including the "diet Lawrence" patented in the United States, a formula developed at the Biological Station in St. Andrews, New Brunswick (Dr. Shawn Robinson), a feed developed in Ross Island Salmon Ltd (Dr. Christopher Pearce), a feed (wet) developed by NIWA (based on a diet formulation developed at the Norwegian Research Institute of Fisheries and Aquaculture Ltd) in New Zealand (Dr. Phil James) and a dry food developed the Institute Research Norwegian Fisheries and Aquaculture Ltd in Tromsø (NIFA-feed) (Woods et al., 2008).
To date, most studies that used feed formulations have examined the effects of these diets on gonadal growth (de Jong-Westman *et al*., 1995a, b; McBride *et al*., 1997, Pearce *et al*. 2002a, b, Pearce *et al*., 2003; James, 2007; Woods *et al*., 2008), the ingestion rate and somatic growth (Klinger *et al*., 1998;. Kennedy *et al*., 2007). For a number of sea urchin species, the gonadal growth has been shown to be faster with formulated feed than those obtained with natural foods, such as algae (reviewed by Lawrence *et al.*, 2001). However, little research has focused on the effects of feed on organoleptic characteristics of the gonads, such as color (Goebel and Barker, 1998; McLaughlin and Kelly, 2001; Robinson *et al*., 2002., Watts *et al*., 1998), taste (Pearce *et al*., 2003; McBride *et al*., 2004, Siikavuopio *et al*., 2007, Woods *et al*., 2008) and the consistency (Pearce *et al*., 2003).
In order to allow a further development of formulated feed and an accurate knowledge of the feeding regimes to be kept in rearing system, we should try to identify what are, the biochemical components of a feed which affect the sea urchin growth and how they can influence the quality of the gonads. At the same time it is important to understand how environmental factors (such as temperature and photoperiod) can influence the growth of the gonads of sea urchins (Basuyaux and Mathieu, 1999; Spirlet *et al*., 2000; Shpigel *et al*., 2004 , McCarron *et al*., 2009).

1.3.1 Maize and Spinach

Maize, also known as “frumentone”, and “granone” is native to America and was introduced in Italy in the 16th century where it had a strong diffusion, at least initially, especially in the Veneto region. The most common and most widespread use of maize is the zootechnical application, especially in poultry, cattle, calves, swine, horses and sheep both in the form of grains, flours, or other feed. The main characteristics that make it particularly suitable to be used as the basis of feed of many animal species are its high productivity, high nutritional value (although substantially save), the presence of easily digestible starch, the contents of xanthophylls and cultivation "easy" and completely mechanized.
With regard to spinach (*Spinacia oleracea* L.), have always been considered of vegetables with a high nutritional value. Spinach are a rich source of carotenoids (yellow, orange or red) even if masked by the presence of green chlorophyll.
Carotenoids are a large class of compounds present ubiquitary in plants, algae and various microorganisms and from chemical point of view can be divided into two large groups: carotenoids and xanthophylls. Carotenoids have a protective effect against certain chronic diseases, cancers and cardiovascular diseases (Britton *et al*., 2008); carotenoids are powerful antioxidants that can effectively remove reactive oxygen species (ROS) by the presence in their structure of unsaturated groups (Cao *et al*., 1997).

1.3.1.1 Applications in zootechnics and beyond ...

Maize since its introduction in Italy has always been employed in the breeding of many animal species; briefly below its main zootechnics applications:

Cattle

Maize is administered to livestock in various forms: as cob, whole grain or wheat, hulled vaporized and crushed. Maize is an excellent food for cows, as for any other type of cattle, although the implementation with other foods that can compensate the protein deficiency is necessary.

Swine

Maize in our country is employed for pig fattening. Maize is particularly suitable for sows, provided that the deficiencies of protein, minerals and vitamins are correct.

Poultry

Also in feeding poultry, maize is considered as the most important cereal and is an irreplaceable basic feed for all categories of poultry; from chicks to hens, broilers to breedings chicken.

Horses

Besides oats, maize is the largest cereal given to horses. In some cases, horses maintain their weight better if fed with hay and maize, instead of just hay and oats.

Aquaculture

In the formulation of diets for fish species, cannot be obviously apart from the economic aspects. Considering that diets contributes for 50% to the cost production of aquaculture system, considerable savings can be achieved by replacing part of the animal-based protein meal (fish) with plant products such as the maize gluten and soybean.

Formulated feed based on vegetable flours seem to be nutritionally adequate, but not very attracting to some species of fish, which respond with a decrease in food intake. For example, diets containing high levels of soy are poorly accepted by salmonids; In contrast, maize is a protein source, extremely appetizing for salmonids. Recent findings show that feed based on mais gluten or a resulted from a combination of maize gluten and soybean flour can replace most of fish meal, without having any effect on productive performance (Tufarelli and Laudadio, 2006).

Regarding the echinocoltura, in the literature have already been reported data concerning the use of maize both for inducing gonadal growth both as regards its use in promoting the maturation of gametes (Basuyaux and Blin, 1998; Luis *et al*., 2005).

Chapter 2
Aim of the Study

2.1 Objectives

The object of this experimental work has been focused on the following aspects:

✓ Defining an acclimatization protocol for *Paracentrotus lividus* in Recirculating Aquaculture System (RAS);
✓ Defining a maintenance protocol for mature *Paracentrotus lividus* in RAS in order to achieve a continuous availability of gametes and embryos of that species, aiming to ensure the execution of ecotoxicological bioassay, over the spawning period of this species. Within this research theme have been tested seaweed and mais and spinach diets;
✓ Identification of optimal diets to ensure rapid gonadal growth and promote maturation of gametes of *Paracentrotus lividus* in RAS system. Under this research theme three diets were tested; a maize and spinach diet, a seaweed diet and a pellet (Classic K®) normally used in fish farming activities.

2.2 Experimental plan

The research project has provided during the three years following three experimental phases:

I. Defining an acclimatization protocol for adult Paracentrotus lividus organisms in Recirculate Aquaculture System (RAS):

In this preliminary phase, that preceded the testing of maturation protocol and the experiment for the maintenance of *Paracentrotus lividus* mature stage, were put in place all the procedures to minimize stress on organisms both during the transport from their natural habitat to the rearing tanks both during the acclimatization phases which preceed the experimental trials.

II.Determination of ingestion rates for tested diets:

In order to avoid an excessive waste of food to be used in the experiments, for the optimization of potential future trials and to avoid an excessive load of nutrients that could compromise water quality in the rearing aquarium were estimated the ingestion rates of tested foods.

III. Development of protocols for the maintenance of mature Paracentrotus lividus:

Were tested two different diets to ensure the maintenance of mature stage in adults *P. lividus* in RAS. 100 organisms, ranging in size between 40 and 45 mm in diameter, were collected from field and divided into two pools (three replicates each). Two different diets were tested on organisms: the first based on macrophytes, collected from the sampling site of sea urchin, and maize and the second composed of maize and spinach.
As already reported in literature, maize seems to have a positive effect in encouraging the gametogenesis and gonadal growth (Basuyaux and Blin, 1998; Luis and Gago, 2005). Spinach were employed in diet to assess any beneficial effects of carotenoids on gonadal maintenance. On N = 10 sea urchins were assessed weight, diameter, gonadal index and the quality of the gametes at the beginning of trials (T = 0) by means ecotoxicological test with reference toxicant ($Cu(NO_3)_2*3H_2O$). The evaluation of the quality of the gametes of reared organisms was performed every 30 days.

IV Development of protocols to induce gonadal growth, gametes maturation and for the maintenance of sexual maturity:

La crescita gonadica e l'avanzamento del processo di maturazione degli individui è stato valutato ad intervalli di 3 settimane.
With regard this experimental phase, sea urchin collected (N=120) were starved for 6 weeks in order to leads to consumption of the possible content of the gonads, which also act as storage organ, and to get in phase sea urchin regarding their reproductive cycle. Subsequently, organisms were divided in four aquaria (three replicate each) and the following three artificial diets were tested:

- ✓ Maize&Spinach,
- ✓ Macrophytes,
- ✓ Pellet Classic K®.

On N = 10 sea urchins were assessed weight, diameter, gonadal index and the quality of the gametes at the beginning of trials (T = 0) by means ecotoxicological test with reference toxicant ($Cu(NO_3)_2*3H_2O$). Gonadal growth, gametes quality and sexual maturation were assessed every three weeks.

V Validation of protocols for maintaining mature organisms and induce sexual maturation in Recirculating Aquaculture System by assessing the quality of gametes and embryos:

Validity of protocol for the maintenance of mature stage in *P. lividus* reared in RAS was evaluated by means the following analysis:

- ✓ Spermio and embryotoxicity test with reference toxicant ($Cu (NO_3)_2*3H_2O$),
- ✓ Evaluation of gonadal weight and gonadosomatic index (GI),

Results obtained were compared with those obtained from sea urchin belonging to natural population.
Validity of protocol for the induction of maturation of *P. lividus* in RAS with artificial diets was performed by:

- ✓ Spermio and embryotoxicity test with reference toxicant ($Cu (NO_3)_2*3H_2O$),
- ✓ Evaluation of temporal trends of gonadal weight and gonadosomatic index (GI),
- ✓ Evaluation of maturation by histological examination,
- ✓ Determination of Righting Activities Coefficient (RAC),
- ✓ Evaluation of Sperm motility,
- ✓ Analysis with harmonic generation microscopy (HGM) on plutei obtained from reared in order to evaluate apoptotic effect.

Even in this case, results obtained were compared with those obtained from sea urchin belonging to natural population.

Chapter 3
Materials and Methods

3.1 Echinoculture facility

Four Aquarium, 100 liters each, were set up in and filled with filtered sea water (45μm), collected along the Tyrrhenian coast near Castiglioncello (LI) [43 ° 25'31 .79" N, 10 ° 23 '37.51" E] from an area free from human impacts. Each tank was equipped with a refrigerator HAQUOSS ARTIKA 600 and skimmer pump Blue Bios 300. In order to remove most of the catabolite resulting from sea urchin food, skimmer was oversized compared to the size of the tank. The rearing system is also equipped with a double UV system (Tetratec UV 400) equipped with 10 watt low pressure mercury vapor lamp which emits at a wavelength of 254 nm to optimize the germicidal effect. Each UV system ensures the sterilization of two tanks.
Each tank has a biological filter compartment composed of three chamber. The first chamber contains perlon wool as mechanical pre-filter, the next chamber contains support for bacteria (ceramic razor clams), the third chamber host the pump that circulates the water in the aquarium. Circulation of water inside aquarium is the most critical factor, because it must assure gas exchange to the entire water volume. In order to assure the maturation of biological filter, during the first 5 weeks no organisms were placed into the aquarium. In this early phase a photoperiod of 8-10h L:14-16h D was employed by using fluorescent T8 by 30 watt.

3.1.1 Chemical and physical parameters

During all the experimental phase periodic checks of temperature, salinity, pH, dissolved oxygen, ammonia, nitrite, nitrate and phosphate, at least every 48 hours were performed. The analyzes on the water samples were performed by means of spectrophotometer Hach Lange D3900 equipped with thermoreactor LT 230. Dissolved oxygen, pH and temperature were monitored by directly immersing the sensor EUTECH PCD 650 in the tub. The salinity was measured by refractometer. The parameters were monitored every 48h. during the phase of maturation of biological filter and weekly during the phases of the testing of various protocols.

3.2 Organisms collection

The organisms were collected in Fortullino, in the province of Livorno (LI), at the same sampling site where water were collected for the preparation of the aquarium. The sampling operation was carried out under optimal environmental conditions. The objective is avoiding to sample the organisms during or immediately after the storms, conditions that may increase the stress of organisms and induce the spawning. Moreover, to avoid any problem during transport to the laboratory or at the time of placing animals in the aquarium, organisms were transported, in a suitable refrigerated container, wrapped in absorbent paper soaked in sea water. Once sampled, was measured by calliper (accuracy 0.05 mm) the size of the sea urchin , in order to select those with a diameter theca (spines excluded) ranging from 40 to 45 mm. As demonstrated by Fernandez and Boudoresque (2000), organisms ranging from 35 to 45 mm, are the size class which has a greater energy investment in the reproduction, unlike the juvenile stages where one has a greater use of energy in the growth of test and the lantern of Aristotle. The collection of organisms during all phases of experimentation has been carried out in the period January-April, the period during which the temperature of the water in the sampling site varies from 13 to 16 °C. At the time of collection, to avoid thermal shock following the placing of the sea urchin in the aquaria, the water temperature was measured in the sampling site so as to set the temperature of the rearing tanks at the same temperature.

3.3 Acclimatization in Recirculating Aquaculture System (RAS)

Once transported to the laboratory organisms were slowly introduced into the aquaria. The water temperature in this early phase has been maintained equal to that present at the sampling site (14 °C) to avoid thermal shock. For the first five days organisms were maintained at the temperature of 14 °C, without food and with a photoperiod of 8h L: 16h D. Subsequently, the organisms were fed for two weeks, with a diet consisting exclusively of algae maintaining a photoperiod of 8h L: 16h D. Every five days the water temperature has been changed 1 °C up to bring it to the desired temperature of breeding. Daily fecal pellets were removed from the bottom of each tank.

3.4 Maintenance of *Paracentrotus lividus* in mature stage in RAS

Adults of *Paracentrotus lividus* (N = 100), with test diameter ranging from 40 to 45 mm were collected in a sub-littoral zone along the coast of Livorno (Fortullino). At the time of sampling, the water temperature was found to be 16 °C. Once arriving at the laboratory, N = 15 organisms were dissected to evaluate the weight of the gonads and gonadal index (GI) of the natural population at the time of sampling (T = 0). Remaining sea urchins were acclimated for 5 days at 16 °C without food (Fernandez and Pergent, 1998). The following two weeks, sea urchins were fed with seaweed taken from the sampling site; the photoperiod was set to 8H L: D 16H (Short Day). Water temperature was varied by 1 °C every 5 days until reaching the rearing conditions (T = 14 °C). Photoperiod was increased daily about 9 minutes until reaching L 10H: 14H D condition. Subsequently organisms were divided into two groups, three replicates each, and fed with two different diets. The two diets were administered *ad libitum*, (Garmendia, 2009; Shpiegel, 2004; Spirlet *et al*., 1998), and daily were removed from the aquaria the fecal pellets. Every 30 days, N = 10 organisms, were dissected to evaluate the weight and the GI. The quality of the gametes was assessed through the preparation of spermio and embryotoxicity test with reference toxicant [$CuNO_3$*$2H_2O$].
With the same frequency in time, where possible, the same analyzes were conducted on natural populations of organisms belonging to Fortullino (Livorno).

3.4.1 Use of diet based on maize and seaweed for the maintenance of sexual maturity of Paracentrotus lividus

For the maintenance of adult of *Paracentrotus lividus* in stage of sexual maturity we employed a diet consisting of 50% Maize, previously reduced to grains of a few millimeters, and the remaining 50% of algae collected from the sampling site of sea urchins. The seaweed mixture, was essentially composed, in varying amounts depending on the availability in the sampling period, by the following species:

- ✓ *Dyctiopteris sp.*
- ✓ *Padina Pavonica*
- ✓ *Dyctiota sp.*
- ✓ *Ulva lattuga*
- ✓ *Halopteris scoparia*
- ✓ *Flabellia petiolata*
- ✓ *Laurencia sp.*
- ✓ *Corallina elongata*
- ✓ *Codium sp.*

The diet was administered twice a week for a quantity of 15 grams of maize (wet weight) and 15 grams of algae (wet weight) per replica. The seaweeds before being weighed were dried on absorbent paper for five minutes. Prior to administration of food, from each aquarium, were removed fecal pellets and food not consumed in the preceding days, taking care to remove, from the sea urchin aboral surface, fragments of maize and algae, without impair organisms.

3.4.2 Use of diet based on maize and spinach for the maintenance of sexual maturity of Paracentrotus lividus

Second diet tested in this experimental phase was a diet consisting of 15 grams (w.w.) of maize and 15 grams (w.w.) of fresh spinach leaves chopped, for each of the three replicates of treatment.

3.5 Experimentation of diets stimulating gonadal growth and sexual maturation

To promote the maturation of adult *Paracentrotus lividus* have been tested in combination with a photoperiod of 10h L: D 14h and water temperature of 16 °C, the following three diets: a pellet used in fish farming (Classic K® Hendrix S.p.A.), a natural diet based on algae and a diet based on maize and spinach.
Adults P. lividus (N = 120) with test diameter ranging from 40 to 45 mm (Fernandez and Boudouresque, 2000) were sampled from an intertidal rocky site along the coast of Livorno (Italy), far from industrial or agricultural discharges. Once arrived in laboratory N = 15 organisms were dissected to determine the weight of the gonads and their gonadal index (GI) at the beginning of the experimental trials (T = 0). Sea urchins were starved for 2 months, before being fed *ad libitum*. This leads to consumption of the possible content of the gonads, which also act as storage organs, in order for the animals to get in phase regarding their reproductive cycle (Spirlet *et al*., 1998a). Sea urchins were later located in 4 aquaria within which 3 replicates were arranged with glass plates.

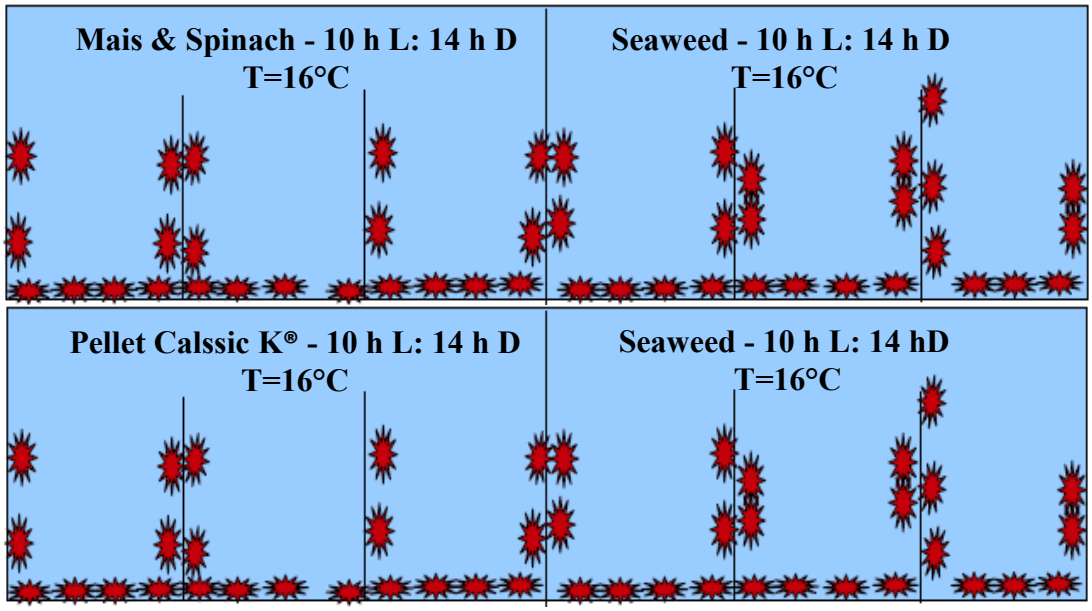


Fig. 3.5.1. Schematic description of rearing system employed during experimental trial to induce sexual maturation of *Paracentrotus lividus.*

Every 3 weeks were evaluated the sensitivity of gametes towards the reference toxicant and the gonadal index progression in organisms reared with three different diets and in those belonging to the natural population. Were also determined the maturation stage by histological analysis, evaluated the sperm motility, estimated the Righting Activities coefficient (RAC) and the presence of any apoptotic signals in plutei obtained from reared organisms by means of harmonic generation microscopy technique (HGM).
Subsequently, in order to better evaluate the progression of maturational stages of *P. Lividus,* reared in Recirculating Aquaculture System, in the light of the results gonadosomatic index (GI), have been conducted for the diet Maize & Spinach, histological analyzes on adults *P. lividus* reared in the same manner used in this experimental phase. In this regard, forty sea urchins, with diameter between 40 and 45 mm, are been subjected to six weeks starving period and subsequently fed with maize and spinach.
Every three weeks, N = 10 sea urchins were dissected to carry out histological analysis of gonadal tissue and for the calculation of gonadal index (GI).

3.5.1 Starving

Once sampled, adult *P. lividus* (test diameter between 40 and 45 mm) were subjected to a starving periods for 6 weeks. Sea urchins were kept in the aquaria with 12 °C water temperature, and photoperiod 12H L: 12H B completely devoid of food (Grosjean *et al*., 1998). After 6weeks N = 10 organisms were dissected and were evaluated, by histological analysis, the maturation stage.

3.5.2 Pellet diet

In assessing the induction of maturation of *P. lividus* was employed pellet Classic K® (Hendrix S.p.A), with the following biochemical composition:

Table 3.5.1. Biochemical Composition (%) of pellet Classic K® (Hendrix S.p.A.)

	Composizione %
Protein	43.0
Crude Fat	11.5
Crude Fiber	3.2
Ash	8.0
Phosphorus	0.8
Digestible Energy (MJ/kg)	14.8

And the following ingredients:

Table 3.5.2. Main ingredients of pellet Classic K® (Hendrix S.p.A.)

Dehulled toasted soybean meal
Corn gluten
Fish meal
Wheat
Fish oil
Rape seed oil
Sunflower meal
Soybean oil
BHT

The pellet was administered daily in an amount equal to 1.8 to 2% of the mass of the individuals present in the aquaria, as recommended by the manufacturer. Food administered, as demonstrated in the course of the trial, has proved to be a significantly more than the requested food organisms, that so they could have plenty of food. The biomass of organisms present in the tank was initially estimated on the basis of the following relationship:

$$FW\ (g) = 5.50*10\text{-}4\ D^{2,94} \qquad R^2 = 0.997$$ (Grosjean, 2001).

where FW is the fresh weight of the organism estimated on the basis of the diameter (D), expressed in millimeters of the organism. The pellets not consumed daily has been removed from aquaria.

3.5.3 Maize and Spinach Diet

The diet used in this phase is the same used in the testing the protocol for the maintenance of sexual maturity of *Paracentrotus lividus* . The diet is composed of 50% by Spinach and 50% of granules of maize; however, in this experimental phase, since the food resource should not be, in any way a limiting factor, diet was administered *ad libitum*. Daily were removed residues not consumed.

3.5.4 Macrophytes Diet

This diet is entirely made up of algae taken from the sampling site of *Paracentrotus lividus* and belonging to genus reported in section 3.4.1. As for diet "Maize&Spinach" algae were administered *ad libitum* and seaweed not consumed the previous day were removed from the tanks.

3.6 Ingestion rates

The ingestion rates were estimated, each month, during the testing of the protocol for the maintenance of sexual maturity of *Paracentrotus lividus*. Over a 3 days, for each type of given food, were evaluated the difference, in dry weight, of the given food and that not consumed. The food over this three days period was administered daily and every 24 h, the uneaten food was removed, dried in an oven and weighed. Beforehand, for each type of administered food, was estimated, by means drying at 60 °C and weighted, the dry weight.

3.7 Validation of protocols for the maintenance of sexual maturity and the induction of maturation in *Paracentrotus lividus*

To assess the effectiveness of the protocol for acclimatization and maintenance of sexual maturity in adults *Paracentrotus lividus* were performed the following analysis:

- ✓ Spermiotoxicity tests with sea urchin *Paracentrotus lividus*,
- ✓ Embryotoxicity test with sea urchin *Paracentrotus lividus*,
- ✓ Calculation of Gonadosomtic index (GI),

to verify the validity of the protocol for inducing sexual maturation of *P. lividus* in Recirculate Aquaculture System were carried out the following analysis:

- ✓ Spermiotoxicity tests with sea urchin *Paracentrotus lividus*,
- ✓ Embryotoxicity test with sea urchin *Paracentrotus lividus*,
- ✓ Determination of sperm motility,
- ✓ Calculation of Gonadosomatic index (GI),
- ✓ Evaluation of the Righting Activities Coefficient (RAC),
- ✓ Histological examination
- ✓ Analysis by Harmonic Generation Microscopy (HGM) and Two Photon (2PF) technique.

3.7.1 Spermiotoxicity tests

To assess the fertilization success of sperm obtained from organisms reared in Recirculating Aquaculture System were carried out tests with reference toxicant. The EC_{50} values thus obtained were compared with the results obtained from organisms belonging to natural population and with the control chart of the STS ISPRA laboratory of Livorno where the tests were performed.
The test involves exposure of 100 µl diluted sperm solution toward the reference toxicant [$Cu(NO_3)_2*3H_2O$], in order to evaluate the fertilization success with respect to a negative control.

The sperm solution was added to each test chamber and then were incubated at 18 ± 1 ◦C for 1 hour. Subsequently, 1 ml of eggs suspension was added to each test tube and after 20 min (time allowed for eggs fertilization) the test was stopped by adding 1 ml of 40% formalin.
The sperm: egg ratio employed was 15000:1 with 1000 eggs in 10 ml of test solution.
At least 100 eggs from each tube were examined and scored for the presence or absence of a fertilization membrane. The decrease in fertilization rates, with respect to control of natural filtered seawater, was evaluated.

3.7.1.1 Test preparation

Four replicates were set up for each of the following samples:

- ✓ a negative control;
- ✓ a positive control represented by increasing concentrations of the reference toxicant (Cu $(NO_3)_2$*$3H_2O$).

The temperature of each aqueous solution has been maintained at 18 ± 1 °C and the final volume in each test chamber was 10 ml.

3.7.1.2 Gametes collection

The recovery of the animals from the aquarium has been carried out with great care to avoid damaging the tube feet. Once recovered, sea urchins were placed in a bowl covered with tissue paper soaked in sea water, to avoid the animals will stick at container. To prevent the accidental fertilization of eggs were used two different operators for the independent collection of sperm and eggs.
Sea urchins were induced to spawn by injecting 1 ml of 0.5 M KCl solution into the coelom through the peristome, as suggested by Tyler (1949). Animals were vigorously shacked in order to stimulate the spawning and to ease the distribution of KCl on gonads. If after the first injection the gametes have not been released, a second injection has been executed. If after the second injection weren't collected gametes the animal was discarded.
Eggs from each female were shed into 50-ml beakers previously filled with filtered (45μm) sea water (FSW), by positioning the female genital with the pore towards the water. Later the eggs of each female were collected with a 2 ml pipette and examined under a microscope to determine their maturity (were discarded vacuolated eggs, irregular or small). A preliminary fertilization test was also carried out, adding to each sub-sample of eggs, representative of each female, a small amount of sperm solution. Eggs that were not fertilized in a short time (20-80 sec) have been discarded. Secondly, mature eggs were pooled and left decanted into a 1-l beaker and washed with natural filtered seawater. Decanting, rinsing and settling processes has been repeated several times to remove damaged eggs, which tend to float, and to reduce the amount of egg jelly, which could interfere with fertilization (Chapman, 1995). During and after washing, the eggs were stored at 18 ± 1 ° C.
The sperm was collected "dry" directly from the surface of the sea urchins using a Pasteur pipette and stored in eppendorf at 4 ° C.

3.7.1.3 Gametes counting

The eggs concentration was determined in a 10 ml test tube, adding 0.1 ml of the final suspension of the eggs (maintained in suspension) and bringing to volume with sea water (FD = 100). The eggs

count was performed by means of an optical microscope (10x), taking 1 ml from the solution thus prepared. Based on this count, the eggs suspension was concentrated or diluted until reaching a fixed number of 1000 eggs / ml. The eggs were stored at 18 ± 1 ° C until test execution. The sperm concentration was determined adding 50µl of sperm in a 25 ml test tube, and bringing to volume with fresh water (FD = 500). The sperm count was performed on a hemocytometer (Thoma chamber) under a microscope at 40×. Then, once the dilution of semen necessary to obtain a 15.000:1 sperm/egg ratio per test chamber was determined, the necessary aliquot of semen was accordingly diluted.

3.7.1.3.1 Sperm counting by Thoma chamber

For the count of sperm has been used a Thoma chamber of double-grating. The flat base of this special optical glass has the size of the slide of the microscope. The chamber has two sets of etched gratings for counting, whose depth is 0.1 mm. When a coverslip is placed above, there is a difference of 0.1 mm between the glass and the central chamber. Each grating has 16 square fields with side of 0.2 mm in turn divided into 16 mini-squares with an area of 0.0025 mm^2 (Fig. 3.7.1.3.1).

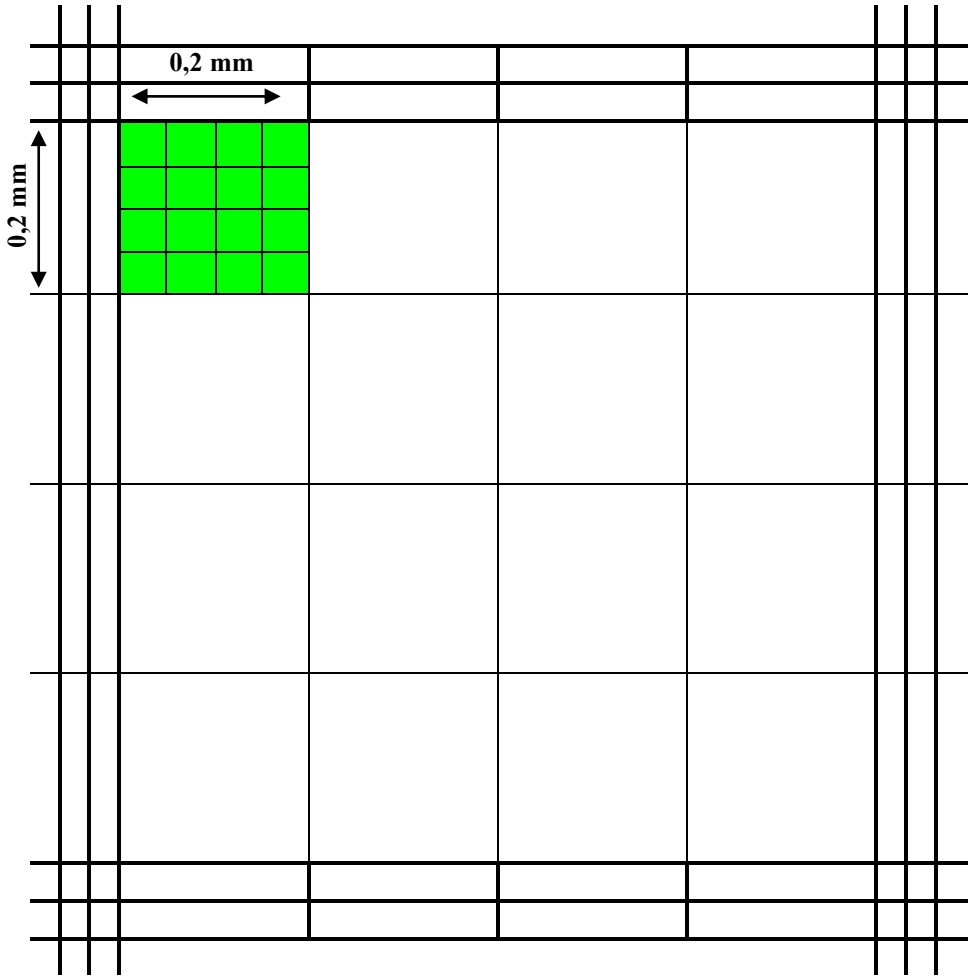


Fig. 3.7.1.3.1. Schematic representation of a field in the grating of Thoma chamber.

The volume of each field is equal to:

$$Voume\ di\ 1\ field\ (V) = 0{,}2 * 0{,}2 * 0{,}1 = 0{,}004 = 4 * 10^{-3}\ mm^3$$

For each determination of sperm density, a suspension was prepared by diluting 50µl of sperm in a 25 ml test tube with fresh water (FD = 500). Thus, sperms present in 5 fields were counted and it was determined the mean value of sperm per field . The number of spermatozoa per mm3 was determined by the following formula:

$$N° \text{ sperms for 1 } mm^3 \text{ (x)} = \frac{M \times 1}{V}$$

where M is the average of sperm per field and V is the volume of a single field of the Thoma chamber reticle
The number of sperm in the starting solution was so determined:

$$N° \text{ sperms in the starting solution (Y)} = x \times 1000 \times FD$$

where x is the number of spermatozoa in the starting solution and FD the dilution factor. In our case the diluiction factor is FD = 500.
The dilution factor to be used to prepare a solution with 15000:1 sperm:egg ratio was determined by the formula:

$$\text{Sperm Diluiction Factor (fd)} = \frac{Y}{15x10^6} x\ 0,1$$

Where:

- ✓ $15x10^6$ is the total amount of sperm to be introduced in each test chamber in such a way that the final sperm / egg both ratio of was 15.000:1.
- ✓ = 100 µl final solution of sperm to be introduced into each tube.

3.7.1.4 Test Execution

The test was performed the same day of gametes collection. The sperm cell test was performed by exposing 100 µl of the final sperm solution in 10 ml of test solution. The semen was exposed to the tested solutions for 1 hour at a temperature of 18 ± 1 ° C, then 1 ml of natural filtered seawater containing 1000 eggs was added and after 20 min, time allowed for eggs fertilization, the test was stopped by the addition of 1 ml of paraformaldehyde.

3.7.1.5 Reading of results

Once the test was stopped and eggs were settled, samples were concentrated by pipetting off most of the overlying solution and a subsample of the concentrated eggs was placed into a counting slide. At least 100 eggs from each tube were examined and were evaluated the percentage of fertilized eggs (eggs, whose membrane fertilization is completely or partially visible). Have not been considered immature eggs or damaged ones.

3.7.1.6 Results validity

The bioassay was considered invalid if the following conditions were not achieved.

- ✓ The fertilization percentage in the negative control ≥ 70% but less than 100%.
- ✓ The standard deviation between replicates of the same sample was less than 5%

The Abbott's formula has been applied, in order to consider the number of unfertilized eggs in the control (Finney, 1971) and according to it, the relative percentage of unfertilized eggs in each treatment was compared and normalized to that in the control.

$$Abbott = (x - y) \times 100 \,/\, 100 - y$$

where:
x=% Effect in the tested sample;
y =% effect in the control.

The values thus obtained were used in the automatic calculation of the EC50 value by using the Trimmed Spearman-Karber method (Hamilton *et al*., 1978).

3.7.2 The embryotoxicity test

Similarly to fertilization tests, the embryotoxicity test, with reference toxicant, was performed to assess the quality of embryos obtained from gametes collected from reared organisms .
The test involves the exposure of a defined number of zygotes to the toxic substance, in order to evaluate the success of embryonic development until the stage of larva (pluteus) compared to a negative control. The test was performed in polystyrene six-well plates (Ø 34.6 mm) IWAKI® . Zygotes were incubated in a dark room at 18°C for 72 h. The sperm: egg ratio chosen was 20,000:1 with 2000 eggs in 10 ml of test solution. The percentage of plutei with normal development in each treatment was determined by observing 100 larvae. The gametes collection and the counting procedure were performed as reported in sections 3.6.1.2 and 3.6.1.3.

3.7.2.1 Test Execution

Four replicates for each of the following samples were set up:

- ✓ a negative control;
- ✓ a positive control represented by increasing concentrations of the reference toxicant

The test was performed at 18 ± 1 ° C.
.

3.7.2.2 Reading of results

The percentage of normal plutei was determined, by means of an inversion microscope. At least 100 larvae were counted for each well of the test chamber..

3.7.2.3 Results validity

Test was considered valid when the following conditions are verified:

- ✓ The percentage of larvae in the negative control was greater than 70% but less than 100%,
- ✓ The standard deviation between replicates of the same sample, was lower than 5%.

Percentage of normal plutei have been corrected according to Abbott's formula (see par. 3.6.1.6).

3.7.3 Evaluation of sperm quality

The sperm motility of *Paracentrotus lividus* was carried out by analyzing the sperm motility, expressed in classes (0 to 5), based on the percentage of spermatozoa with rapid, vigorous and linear movement (RVL), according to the correlation given by Fabbrocini *et al*., (2000) and reported in (Tab.3.6.1).
Sperm motility parameters of reared urchins were therefore compared to those of urchins collected from wild population during the execution the experimental trial.

Table 3.6.1. Classes of Motility in Relation to the Percentages of Spermatozoa with Rapid, Vigorous, and Forward Motility (da Fabbrocini *et al.,* 2000).

% spermatozoa RVL	0	5	10	15	20	30	50	65	80	90	100
Classes of motility	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5

The analysis of sperm motility was conducted by analyzing the following parameters:

- ✓ activation time: semen behavior in the first few minutes post-activation, recording the achievement of the highest class of motility;
- ✓ maximum class motility: maximum class of sperm motility achieved;
- ✓ maximum sperm motility duration: time period during which sperm shows a motility class≥ 3 (span class 3);
- ✓ Total motility duration: monitoring of sperm motility activation to loss of motility (achieving class 0);

3.7.4. Righting response

The righting behaviour is a well documented characteristic of echinoderm (Hyman, 1955) and reflect the functioning level of neuromuscular systems regulating locomotion. The righting activities coefficient (RAC) has been used widely used to assess stress condition in response to environmental variables (Diehl *et al*., 1979; Himmelman *et al*., 1984; Forcucci and Lawrence 1986; Lawrence and Cowell, 1996). The righting time corresponded to the time required by sea urchin to turn back over once it had been placed on its aboral face. The righting activities coefficient (RAC) was determined through the formula of Percy (1973):

$$RAC = \frac{1000}{righting} time\ (s)$$

Those individuals that after 10 minutes showed no response has been assigned, in accordance with Turquin-Joly *et al*., (2009), a RAC value of 1000 / (2 * 600).
The righting activities coefficient was assessed for organisms reared in the aquarium during the validation of the protocol for the induction of sexual maturation. RAC Values from reared organisms were compared to those obtained from natural populations in order to evaluate the state of health of organisms.

3.7.5 Gonadal weight and gonadosomatic index (GI)

For each specimen was measured:

- ✓ diameter (excluding spines) with calliper;
- ✓ total wet weight of the individual (g);
- ✓ wet weight of the five gonads (g).

The wet weight of individuals and the wet weight of the gonads were used to calculate the gonad index (GI) (Lozano *et al*., 1995):

GI = (*wet weight of 5 gonads / wet weight of the sea urchin*)*100.

3.7.6 Histological examination

The analysis of the gonads maturation stages have been made through the preparation of histological sections, a technique that has provided the following steps:

- ✓ Sample taking of tissue and fixation;
- ✓ Rinsed;
- ✓ Dehydration;
- ✓ Clarification;
- ✓ Inclusion in paraffin;
- ✓ Slicing by microtome;
- ✓ Stretching of tapes of paraffin;
- ✓ Coloring;

3.7.6.1 Sample collection and fixation

Small pieces of sea urchins gonads, have been taken by the dissected organisms and fixed in 10% paraformaldehyde solution. Fixation is used to block the vital activity of the cell, making insoluble structural components, stabilizing the proteins and inactivating the hydrolytic enzymes.

3.7.6.2 Rinsing

The following day the samples were placed in fresh water for 24h, changing the solution at least 2-3 times over the course of 24h. At the end of this phase the samples were placed in a solution of ethyl alcohol 70° for their conservation.

3.7.6.3 Dehydration

The dehydration, functional to remove the aqueous component that would not allow the entry of the paraffin in the tissues, was performed by exposure of the samples to the following scale ascending alcohol:

- ✓ 1 h ethyl alcohol 70°;
- ✓ 1 h ethyl alcohol 85°;
- ✓ 1 h ethyl alcohol 96°;
- ✓ 1 h absolute ethyl alcohol;
- ✓ 1 h absolute ethyl alcohol.

3.7.6.4 Clarification

This phase, which is necessary to make the dehydrated piece of tissue, diaphanous and penetrable to the paraffin, was performed by exposing the sample to solutions of Histolemon according to the following recipe:

- ✓ 1 h absolute ethanol / histolemon 1:1;
- ✓ 30 min. histolemon;
- ✓ 30 min. histolemon 57 ° C.

3.7.6.5 Inclusion in paraffin

The sample, dehydrated and clarified, was placed in an oven at 57 °C in tubs of steel containing liquid paraffin. The sample was left overnight immersed in the paraffin at 57 °C. The following morning on each sample was mounted an ABS stirrup in order to ease the cutting operation at the microtome. The included tissue was allowed to cool.

3.7.6.6 Cutting and colouring operations

Samples were cut by using microtome into 7μm thickness slices and mounted on slides wet with solution of Albumin glycerol (12 drop/100ml bidistilled water). The slides were allowed to dry at 37 ° C and subsequently we proceeded to staining on the basis of the following protocol:

- ✓ 8 Min histolemon 1;
- ✓ 8 Min histolemon 2;
- ✓ min absolute ethyl alcohol;
- ✓ min 96 ° ethyl alcohol;
- ✓ 2 min 75 ° ethyl alcohol;
- ✓ 2 min 50 ° ethyl alcohol;
- ✓ 2 rinses in distilled water bi;
- ✓ 10 min Mayer's haemalum;
- ✓ 2 rinses in distilled water bi;
- ✓ 15 min water fountain current;
- ✓ 2 distilled water rinse;
- ✓ 7 sec eosin (0.5% acetic acid added 1 drop per 20 ml);
- ✓ rinsing in distilled water bi;
- ✓ Quick Step ethyl alcohol 75 °;
- ✓ Quick Step in ethyl alcohol 96 °;
- ✓ Quick Step in absolute ethanol;
- ✓ 2 min absolute ethyl alcohol 1;
- ✓ 2 min absolute ethyl alcohol 2;
- ✓ 2 min absolute ethyl alcohol 3;
- ✓ 2 min histolemon;
- ✓ 2 min histolemon.

On stained sample has been mounted a coverslip with a drop of Lemonvitrex. The slides were allowed to dry at 37 ° C and then observed with an optical microscope.

3.7.7 Harmonic generation (HGM) and two photons (2PF) microscopy

The second and third harmonic generation microscopy (SHG-THG) and the 2-photon microscopy (2PF) are nonlinear microscopy techniques which base their optical resolution on the interaction of the wavelength of light with matter. The microscope used for this work is based on a femtosecond laser Cr: forsterite which operates around 1230 nm. This laser are able to penetrate deep into the tissue causing little damage compared with the common Ti: sapphire laser used in fluorescence microscopy (700-1000nm). The laser was mounted on an Olympus BX51 microscope, and plutei obtained with gametes of reared organisms in recirculating aquaculture system were observed with an objective 60X immersion and numerical aperture (NA) of 1.2. (Fig. 3.11.1) at the Molecular Imaging Center, National Taiwan University, Taiwan.

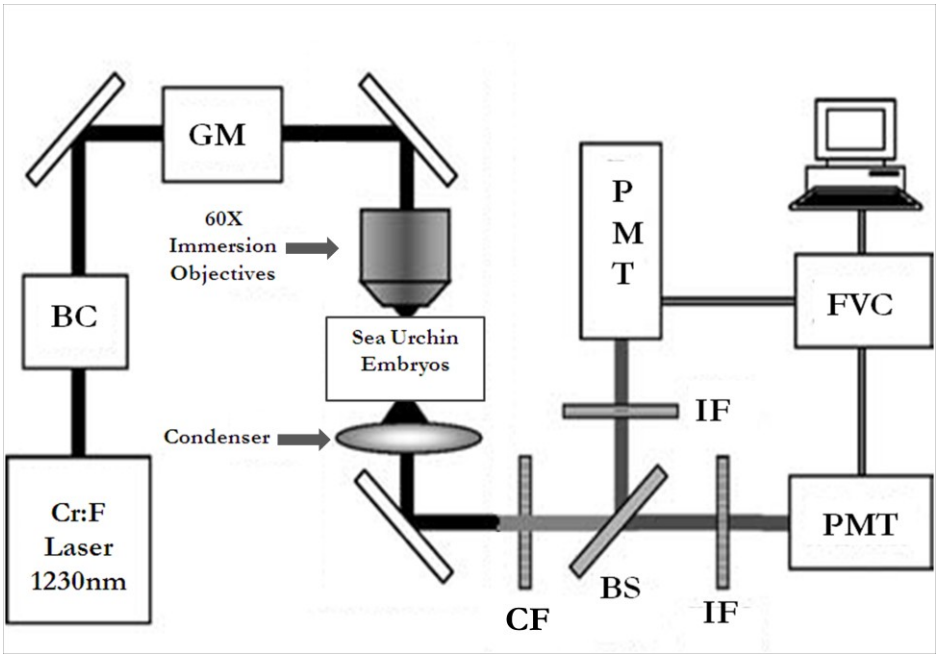


Fig. 3.7.7.1. Schematic diagram of the microscope: BC beam collimator; GM, galvanometric mirrors; CF, color filter; BS, beamsplitter; IF, interference filter; PMT, photomultiplier tube; FVC, Fluoview control unit. (By C.-K. Sun *et al.* 2004/ Journal of Structural Biology 147 (2004) 19–30, modified).

The harmonic generation (HGM) and the 2-photons microscopy techniques are non-invasive methodologies of laser scanning microscopy that allow to acquire signals coming from autofluorescent samples, with submicron spatial resolution without the use of fluorescent markers (Sun *et al*., 2004). In copepods and zebrafish, these techniques have been shown to be able to reveal the onset of cell death mechanisms (apoptosis) (Sun *et al*., 2004, Chen *et al*., 2006; Buttino *et al*., 2011).

In particular, the signals obtained by SHG and THG microscopy fall within the range of visible wavelengths, making this technique compatible with optical microscopy. In particular, the signal of the SHG can reveal the distribution of structural proteins such as collagen, the microtubules, neurons and muscle fibers, while the signal of the THG can highlight, through the discontinuity of the refractive index, the morphology of cell membranes and of lipid vesicles. In two-photon microscopy the signal obtained is linked to the presence of biline, porphyrins, chlorophyll and their metabolites.

In this study, for the first time, these innovative techniques have been applied on sea urchin plutei obtained from gametes of reared organisms. Results obtained from breaded organisms were compared with those obtained from organisms belonging to the natural population in order to highlight possible events of cell death or abnormalities development caused by the rearing conditions and diets employed.

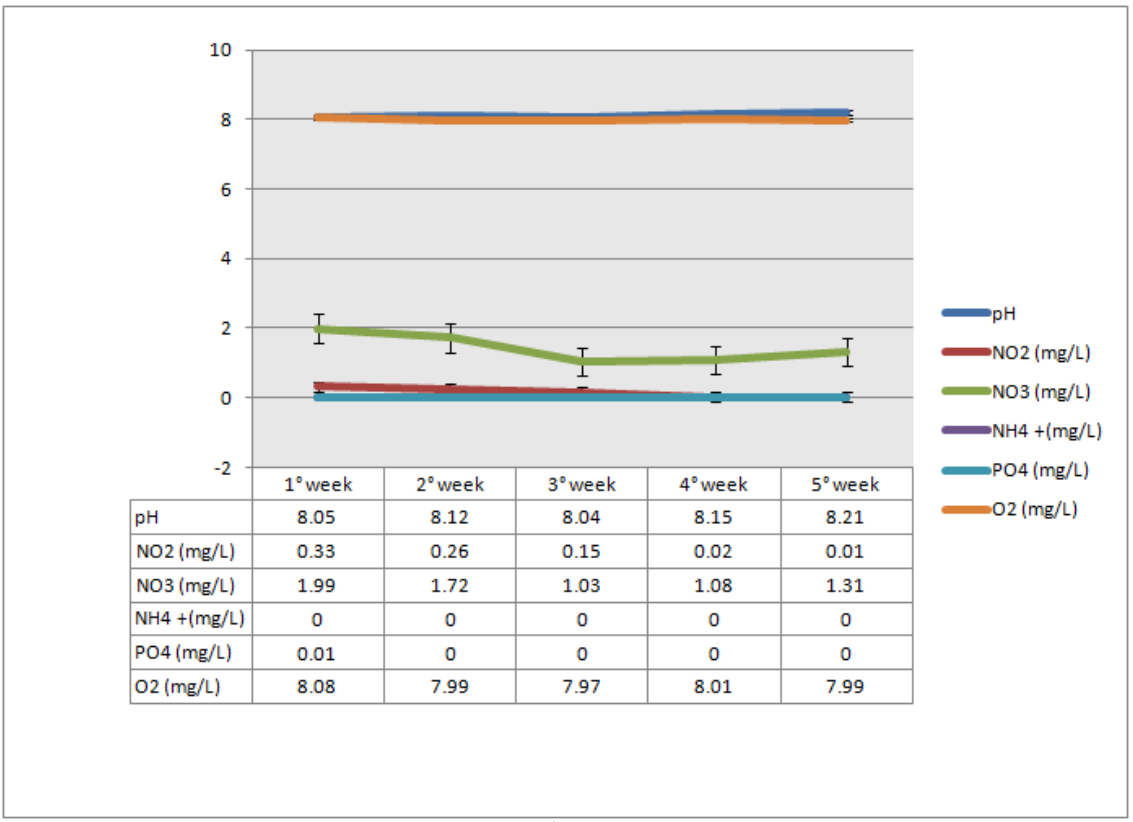
3.7.8 Statistical analysis

The tests with reference toxicant conducted on gametes obtained from farmed organisms in RAS were performed at least 3 times with 4 replicates for each concentration tested ($N \geq 12$)
For each treatment, within each aquarium were identified by means of movable septum $N = 3$ replicates. The gonads weight and the GI values for each treatment were conducted at least on $N \geq 10$ organisms.
The statistical analysis was conducted by performing one way and two-way ANOVA, in relation to the type of data to be analyzed, for the calculation of any differences between treatments (diets), between the time of exposure and for the interaction "time x treatment".
Where it was not possible to perform an ANOVA analysis, the t test was used to check significant differences between two groups of samples. A value of $p < 0.05$ was chosen as level for significance.

Chapter 4
Results and Discussion

4.1 Acclimatization in Recirculating Aquaculture System (RAS)

The aim in conducting this experimental phase was to identify a suitable protocol for acclimatization of adults *Paracentrotus lividus* in Recirculating Aquaculture System (RAS). This is a critical phase because, the sampling, the transport operation and placing in the aquaria brings a high stress condition for organisms, which can lead to the "accidental" release of gametes in the tank, determining in RAS a rapid deterioration of water quality, which can sometimes lead to the death of organisms present in the aquaria. This phase, preliminary conducted for all the experimental trials carried out in these three years of research, pointed out that the protocol used for sea urchin acclimation proved to be appropriate and adequate. During the three years, the acclimatization so conduct resulted in the death of any organism and did not cause the unintentional release of gametes within the aquaria. Before the acclimation operation and placing of sea urchins, the maturation of the biological filter of tanks has been very important and supported by the mechanical skimmers, has allowed, after the introduction of *P. lividus*, an efficient reduction of the organic load. To evaluate the maturation process of the biological filter were monitored nutrients, dissolved oxygen and pH. The trend of parameters during the weeks of maturation of the tank was reported below (Fig. 4.1.1).



	1° week	2° week	3° week	4° week	5° week
pH	8.05	8.12	8.04	8.15	8.21
NO2 (mg/L)	0.33	0.26	0.15	0.02	0.01
NO3 (mg/L)	1.99	1.72	1.03	1.08	1.31
NH4 +(mg/L)	0	0	0	0	0
PO4 (mg/L)	0.01	0	0	0	0
O2 (mg/L)	8.08	7.99	7.97	8.01	7.99

Fig. 4.1.1. Temporal trends of NO_2, NO_3, NH_4^+, PO_4, O_2(mg/L), and pH during the 5 weeks of biological filter conditioning in the rearing.

As can be seen from the graph parameters such as NO_3, NO_2 and PO_4 responsible for water eutrophication, during 5 weeks falling significantly stabilizing at very low concentrations, which in the case of nitrite were less than 100 µg/L, a sign of an effectiveness good functioning of the biological filter. With regard to the concentration of oxygen, important role in identifying both the self-purifying capacity of water, this has remained stable around the value of 8 mg / L that we can reasonably consider the level of saturation (100 %) for surface water marine (Cognetti *et al*., 1999).

The pH values were in line with the average value of 8.2 for the sea water (Cognetti *et al*., 1999) and in agreement with the values recorded for the waters of the sampling site.

4.2 Maintenance of mature stage in *Paracentrotus lividus* reared in RAS

Below are reported the EC values for spermio and embryotoxicity test with reference toxicant with *Paracentrotus lividus*. Tests were performed to evaluate the quality of gametes and embryos obtained from organisms maintained in mature stage by means of two different diets. The gonadosomatic index values (GI) for the two different treatments were also reported.

4.2.1 Spermiotoxicity test

The following section summarizes the results of fertilization tests (spermiotoxicity test) with reference toxicant for adults *Paracentrotus lividus* kept in mature stage by using two different diets. Results were compared with values reported for the natural population.
In the four months during which *P. lividus* has been maintained in the laboratory, with a diet based on maize and algae, sea urchins have provided gametes whose EC_{50} value with reference toxicant ($Cu(NO_3)_2*3H_2O$) is found to be 43.61µg/L (34.17 - 49:26µg/L) (Fig.4.1.1.1).

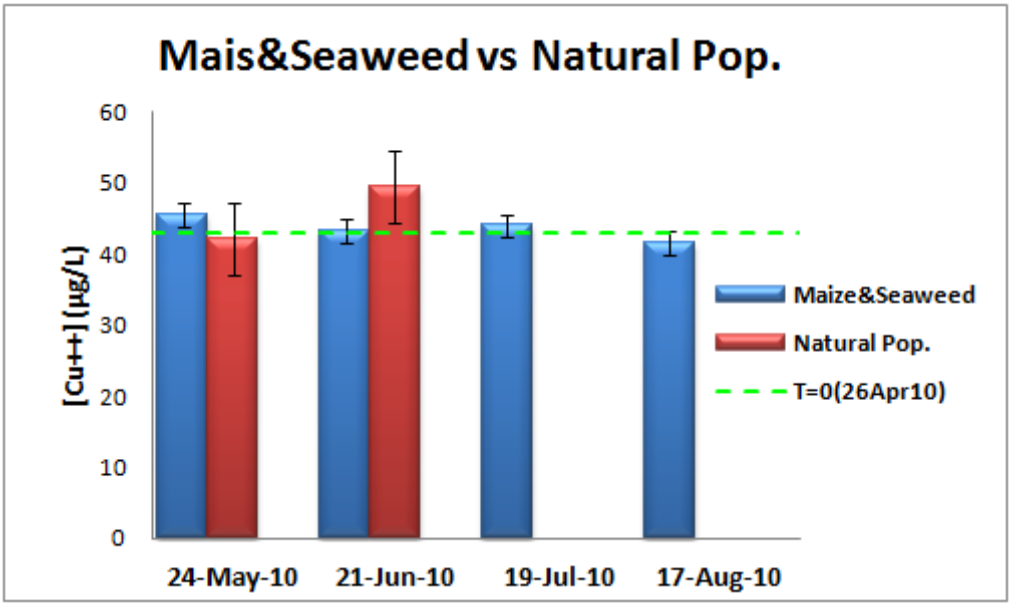


Fig. 4.2.1.1. EC_{50} (µg/L) values for reference toxicant[$Cu(NO_3)_2*3H_2O$] obtained with spermiotoxicity test performed on mature *Paracentrotus lividus* maintained in Recirculating Aquaculture System (RAS) for four month (April 2010- August 2010) with artificial diet Maize&Seaweed . EC_{50} values obtained are compared with those obtained from *P. lividus* belonging to natural population (Natural Pop.). EC_{50} (µg/L) values obtained at T=0(April 2010) for the wild population are reported. The values are mean and standard deviation.

Values are in good agreement with those reported in literature for *P. lividus* and for other species of echinoderms (Nacci *et al*., 1986; Dinnel *et al*., 1987; Volpi Ghirardini and Arizzi Novelli, 2001; Lera and Pellegrini, 2006). Values reported are also included in the laboratory control chart [68.18 to 21.69 µg/L] (Fig. 4.1.1.2) showing no significant differences ($p > 0.05$) from the EC50 value obtained from adults *P. lividus* belonging to the natural population [45.84 (49.11-42.79) µg]. As shown in the figure, for sea urchins natural population, has not been possible to perform the fertilization test since July 2010. Actually, in accordance with the data concerned the reproductive

cycle of this species in the Mediterranean (Gianbartolomei, 1990), has not been possible to obtain suitable gametes.

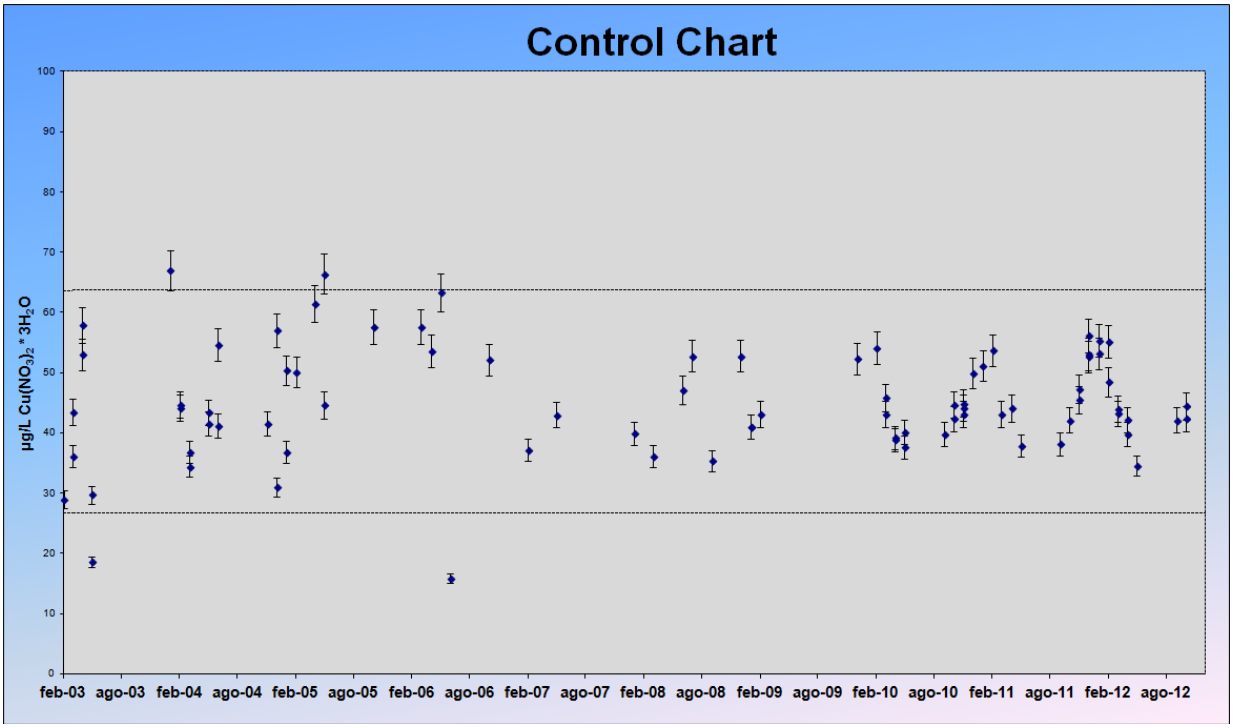


Fig. 4.2.1.2. Graphical representation of Control Chart from STS ISPRA Livorno laboratory where test with *Paracentrotus lividus* where performed. The values are mean and standard deviation.

Differences between EC_{50} values obtained from natural population [45.84 µg/L(49.11-42.79)] and those recorded from reared organisms resulted not significant ($p > 0.05$).
Similar to that recorded for the diet based on maize and algae even *P.lividus* reared with maize and spinach have provided gametes whose EC_{50} values [42.08 µg/L] (ranging from 45.74 to 39.18 µg/L)] are in agreement with published data. As well as for the Maize&Seaweed diet the EC_{50} values fall within the range of the laboratory control chart.

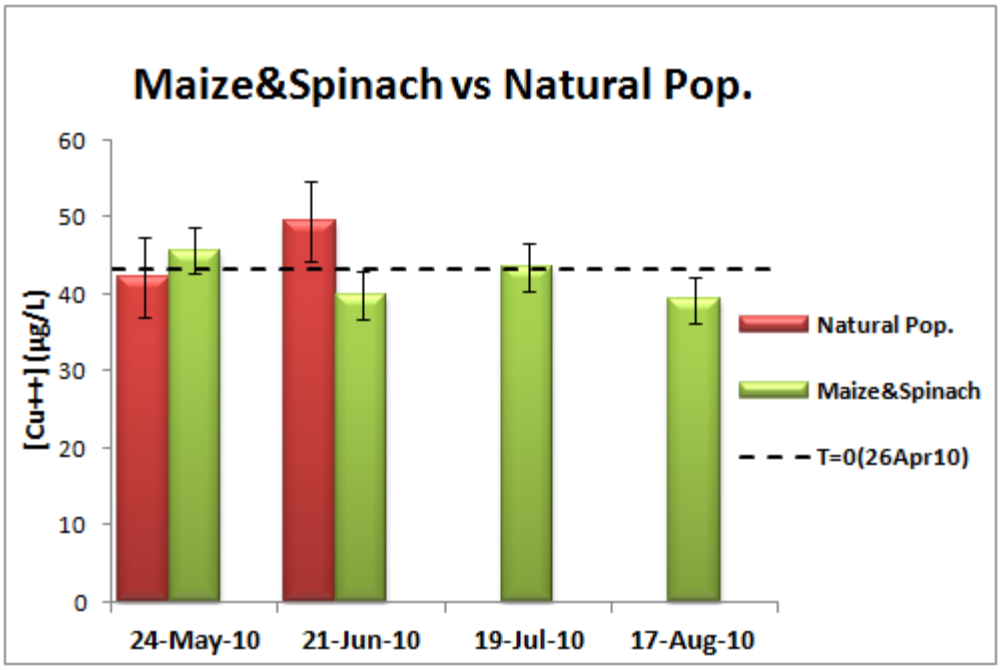


Fig. 4.2.1.3. EC_{50} (µg/L) values for reference toxicant[$Cu(NO_3)_2*3H_2O$] obtained with spermiotoxicity test performed on mature *Paracentrotus lividus* maintained in Recirculating Aquaculture System (RAS) for four month (April 2010- August 2010) with artificial diet Maize&Spinach . EC_{50} values obtained are compared with those obtained from *P. lividus* belonging to natural population (Natural Pop.). EC_{50} (µg/L) values obtained at T=0(April 2010) for the wild population are reported The values are mean and standard deviation.

The recorded values are not significantly different neither from the values obtained from sea urchins natural population nor from those recorded for adults *P. lividus* reared with a maize and seaweed diet (Fig. 4.2.1.4).

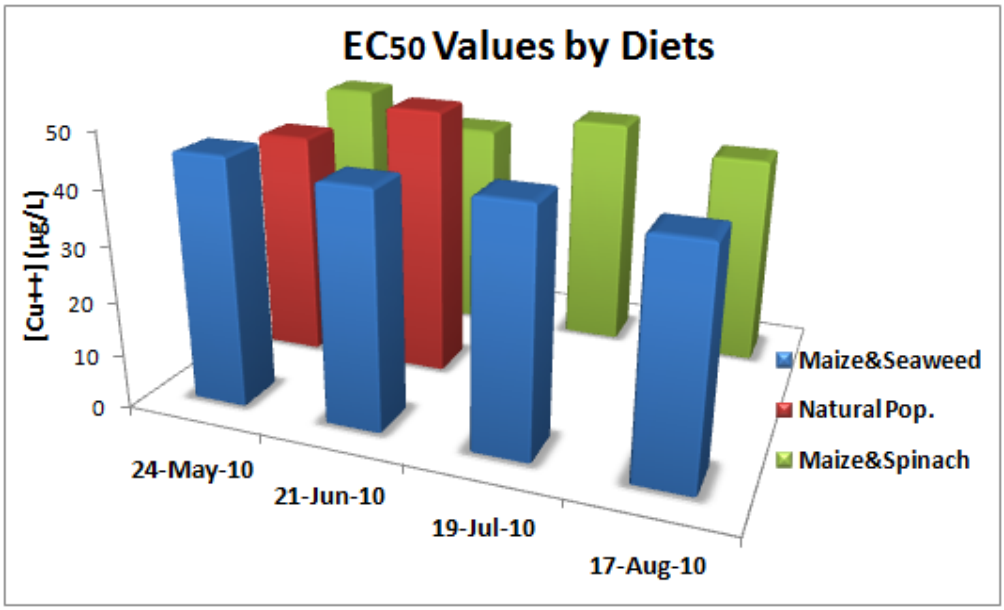


Fig. 4.2.1.4. Comparison between EC_{50} (µg/L) values obtained with spermiotoxicity test performed on *Paracentrotus lividus* reared with the artificial diets (Maize&Spinach; Maize&Seaweed) and those obtained from *P. lividus* belonging to natural population (Natural Pop.)

4.2.2 Embryotoxicity test

Below are reported the results of embryotoxicity test for organisms kept in mature stage with the following diets: Maize and Spinach; Maize ad Seaweed. Results from reared organisms were compared with those obtained from the natural population.
The EC_{50} values recorded during 4 months trials showed, for the diet based on Maize and seaweed, a moderate variability (43.94 to 56.24 µg/L) when compared with those registered for natural population (Fig. 4.2.2.1). Anyway any significant difference were recorded ($p > 0.05$) among the breeding sea urchins and those taken from the natural population. The EC_{50} values for embryotoxicity test are consistent with literature data, which report for copper values ranging from 20 to 110 µg/L (Arizzi Novelli *et al*., 2003; Fernández and Beiras 2001; Warnau et al., 1996). However it is interesting to stress that EC_{50} values for organisms keep in RAS present a sinusoidal trend. Value increase after two months of diets administration (June 2010), then return gradually, between July and August 2010 to EC_{50} values similar to those reported at T = 0 (April 2010) for the wild population.

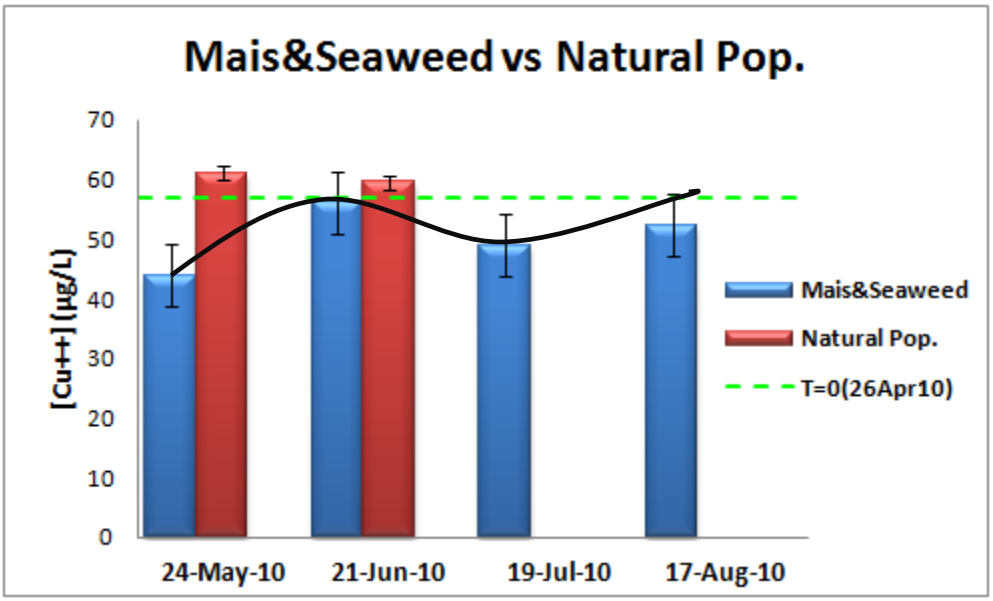


Fig. 4.2.2.1. EC_{50} (µg/L) values for reference toxicant[$Cu(NO_3)_2*3H_2O$] obtained with embryotoxicity test performed on mature *Paracentrotus lividus* maintained in Recirculating Aquaculture System (RAS) for four month (April 2010- August 2010) with artificial diet Maize&Seaweed . EC_{50} values obtained are compared with those obtained from *P. lividus* belonging to natural population (Natural Pop.). EC_{50} (µg/L) values obtained at T=0(April 2010) for the wild population are reported. The values are mean and standard deviation.

Embryos obtained from *P. lividus* reared with maize and spinach provide EC_{50} values towards reference toxicant almost constant over time and similar to those reported for the natural population (Fig.4.2.2.2).

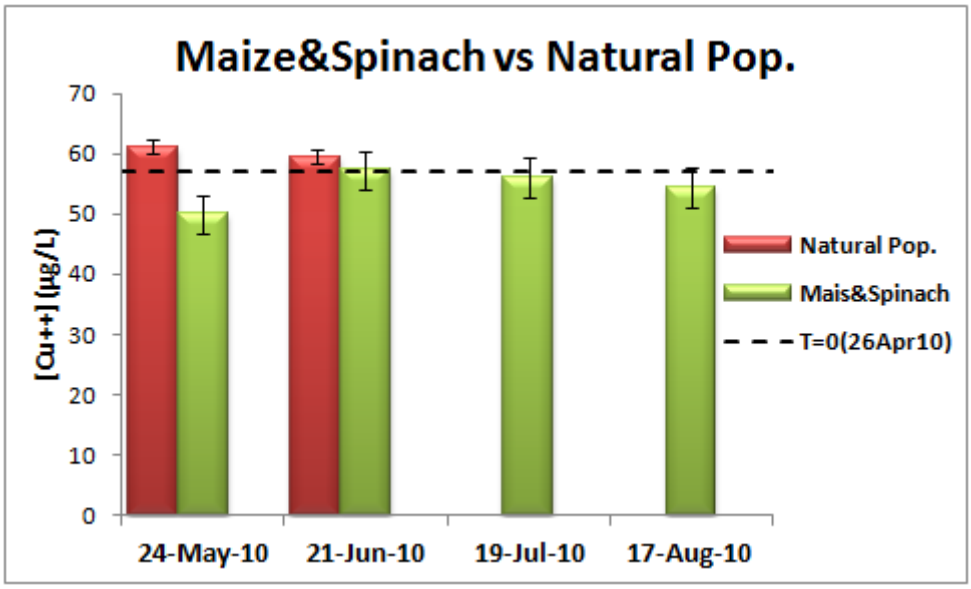


Fig. 4.2.2.2. EC_{50} (µg/L) values for reference toxicant[$Cu(NO_3)_2*3H_2O$] obtained with spermiotoxicity test performed on mature *Paracentrotus lividus* maintained in Recirculating Aquaculture System (RAS) for four month (April 2010- August 2010) with artificial diet Maize&Spinach . EC_{50} values obtained are compared with those obtained from *P. lividus* belonging to natural population (Natural Pop.). EC_{50} (µg/L) values obtained at T=0(April 2010) for the wild population are reported The values are mean and standard deviation

Even in this case, there were no significant differences between the two diets used and between diets and organisms belonging to the natural population (Fig.4.2.2.3).

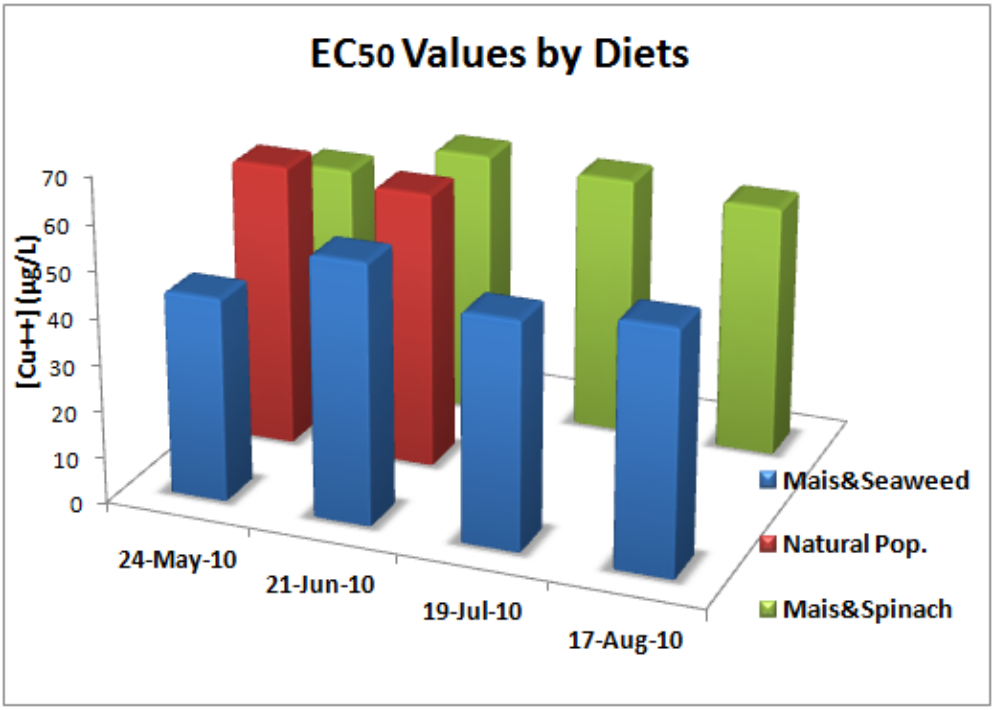


Fig. 4.2.2.3. Comparison between EC_{50} (µg/L) values obtained with spermiotoxicity test performed on *Paracentrotus lividus* reared with the artificial diets (Maize&Spinach; Maize&Seaweed) and those obtained from *P. lividus* belonging to natural population (Natural Pop.)

4.2.3 Gonadal weight and gonadosomatic index (GI)

Since the first month of treatment for both diets, a marked increase in the weight of the gonads were recorded particularly as regards the sea urchins fed with maize and algae (Fig.4.2.3.1). Starting from the second month of treatment, the weight values reported for the two diets were similar. In the last two months of trials the weight of the gonads has remained fairly constant for both diets with an average values of 7g per individual. In the wild stock the weight of the gonads was significantly lower ($p < 0.05$) than farmed organisms. Since the second month of treatment differences between reared and wild organisms were considerable; lowest value were recorded in July and August. In fact, as reported by many authors (Azzolina, 1988; Fernandez and Caltagirone, 1994; Turon *et al*., 1995, Shpigel *et al*., 2004) the optimal temperature conditions for the growth of this species in the Mediterranean area ranging between 12 and 18 ° C, temperatures that are much lower than those recorded during summer time in the natural habitat, where in August the water temperature exceed 25 °C.

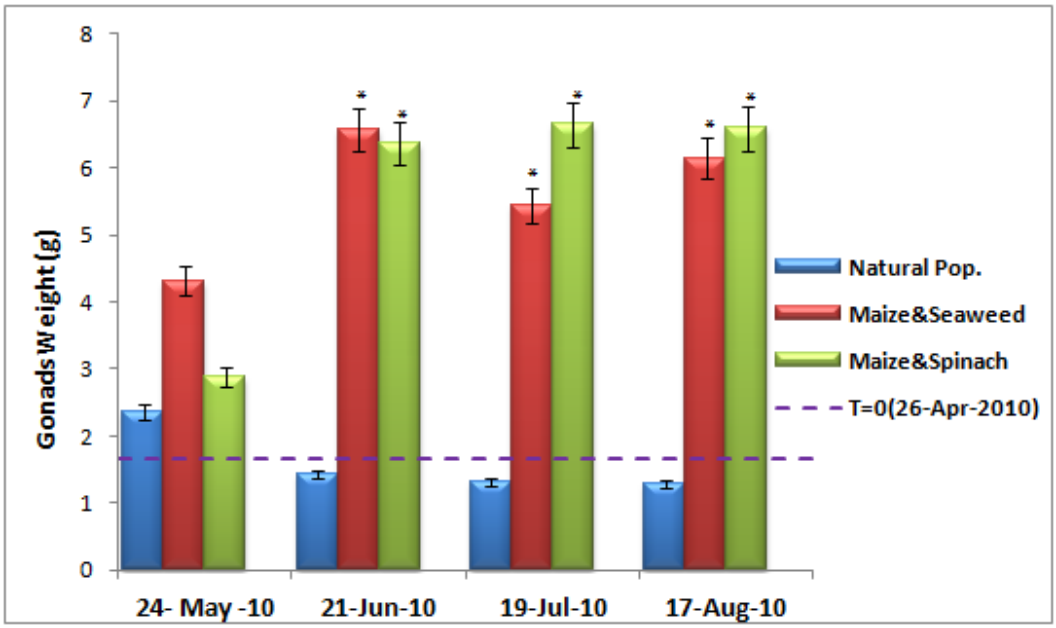


Fig. 4.2.3.1. Average gonadal wet weight temporal trend in natural population's sea urchin popolazione (Natural Pop.), and for *Paracentrotus lividus* reared in RAS with artificial diets (Maize&Seaweed, Maize&Spinach). On the graph is reported the average gonadal weight at the beginning of the experiments (T=0 (26-Apr-2010)). The values are mean and standard deviation

As for the weight, gonadosomatic index (GI) has shown for the two diets a similar trend. As from the first month, a significant increase of GI ($p < 0.0001$) for the tested diets compared with the natural population was recorded (Fig. 4.2 .3.2). The highest value of GI was recorded in July 2010 for the maize and spinach diet (GI = 12.84) while the lowest values were recorded in the natural population in July and August with a value the GI of about 2.9.

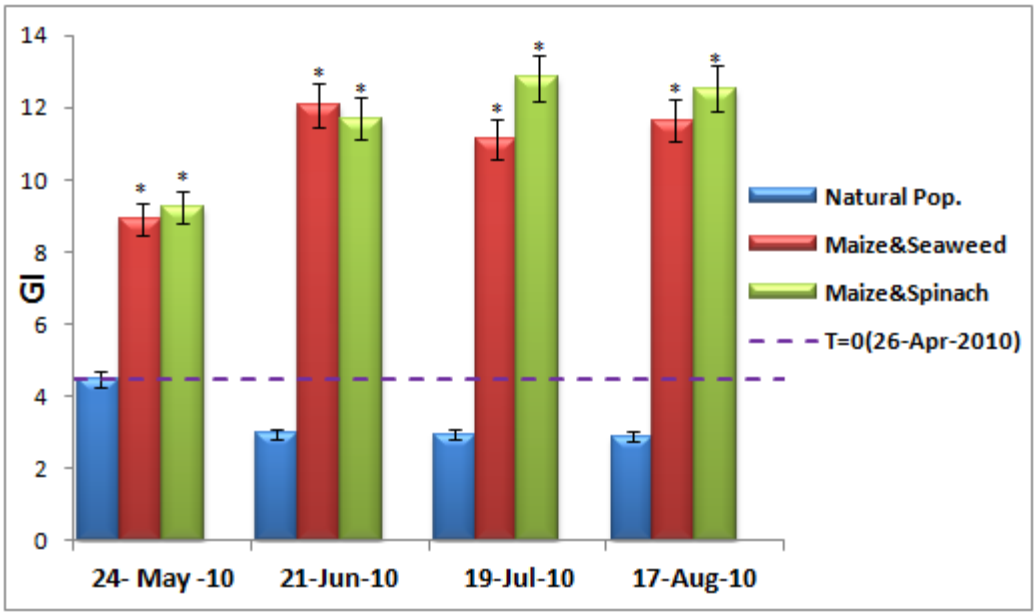


Fig. 4.2.3.2. Temporal trends of the average Gonadosomatic Index (GI) recorded during experiments for each diets (Maize&Spinach, Maize&Seaweed,). GI values obtained for each diet are compared with GI values obtained for *Paracentrotus lividus* belonging to natural population (Natural Pop.) and those reported at T=0 (April 2010) for the wild population. The values are mean and standard deviation.

4.3 Experimentation of diets stimulating gonadal growth and sexual maturation

Following are presented the results of diets used in promoting gonadal growth and sexual maturation of adults *Paracentrotus lividus* subjected to a preliminary phase of starving.

4.3.1 Starving

Starving operation was carried out on over 100 adult organisms of *Paracentrotus lividus*. After the first three days of acclimatization sea urchins were kept at 12 °C with a photoperiod of 12h L: 12h D completely devoid food for 6 weeks. Starving resulted in the death of one individual. There were no unusual water quality parameters to explain this death. Compared to the starting number of specimens, at the beginning of the experiments, the death of one sea urchins can be considered an acceptable result.
Except for the death of an individual of *P. lividus* during the starving there were no further problems.

4.3.2 Spermiotoxicity test

The EC_{50} values for the test fertilization with $Cu(NO_3)_2*3H_2O$ for sea urchins reared in RAS with diets ranging from 25.65 to 45.38 µg/L (Fig. 4.3.3.1). These values are in agreement with those reported in the literature (Nacci *et al*., 1986; Dinnel *et al*., 1987; Volpi Ghirardini and Arizzi Novelli, 2001; Lera and Pellegrini, 2006). However, if for the two artificial diets (Maize&Spinach, Pellets Classic K®) the spermiotoxicity test showed similar EC_{50} values ($p > 0.05$), with regard seaweed diet (Control) the EC_{50} values obtained were significantly lower than those obtained for "artificial " diets. Moreover, differences in terms of EC_{50} values, between organisms reared with maize and spinach and those reared with algae were statistically significant ($p = 0.045$). The EC_{50} values observed for wild population were similar with those obtained for "artificial" diets .

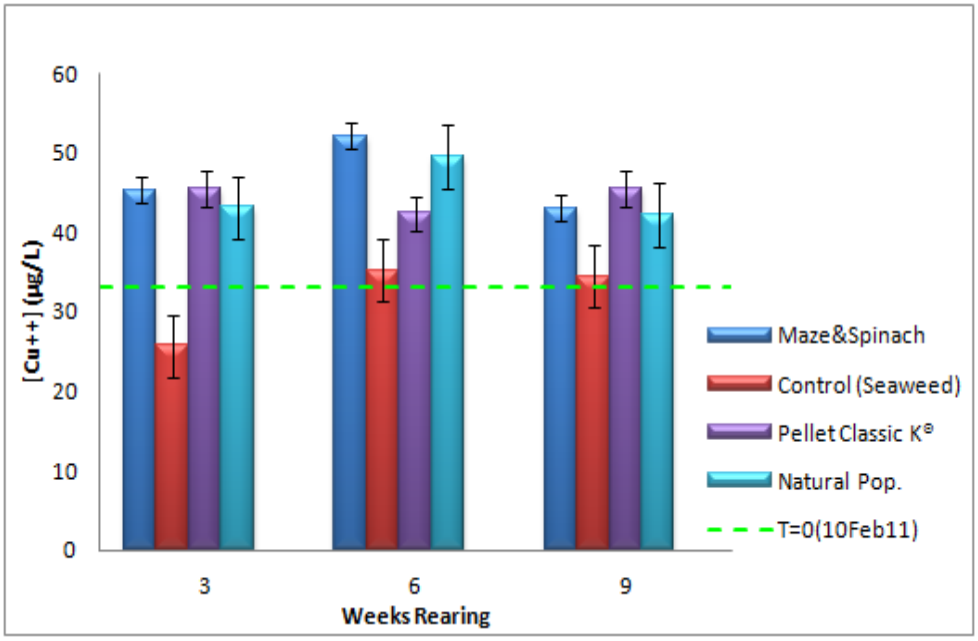


Fig. 4.3.2.1. Temporal trends of EC_{50} (µg/L) values, with reference toxicant[$Cu(NO_3)_2*3H_2O$], obtained with spermiotoxicity test performed on *Paracentrotus lividus* reared in RAS with artificial diets. EC_{50} values obtained are compared with those obtained from *P. lividus* belonging to natural population

(Natural Pop.). EC_{50} (µg/L) values obtained at T=0(10 Feb 2011) for the wild population are reported. The values are mean and standard deviation.

4.3.3 Embryotoxicity test

As regards the embryotoxicity tests the EC_{50} values are in agreement with values reported by other authors such Arizzi Novelli *et al*., (2003), and Fernandez Beiras (2001), His *et al*. (1999) Warnau *et al*., (1996) reported for copper EC_{50} values ranging from 20 to 110 µg/L.

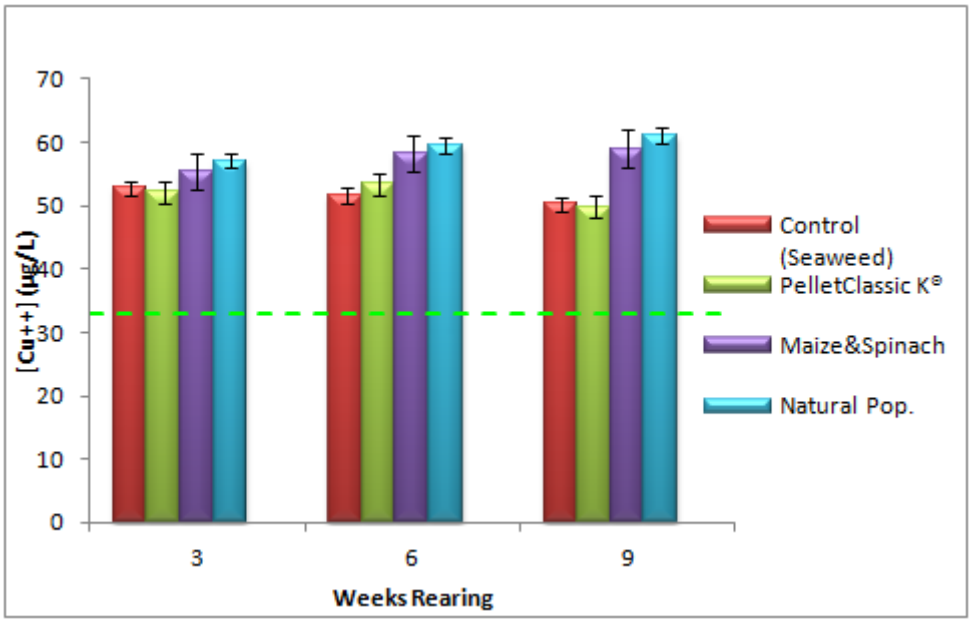


Fig. 4.3.3.1. Temporal trends of EC_{50} (µg/L) values, with reference toxicant[$Cu(NO_3)_2$*$3H_2O$], obtained with embryotoxicity test performed on *Paracentrotus lividus* reared in RAS with artificial diets. EC_{50} values obtained are compared with those obtained from *P. lividus* belonging to natural population (Natural Pop.). EC_{50} (µg/L) values obtained at T=0(10 Feb 2011) for the wild population are reported. The values are mean and standard deviation.
***a= Statistically significant with respect to P. lividus reared with seaweed (Contol (Seaweed)).**

The EC_{50} values referred to the three treatments did not showed significant differences even though the values for the diet based on maize and spinach were, on average, higher than those found for the other two diets. As regards EC_{50} values referred to the natural population, there were no significant differences compared to the three diets employed. At the end of 9 weeks of trial, gametes obtained from wild population were more sensitive to the toxic reference (EC_{50} = 62.81 µg/L), this result could be explained by the period during which it was performed the embryo assay. In fact in the 9th week of experimentation we were already in late May, period during which there is a rapid rise in water temperature and the organisms occur mostly without gametes as a result of spawning season (Fenaux, 1968; Lozano *et al*., 1995) or with a poorly quality of gametes because in *spent* or *recovering* stage (Byrne, 1990).

4.3.4 Evaluation of sperm motility

The rearing system together with the three different diets provided, good quality gametes with values of sperm motility comparable to those reported for the natural population (Fig. 4.3.4.1).
For a better assessment of sperm quality is, however, more important to assess the value of the curvilinear velocity (VCL) by Sperm Class Analyzer ® system as described by Fabbrocini *et al*., (2010) since this is the parameter that determines the success of fertilization in *P. lividus* and other species of echinoids (Bracho *et al.*, 1997; Au *et al.*, 2001; Fabbrocini and D'Adamo, 2010).

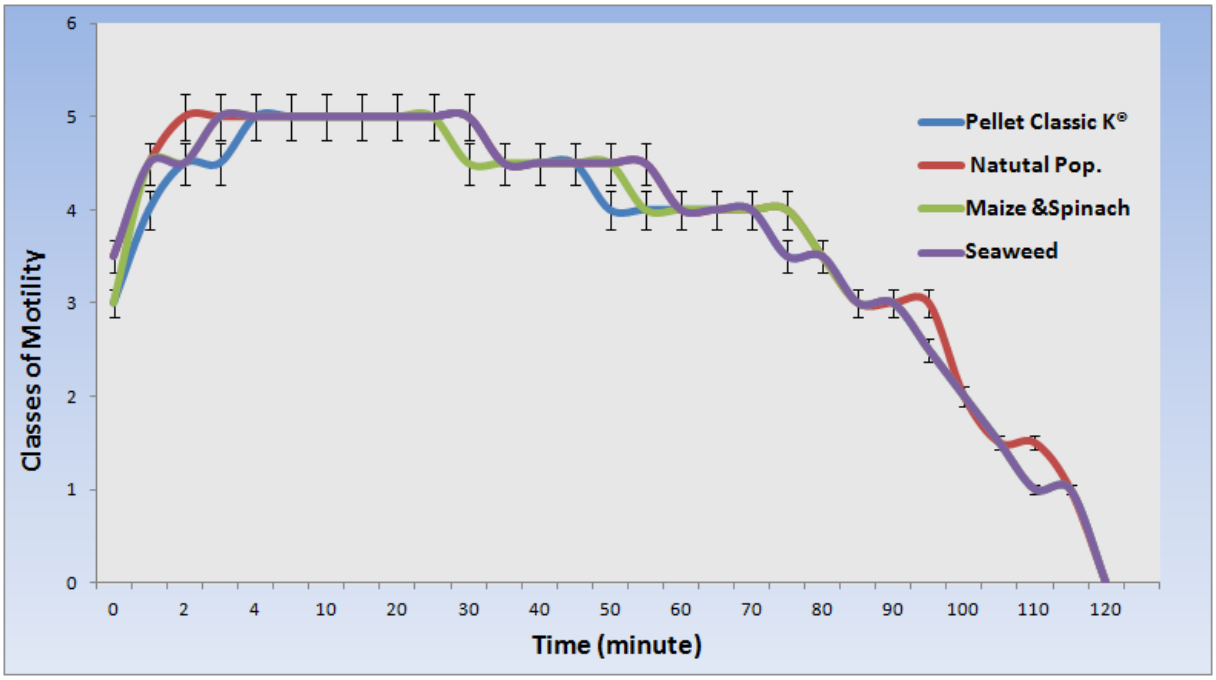


Fig. 4.3.4.1. Pattern of motility expressed in classes for *Paracentrotus lividus* reared with three different diets. Values are compared with those obtained from specimen belonging to natural population (Natural Pop.)

The results are the average of trials made periodically throughout the experimental period, the graph highlights the achievement of the highest class of motility for farmed organisms regardless of diet used.

4.3.5 Righting response

With regard the righting activities coefficient (RAC), there were no significant differences between the adults *Paracentrotus lividus* belonging the natural population and those reared in aquaria with three different diets (Fig.4.3.5.1).

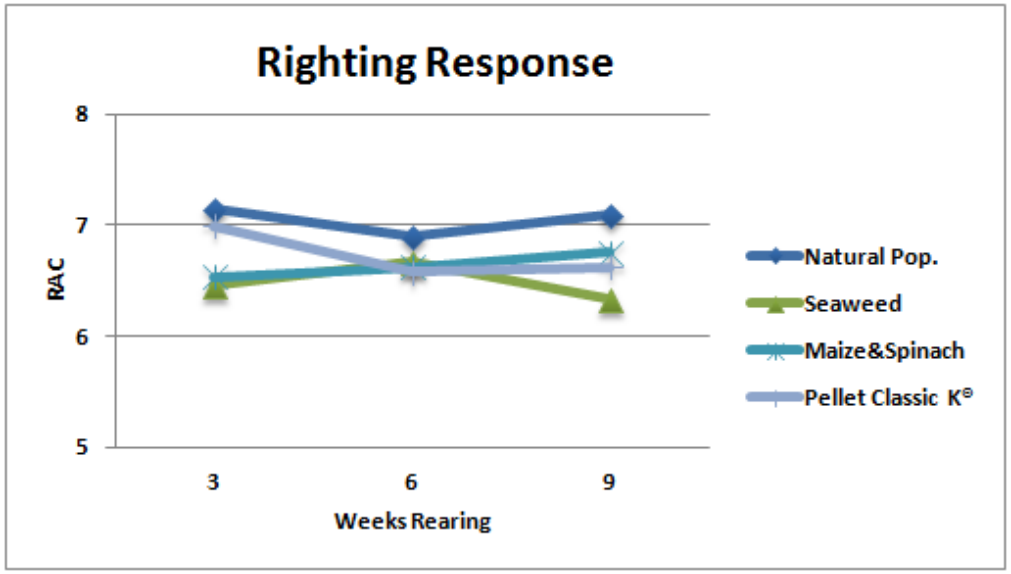


Fig. 4.3.5.1. ***Righting Activities Coefficient*** **(RAC) representation for** ***Paracentrotus lividus*** **reared with three diets in RAS. RAC values are compared with those obtained from specimen belonging to natural population (Natural Pop.) .**

The highest RAC values were recorded for *P. lividus* belonging natural population, which showed a major ability (shorter time) to bring the oral surface in contact with the substrate once reversed compared with reared organisms. With regard the published data, the average time to capsize of reared sea urchins is in good agreement with those reported from Bayed *et al*., (2005), for the natural population of *P. lividus* of the Atlantic coasts of North Morocco, and Axiak and Saliba (1981). Moreover, RAC values recorded were lower with those reported for *Lytechinus variegatus* by Böttger *et al*., (2001).

4.3.6 Gonadal weight and gonadosomatic index (GI)

As shown in table 4.3.6.1, starving led to a sharp reduction in gonadal weight and consequently the reduction of gonadosomatic index value (GI). These data thus confirm the success, of starving operation, in leading to consumption of the possible content of the gonads before the diets were tested. However, after 3 weeks of rearing, regardless of the diet used (Maize&Spinach, pellets, seaweed) GI values and gonadal weight have returned to levels found in *P. lividus* natural populations at time of collection (T = 0). In the following three weeks, the gonadal growth of reared sea urchins continued constantly and were especially high both for sea urchins bred with maize and spinach and for those reared with pellets.

Table 4.3.6.1. Temporal trends of gonadal wet weight (g) and gonoadosomatic index (GI) obtained from three different diets. The values are mean and standard deviation.

Diet	Diet	Gonadal wet weight (g) [Mean ± sd]	GI [Mean ± sd]
Natural population (T=0)	-	4.56 ± 0.77	9.62 ± 1.54
Starving (6 weeks)	Starving	2.14 ± 0.14	4.36 ± 0.42

Table 4.3.6.1. continued

Time	Diet	Gonadal wet weight (g) [Mean ± sd]	GI [Mean ± sd]
	Seaweed	3.22 ± 0.25	9.39 ± 0.37
3 rearing weeks	Maize&Spinach	3.71 ± 1.19	10.26 ± 3.17
	Pellet (Classic K®)	3.62 ± 051	9.47 ± 1.14
	Seaweed	4.77 ± 0.67	12.03 ± 1.19
6 rearing weeks	Maize&Spinach	7.31 ± 0.80	16.08 ± 2.84
	Pellet (Classic K®)	6.80 ± 0.79	14.79 ± 1.73
	Seaweed	4.19 ± 0.51	10.25 ± 1.32
9 rearing weeks	Maize&Spinach	9.13 ± 1.07	19.24 ± 2.95
	Pellet (Classic K®)	4.59±1.05	11.03 ± 2.41

In the last three weeks of trials the weight of the gonads and the GI has continued to grow significantly for *P. lividus* fed with Maize & Spinach, while for the organisms farmed with seaweed collected from the sampling site of sea urchins and organisms fed with pellet, there was a reversal trend of gonadal growth (Fig. 4.3.6.1).

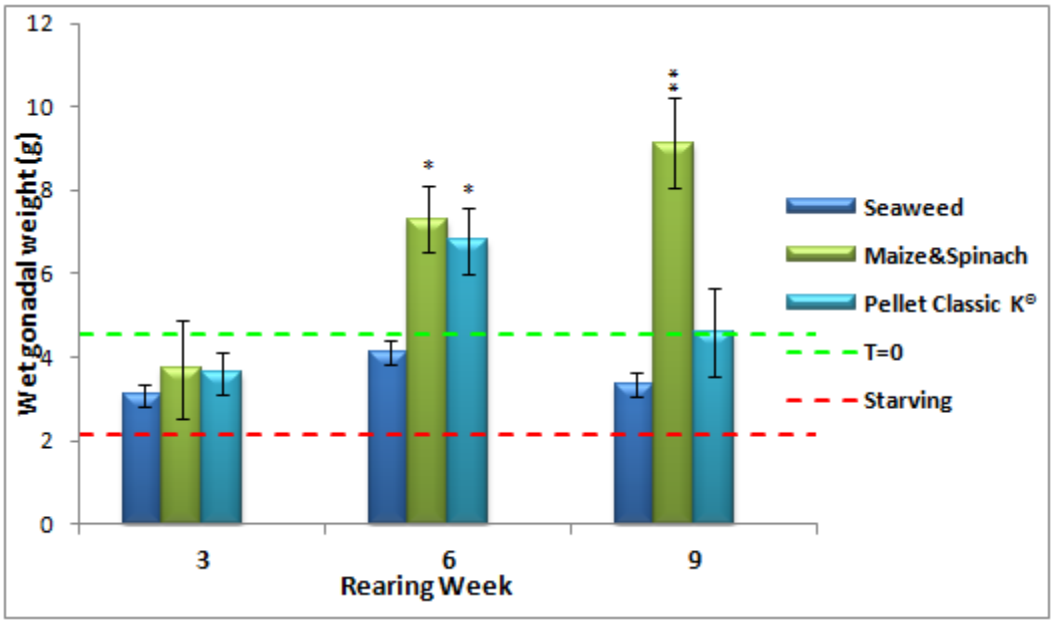


Fig. 4.3.6.1. Temporal trends of gonadal wet weight for the three different diets (Seaweed, Maize&Spinach, Pellet Classic K®). On the graph are reported the gonadal weight for the natural population at the time of organsim collection (T=0) and after 6 weeks starving (Starving). Note: *, ** : statistically significant.

As regards the GI value, statistical analysis, showed significant differences between the seaweed and the artificial diets (Maize&Spinach and pellets Classic K®) ($p < 0.0001$). Analyzing in detail the GI values recorded after 3, 6 and 9 weeks using the Newman-Keuls test (SNK), with a 95% interval of confidence, can be stressed as after 9 weeks the GI values obtained for sea urchin reared with

pellet were significant different ($p < 0.0001$) from those recorded for *P. lividus* fed with maize and spinach or seaweed (Figure 4.3.6.2).

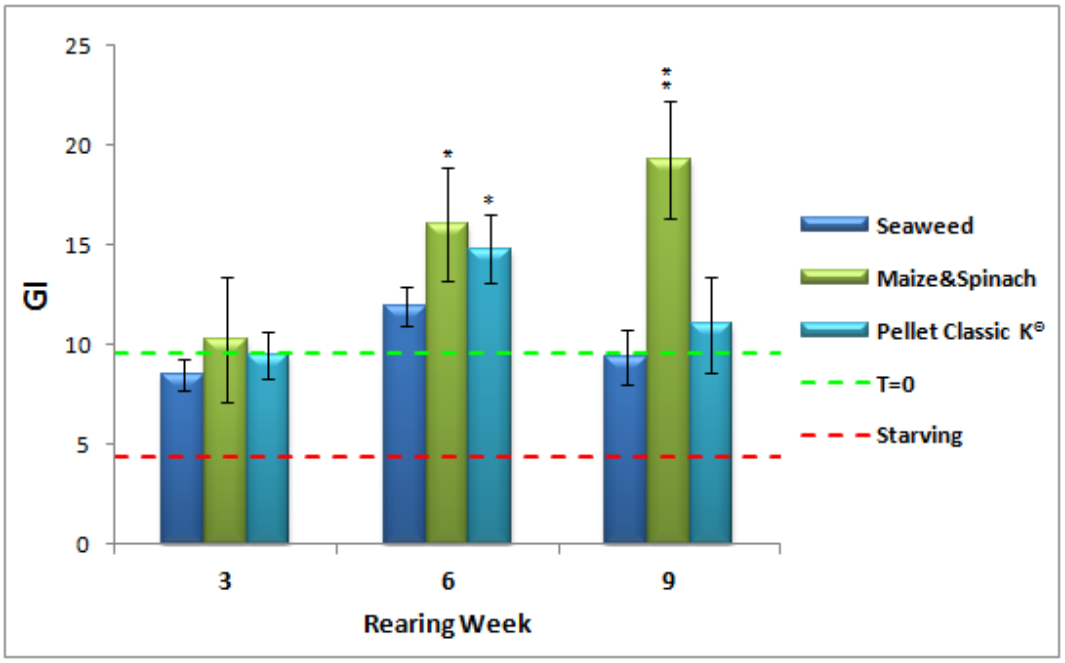


Fig. 4.3.6.2. Temporal trends of gonadosomatic index (GI) for the three different diets (Seaweed, Maize&Spinach, Pellet Classic K). On the graph are reported the GI values for the natural population at the time of organsim collection (T=0), and after 6 weeks starving (Starving). Note: *, ** : statistically significant.

Similar results were obtained from the analysis of the gonad fresh weight trend after 9 weeks of treatments. Significant differences were found between the two "artificial" diets (Maize& Spinach and Pellett Classic K®) ($p < 0.0001$) while no differences were recorded between seaweed farmed organisms and those brought up with Pellet Classic K® ($p > 0.05$).
Considering the good results, both in terms of gonadal growth both as regards the quality of gametes obtained, in the light of the histological analysis results reported below, and given the low cost of maize and spinach diet, further experimentation using the diet Maize & Spinach was carried in order to confirm the gonadal growth rate (GI) and to evaluate the sexual maturation cycle by histological analysis. .
Referring to this further experimental phase with "Maize&Spinach" diet, the GI values and gonad weight reported in Table 4.3.6.2 confirm the soundness of the starving period in determining the "resorption" of the gonads. Subsequently has been recorded a steady increase in the weight of the gonads and related GI that has been reached, at the end of 9 weeks of rearing, the average value of 17.77 ± 1.90.

Table 4.3.6.2. Average values of gonadal wet weight (g) and gonadosomatic index obtained during the rearing of *Paracentrotus lividus* with Maize&Spinach. The values are mean and standard deviation

Time	Gonadal Wet Weight (g) [Mean ± sd]	GI) [Mean ± sd]
T=0	3.91 ± 0.15	8.93 ± 0.36
Starving (6 weeks)	1.07 ± 0.10	2.56 ± 0.25
3 rearing weeks	2.12 ± 0.07	5.29 ± 0.20
6 rearing weeks	3.48 ± 0.17	8.69 ± 0.30
9 rearing weeks	7.84 ± 1.94	17.77 ± 1.90

4.3.7 Histology of gonads

On the basis of histological analysis the organisms were classified into 6 different stages as reported by Byrne (1990). Analysis showed that the three diets, combined with a temperature of 16 °C under a 10 h L :14 H D regime have led in 3 weeks (Fig. 4.3.7.1 and Fig. 4.3.7.2), to the maturation of *P. lividus* specimen.

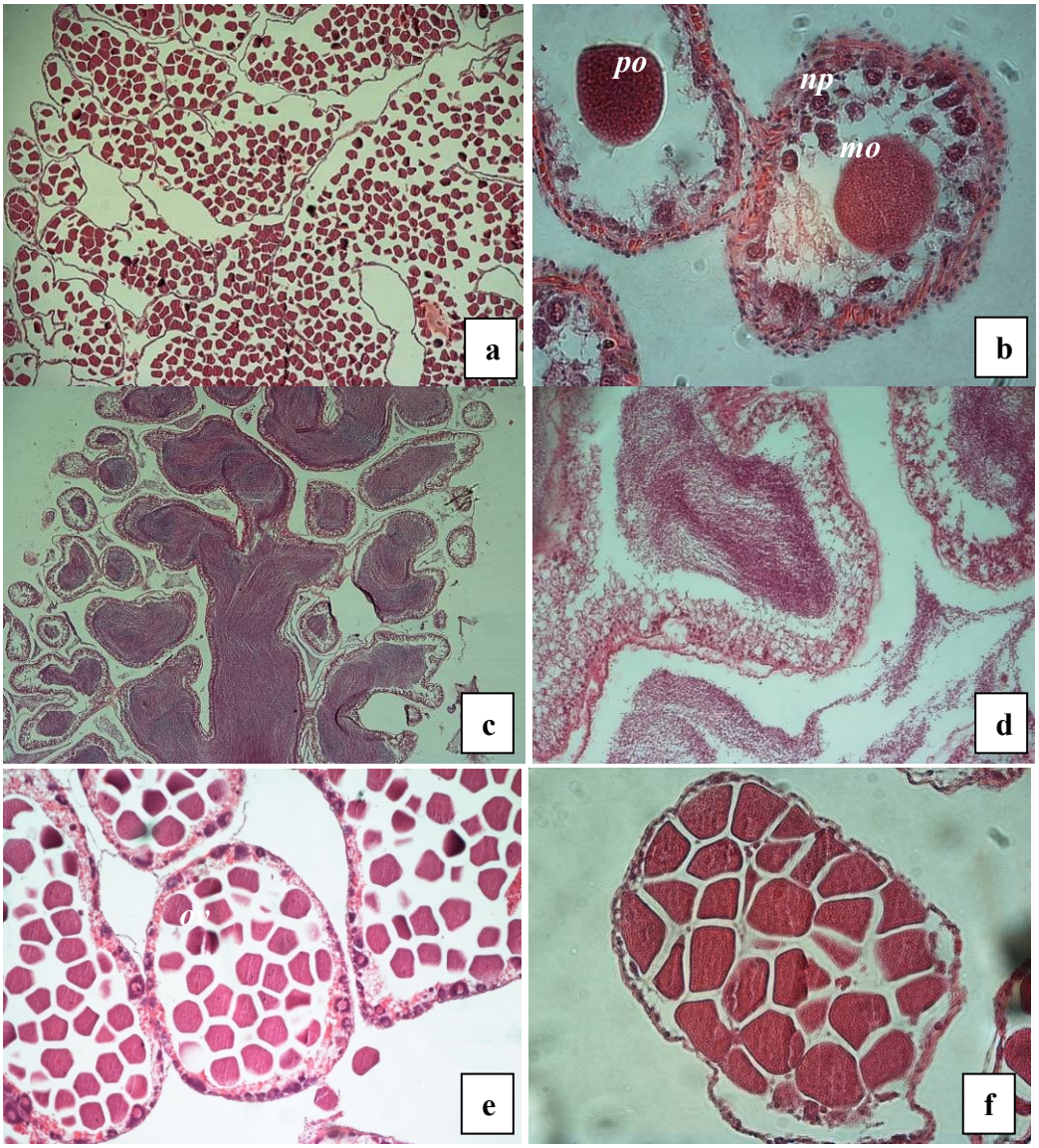


Fig. 4.3.7.1. Representative histological section of Paracentrotus lividus reared with three diets (Seaweed, Maize&Spinac, Pellets Classic K) in RAS stained with Mayer's haemalum-eosin techinique. a) female gonad (4x) in mature stage after 3 weeks of seaweed diet. b) Detail (40x) of ascini after 3 weeks of seaweed diet; premature oocytes (op) beside an oocytes in maturation (om) surrounded by nutritive phagocytes c) male testis in partly spawned stage (4x) after 9 weeks of seaweed diet. d) Detail of testis (20x) after feeding seaweed for 9 weeks. e) female gonad in mature stage (4x) rerared with Maize&Spinach for 3 weeks; detail of ascini (f) (20x) with ova (ov) closely packed.

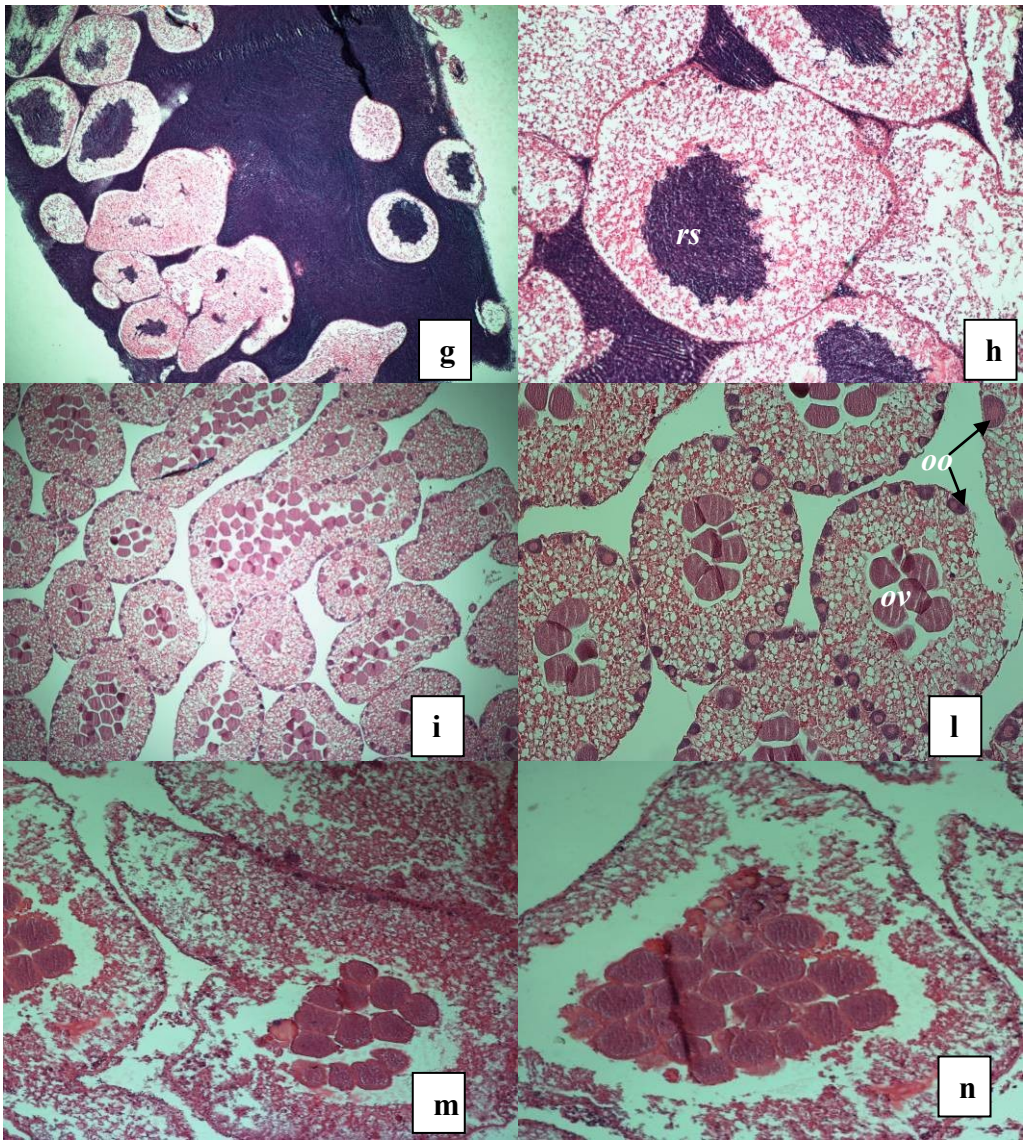


Fig. 4.3.7.2 Representative histological section of *Paracentrotus lividus* reared with three diets (Seaweed, Maize&Spinac, Pellets Classic K) in RAS stained with Mayer's haemalum-eosin techinique. g) Male testis (4x), reared with Maize&Spinach for 9 weeks, in recovering stage. Ascinal wall (h) (10x) presents a thick layer of nutritive phagocytes; relict spermatozoa (rs) are present in the center of ascini. i) Female gonad in premature stage (4x) after 3 weeks pellet Classic K® diet. Detail of ascini (l) (10x); whereas ova accumulate in the ascinal lumen, nutritive phagocytes are displaced from the center along ascinal wall. Along ascina wall are present oocytes (oo) inside nutritive phagocytes' incubation chambers circondati da fagociti nutritivi. m) , n) female gonad (10x) after feeding pellets Classic K® for 9 weeks in recovering stage. Unspawned ova are present, these relict ova undergoing lysis.

Based on the results obtained we can affirm that only diets Maize&Spinach or pellet Classic K® are able to make, in terms nutrients and energy, such a contribution from 60 ensure that bodies move rapidly to a *mature* stage, reached in three weeks and still present even after six weeks of diet, a recovery phase (stage I-*recovering*) to start a new process of gametogenesis. For these diets were not observed organisms in stage VI (*spent*) nor after three or six or even nine weeks of treatment. To *mature* stage seems to follow directly *recovering* stage, dramatically reducing the time during which the organisms do not are in an active phase of gametogenesis (stage II-V).

The farmed organisms with sun algae at the end of the nine weeks are results largely in a stadium

partly spawned or *spent* and therefore, for such a diet, time will be more resistant than the farmed organisms with artificial diets, for the initiation of a new process of gametogenesis.
The histological analysis carried out in the next experiment, performed with individuals of Paracentrotus lividus reared only with maize and spinach, confirmed the results obtained in the previous analyzes, certifying as diet based on maize appears excellent both in promoting gonadal growth and gametogenesis.
In particular, as a result of the period of starving the duration of 6 weeks 90% of the organisms tested (N = 10) was in a maturational stage VI (*spent*) (Fig.4.3.7.3 a) and only one individual is presented in stage of maturation between stage VI (*spent)* and stage I (*recovering*) (Fig.4.3.7.3 b). After three weeks of breeding based on maize and spinach at a temperature of 16 ° C, more than 50% of the organisms were fully mature (Fig.4.3.7.3 c, d) and overall, 80% of the urchins was in a phase of active gametogenesis (stage II-V) (Fig.4.3.7.4 e, f). At the sixth week of breeding while remaining 50% the number of mature organisms increased significantly the number of organisms in Stage V (*partly spawned*) (Fig.4.3.7.4 g) which formed the end of the 6 weeks of diet for 40% of the organisms treated. Remaining 10% of sea urchin were in *recovering* stage (Fig.4.3.7.4 h).
After nine weeks, 60% of the organisms was in a stage V (*partly spawned*) (Fig.4.3.7.4 i) while the remaining 40% of sea urchins was in a stage between stage I and stage II (*recovering- growing*) (Fig.4.3.7.4 l).

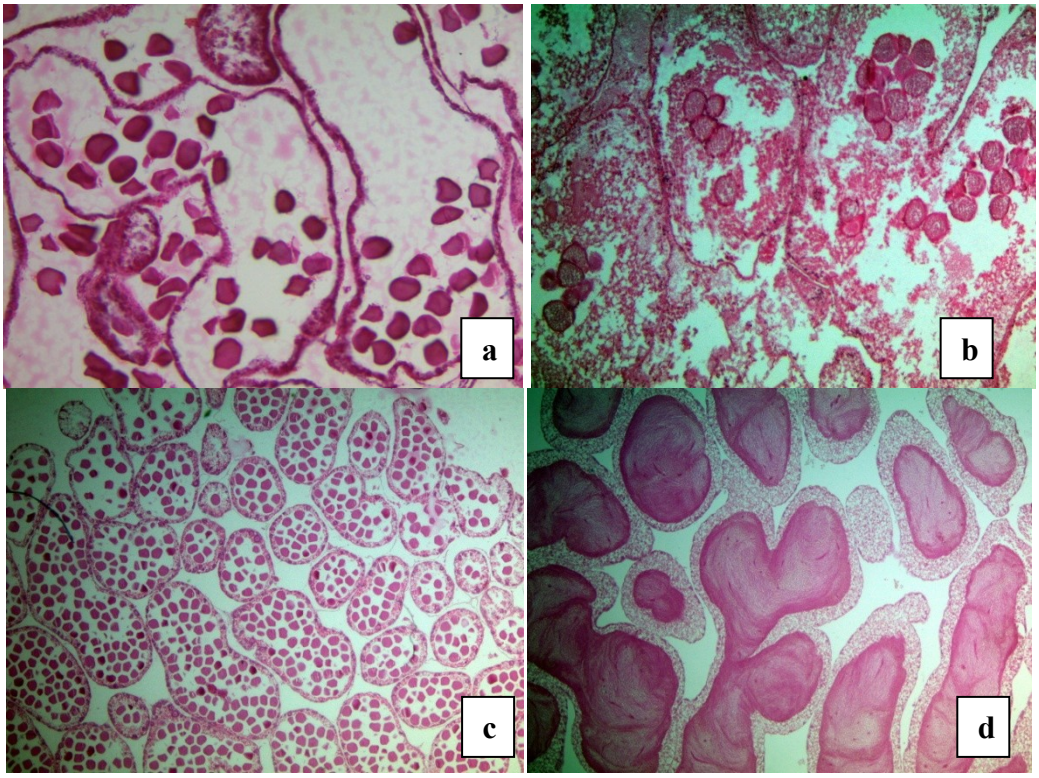


Fig. 4.3.7.3. Representative histological section of *Paracentrotus lividus* reared with Maize&Spinach for 9 weeks in RAS and stained with Mayer's haemalum-eosin techinique. a) female gonad (10x) in spent stage after 6 weeks starving. b) Ovaries (10x) in recovering-spent stage after 6 weeks starving. c,d) ovaries and testis in mature stage (4x) after 3 weeks rearing.

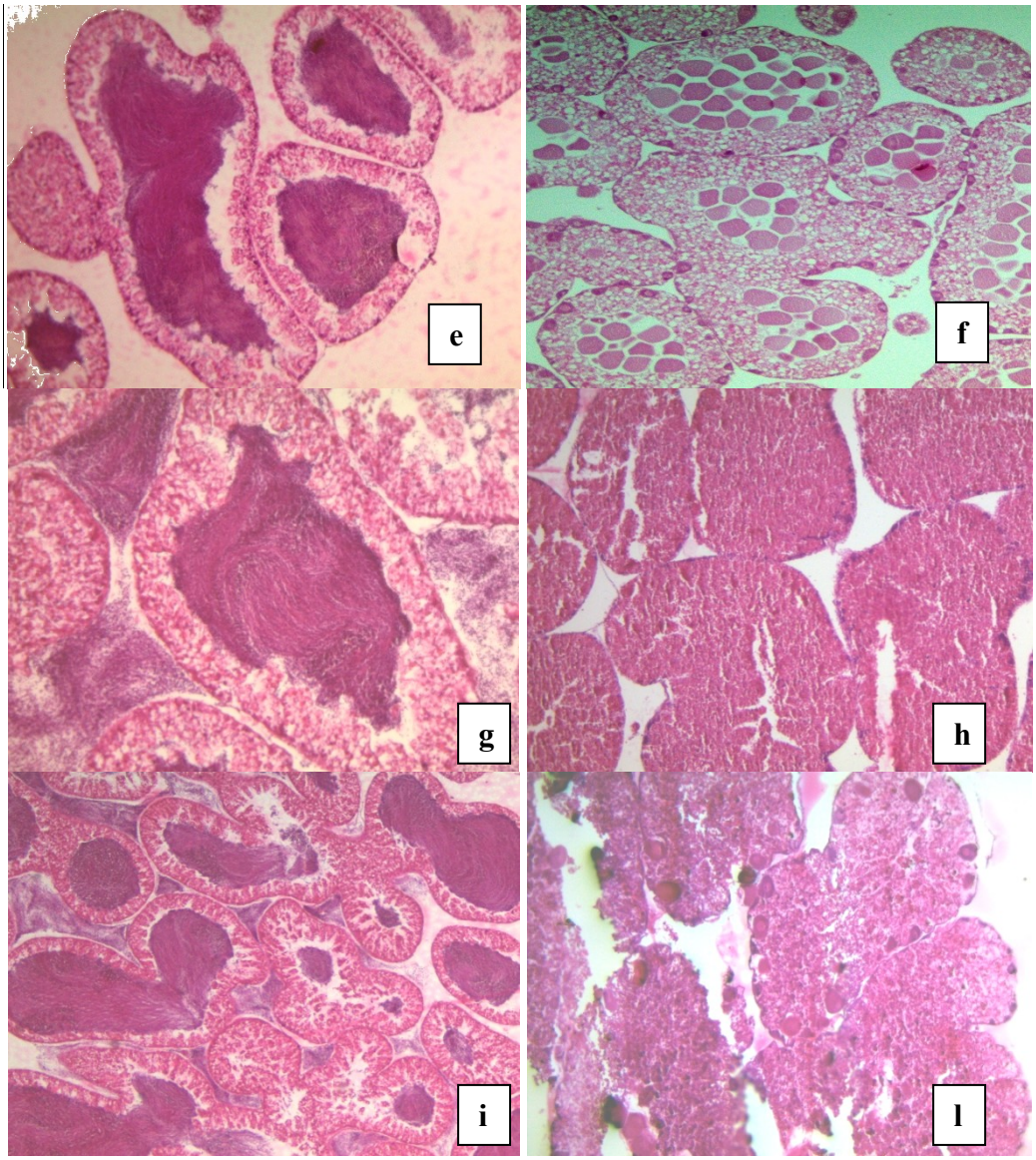


Fig. 4.3.7.4. Representative histological section of *Paracentrotus lividus* reared with Maize&Spinach for 9 weeks in RAS and stained with Mayer's haemalum-eosin techinique. e), f) male and female gonads in premature stage (10x) after 6 weeks rearing. g) testis (10x) in partly spawned stage after 6 weeks rearing. h) Ovaties (10x) in recovering stage after 6 weeks rearing. i) Testis in partly spawned stage (10x) and l) ovaries (10x) in Recovering-Growing after 9 weeks rearing..

As it is possible to deduce from results, the Maize&Spinach diet with a rearing temperature of 16 ± 1 °C and 10H L: 14H D light regime, has produced in three weeks, the maturation from spent stage of adult *P. lividus*.

The overall percentage of mature organisms remained high even after six weeks of breeding and at the 9th week the whole amount of specimens were in an active phase of gametogenesis (between stage II and stageV). Any organisms in Spent stage has been found neither after six, nor after nine weeks of experiments, confirming that the light regime, temperature and diet have allowed *P. lividus* to pass quickly from an inactive gametogenesis stage (stage IV) to an active stage of gametogenesis once reached the maturity stage ensuring the nearly constant presence of mature organisms within the tanks. Moreover, treatment have ensured to bypass the summer period during which, along the Tyrrhenian coast the temperatures rise up to 25 °C and sea urchins are unable to produce gametes (Gianbartolomei, 1990).

4.3.8 Analysis by using Harmonic Generation (HGM) and Two Photon (2PF) microscopy

As regards the assessment of apoptotic effects induced by farming conditions the results obtained with techniques of Second and Third Harmonic Generation (HGM) microscopy highlight differences between plutei obtained from the three diets. In particular, plutei obtained from the diet "Maize & Spinach" , although did not showed morphological abnormalities presented an increase in fluorescence signal both with two photon microscopy (2PF) both with the Third Harmonic Generation (THG) technique (Fig.4.3.8.1) and thus a potential apoptotic signal. Whether specimens belonging to natural populations or reared with the other two diets (pellet Classic K® or seaweed) did not show any apoptotic signal organisms.
In addition, in plutei obtained by *P. lividus* fed with Maize&Spinach, nonspecific signal of the skeletal rods were not highlighted. Relate Skeletal roads signal were evident in plutei reared with the other two diets and the natural population. The effect on plutei "skeletal parts" were also noted in plutei treated with $HgCl_2$ (data to be published).
The HGM microscopy technique, applied in this study for the first time, allowed the observation of abnormalities in the development of sea urchin plutei obtained from *P. lividus* kept in RAS with different diets. This technique is certainly a valuable and promising tool for applications in ecotoxicological studies, as confirmed by other *in vivo* studies on zebrafish embryos (*Danio rerio)* (Chen *et al*., 2006; Sun *et al*., 2004) where, the strong THG signal was associated with the presence of apoptotic body in the zebrafish hindbrain. The same apoptotic body were stained positively through the fluorescent marker acridine orange. Further studies conducted on nauplii of *Acartia tonsa* (Buttino *et al.*, 2011) have shown that the strong fluorescent signal detected with the 2PF and with the THG was associated with the onset of apoptosis in the digestive system of this copepod, data once again confirmed by the classical staining technique of TUNEL. We must also emphasize that, the non-invasive nature of the SHG and THG technique has permitted a 3-dimensional observation of the cellular structures of sea urchin pluteus allowing the observation of morphological changes, in the complex development processes, related to the rearing condition. These technique has also stressed results, without the use of fluorescence markers, getting through the common phenomena of photo-damage, photo-toxicity and photo-bleaching linked to the use of fluorescent probes. So despite Maize& Spinach diet result suitable in ensuring short time (3 weeks) gametes maturation and for gonadal growth, for which have been recorded the higher GI values, this diet didn't seem proper to provide gametes to be employed in echinocolture.

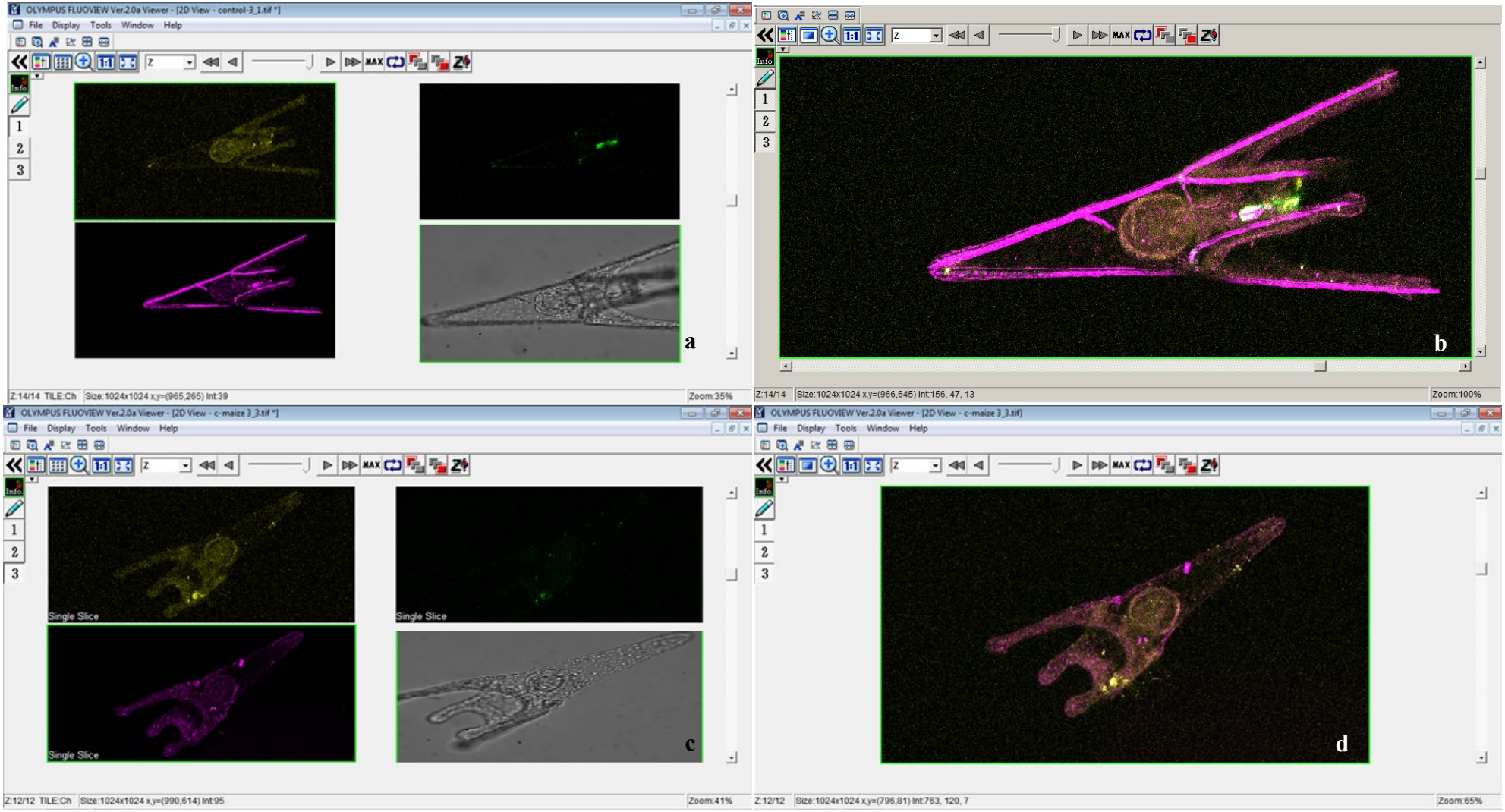


Fig. 4.3.8.1 A. Sea urchin plutei obtained fronm different diets observed with light microscopy, two photon fluorescence (2PF) microscopy and second (SHG) and third (THG) harmonic generation microscopy. On the left side (a, c, e, g) are reported , in a clock wise turn, starting the observation from the low left corner, the images obtained with THG, 2PF, SHG and light microscopy technique. On the right side (b, d, f, h) are presented the images obtained merging the THG, 2PF and SHG signals. Plutei observed are obtained from *Paracentrotus lividus* reared with Maize&Spinach (c, d), pellet Classic K®(e, f), Macrophytes (g,h) and those belonging to natural population (a,b).

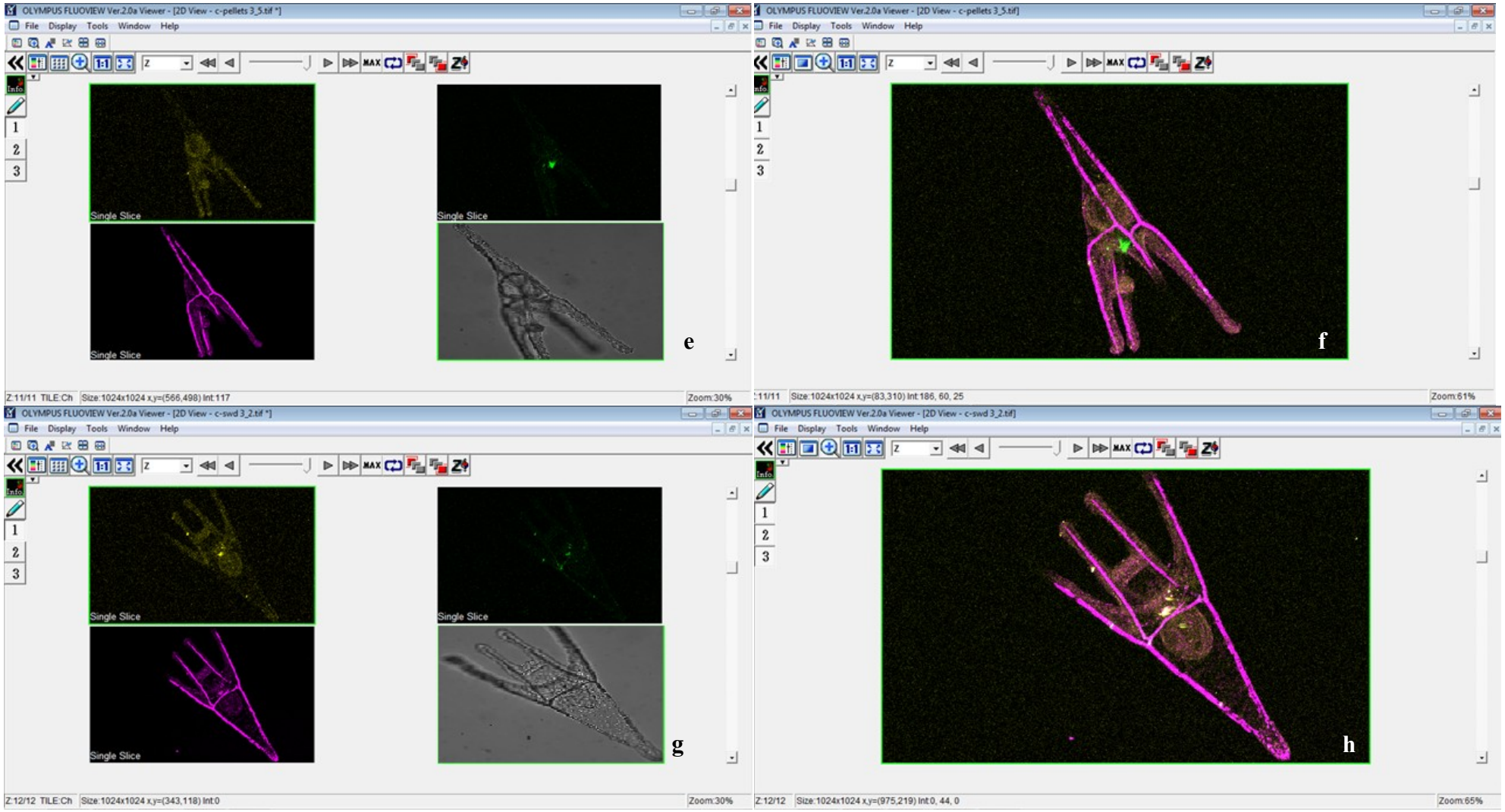


Fig. 4.3.8.1 B. continued Sea urchin plutei obtained fronm different diets observed with light microscopy, two photon fluorescence (2PF) microscopy and second (SHG) and third (THG) harmonic generation microscopy. On the left side (a, c, e, g) are reported , in a clock wise turn, starting the observation from the low left corner, the images obtained with THG, 2PF, SHG and light microscopy technique. On the right side (b, d, f, h) are presented the images obtained merging the THG, 2PF and SHG signals. Plutei observed are obtained from *Paracentrotus lividus* reared with Maize&Spinach (c, d), pellet Classic K®(e, f), Macrophytes (g,h) and those belonging to natural population (a,b).

4.4 Ingestion Rates

Data concerning ingestion rates of *Paracentrotus lividus* are given in Table 4.4.1.In order of preference, the most appreciated food were:Maize, *Dyctiopteris sp*, *Spinach*, pellets Classic K® and *Codium sp*.

Table 4.4.1. Ingestion rates by for the macrophyte, maize, spinach and pellet classic K® tested. Ingestion rates are expressed in terms of dry weight per day. The values are mean and standard deviation.

Species	***Ingestion rates (g/day d.w.)***
Codium sp.	0.137 ± 0.32
Corallina elongata	0.025 ± 0.68
Dyctiopteris sp	0.141 ± 0.45
Dyctiota sp	0.076 ± 1.16
Flabellia petiolata	0.030 ± 2.08
Halopteris scoparia (=Stypocaulon scoparium)	0.035 ± 2.13
Laurencia sp.	0.115 ± 0.89
Mais	0.281 ± 0.93
Padina Pavonica	0.085 ± 0.56
Pellet Classic K®	0.137 ± 0.89
Spinaci	0.139 ± 1.18
Ulva Lactuga	0.030 ± 1.06

If it was expected this kind of results for the brown alga *Dyctiopteris sp*, considering that brown algae together with *Posidonia oceanica* leaves are among the main components of adult *P. lividus* diet (Verlaque and Nedelec 1983b, Verlaque 1984, 1987a, 1987b), is not as clear the poor desirability for the other brown algae administered. The low liking of red algae, with the exception of *Laurencia sp*, could be related to the presence of brominate substances as reported by Codomier *et al*., (1977)for the red alga *Asparagopsis armata*. Among the seaweed provided, *P. lividus* clearly has been showed any preference towards algae that have a coriaceous consistency due to the presence of precipitates of calcium carbonate in the structure of the alga as for *Corallina elongata*, *Padina pavonica* and *Flabellia petiolata*. The poor desirability towards *Halopteris scoparia*, could be due to the presence of phenolic compounds which act as a deterrent for *P. lividus* (Traer 1980). However, *P. lividus* didn't seem prefer much either *Ulva lactuca*, a green alga, that as reported in the literature, would have seem to be the most preferred to *P. lividus*. As already mentioned above, among the red algae, a good consumption has been registered for *Laurencia obtusa*. These data are in contrast with those reported for this alga from Boudouresque and Verlaque (2007). The consumption of spinach is comparable to that recorded for the "most preferred algae" However, the consumption of maize is significantly higher than those recorded for macrophytes and for pellet Classic K® (Table 4.4.1). This unusual feeding behavior could be partly explained by the high content of carbohydrates and proteins of maize with respect to algae and spinach, which exhibit between them a very similar biochemical composition, characterized by a high water content and a moderate intake of proteins and lipids (Figure 4.4.1). Moreover, this odd food selection is in part due to the generalist and opportunistic feeding behavior of *P. lividus* that makes him able to take advantage of any food source (Boudouresque and Verlaque, 2007).

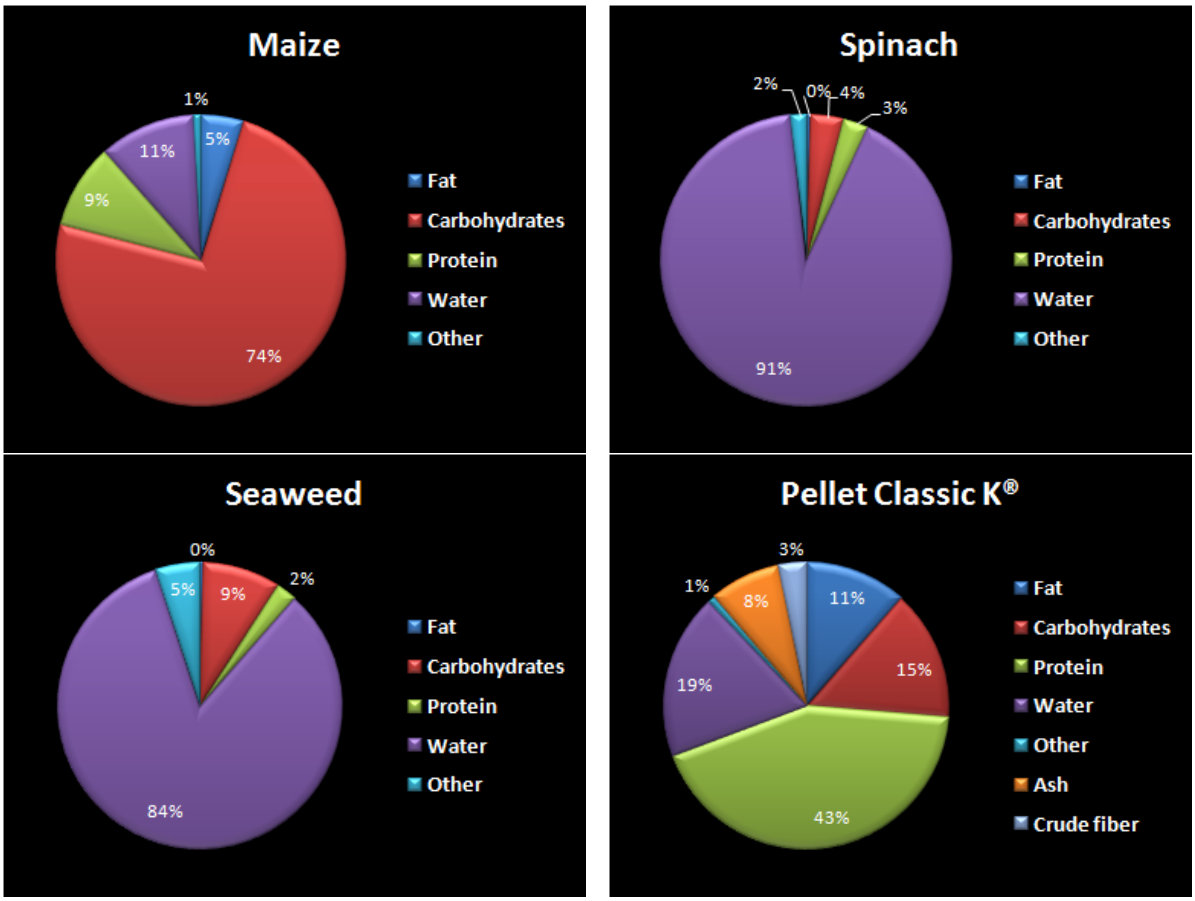


Figure 4.4.1: Biochemical Composition (%) of main ingredients employed in rearing *Paracentrotus lividus* in RAS.
Source: Data are available on the web site on the National Research Institute for Food and Nutrition (INRAN) and on the web site: http://www.valori-alimenti.com/nutrizionali/.

With regard the ingestion rates of pellet, the calculated values are similar to those obtained for quite preferred algae and spinach but are noticeably lower than those recorded for the maize. This result may in part be due to the fact that increasing the quality of the diet, and then the content of proteins and lipids, as in the case of administration of pellet, increase the energy required to assimilate these nutrients (Marsh and Watts, 2007) or, more simply, *P.lividus* show a greater preference for foods with a high content of carbohydrates as maize.

Chapter 5
Conclusion

The protocol employed for acclimatization of adults *Paracentrotus lividus* has enabled the successful transfer of organisms, from the natural habitat to the rearing tanks, minimizing stress on organisms and thus eliminating the possibility that they could accidentally spawn compromising irremediably the water quality of Recirculating aquaculture system.
The protocol of maintenance of the organisms in stand-by of sexual maturity has ensured maintenance of mature organisms in aquaria for a period of 4 months; time that has permit to overcome the summer months during which it is not possible to obtain gametes from organisms belonging to wild population.
The maintenance period was limited to 4 months only because the stock of organisms employed in this experimental phase has been finished . Therefore would be interesting to study what is temporal limit for the maintenance of organisms in maturity stage in RAS with artificial diet, fixed light regime and constant temperature without affecting the health of specimens and compromising the production of quality gametes.
Among the diets tested, to induce gonadal growth and sexual maturation of *P. lividus* in spent stage, those based on Maize and spinach gave the best results in terms of gonadosomatic index values(GI). However, tested diets, administered *ad libitum* with light regime 10h: L 14h D and 16 °C water temperature were found suitable to stimulate gametogenesis and ensure the production of gametes for ecotoxicological tests.
Among the treated diets, on the basis of histological analysis, we can affirm that, although all three diets are adequate in producing in a short time mature organisms, as regards the maintenance of specimens for a long period and thus to ensure constantly the presence of mature organisms in the tanks, only the diets Maize & Spinach and pellet Classic K® were found suitable, from the nutritional point of view, to guarantee the organism, a rapid transition from an inactive phase (stage VI-Spent) to an active phase of gametogenesis (stage II-V).
As regards the induction to the sexual maturation, plutei obtained from farmed organisms with the macrophytes are results, at the end of 9 weeks of rearing, significantly less sensitive (in terms of EC_{50} value to the toxic reference) compared with gametes obtained from farmed organisms with maize & Spinach and / or pellets Classic K®, although the values of EC_{50} for all three diets to the toxic reference are in line with the values reported for natural populations and correspond to the data of the literature (Lera and Pellegrini, 2006; Arizzi Novelli *et al*., 2003; Fernández and Beiras 2001; Volpi Ghirardini and Arizzi Novelli, 2001; Warnau *et al*., 1996; Dinnel *et al*., 1987; Nacci *et al.*, 1986).
With regard to gonadal growth is significant that both the farmed organisms with pelleted by fish farming, both for those brought up on sun gonadal algae growth has been reversed during the last three weeks of treatment (from 6th to 9th th week). If organisms reared with algae, we could speculate that the gonadal growth reached within six weeks of its peak beyond which you can not go without an improvement in the quality of the diet, for *P. lividus* raised pellet, it is likely that the large amount of proteins and lipids introduced with this type of diet has led to the absorption of these nutrients, a high energy cost (Marsh and Watts, 2007) which resulted in a regression of gonadal growth. Still referring to diets tested, the analyzes conducted, using microscopy techniques of second and third harmonic generation (HGM), on plutei obtained from organisms reared in the laboratory show significant differences between the diets used: in particular the plutei obtained from the diet Maize&Spinach while not presenting morphological abnormalities showed a clear signal "apoptosis" (increase in fluorescence) higher than plutei curly obtained from natural populations and farmed organisms with pellets and / or algae. We can therefore say that the diet Maize&Spinach, although it is good for both growth and gonadal
particularly suited to induce the maturation of organisms rapidly, leading to the production of gametes not as suitable for the generation of new individuals considering that the results obtained with innovative techniques of microscopy (HGM) indicate that the parapets generated with this diet may go undergo a process of programmed cell death (PCD). However, if we consider that the PCD is a physiological process necessary to sculpt the tissues of larvae of Paracentrotus lividus before

metamorphosis and apoptotic cells were found for this species in the arms and ciliary bands of competent larvae and juvenile (Roccheri *et al*., 2010) it is necessary to explore this further. An analysis of the rates of ingestion, brown seaweed Dyctiopteris sp, as was expected from the data reported in the literature (Verlaque and Nedelec 1983b, Verlaque 1984, 1987a, 1987b) was the seaweed most pleasing, however, is not as clear the poor palatability for the other brown algae administered. The low liking of the red algae, with the exception of Laurencia sp, could be related to the presence of substances brominate (Codomier *et al*., 1977) while the poor palatability of *Padina pavonica*, *Corallina elongata* and *Flabellia petiolata* could be due to the presence of precipitated calcium carbonate in the structure of seaweed. The consumption of spinach by the sea urchin was good and comparable to those observed during the experiments for "more like algae", but the maize was found to be the most consumed food from *Paracentrotus lividus*. This unusual feeding behavior could be partly explained by the high content of carbohydrates and protein than seaweed and spinach, which instead have a very similar biochemical composition characterized by a high water content and a moderate intake of proteins and lipids.

The extreme versatility of the sea urchin to adapt quickly to take advantage of all sources of food are administered is the one aspect that makes this species particularly suitable for use in plant breeding, especially for what could be called the fattening stage, ie the induction of gonadal growth of organisms taken from the wild before they are marketed.

In this regard, the analyzes conducted on diets during this period of experimentation, indicate that for the rearing of sea urchin, maize, in combination with spinach, is a food that provides high gonadal "yield" in a very short time and is able to promote gametogenesis. However, the good results obtained with the other diets, at least as regards the production of gametes, suggest that in a closed-loop system, where the food is not a limiting factor, the suitable temperature of breeding is the most important parameter in ensuring the maturation of individuals.

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Bibliografia

AA VV Acquacoltura Responsabile. In: Cataudella S & Bronzi P, eds., Unimar-Uniprom, Roma 2001, 683 pp.

Adams NL (2001) UV radiation evokes negative phototaxis and covering behaviour in the sea urchin *Strongylocentrotus droebachiensis*. Mar Ecol Prog Ser 213: 87-95.

Allain JY (1971) Note sur la pêche et la commercialisation des oursins en Bretagne nord. Trav Lab Biol Halieutique Univ Rennes 5: 59-69.

Allain JY (1975) Structure des populations de *Paracentrotus lividus* (Lamarck) (Echinodermata, Echinoidea) soumises à la pêche sur les côtes Nord de Bretagne. Rev Trav Inst Pech Marit 39 (2): 171-212.

Andrew NL, Agatsuma Y, Ballesteros E, Bazhin AG, Creaser EP, Barnes DKA, Botsford LW, Bradbury A, Campbell A, Dixon JD, Einarsson S, Gerring PK, Hebert K, Hunter M, Hur SB, Johnson CR, Juinio-Menez MA, Kalvass P, Miller RJ, Moreno CA, Palleiro JS, Rivas D, Robinson SML, Schroeter SC, Steneck RS, Vadas RL, Woodby DA, Xiaoqi Z . Status and management of world sea urchin fisheries. In: Oceanography and Marine Biology: an annual review, 40, Gibson R, Barnes M & Atkinson RJA, eds., CRC Press-Taylor & Francis Group 2002, pp. 343-425.

Arizzi Novelli A, Losso C, Ghetti PF, Volpi Ghirardini A (2003) Toxicity of heavy metal using sperm cell and embryo toxicity with *Paracentrotus lividus* (Echinodermata: Echinoidea): comparison with exposure concentration in the lagoon of Venice, Italy. Environ Toxicol Chem 22(6): 1295–1301.

Au DWT, Lee CY, Chan KL,Wu RSS (2001) Reproductive impairment of the sea urchins upon chronic exposure to cadmium. Part I: effects on gamete quality. *Environ Pollut*111: 1–9.

Aubin G (2004) Régime alimentaire des deux oursins réguliers *Paracentrotus lividus* et *Sphaerechinus granularir* dans une zone colonisée par *Caulerpa racemosa* var. *cylindracea* (Sonder) Verlaque, Huiman et Boudoursque du golfe de Marseille. Rapport stage Maîtrise, Univ Aix-Marseille 2.

Augier H, Ramonda G, Rolland J, Santimone M (1989) Teneurs en métaux lourds des oursin comestibles *Paracentrotus lividus* (Lamarck) prélevés dans quatre secteurs tests du littoral de Marseille (Méditerranée, France). Vie Mar HS 10: 226-239.

Axiak V, Saliba LJ (1981) Effects of surface and sunken crude oil on the behaviour of a sea urchin. Mar Pollut Bull 12: 14-19.

Azzolina JF (1988) Contribution à l'étude de la dynamique des populations de l'oursin comestibles *Paracentrotus lividus*. Thèse Doct, Univ Aix- Marseille 2.

Azzolina JF , Willsie A. Abondance des juveniles de *Paracentrotus lividus* au sein de l'herbier à *Posidonia oceanica*. In: Coloque international sur *Paracentrotus lividus* et les oursins comestibles, Boudouresque CF, eds., GIS 1987, Posidonie, Marseille, pp. 159-167.

Barille-Boyer AL, Barille L, Grue Y , Harin N (2004) Temporal changes in community structure of tide pools following the "Erika" oil spil. Aquat Living Resour 17 (3): 323-328.

Barker MF, Keogh JA, Lawrence JM , Lawrence AL (1998) Feeding rate, absorption efficiencies, growth and enhancement of gonad production in the New Zealand sea urchin *Evechinus chloroticus valenciennes* (Echinoidea: Echinodermata) fed prepared and natural diets. J Shellfish Res 17: 1583-1590.

Barnes DKA , Crook AC (2001) Quantifying behavioural determinants of the costal European sea-urchin *Paracentrotus lividus*. Mar Biol 138: 1205-1212.

Basuyaux O , Blin JL (1998) Use of maize as a food source for sea urchins in a recirculating rearing system. Aquac Int 6: 233–247.

Bayed A, Quiniou F, Benrha A , Guillou M (2005) The *Paracentrotus lividus* population from the northern Moroccan Atlantic coast: growth, reproduction and health condition. J Mar Biol Assoc U K 85: 999-1007.

Benedetti-Cecchi L , Cinelli F (1995) Habitat heterogeneity, sea urchin grazing and distribution of algae in littoral rock pools on the west coast of Italy (western Mediterranean). *Mar Ecol Prog Ser* 126: 203-212.
Bernádez C, Freire J , González-Gurriarán E (2000) Feeding of the spider crab *Maja squinado* in rocky subtidal area of the Ría de Arousa (north-west Spain). J Mar Biol Assoc U K 80:95-102.
Beyer D, Pearse JS , Steele ME Both photoperiod and diet influence resource partioning between somatic and gonad growth in sea urchin, *Strongylocentrotus franciscanus*. In: *Echinoderms: San Francisco,* Mooi & Telford, eds., Balkema Press 1998, Rotterdam, the Netherlands, pp. 577-590.
Bonnet A (1925) Documents pour servir à l'étude des variations chez les Echinides. Bull Inst Oceanogr (Monaco) 462: 1-28.
Boolootian RA. Reproductive physiology. In Physiology of Echinodermata, Boolotian RA, eds., Interscience Press 1996, New York, USA, 822 pp.
Boudouresque CF, Verlaque M, Azzolina JF, Meinesz A, Nédélec H, Rico V (1989) Evolutions des populations de *Paracentrotus lividus* et d'*Arbacia lixula* (Echinoidea) le long d'un tansect permanent à Galeria (Corse). Trav Sci Parc Nat Rég Rés Nat Corse 22: 65-82.
Boudouresque CF, Rodríguez-Prieto C , Arrighi F (1993) Place de *Caulerpa prolifera* (Chlorophyta) dans les preferences alimentaires de l'oursin *Paracentrotus lividus. Trav Sci Parc Nat Rég Rés Nat Corse* 41: 41-51.
Boudouresque CF, Verlaque M. Ecology of *Paracentrotus lividus* In Edible sea urchins: biology and ecology, Second edition, Lawrence J, eds., Elsevier Press 2007, Amsterdam, pp. 243-285.
Böttger SA, McClintock JB , Klinger TS (2001) Effect of inorganic and organic phosphates on feeding, feeding absorption, nutrient allocation, growth and righting response of the sea urchin *Lytechinus variegates. Mar Biol* 138: 741-751.
Bracho GE, Fritch JJ , Tash JS (1997) A method for preparation, storage andactivation of large population of immotile sea urchin sperm. Biochem Biophys Res Commun 237(1): 57–62.
Britton G (2008) Functions of intact carotenoids. In Carotenoids Vol.4 Natural Functions, Britton G, Liaaen-Jensen S & Pfander H, eds., Birkhäuser Verlag Press, Basel pp. 189–211.
Bulleri F, Benedetti-Cecchi L, Cinelli F (1999) Grazing by the sea urchin *Arbacia lixula* L. and *Paracentrotus lividus* Lam. in the Northwest Mediterranean. J Exp Mar Biol Ecol 241:81–95.
Buttino I, Pellegrini D, Romano G, Hwang JS, Liu TM, Sartori D, Sun CK, Macchia S, Ianora A (2011) Study of apoptosis induction in *Acartia tonsa* nauplii exposed to chronic concentration of Nickel. Chem Ecol 27(2):97-104
Byrne M (1990) Annual reproductive cycles of the commercial sea urchin *Paracentrotus lividus* from an exposed intertidal and a sheltered subtidal habitat on the west coast of Ireland. Mar Biol 104:275-289.
Caltagirone A, Francour P , Fernandez C. Formulation of an artificial diet for rearing of the sea urchin *Paracentrotus lividus*: I. Comparison of different binding agents. In Echinoderm Research, Scalera-Liaci L & Canicatti C, eds., Balkema Press 1992, Rotterdam, pp.115-119.
Cao G, Sofic E, Prior RL (1997) Antioxidant and prooxidant behavior of flavonoids: structure–activity relationships. Free Radic Res 22 (5):749–760.
Cellario C, Fenaux L (1990) *Paracentrotus lividus* (Lamarck) in culture (larval and benthic phases): parameters of growth observed two years following metamorphosis. Aquaculture 84:173-188.
Chapman GA. Sea urchin sperm cell test. In Fundamentals of AquaticToxicology: Effects, Environmental Fate and Risk Assessment:2nd Edition, Rand GM, eds.,Taylor and Francis Press 1995, pp. 189–205.
Chen SY, Hsieh CS, Chu SW, Lin CH, Ko CY, Chen YC, Tsai HJ, Hu CH , Sun CK (2006) Non invasive harmonics optical microscopy for long-term observation of embryonic nervous system development in vivo. J Biomed Opt 11:1-8.
Cherbonnier G (1954) Le roman des Echinodermes. Les beaux Livres, Rennes.
Cherbonnier G (1956) Les Echinodermes de Tunisie. Bull Stati océanogr Salammbô 53:1-23.

Codomier L, Bruneau Y, Combaut G, Teste J (1977) Etude biologique et chimique *d'Asparagopsis armata* et de *Falkenbergia rufolanosa* (Rhodophycées, Bonnemaisoniales). CR Acad Sci Paris 284 D:1163-1165.
Cognetti G, Sarà M , Magazzù G. In Biologia Marina,1a edizione, Calderini, eds., 2008, Bologna, Italy. pp.596.
Cook EJ , Kelly (2007) Enhanced production of the sea urchin *Paracentrotus lividus* in integrated open-water cultivation with Atlantic salmon *Salmo salar.* Aquaculture 273:573-585.
Crapp GB, Willis ME (1975) Age determination in the sea urchin *Paracentrotus lividus* (Lamarck) with notes on the reproduction cycle. J Exp Mar Biol Ecol 20:157-178.
Crook AC (2003) Individual variation in the covering behaviour of the shallow water sea urchin *Paracentrotus lividus*. PSZN: Mar Ecol 24 (4):275-287.
Crook AC, Barnes DKA (2001) Seasonal variation in the covering behaviour of the echinoid *Paracentrotus lividus.* PSZN: Mar Ecol 22 (3): 231-239.
Crook AC, Verling E, Barnes DKA (1999) Comparative study of the covering reaction of the purple sea urchin *Paracentrotus lividus*, under laboratoryand held conditions. J Mar Biol Assoc U K 79:1117-1121.
Cuomo V, Vanzanella F, Fresi E, Mazzella L , Scipione MB (1982) Microflora delle fanerogame marine dell'isola d'ischia: *Posidonia oceanica* (L.) Delile e *Cymodocea nodosa* (Ucria) Aschers. Boll Mus Ist Biol dell' Univ Genova suppl 50:162-166.
de Jong-Westman M, March BE , Carefoot TH (1995a). The effect of different nutrient formulations in artificial diets on gonad growth in the sea urchin *Strongylocentrotus droebachiensis*. Can J Zool 73:1495 – 1502.
de Jong-Westman M, Qian PY, March BE , Carefoot TH (1995b) Artificial diets in sea urchin culture: effects of dietary protein level and other additives on egg quality, larval morphometrics, and larval survival in the green sea urchin, *Strongylocentrotus droebachiensis.* Can J Zool 73:2080 – 2090.
Dambach M , Hentschel G (1970) Die Bedeckungsreaktion von Seeigeln. Neue Versuche und Deutungen. Mar Biol 6: 135-141.
Delmas P, Régis MB (1985) Impact de la pollution domestique sur la biologie et la morphométrie de l'échinoïde *Paracentrotus lividus* (Lamarck). Données preliminaries. CR Acad Sci Paris 300 (3, 4):143-146.
Delmas P, Régis MB (1986) Données preliminaries sur le contnu digestif de l'oursin comestibles *Paracentrotus lividus* (Lamarck) soumis à l'influence d'effluents domestiques. Mar Environ Res 20:197-220.
Delmas P (1992) Etude des populations de *Paracentrotus lividus* (Lam.) (Echinodermata: Echinoidea) soumises à une pollution complexe en Provence nord-occidentale: densités, structure, processus de détoxication (Zn, Cu, Pb, Cd, Fe). Thése Doct, Univ Aix- Marseille 3.
Devin MG. Land-based echinoculture: a novel system to culture adult sea urchins. In The sea urchin: From basic biology to aquaculture, Yokota Y, Matranga V & Smolenicka Z, eds., Swets & Zeitlinger B.V. Press 2002, Lisse, The Netherlands, pp. 145-159.
Diehl WJ III, McEdward L, Proffitt E, Rosenberg V, Lawrence JM (1979) The response of *Luidia clathrata* (Echimodermata: Asteroidea) to hypoxia. Comp Biochem Physiol A 61: 669-671.
Dincer T, Cakli S (2007) Chemical composition and biometrical measurements of the Turkish sea urchin (*Paracentrotus lividus,* Lamarck, 1816). Crit Rev Food Sci Nutr 27 (1):21-26.
Dinnel PA, Link JM, Stober QJ (1997) Improved methodology for a sea urchin sperm cell bioassay for marine waters. Arch Environ Contam Toxicol 16: 23-32.
Dix TG (1970) Covering response of the echinoid *Evechinus chloroticus* (Val.). Pac Sci 4:187-194.
Dominique F (1973) Contribution a l'ètude du cycle annuel de reproduction de deux éspeces d'echinoides (echinodermata) des cotes de Bretagne. B Sc Thesis, Université Libre de Bruxelles.
Drzewina A, Bohn G (1924) Un nouveau cas d'hermaphrodisme chez l'oursin, *Strongylocentrotus lividus*. CR Acad Sci Paris 178:662-663.

Ebling FJ, Hawkins AD, Kitching JA, Muntz L , Pratt WM (1966) The Ecology of Lough Inc. XVI. Predation and diurnal migration in *Paracentrotus* community. J Anim Ecol 35: 559-566.
Fabbrocini A, Lubrano Lavadera S, Rispoli S , Sansone G (2000) Cryopreservation of sea bream (*Sparus aurata*) spermatozoa. Cryobiology 40: 46-53.
Fabbrocini A, Di Stasio M , D'Adamo R (2010) Computerized sperm motility analysis in toxicity bioassays: a new approach to pore water quality assessment. Ecotoxicol Environ Saf 73:1588-1595.
FAO (2002) Fishery statistics: Capture production. FAO Yearbook Vol. 94/1.
FAO (2008) Fishery and Aquaculture statistics. FAO yearbook 2008. http://www.fao.org/docrep/013/i1890t/i1890t.pdf
FAO (2009) Fishery and Aquaculture Statistics. FAO Yearbook 2009. 78pp.
FAO (2012) The State of World Fisheries and Aquaculture 2012. Rome. 209 pp.
Fenaux L (1968) Maturation des gonade set cycle saisonnier des larves chez *A. lixula, P. lividus* et *P. microturberculatus* (echinides) à Villefranche-Sur-Mer. Vie Milieu 13:1-52.
Fernandez C (1989) Contribution à l'élaboration des bases scientifiques de l'aquaculture de l'oursin *Paracentrotus lividus.* Mém Maîtrise Sci Techn, Univ Corse.
Fernandez C (1990) Recherches preliminaries à la mise en placed'un ppilote d'aquaculture de l'oursin *Paracentrotus lividus.* Mém Maîtrise Sci Techn, Univ Corse.
Fernandez C (1996) Croissance et nutrition de *Paracentrotus lividus* dans le cadre d'un projet aquacole avec alimentation artificielle. PhD Thesis, Université de Corse, France.
Fernandez N , Beiras R (2001) Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the *Paracentrotus lividus* sea-urchin. Ecotoxicology 10:263–271.
Fernandez C , Boudouresque CF (1997) Phenotypic plasticity of *Paracentrotus lividus* (Echinodermata: Echinoidea) in a lagoonal environment. Mar Ecol Prog Ser 152(1-3):145-154.
Fernandez C, Caltagirone A. Growth rate of adult sea urchins, *Paracentrotus lividus* in a lagoon environment: The effect of different diet types. In Echinoderms through time, David B, Guille A, Féral JP & Roux M, eds., AA Balkema Press 1994, Rotterdam, pp. 655-660.
Fernandez C , Pergent G (1998) Effect of different formulated diets and rearing conditions on growth parameters in the sea urchin *Paracentrotus lividus*. J Shellfish Res 17:1571-1581.
Fernandez N, Beiras R (2001) Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the *Paracentrotus lividus* sea-urchin. Ecotoxicology 10:263–271.
Fernandez C, Pasqualini V, Johnson M, Ferrat L, Caltagirone A , Boudouresque CF. Stock evaluation of the sea urchin *Paracentrotus lividus* in a lagoonal environment. In Echinoderm research 2001, Féral JP & David B, eds., AA Balkema Press 2003, Lisse, pp. 319-323.
Finney DJ (1971) *Probit Analysis*, Cambridge University Press, London, UK.
Forcucci D, Lawrence JM (1986) Effect of low salinity on the activity, feeding, growth and asorption efficiency of *Luidia clathrata* (Echinodermata: Asteroidea). Mar Biol 92: 315-321
Frantzis A, Berthon JF, Maggiore F (1988) Relations trophiques entre les oursins *Arbacia lixula* et *Paracentrotus lividus* (Echinoidea Regularia) et le phytobenthos infralittoral superficiel dans la baie de Port-Cros (Var, France). Sci Rep Port-Cros Nation Park 14:81-140.
Frantzis A ,Grémare A (1992) Ingestion, absorption, growth rate of *Paracentrotus lividus* (Echinodermata: Echinoïdea) fed different macrophytes. Mar Ecol Prog Ser 95: 169–183.
Fuji A (1967) Ecological studies on the growth , food consumption of Japanese common littoral sea urchin, *Strongylocentrotus intermedius* (A. Agassiz). Mem Fac Fish Hokkaido Univ 15(2):83-160.
Fuji A, Kawamura K (1970) Studies on the biology of the sea urchin. IV. Habitat structure and regional distribution of *Strongylocentrotus intermedius* on a rocky shore of southern Hokkaido. Bull Jpn Soc Fish Oceanogr 36(8):755-762.
Gago J, Range P, Luis O. Growth, reproductive biology and habitat selection of the sea urchin *Paracentrotus lividus* in the coastal waters of Cascais, Portugal. In Echinoderm research 2001, Féral JP & David B, eds., AA Balkema Press 2003, Lisse, pp. 269-276.

Gamble JC. Ecological studies on *Paracentrotus lividus* (Lmk.). In Underwater association Report. TGW Industrial & Research Promotion Press 1966-1967, Carshalton, England, pp. 47-50.
Garmendia JM, Menchaca I, Belzunce MJ, Franco J , Revilla M (2009) Induction to maturation of the sea urchin *Paracentrotus lividus* (Lamarck, 1816) under laboratory conditions. Environ Technol 30 (13, 1): 1441–1446.
Giambartolomei FM (1990) Nuove acquisizioni sul test di fertilità dello sperma di riccio di mare *Paracentrotus lividus*, Thesis, University of Padova, Italy.
Goebel N, Barker M. Artificial diets supplemented with carotenoid pigments as feeds for sea urchins. In Echinoderms, Telford M, eds., Balkema Press 1998, pp. 667-672.
Grosjean P, Jangoux M. Effect of light on feeding in cultivated echinoids (*Paracentrotus lividus*). In Echinoderms Through Time, David B, Guille A, Feral JP & Roux M, eds., Balkema Press 1994, Rotterdam, the Netherlands, pp. 259-262.
Grosjean P, Spirlet C, Gosselin P, Vaitilingon D , Jangoux M (1998) Land-based, closed-cycle echiniculture of *Paracentrotus lividus* (Lamarck) (Echinodermata: Echinoidea): a long-term experiment at a pilot scale. J Shellfish Res17:1523-1531.
Grosjean P, Spirlet C, Jangoux M (1996). Experimental study of growth in the echinoid *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata). J Exp Mar Biol Ecol 201(1-2): 173-184.
Grosjean P, Spirlet C, Jangoux M (2003) A functional growth model with intraspecific competition applied to a sea urchin, *Paracentrotus lividus*. Can J Fish Aquat Sci 60(3):237-246.
Guerriero A, Meinesz A, D'ambrosio M, Pietra F (1992) Isolation of toxic and potentially toxic sesqui- and monoterpenes from the tropical green seaweed *Caulerpa taxifolia* which has invaded the region of Cap Martin and Monaco. Helv Chim Acta 75:689-695.
Guettaf M (1997). Contribution a l'étude de la variabilité du cycle reproductif (indice gonadique et histologie des gonades) chez *Paracentrotus lividus* (Echinodermata: Echinoidea) en Méditerranée sud occidentale (Algérie). These, Universite de la Mediterranee Aix-Marseille III.
Guettaf M, San Martin GA (1995) Etude de la variabilité de l'indice gonadique de l'oursin comestible *Paracentrotus lividus* (Echinodermata : Echinidae) en Méditerranée nord-occidentale. Vie Milieu 45 (2):129-137.
Guettaf M, San Martin GA, Francour P (2000) Interpopulation variability of the reproductive cycle of *Paracentrotus lividus* (Echinodermata: Echinoidea) in the south-western Mediterranean. J Mar Biol Ass U K 80:899-907.
Hagen NT (1996) Echinoculture: from fishery enhancement to closed cycle cultivation. World Aquac 27:6-19.
Hamilton MA, Russo RC, Thurston RV (1978) Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ Sci Technol 12:714–720.
Harmelin JG, Bouchon C, Hong JS (1981) Impact de la pollution sur la distribution des echinoderms des substrats durs un Provence (Méditerranée Nord-occidentale). Téthys 10 (1):13-36.
Haya D, Regis MB (1995) Comportement trophique de *Paracentrotus lividus* (Lam.) (Echinodermata: Echinoidea) soumis à six régimes alimentaires dans des conditions expérimentales. Mésogée 54:35-42.
Hayward PJ, Ryland JS. The Marine Fauna of British Isles and North-West Europe, 2. Clarendon Press 1990, Oxford, UK.
Hereu B, Zabala M, Linares C, Sala E (2005) The effect of predator abundance and habitat structural complexity on survival of juvenile sea urchin. Mar Biol 146:293-299.
Himmelman JH, Gederley H, Vignault G, Drouin G, Wells PG (1984) Response of the sea urchin, *Strongylocentrotus droebachiensis,* to reduce salinities: importance of size, acclimation and interpopulation differences. Can J Zool 62: 1015-1021.
His E, Heyvang I, Geffard O, De Montaudouin X (1999) A comparison between oyster (*Crassostrea gigas*) and sea urchin (*Paracentrotus lividus*) larval bioassay for toxicological studies. Water Res 33(7):1706–18.
Hyman LH. The invertebrates. IV. Echinodermata. McGraw-Hill Press 1955, New York.

ICES (1997) Report of the ICES Advisory Committee on the Marine Environment.
Ivlev VS. Experimental ecology of the feeding of the fishes. Yale University Press 1961, New Haven.
James P, Heath P, Unwin MJ (2007) The effects of season, temperature and initial gonad condition on roe enhancement of the sea urchin *Evechinus chloroticus*. Aquaculture 270: 115-131.
James P (2007) The Effects of Environmental Factors and Husbandry Techniques on Roe Enhancement of the New Zealand Sea Urchin, *Evechinus chloroticus* PhD Thesis Victoria University of Wellington.
Jangoux M, Lawrence JM. Echinoderm Nutrition, In Jangoux M , Lawrence JM, eds., Balkema Press 1982, Rotterdam, the Netherlands.
Joly-Turquin G, Dubois P, Coteur G, Danis B, Leyzour S, Le Menach K, Budzinski H , Guillou M (2009) Effects of the Erika oil spill on the common starfish *Asterias rubens*, evaluated by field and laboratory studies. Arch Environ Contam Toxicol 56 (2):209-220.
Keegan BF, Könnecker G. Aggregation in echinoderms on the west coast of Ireland. An ecological perspective. In Echinoderms: present and past, Jangoux M, eds., AA Balkema Press 1980, Rotterdam, pp. 199.
Kečkeš S, Ozretić B, Lucu Č (1966) About a possible mechanism involved in the shedding of sea-urchins. Experentia 22:146-147.
Keesing JK, Hall KC (1998) Review of harvests and status of world sea urchin fisheries points to opportunities for aquaculture. J Shellfish Res 17:1597-1604.
Kelly MS, Brodie C, McKenzie JD (1998) Somatic and gonadal growth of the sea urchin *Psammechinus miliaris* (Gmelin) maintained in polyculture with the Atlantic salmon. J Shellfish Res 17:1557-1562.
Kelly M (2001) Environmental parameters controlling gametogenesis in the echinoid *Psammechinus miliaris*. J Exp Mar Biol Ecol 266:67-80.
Kempf M (1962) Recherches d'écologie compare sur *Paracentrotus lividus* (Lmk.) et *Arbacia lixula* (L.). Rec Trav Stat Mar Endoume 25(39): 47-116.
Kennedy EJ , Robinson SMC (2007) Effects of dietary minerals and pigment on somatic growth of juvenile green sea urchins, *Strongylocentrotus droebachiensis.* J World Aquac Soc 38:36-48.
Kitching JA, Ebling FJ (1961) The ecology of Lough Ine. CI. The control of algae by *Paracentrotus lividus* (Echinoidea). J Anim Ecol 30: 373-383.
Kitching JA, Ebling FJ (1967) Ecological studies at Lough Ine. Adv Ecol Res 4:197-291.
Kitching JA, Thain VM (1983) The ecological impact of the sea urchin *Paracentrotus lividus* (Lamarck) in Lough Ine, Ireland. Phil Trans Roya Soc London B 300: 513-552.
Klinger TS (1997) Gonad and somatic production of Strongylocentrotus droebachiensis fed manufactured feeds. Bull Aquac Assoc Can 1:35-37.
Klinger TS, Lawrence JM, Lawrence AL. Digestion, absorption, and assimilation of prepared feed by echinoids. In Echinoderms:San Francisco, Mooi R & Telford M, eds., Balkema Press 1998, Rotterdam, pp. 713-721.
Knoepffler-Péguy M, Maggiore F, Boudouresque CF, Dance C. Compte Rendus d'une experience sur les preferenda alimentaires de *Paracentrotus lividus* (Echinoidea) à Banyuls-sur-Mer. In Colloque international sur *Paracentrotus lividus* et les oursins comestibles, Boudouresque CF, eds., GIS Posidonie Publication 1987, Marseilles, pp. 59-64.
Knoepffler-Péguy M, Nattero MJ. Comportement de *Paracentrotus lividus* en présence de la souche de *Caulerpa taxifolia* du port de St Cyprien (Fr. Pyr. Or.) In Second International workshop on Caulerpa taxifolia, Ribera MA, Ballesteros E, Boudouresque CF, Gómez A & Gravez V, eds., Publ Univ Barcellona (Spain) 1996, pp. 309-314.
Lambertson JQ, Dewitt TH, Swartz C. Assessment of sediment toxicity to marine benthos. In Sediment toxicity assessment, Burton GA, eds., Lewis Publishers 1992, pp. 183-211.

Lawrence J, Fenaux L, Corre MC, Lawrence A. The effect of quantity and quality of prepared diets on production in *Paracentrotus lividus* (Echinodermata: Echinoidea). In Echinoderm research 1991, Scalera-Liaci L & Canicatti C, eds., AA Balkema Press 1992, Rotterdam, pp. 107-110.
Lawrence JM (1976) Covering response in sea urchins. *Nature* 262: 490-491.
Lawrence JM, Cowell BC (1996) The righting response as an indication of stress in *Stichaster striatus* (Echinodermata: Asteroidea). Mar Freshw Behav Physiol 27: 239-248.
Lawrence JM, Olave S, Otaiza R, Lawrence AL , Bustos E (1997) Enhancement of gonad production in the sea urchin *Loxechinus albus* in Chile fed extruded feeds. J World Aquac Soc 28:91-96.
Lawrence JM. The edible sea-urchin. In Edible Sea Urchins: Biology and Ecology, Lawrence JM, eds., Elsevier 2001, Amsterdam, pp. 1-4.
Lawrence JM, McBride SC, Plank LR, Shpigel M. Ammonia tolerance of the sea urchins *Lytechinus variegates*, *Arbacia punctulata*, *Strongylocentrotus franciscanus*, and *Paracentrotus lividus*. In Echinoderm research 2001, Féral JP & David B, eds., AA Balkema 2003, Lisse, pp. 233-236.
Ledireac'h JP. La pêche des oursins en Méditerranée: historique, techniques, législation, production. In Colloque international sur Paracentrotus lividus et les oursins comestibles, Boudoresque CF, eds., GIS Posidonie Publication 1987, Marseille pp. 335-362.
Le Gall P. La pêche des oursins en Bretagne. In Colloque international sur *Paracentrotus lividus* et les oursins comestibles, Boudoresque CF,eds., GIS Posidonie Publication 1987, Marseille, pp. 311-324.
Le Gall P. Culture of Echinoderms. In Aquaculture, Vol. 1, Barnabé G, eds., Ellis Horwood Press1990, NewYork, USA, pp. 443-462.
Le Gall P, Bucaille D, Dutot P (1989) Rèsistance aux variations de salinité chez *Paracentrotus* et *Psammechinus.* Vie Mar HS 10: 83-84.
Lemée R, Boudouresque CF, Gobert J, Malestroit P, Mari X, Meinesz A, Ménager V, Ruitton S. Feeding behaviour of *Paracentrotus lividus* et *Psammechinus miliaris*. In Echinoderm research, De Ridder C, Dubois P, Lahaye MC & Jangoux M, eds., AA Balkema Press1996, Rotterdam, pp 183-188.
Lera S, Pellegrini D (2006) Evaluation of the fertilization capability of *Paracentrotus lividus* sea urchin storaged gametes by the exposure to different aqueous matrices. Environ Monit Assess 119:1-13.
Lozano J, Galera J, Lopez S, Turon X, Palacin C, Morera G (1995) Biological cycles and recruitment of *Paracentrotus lividus* (Echinodermata: Echinoidea) in two contrasting habitats. Mar Ecol Prog Ser 122: 179-191.
Luis O, Delgado F, Gago J (2005) Year-round captive spawning performance of the sea urchin *Paracentrotus lividus*: Relevance for the use of its larvae as live feed. Aquat Living Resour 18:45–54.
Maggiore F, Berthon JF, Boudouresque CF, Lawrence J. Données préliminarires sur les relations entre *Paracentrotus lividus*, *Arbacia lixula* et le phytobenthos dans la baie de Port-Cros (Var, France, Méditerranée). In Colloque international sur Paracentrotus lividus et les oursin comestibles, Boudouresque CF, eds., GIS Posidonie Publication 1987, Marseilles, pp. 65-82.
Marsh AG, Wats SA. Biochemical and energy requirements of gonad development. In Edible sea urchins: biology and ecology, Second edition, Lawrence J, eds., Elsevier 2007, Amsterdam pp. 35-53.
Martinelli J (1981) Actividad erosive de *Paracentrotus lividus* (Lmk.) (Echinodermata, Echinoidea) en el litoral gerundense. Oecol Aquat 5: 219-225.
Martínez I, García FJ, Sànchez AI, Daza JL, del Castillo F. Biometric parameters and reproductive cycle of *Paracentrotus lividus* (Lamarck) in three habitats of the southern Spain. In *Echinoderm research 2001*, Féral JP & David D, eds., AA Balkema Press (2003), Lisse, pp.281-287.

Martinez-Pita I, Garcia F J , Pita ML (2010) The effect of seasonality on gonad fatty acids of the sea urchins *Paracentrotus lividus* and *Arbacia lixula.* J Shellfish Res 29 (2): 517-525.
Mastaller M (1974) Zerstörung des Makrophytals an der nordadriatischen Küste durch intensive Abweiden durch Seeigel; eine Untersuchung über pipulationnsstrukturen, nahrung und Fressverhalten be idem Echinoidem *Paracentrotus lividus* (Lmrck). Dipl Arbeit, Zool Inst Uni München.
Mazzella L, Buia MC, Gambi MC, Lorenti M, Russo GF, Scipione MB, Zupo V (1992) Plant-animal relationship in the marine benthos. Oxford Science, Oxford, pp. 165-187.
McBride SC, Pinnix WD, Lawrence JM, Lawrence AL, Mulligan TM (1997) The effect of temperature on production of gonads by the sea urchin *Strongylocemtrotus franciscanus* fed natural and prepared diets. J World Aquac Soc 28: 357-365.
McBride SC, Price RJ, Tom PD, Lawrence JM, Lawrence AL (2004) Comparison of gonad quality factors: colour, hardness and resilience, of *Strongylocentrotus franciscanus* between sea urchins fed prepared feed or algal diets and sea urchins harvested from the Northern California fishery. Aquaculture 233: 405-422.
McCarron E, Burnell G, Kerry J, Mouzakitis G (2009) An experimental assessment on the effects of photoperiod treatments on the somatic and gonadal growth of the juvenile European purple sea urchin *Paracentrotus lividus*. Aquac Res: 1-10.
McLaughlin G, Kelly MS (2001) Effect of artificial diets containing cartenoid-rich microalgae on gonad growth and color in the sea urchin *Psammechinus miliaris* (Gmelin). J Shellfish Res 20: 377-382.
Millott N (1954) Sensitivity to light and the reactions to changes in light intensity of the echinoid *Diadema antillaum*. Phil Trans R Soc Lond B 238:187-220.
Millott N. The photosensitivity of echinoids. In Advances in Marine Biology, Russell FS & Yonge M, eds., Academic Press1975, NewYork, USA pp.1-52.
Minor MA , Scheibling RE (1997) Effects of food ration and feeding regime on growth and reproduction of the sea urchin *Strongylocentrotus droebachiensis*. Mar Biol 129:159-167.
Mol S, Baygar T, Varlik C, Tosun SY (2008) Seasonal variations in yield, fatty acids, amino acids and proximate compositions of sea urchin (*Paracentrotus lividus*) roe. *J* Food Drug Anal 16 (2):68-74.
Moore HB (1934) Acomparison of the biology of *Echinus esculentus* in different habitats. Part I. J Mar Biol Assoc U K 19:869-881.
Mortensen T. Handbook of the echinoderms of the British Isles. University Press 1927, Oxford.
Mortensen T (1943) A monograph of the Echinoidea, III.3 Camarodonta. II. Echinidae, Strongylocentrotidae, Paraseleniidae, Echinometridae. CA Reitzel, Copenhagen.
Mortensen A, Siikavuopio SI, Raa J. Use of transglutaminase to produce a stabile sea urchin feed. In Sea Urchins-Fisheries and Ecology: Proceedings of the International Conferences on Sea Urchin Fisheries and Aquaculture, Lawrence JM & Guzman O, eds., Puerto Varas, Chile March 25-27, DEStech Publications Inc./ Lancaster 2003, PA, USA, pp. 203-213.
Muntz L, Ebling FJ, Kitching JA (1965) The ecology of Lough Ine. XIV. Predatory activity of large crabs. J Anim Ecol 34:315-329.
Nacci D, Jackim E, Walsh R (1986) Comparative evaluation of three rapid marine toxicity tests: sea urchin early embryo growth test, sea urchin sperm cell toxicity test and Microtox. Environ Toxicol Chem 5:521 – 525.
Neefs Y (1937) Sur divers cas d'hermaphrodisme fonctionnel chez l'oursin *Strongylocentrotus lividus*. CR Acad Sci Paris 204 (11):901-902.
NEF (2012) Fish Dependence – 2012 Update. The increasing reliance of the EU on fish from elsewhere. www.ocean2012.eu.
Neill SRSJ, Larkum H. Ecology of some echinoderm in Maltese waters. In Symposium underwater association, Lythgoe JN & Woods JD, eds., Underwater Ass Press 1966, Malta, pp. 51-55.

Neill FX, Pastor R (1973) Relaciones tróficas de *Paracentrotus lividus* (Lmk.) en la zona litoral. Invest Pesq 37 (1):1-7.
Nédélec H (1982) Ethologie alimentaire de *Paracentrotus lividus* dans le baie de Galeria (Corse) et son impact sur les peuplements phytobenthiques. Thèse Doct 3° cycle, Univ Aix-Marseille 2.
Odile F, Boudouresque CF, Knoepffler-Péguy M. Etude expérimentale des preferenda alimentaires de *Paracentrotus lividus* (Echinoidea). In Actes du 6° Séminaire international sur les Echinodermes, Régis MB, eds., Ile des Embiez 1988 (Var-France), p. 85.
Olave S, Bustos E, Lawrence JM, Cárcamo P(2001) The effect of size and diet on gonad production by the Chilean sea urchin *Loxechinus albus*. J World Aquac Soc 32:210-214.
Palacin C, Giribert G, Turon X (1997) Patch recolonization through migration by the echinoid *Paracentrotus lividus* in communities with high algal cover and low echinoid densities. Cah Biol Mar 38:267-271.
Pantazis PA, Kelly MS, Connolly JG , Black KD (2000) Effect of artificial diet on growth, lipid utilization and gonad biochemistry in the adult sea urchin *Psammechinus miliaris*. J Shellfish Res 19:995-1001.
Pantazis PA (2009) The culture potential of *Paracentrotus lividus* (Lamarck 1816) in Greece: a preliminary report. Aquac Int 17:545–552.
Pastor R (1971) Distribución del erizo de mar, *Paracentrotus lividus* (Lmk), en la Ría de Vigo. Publ Técn Dir Gen Pesca Marít, Spain 9:255-270.
Pearce CM, Daggett TL, Robinson SMC (2002a) Effect of binder type and concentration on prepared feed stability and gonad yield and quality of the green sea urchin, *Strongylocentrotus droebachiensis*. Aquaculture 205:301–323.
Pearce CM, Daggett TL, Robinson SMC (2002b) Effect of protein source ratio and protein concentration in prepared diets on gonad yield and quality of the green sea urchin, *Strongylocentrotus droebachiensis*. Aquaculture 214:307–332.
Pearce CM, Daggett TL, Robinson SMC (2002c) Optimizing prepared feed ration for gonad production of the green sea urchin *Strongylocentrotus droebachiensis*. J World Aquac Soc 33:268–277.
Pearce CM, Daggett TL, Robinson SMC (2003) Effects of starch type, macroalgal meal source, and beta-carotene on gonad yield and quality of the green sea urchin, S*trongylocentrotus droebachiensis* (Müller), fed prepared diets. J Shellfish Res 22:505-519.
Pearce CM, Daggett TL, Robinson SMC (2004) Effect of urchin size and diet on gonad yield and quality in the green urchin (*Strongylocentrotus droebachiensis*). Aquaculture 233:337-367.
Pearse JB, Pearse VB , Davis KK (1986) Photoperiodic regulation of gametogenesis and growth in the sea urchin, *Strongylocentrotus purpuratus*. J Exp Biol 237:107-118.
Pearse JS, Cameron RA. Reproduction of marine invertebrates. In Echinodermata: Echinodea Vol.VI, Echinoderms and Lophophorates, Giese AC, Pearse JS & Pearse VB, eds., The Boxwood Press 1991, Pacific Grove, pp.435-448.
Pedrotti ML (1993) Spatial and temporal distribution and recruitment of echinoderm larvae in the Ligurian Sea. J Mar Biol Ass U K 73:513-530.
Percy JA (1973) Thermal adaptation in the boreo-aarctic *Strongylocentrotus droebachiensis* (O.F. Mueller, 1776). II. Seasonal acclimatization and urchin actvity. Physiol Zool 46:129-138.
Powis de Tenbossche T (1978) Comportement alimentaire et structures digestive de *Paracentrotus lividus* (Lamarck) (Echinodermata – Echinoidea). Mém Licence Sci biol, Université libre de Bruxelles
Régis MB (1978) Croissance de deux échinoïdes du golfe de Marseille (*Paracentrotus lividus* (Lmk) et *Arbacia lixula* L.). Aspects écologiques de la microstructure du squelette et de l'évolution des indeces physiologiques. Thèse Doct Sci, univ Aix-Mairselle 3.
Régis MB, Arfi R (1978) Etude compare de la croissance de trios populations de *Paracentrotus lividus* (Lamarck), occupant des biotopes different, dans le golfe de Marseille CR Acad Sci, Sér D 286:1211-1214.

Régis MB (1979) Analyse des fluctuation des indices physiologiques chez deux échinoides (*Paracentrotus lividus* (Lmck) et *Arbacia lixula* L.) du Golfe de Marseille. Téthys 9: 167-181.
Régis MB (1986) Microstructure adaptative des radioles de *Paracentrotus lividus* (Echinodermata: Echinoidea) en milieu eutrophisé par des eaux uses. Mar Biol 90: 271-277.
Richner H , Milinski M (2000) On the functional significance of masking behaviour in sea urchin – an experiment with *Paracentotus lividus.* Mar Ecol Prog Ser 205:307-308.
Rico V (1989) Contribution à l'ètude des preferenda alimentaires et du comportament moteur de l'oursin régulier *Paracentrotus lividus.* Dipl Études Approf en Océanogr, Univ Aix-Marseille 2.
Robinson SMC, Castell JD , Kennedy EJ (2002) Developing suitable colour in the gonads of cultured green sea urchins (*Strongylocentrotus droebachiensis*). Aquaculture 206:289-303.
Robinson SM The evolving role of aquaculture in the global production of sea urchin. In Sea Urchins-Fisheries and Ecology:Proceedings of the International Conferences on Sea Urchin Fisheries and Aquaculture, Lawrence JM & Guzman O, eds., Puerto Varas, Chile March 25-27, DEStech Publications Inc./Landcaster 2003, PA, USA, pp.343-357.
Roccheri MC, Tipa C, Bonaventura R , Matranga V (2002) Physiological and induced apoptosis in sea urchin larvae undergoing metamorphosis. Int J Dev Biol 46(6):801–806.
Ruiz- Fernandez JM (2000) Respusta de la fanerógama marina *Posidonia oceanica* (L.) Delile a perturbaciones antrópicas. Tesis Biología Univ Murcia, Spain.
Saito K (1992) Japan's sea urchin enhancement experience. In The Management and enhancement of sea urchins and other kelp bed resources: a Pacific rim perspective, Dewees CM, eds., California Sea Grant College Publication, University of California, La Jolla, CA92093-0232, pp. 1-38.
Sala E (1996) The role of fishes in the organization of a Mediterranean sublittoral community. Thèse Doct, Univ Aix-Marseille 2.
Sala E (1997) Fish predators and scavengers of the sea urchin *Paracentrotus lividus* in protected areas of the nort-west Mediterranean Sea. Mar Biol 129:531-539.
Sala E, Zabala M (1996) Fish predation an the structure of the sea urchin *Paracentrotus lividus* population in the NW Mediterranean. Mar Ecol Prog Ser 140:71-81.
San Martin G (1995) Contribution à la gestion des stocks d'oursins: etude des populations et transplantations de *Paracentrotus lividus* à Marseille (France, Méditerranée) et production de *Loxechinus albus* à Chiloe (Chili, Pacifique). Ph. D. Thesis, Université d'Aix – Marseille, 166 pp.
Sánchez-España AI, Martínez-Pita I, García FJ (2004) Gonadal growth and reproduction in the commercial sea urchin *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata: Echinoidea) from southern Spain. Hydrobiologia 519:61-72.
Sellem F, Langar H, Pesando D (2000) Age et croissance de l'oursin *Paracentrotus lividus* Lamarck, 1816 (Echinodermata-Echinoidea) dans le golfe de Tunis (Méditerranée). Oceanol Acta 23(5):607-613.
Semroud R, Kada H. Contribution à l'étude de l'oursin *Paracentrotus lividus* (Lamarck) dans la région d'Alger (Algérie): indice de réplétion et indice gonadique. In Colloque international sur Paracentrotus lividus et les oursin comestibles, Boudouresque CF, eds., GIS Posidonie Publication 1987, Marseille, pp.117-124.
Sharp DT, Gray JE (1962) Studies on factor affecting the local distribution of two sea urchin *Arbacia punctulata* and *Lytechinus variegates.* Ecology 43(2):309-313.
Shpigel M, McBride S, Marciano S, Lupatsch I (2004) The effect of photoperiod and temperature on the reproduction of European sea urchin *Paracentrotus lividus*. Aquaculture 232:343-355.
Siikavuopio SI, Dale T, Mortensen A , Foss A (2007) Effects of hypoxia on feed intake and gonad growth in the green sea urchin, *Strongylocentrotus droebachiensis*. Aquaculture 266:112-116.
Spirlet C, Grosjean Ph, Jangoux M (1998a) Reproductive cycle of the echinoid *Paracentrotus lividus*: analysis by means of the maturity index. Invertebr Reprod Dev 34:69-81.
Spirlet C, Grosjean Ph, Jangoux M (1998b) Optimizing food distribution in closed-circuit cultivation of edible sea urchins (Paracentrotus lividus: Echinoidea). Aquat Living Resour 11(4):273-277.

Spirlet C, Grosjean Ph, Jangoux M (2000) Optimisation of gonad growth by manipulation of temperature and photoperiod in cultivated sea urchins, *Paracentrotus lividus* (Lamarck) (Echinodermata). Aquaculture 185:85-99.
Sun CK, Chu SW, Chen SY, Tsai TH, Liu TM, Lin CH, Tsai HJ (2004) Higher harmonic generation microscopy for developmental biology. J Struct Biol 147: 19–30.
Tertsching W (1989) Diel activity patterns and foraging dynamics of the sea urchin *Tripneustes ventricosus* in a tropical seagrass community and a reef environment (Virgin Islands). Mar Ecol 10: 3-21.
Tortonese E (1965) Fauna d'Italia; Echinodermata. Edizioni Calderini, pp. 422. Bologna.
Torunski H (1979) Biological erosion and its significance for the morphogenesis of limestone coasts and nearshore sedimentation (Northern Adriatic). Senckenb Marit 11:193-265.
Traer K.The consumption of *Posidonia oceanica* Delile by Echinoids at the Isle of Ischia. In Echinoderms: present an past, Jangoux M, eds., AA Balkema Press 1980, Rotterdam, pp. 241-244.
Tufarelli V e Laudadio V (2006) Il consumo di alimento nei pesci d'acquacoltura. *Il Pesce* 3: 16.
Turon X, Giribert G, López S, Palacín C (1995) Growth and population structure of *Paracentrotus lividus* (Echinodermata: Echinoidea) in two contrasting habitats. Mar Ecol Prog Ser 122: 193-204.
Tyler A (1949) A simple non-injurious method for inducing repeated spawning of sea urchins and sand dollars. Collecting Net 19, 19–20.
Uriz MJ, Turon X, Becerro MA , Galera J (1996) Feeding deterrence in sponges. The role of toxicity, physical defense, energetic contents, and life-history stages. *J Exp* Mar Biol Ecol 205: 187-204.
Vadas RL, Beal B, Dowling T, Fegley JC (2000) Experimental field tests of natural algal diets on gonad index and quality in the green sea urchin, *Strongylocentrotus droebachiensis*: a case for rapid summer production in post-spawned animals. Aquaculture 182:115-135.
Verlaque M (1984) Biologie des juveniles de l'oursin herbivore *Paracentrotus lividus*: sélectivité du broutage et impact de l'espèce sur les communautés algales de substrat rocheux en Corse (Méditerranée, France). Bot Mar 27 (9):401-424.
Verlaque M. Relations entre *Paracentrotus lividus* (Lamarck) et le phytobenthos de Mediterranée occidentale. In Colloque international sur Paracentrotus lividus et les oursin comestibles, Boudouresque CF, eds., GIS Posidonie Publication 1987a, Marseille, pp. 5-36.
Verlaque M (1987b) Contriution à l'étude du phytobenthos d'un écosystème photophile termophile marin en Méditerranée occidentale. Etude structurale et dynamique du phytobenthos et analyse des relations faune-flore. Thèse Doct, Univ Aix-Marseille 2.
Verlaque M, Nédélec H. Relations entre *Paracentrotus lividus* (Lamarck) et le phytobenthos de Méditerranée occidentale. In Colloque international sur *Paracentrotus lividus* et les oursin comestibles, Boudouresque CF, eds., GIS Posidonie Publication 1983a, Marseilles, pp. 5-36.
Verlaque M, Nédélec H (1983b) Biologie de *Paracentrotus lividus* (Lamarck) sur substrat rocheux en Corse (Méditerranée, France): alimentation des adultes. Vie Milieu 33 (3-4):191-201.
Verling E, Crook AC, Barnes DKA (2002) Covering behaviour in *Paracentrotus lividus*: is light important? Mar Biol 140:391-396.
Visconti G, Agnetta D, Gianguzza P, Musmeci MT, Piscopo L, Riggio S. Ciclo riproduttivo e gametogenesi di *Paracentrotus lividus* (Echinodermata: Echinoidea) nell' AMP "Isola di Ustica". *Atti del XVIII Congresso Nazionale SItE,* 2008: 67-73
Volpi Ghirardini A , Arizzi Novelli A (2001) A sperm cell toxicity test procedure for the Mediterranean species *Paracentrotus lividus* (Echinodermata: Echinoidea). Environ Technol 22:439 – 445.
Walker CW , Lesser MP (1998) Manipulation of food and photoperiod promotes out-of-season gametogenesis in the green sea urchin *Strongylocentrotus droebachiensis*: implications for aquaculture. Mar Biol 132:663-676.

Warnau M, Iaccarino M, De Biase A, Temara A, Jangoux M, Dubois P , Pagano G (1996) Spermiotoxicity and embryotoxicity of heavy metals in the Echinoid *Paracentrotus lividus*. Environ Toxicol Chem 15:1931– 1936.
Watts SA, Boettger A, McClintok JB , Lawrence JM (1998) Gonad production in the sea urchin *Lytechinus variegatus* (Lamarck) fed prepared diets. J Shellfish Res 17:1591-1595.
Williams H (2002) Sea Urchin Fisheries of the World: A Review of Their Status, Management Strategies and Biology of the Principal Species – Background Paper, Department of Primary Industries and Water, Tasmania (http://www.dpiw.tas.gov.au/internnsf/Attachments/HMUY-5DA58V/$FILE/World_urchin_%20fisheries.pdf).
Woods CM, James P, Moss G, Wright J , Siikavuopio SI (2008). A comparison of the effect of urchin size and diet on gonad yield and quality in the sea urchin *Evechinus chloroticus* Valenciennes. Aquac Int 16:49-68.
Yamamoto M, Ishine M , Yoshida M (1998) Gonadal maturation independent of photoic conditions in laboratory reared sea urchins, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrinus*. Zool Sci 5:979-998.
Yokota Y. Fishery and consumption of the sea urchin in Japan. In The sea urchin: From basic biology to aquaculture, Yokota Y, Matranga V & Smolenicka Z, eds., Swets & Zeitlinger B.V. 2002, Lisse, The Netherlands, pp. 129-138.
Zavodnik D (1980) Distribution of Echinodermata in the north Adriatic insular region. Acta Adriat 21 (2):437-468.
Zupi V, Fresi E. A study of the food web of the *Posidonia oceanica* ecosystem: analysis of the gut contents of echinoderms. In International workshop on Posidonia oceanica beds, Boudouresque CF, Jeudy de Grissac A & Olivier J, eds., GIS Posidonie Publication 1984, Marseille, pp. 373-379.