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Role of steroid hormones in echinoid reproductive biology

PhD thesis

Silvia Mercurio

R08972

PhD tutor: Prof.ssa M. D. Candia Carnevali

PhD coordinator: Prof. M. Ferraguti

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To my twin sister

for the best advice I have ever received

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Chapter I

ABSTRACT & THESIS SYNOPSIS

1. Abstract

Echinoid reproductive cycle has been extensively studied in several species but the mechanisms regulating gametogenesis processes are still scarcely understood. Apart from environmental factors, different research have suggested a steroid role in gonad maturation and growth. Particularly, in echinoderms steroid involvement in reproduction has been suggested by both studies on seasonal changes of steroid levels during the gonadal cycle and experiments of hormone administration. Nevertheless, the steroid function in echinoid reproductive processes has not been clearly identified, probably due to the low number of studies and the big variability of results reported. Thus, the main aim of this research project was to shed light on echinoid endocrinology and, in particular, to clarify the involvement of sex-steroid hormones in sea urchin reproductive biology. This was achieved employing both *in vivo* and *in vitro* approaches.

First of all, considering the lack of studies on the development of effective cell cultures from echinoderm gonads, primary cell cultures from ovaries of the edible sea urchin *Paracentrotus lividus* were developed. Ovary cell phenotypes, present in culture, were identified and characterized by different microscopic techniques. Although cell cultures could be produced from ovaries at all stages of maturation, the cells appeared healthier and viable, displaying a higher survival rate, when ovaries at early stages of gametogenesis were used. In terms of culture medium, ovarian cells were successfully cultured in modified Leibovitz-15 medium, whereas poor results were obtained in Minimum Essential Medium Eagle and Medium 199. Different substrates were tested but ovarian cells completely adhered only on poly-L-lysine. To improve *in vitro* conditions and stimulate cell proliferation different serum-supplements were tested. Fetal Calf Serum and an originally developed Pluteus Extract resulted to be detrimental to cell survival, apparently accelerating processes of cell death. In contrast, cells cultured with sea urchin Egg Extract appeared larger and healthier, displaying an increased longevity that allowed to maintain them for up to 1 month. Overall this study provides new experimental bases and procedures for producing successfully long-term primary cell cultures from sea urchin ovaries, providing a simple and versatile experimental tool for research in echinoderm reproductive biology.

Subsequently, *in vivo* and *in vitro* experiments, specifically addressed to determine possible 17β-estradiol (E2) and testosterone (T) involvement in echinoid reproduction, were performed. An *in vivo* long-term experiment of steroid dietary administration was performed in adult specimens of *P. lividus*. The experimental plan was specifically designed in order to reduce individual variability and synchronize the experimental animals at the same starting maturative condition. We analysed and compared different reproductive parameters (Gonad Index, Maturative Index and maturative

stages distribution) in 4 experimental groups: control group (CTL), E2 and T groups fed with pellets containing respectively 17β-estradiol and testosterone, and E2-4 weeks group fed with control pellets for the first 4 weeks and then treated with 17β-estradiol. This latter was chosen in order to verify the existence of a specific E2-sensitive gametogenic stage, as proposed in different asteroid species.

Possible steroid effects on *P. lividus* female reproduction was also investigated with an *in vitro* approach. Cells, isolated by ovaries in the same maturative conditions considered in the *in vivo* experiments, were cultured in presence of E2 and T physiological concentrations for 2 weeks. Effects on ovarian cell morphology and behaviour were investigated. In addition, steroid regulation of the Major Yolk Protein (MYP) expression was analyzed 24 and 48 hours after E2 and T exposure. According to our results, E2 and T do not markedly influence echinoid gonad maturation and, particularly, they do not promote gamete maturation. Hormonal dietary administration did not induce striking variations in the considered reproductive parameters and no effect was observed also when males and females were analyzed separately. In addition, no specific maturative stage sensitive to E2 was found, suggesting the existence of different hormonal mechanisms in asteroids and echinoids. Similar considerations could be reported taking into account the *in vitro* experiments. E2 and T exposure did not affect ovarian cell size and behaviour nor MYP expression.

The obtained results suggest that these hormones are not directly involved in either gamete maturation, as demonstrated for vertebrates, or in vitellogenesis processes, as reported for several asteroid species. However a possible involvement of steroids in echinoid physiology cannot be completely excluded and their role in the regulation of lipid metabolism and protein synthesis during the different reproductive stages should be strongly considered as suggested by several authors.

Further specific research on steroid hormone mode of action, physiological function and metabolism are therefore needed to completely understand echinoid reproduction and endocrinology.

2. List of abbreviations

2-DE	Two-dimensional electrophoresis
AR	Androgen Receptor
CFMYP	MYP isoform found in sea urchin Coelomic Fluid
CMFSW	Ca^{2+} Mg^{2+} Free Sea Water
CTL	Control (experimental group)
E1	Estrone
E2	17β-estradiol
EDC	Endocrine Disrupting Compound
EE	Egg Extract
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
EGMYP	MYP isoform found in sea urchin Eggs
ER	Estrogen Receptor
ERR	Estrogen Receptor-related Receptor
FCS	Fetal Calf Serum
GI	Gonad Index
GC-MS	Gas Chromatography Mass Spectrometry
HDMS	Hexamethyldisilazane
L-15	Leibovitz L-15 medium
LLTP	Large Lipid Transfer Protein
M199	Medium 199
MEM	Minimum Essential Medium Eagle
MI	Maturity Index
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MYP	Major Yolk Protein
T	Testosterone
SpSHR2	*Strongylocentrotus purpuratus* orphan steroid receptor 2
vtg	Vitellogenin

3. Aims and thesis synopsis

The general aim of this research project was to shed light on echinoid endocrinology and, in particular, to explore the involvement of steroid hormones in sea urchin reproductive biology. In fact, the current knowledge about these hormones on echinoid reproduction is still fragmentary and most of the studies have reported different and, sometimes, even contrasting results. Considering the ecological and, in some cases, commercial importance of this marine invertebrates, further investigations are certainly needed. This research was therefore addressed to investigate the role of sex-steroid hormones, 17β-estradiol (E2) and testosterone (T), in the reproductive biology of the regular sea urchin *Paracentrotus lividus*, applying both *in vivo* and *in vitro* approaches. In fact, the employment of these different and complementary approaches should provide a wider view of sea urchin endocrinology and help to finally unravel steroid role in echinoids.

In **Chapter II**, a review of the current knowledge regarding steroid hormone involvement in echinoderm reproduction is presented. After an accurate description of the experimental model, the common sea urchin *Paracentrotus lividus*, a detailed summary of previous studies on sex-steroid role in different asteroid and echinoid species is provided. Particular attention is given to E2 and T, whose function in echinoid reproductive processes was investigated in this research. Finally a general overview of available data on primary cell cultures from marine invertebrates is also proposed.

In **Chapter III**, the development of primary cell cultures from sea urchin ovaries is described. Indeed, in echinoids there was no study reporting the development of effective cell cultures from sea urchin gonads. In this work the first attempt to obtain cultures of *P. lividus* ovarian cells was successfully carried out. After an accurate characterization of the cell phenotypes present in culture, our priorities were to optimize the culture conditions, i.e. to define the suitable medium, substrate and possible serum supplements. The obtained results, which are provided in details throughout this chapter, have been already published in international scientific journal.

In **Chapter IV**, investigations on possible E2 and T functions on echinoid reproduction are extensively described. Sex-steroid involvement in *P. lividus* reproductive processes were investigated employing both *in vivo* and *in vitro* experiments. In particular, a long-term experiment of E2 and T dietary administration was performed. The experimental plan was specifically designed in order to obtained reliable results and different reproductive parameters were analyzed. The observed results were then confirmed and deepened with *in vitro* steroid exposure experiments.

Chapter V presents a general and integrated discussion of all the obtained results; new interesting fields and techniques for future investigations are suggested.

Overall, this project was addressed to provide further information on the scarcely known endocrinology of echinoderms and, in particular, of echinoids. Our specific targets were: 1) to analyze thoroughly echinoid reproductive processes and their regulatory mechanisms by focussing on the possible role of 17β-estradiol and testosterone and 2) to provide new information on possible control mechanisms of gonad development in *P. lividus*, an edible and commercially relevant species.

Chapter II

GENERAL INTRODUCTION

1. The experimental model: *Paracentrotus lividus*

Paracentrotus lividus is a regular sea urchin, belonging to the phylum Echinodermata.

Echinoderms are exclusively marine organisms, widespread in all the oceans from shallow waters of intertidal zones to the deep abyssal plains. They are deuterostome invertebrates, being therefore phylogenetically close to chordates (including vertebrates) (Brusca and Brusca, 1990). This phylogenetic position is particularly interesting since it may suggest the existence of common basic mechanisms between the two groups, such as processes of hormonal regulation (Sugni et al., 2007).

At present there are about 7000 extant species of echinoderms, traditionally divided in five classes: Asteroids (starfishes), Crinoids (sea lilies and feather stars), Echinoids (sea urchins and sand dollars), Holoturoids (sea cucumbers) and Ophiuroids (brittle stars) (Fig. 1). In spite of the apparent diversity that characterizes the phylum, echinoderms display several distinctive morphological features. The most typical are:

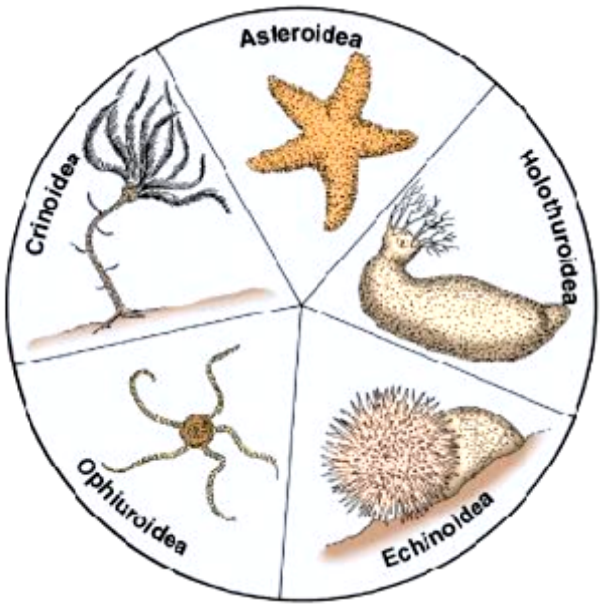

Fig. 1. Echinorderm classes.

- the pentamerous radial symmetry (in adults): all the echinoderms indeed begin their development as bilateral embryos and larvae but during metamorphosis they acquire the characteristic pentamerous symmetry;
- the mesodermal endoskeleton: echinoderm endoskeleton consists of skeletal plates produced and embedded in the dermal layer of the body wall. These plates can be closely connected, forming the test, or differentially scattered and distributed in the connective tissue (ossicles);
- the water-vascular system: this is a complex system of fluid-filled coelomic canals whose numerous projections (tube feet) are involved in a variety of functions, such as feeding, locomotion, respiration and possibly excretion (Brusca and Brusca, 1990).

Paracentrotus lividus (Fig. 2) has a hemispherical body, densely covered by long and sharply pointed spines. The test encloses and protects the internal organs and is composed of connected plates of skeletal tissue, whose inorganic component consists of calcium carbonate, whereas the organic component includes ECM, collagen fibres and sclerocytes (Chia and Harrison, 1994).

This species is widely distributed in the Mediterranean Sea and in the North-East Atlantic Ocean, from Ireland to the Canarians (Mortensen, 1943). In the Mediterranean coasts it is one of the most common sea urchins, widespread in the lower rocky shore, in the shallow sublittoral areas and in beds of seagrass (Riedl, 1991).

Fig. 2. *Paracentrotus lividus*.

The high ecological relevance of this echinoid is related to its impact on the algal community. *P. lividus* is one of the main Mediterranean herbivorous species on rocky bottoms and its foraging activity remarkably affects the composition and the dynamics of algal and rocky littoral pools, making this organism a key element in costal ecosystem maintenance (Lawrence, 1975). It has been demonstrated that a density of four individuals per square meter can reduce to 30-50% of algal cover whereas eleven specimens per square meter can virtually eliminate it at all (Kitching and Ebling, 1961). In addition, this species is commercially relevant since its gonads are an appreciated food in many Mediterranean regions (Gago et al., 2001).

Fig. 3. Gonads of *P. lividus.*

Although some hermaphroditic specimens have been occasionally found (Byrne, 1990), *P. lividus* is a gonocoristic species without a marked sexual dimorphism. Secondary sex differences are related to the shape of the genital papillae and gonopores (Swann, 1954) and they can be easily recognized only under a stereomicroscope when specimens

are close to the spawning event (personal observation). Sex determination mechanism has been scarcely investigated but most of the authors suggested that it is on genotypic basis (Pearse and Cameron, 1991; Lipani et al., 1996).

Regular echinoids have five gonads, internally located in the aboral hemisphere and anchored to the inter-ambulacral plates by thin peritoneal mesenteries (Pearse and Cameron, 1991) (Fig. 3). Gonads have a sac-like elongated shape with an internal alveolar structure. Each gonad is composed by hundreds of acini opening into a central gonoduct; this latter reaches the gonopore, opening in one of the genital plates surrounding the anus. Mature gametes are released through the gonoducts and gonopores directly in the sea water (Hyman, 1955; Piatigorsky, 1975). The larvae, called echinopluteus, are pelagic and planktotrophic until they become competent larvae and undergo metamorphosis (Hyman, 1955).

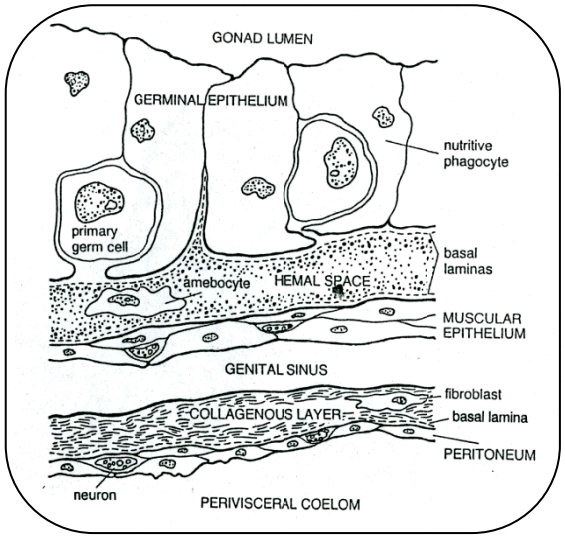

Fig. 4. Schematic representation of the microscopical structure of sea urchin gonad.

P. lividus gonads (Fig. 4) consist of two main types of cells: germinal cells and somatic cells, these latter commonly called nutritive phagocytes (Chia and Bickell, 1983).

Nutritive phagocytes are gonad interstitial cells that vary in both morphological aspect and chemical composition throughout the reproductive cycle. At the beginning of gametogenesis, they are voluminous, with a mean diameter up to 50 μm, and occupy almost the whole acinal lumen surrounding the germinal cells with their long processes (Smiley, 1990). During the cycle they decrease in size, probably because of nutrient transfer to developing gametes, until they are replaced by mature gametes (Nicotra and Serafino, 1988). Generally, they have a round nucleus, often characterized by an evident central nucleolus (Nicotra and Serafino, 1988; Pearse and Cameron, 1991), and large heterogeneous cytoplasmatic inclusions, filled by glyco- and lipoproteins, polysaccharides, proteoglycans and lipids (Chatlynne, 1969; Houk and Hinegardner, 1980). Nutritive phagocytes play multiple roles: they not only provide support, protection and nutriment to the growing germinal cells, functioning as storage, transfer site and active synthesizing place, but they also "clean" the gonad after spawning (Chia and Bickell, 1983; Walker et al., 2000).

The maturation of male germinal cells occurs, as in most of the animals, through a series of meiotic divisions and morpho-functional transformations during the spermatogenesis process. Scattered primitive spermatogonia are present in groups at the base of the germinal layer during non-reproductive months. The (primary and secondary) spermatocytes are distributed closer to the testis

lumen and form several layers converging in spermatogenic columns which infiltrate the nutritive phagocyte layer. The following spermatid stage is characterized by several morpho-functional differentiation events (spermiogenesis) which eventually convert the relatively undifferentiated, early spermatids into highly specialized, functional spermatozoa. Mature spermatozoa are accumulated in the centre of the acinal lumen (Piatigorsky, 1975; Chia and Bickell, 1983).

As spermatogenesis, oogenesis consists of different progressive stages (oogonium, primary oocyte, secondary oocyte and mature ovum), each characterized by several morpho-functional and biochemical modifications. Oogonia are about 5-7 μm in diameter and have a large, oval nucleus with one prominent nucleolus. They tend to align close to the germinal epithelium base, often organised in clusters. The characteristic feature of the young undifferentiated oocytes is their large nucleus (germinal vesicle). The nucleolus, which is in contact with the inner nuclear membrane in the oogonium, in the early vitellogenic oocyte is found in the nucleus centre. In sea urchins meiosis is completed within the ovary and mature eggs are accumulated in the acinus lumen. Besides the plasma membrane and the vitelline membrane, a third transparent layer, called jelly coat, covers the sea urchin eggs and plays important roles in fertilization (Piatigorsky, 1975; Kanatani and Nagahama, 1983).

The maturity stages of the gonadal cycle can be determined by histological analysis. In *P. lividus* previous studies have described a cycle composed of a variable number of stages (Byrne, 1990; Spirlet et al., 1998; Unuma et al., 2003). On the basis of these works and of our previous studies we considered a cycle of five progressive stages (Fig. 5 and 6).

0-Spent: This is the stage after the spawning event. Spent ovaries have thin acinal walls and appear to be empty except for some relict oocytes. The number and type of vitellogenic oocytes and ova present in the ovary is variable and they will be eventually reabsorbed by nutritive phagocytes. The testes seem to be devoid of content although relict spermatozoa may be present. In both sexes nutritive phagocytes appear as a pale meshwork around the gonad periphery.

1-Recovery: Oogonia/spermatogonia and rare young oocytes/spermatocytes are the only germinal cells present along the gonadal walls. The very early gametic stages can make sex identification very difficult. Nutritive phagocytes are large cells full of heterogeneous inclusions, including relic material from phagocytosed gametes.

2-Growing: The gametogenesis processes have begun in both sexes. The ovary contains clusters of primary oocytes along the acinal wall; the testis periphery is underlined by a thin layer of spermatogonia and primary spermatocytes. Nutritive phagocytes are full of nutritive material and form a regular meshwork all over the acinus.

3-Premature: Gametes at all developmental stages are present in the gonads. In the ovary, large primary oocytes start migrating towards the acinus centre and when they reach maximus size, they undergo maturation and early ova can start accumulating in the lumen. Nutritive phagocytes are still present, although displaced from the luminal position by large interposed oocytes. In the testis, columns of spermatocytes can be observed along the acinal wall and mature spermatozoa begin to accumulate in the acinus centre. As in ovaries, nutritive phagocytes are displaced from the luminal position by the mature spermatozoa.

4-Mature: At this stage ovaries are filled with mature ova. The nutritive phagocytes are few in number and confined to the border. Mature testes are filled as well with spermatozoa whereas nutritive phagocytes are restricted to the peripheral area. The spawning event occurs at this stage.

To quantitatively describe the seasonal trend of the reproductive cycle different numerical parameters can be used. One of the most common is the Gonad Index (GI) (Spirlet et al., 1998; Shpigel et al., 2004). In the present study the GI is defined as: GI = (GW / TW) × 100. GW is the wet weight of the five gonads and TW is the wet weight of the whole animal. This index provides information on the different allocation of nutrients to somatic and gonadal production. The GI values tend to increase during the stages before the spawning event and suddenly fall after it.

Another important parameter is the Maturity Index (MI), a numeric value associated to the maturative stage of the gonads. In a population, the mean value of MI numerically describes its reproductive state.

The Tyrrhenian population considered in the present research usually displays a first main reproductive event at the end of the spring, and a further minor and facultative spawning period in early autumn (Fenaux, 1968).

Several exogenous factors can influence gametogenesis: water temperature and photoperiod (Byrne, 1990; Spirlet et al., 1998; Shpigel et al., 2004), food availability (Leoni et al., 2001), environmental hydrodynamics (Fenaux, 1968). Water temperature between 18 and 22 °C seems to enhance growth and gonadal development (Shpigel et al., 2004), whereas higher (24 °C) temperatures appear to inhibit spawning (Spirlet et al., 1998). Photoperiod may affect gonad maturation and the first spawning event appears to be triggered by 15 h-long day (Spirlet et al., 1998). Food appears to play an important role in the regulation of the reproductive cycle too; gametogenesis cannot be initiated until a “critical level” of nutriments is available within the storage tissues (nutritive phagocytes) to ensure gametes growth (Pearse and Cameron, 1991; Spirlet et al., 1998). Apart from these exogenous factors, several endogenous factors, notably hormones, probably play an important role in synchronizing the gonads individually (Spirlet et al., 1998).

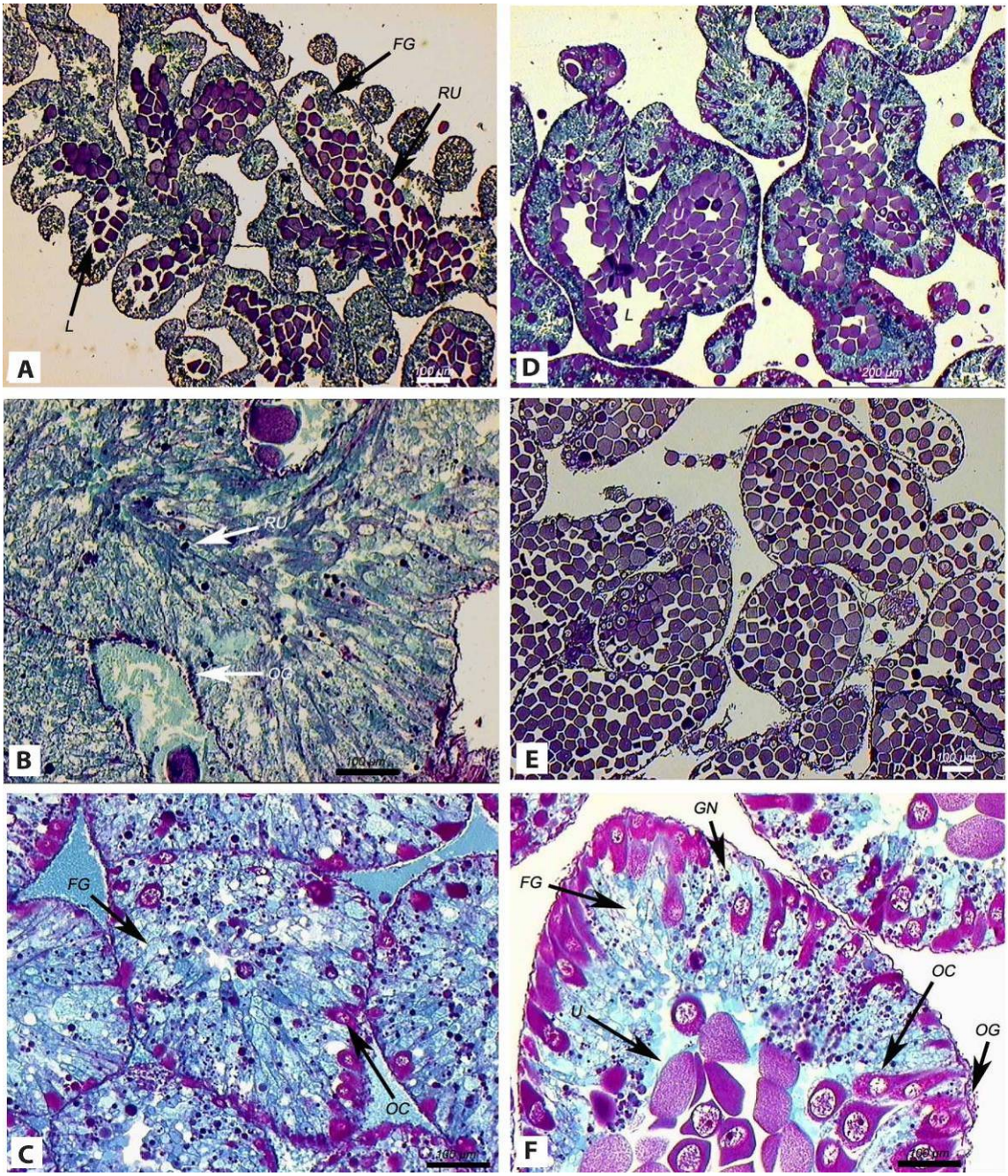

Fig. 5. Maturity stages of the gonadal cycle of *Paracentrotus lividus* female specimens: histological cross-sections of gonads, embedded in paraffin and stained with Milligan's Trichrome. **A)** *Spent*. Ovaries seem to be empty, although relict oocytes and ova (RU) may be present in the acinal lumen (L). Nutritive phagocytes (FG) appear to form a pale meshwork all around the gonad periphery. **B)** *Recovery*. Oogonia (OG) are the only germinal cells present along the gonadal walls. Nutritive phagocytes are large and full of heterogeneous inclusions, including relict material from phagocyted gametes. **C)** *Growing*. The gametogenesis processes have begun. The ovary contains clusters of primary oocytes (OC) along the acinal wall and nutritive phagocytes, full of nutritive material, form a regular meshwork all over the acinus. **D)** *Premature*. Gametes at all developmental stages are present in the ovary. Large primary oocytes start migrating towards the acinus centre, where they undergo maturation. Early ova (U) start accumulating in the lumen. Nutritive phagocytes are still present, although displaced from the luminal position by large interposed oocytes. **E)** *Mature*. At this stage ovaries are packed among mature ova. The nutritive phagocytes are few in number and confined to the border. **F)** *Detail of oogenesis.* Gametes at progressive oogenesis stages (oogonia, oocites and mature ova) can be observed from the acinal wall to the lumen. Phagocytes with cytoplasmatic inclusions (GN) are also evident.

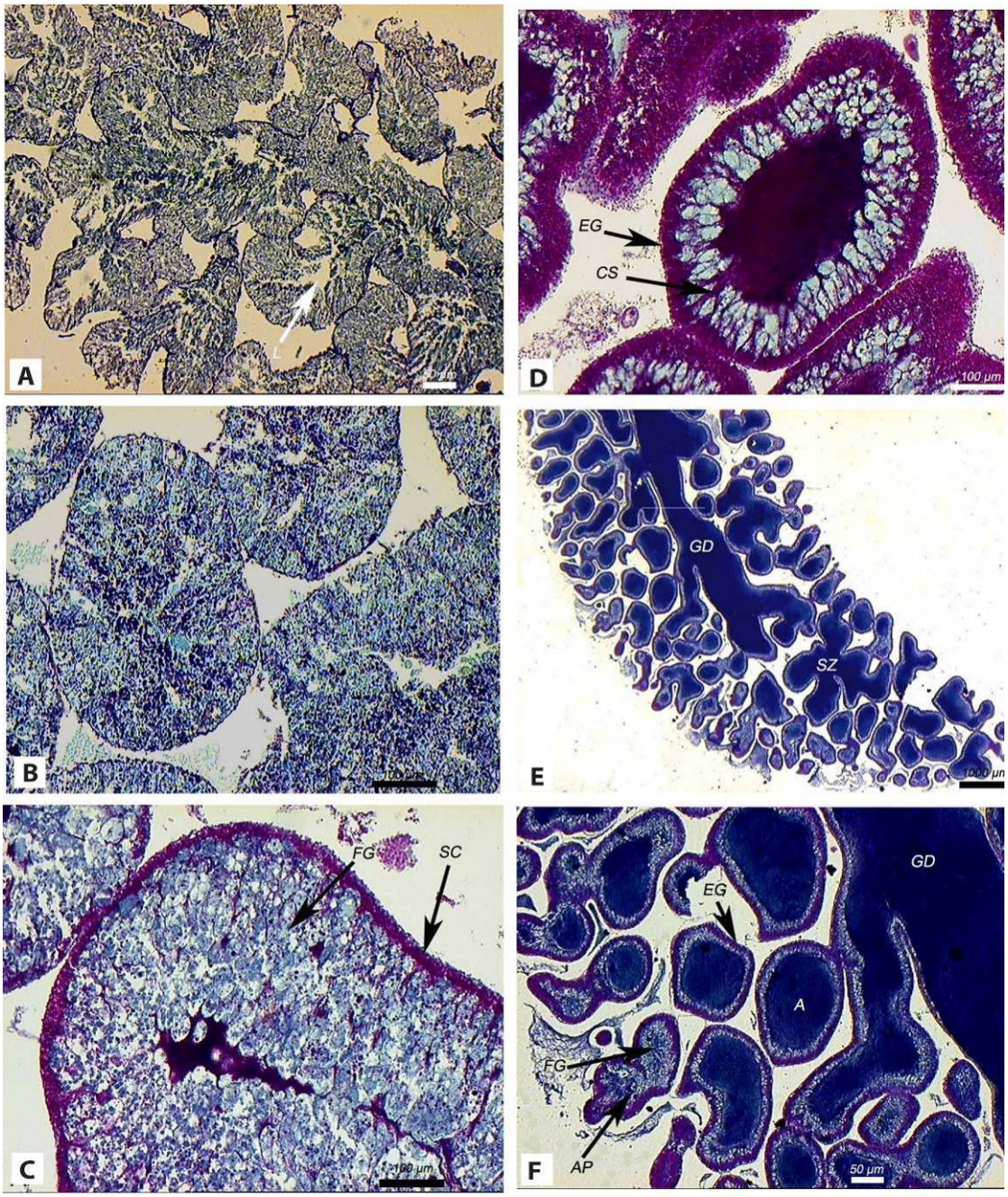

Fig. 6. Maturity stages of the gonadal cycle of *Paracentrotus lividus* male specimens: histological cross-sections of gonads, embedded in paraffin and stained with Milligan's Trichrome. **A)** *Spent*. Testes appear to be empty, although relict spermatozoa may be present in the lumen (L). Nutritive phagocytes appear as a pale meshwork around the gonad periphery. **B)** *Recovery*. Rare spermatogonia are the only germinal cells present along the gonadal walls. Nutritive phagocytes are large and full of heterogeneous inclusions, including relict material from phagocyted gametes. **C)** *Growing*. The gametogenesis processes have begun. The testis periphery is underlined by a thin layer of spermatogonia and primary spermatocytes (SC). Nutritive phagocytes (FG) are full of nutritive material and form a regular meshwork all over the acinus. **D)** *Premature*. Gametes at all developmental stages are present in the gonad. Columns of spermatocytes (CS) can be observed along the testis germinal epithelium (EG) and mature spermatozoa begin to accumulate at the acinus centre. Nutritive phagocytes are displaced from the luminal position by the mature spermatozoa. **E)** *Mature.* Mature testes are packed among spermatozoa (SZ) and nutritive phagocytes are restricted to the peripheral area. The gonoduct (GD) can be observed in the testis centre. **F)** *Acini along the gonoduct*. In each acinus germinal epithelium, spermatozoa and nutritive phagocytes are still evident. Note that peripheral acini appear partially devoid of gametes if compared with acini closer to the gonoduct.

2. Steroid hormones: 17β-estradiol and testosterone

Hormones are substances usually secreted into the circulating fluids and thus distributed to all parts of the organism. Hormones transduce environmental information and regulate physiological processes, particularly reproductive and developmental events such as gametogenesis, maturation, spawning, growth and metamorphosis (Hau, 2007).

In particular, steroid hormones are widespread molecules synthesized from cholesterol. Together with estrone (E1), 17β-estradiol (E2) belongs to the class of estrogens whereas testosterone (T) is one of the most representative androgens (Mathews and Van Holde, 1994).

The role of estrogens in human reproduction is well-known. They are the most important reproductive hormones in women, responsible for the development of secondary sexual characters and the regulation of menstrual cycle (Rosati and Colombo, 2001). Overall, it is generally accepted that estrogens are effectors within the endocrine system of all vertebrates, though their specific physiological role seems to be less conserved. Estrogens regulate metabolic, behavioural and morphological changes during the reproductive cycle of females, and they also play an important role in several processes of males. For example, in Eutherian females, beside effects on endometrium proliferation and cervical mucus, E2 (Fig. 7) is the only substance able to mediate oestrus symptoms. With enlargement of the developing follicle, E2 production rises and brings to ovulation. In males, it is involved in different reproductive activities, such as testicular fluid production or intratesticular sperm transport. In oviparous vertebrates, estrogens are important not only in the regulation of the reproductive cycles but they also have a relevant influence on prenatal development, particularly on sexual differentiation. In addition, estrogens are involved in vitellogenin synthesis as well as in calcium and phosphorus retention and deposition to make them available for egg-shell formation, and influence reproductive behaviour (Lange et al., 2002).

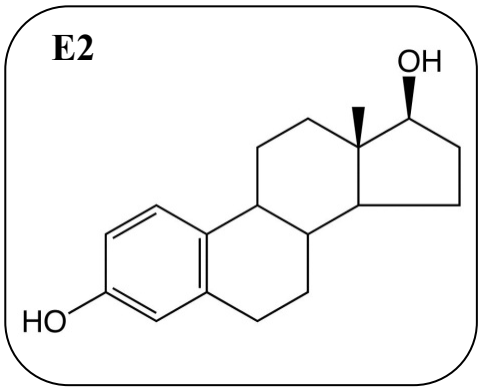

Fig. 7. 17β-estradiol.

Testosterone (Fig. 8) is a sex steroid mainly produced by the testes that is found in all the vertebrates. In men it plays a key role in the development of the male reproductive tissues

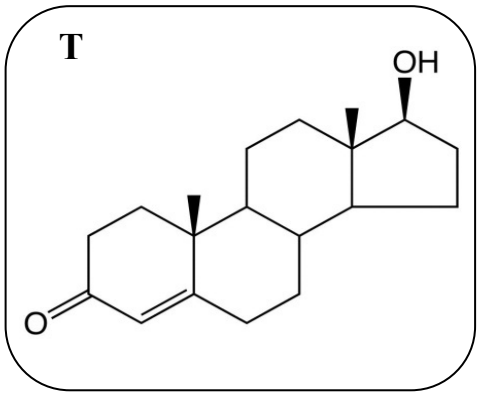

Fig. 8. Testosterone.

as well as promoting secondary sexual characteristics such as increased muscle mass and growth of body hair (Mathews and Van Holde, 1994; Rosati and Colombo, 2001). Across vertebrates, gonadal T secretion occurs typically at the beginning of the breeding season to support reproductive processes. T stimulates male courtship and sexual behaviours, territorial aggression, expression of secondary sexual characters and sperm production. This hormone generally promotes short-term reproductive success whereas it seems to suppress immune function and parental care. In mammals T affects morphological, physiological and behavioural sex differentiation. For example, during embrionic development, T is secreted by testes and is involved in the organization of male accessory sex organs, body development and central nervous system. In non-mammalian vertebrates, sexual differentiation is less dependent on sex steroids and more influenced by geophysical factors (Hau, 2007).

Although the presence of vertebrate-type steroids has been documented in almost all invertebrate groups, the demonstration for an endogenous synthesis is available only in molluscs and echinoderms. In other groups the presence of several key enzymes has not been demonstrated and the identification of these molecules relies only on radioimmunoassay, a quite accurate technique that anyway cannot be considered as definitive proof.

In molluscs most of the vertebrate sex-steroids and, particularly T and E2, are considered to be present. Steroid identification, metabolic conversions, annual variations, sex-related differences, in *vivo* and *in vitro* biological effects and receptor characterization have been demonstrated in the main classes of molluscs. However, although steroid biosynthesis has been strongly investigated, the presence of an aromatase, i. e. the enzyme that catalyzes the conversion of androgens into estrogens, has not been definitely demonstrated.

In other invertebrates the presence of vertebrate-type steroids is poorly documented. These molecules have been identified in some species of annelids, platyhelminthes and cnidarians but the experimental evidences are limited and their origin remains to be determined (Lafont and Mathieu, 2007).

3. E2 and T involvement in echinoderm reproduction

The presence of vertebrate-type steroids and, particularly, of testosterone (T) and estradiol (E2) has been documented in several echinoderm species (Lafont and Mathieu, 2007). Most studies referred to asteroids and echinoids, where these molecules have been detected by several techniques, such as bioassay, radioimmunoassay and gas chromatography-mass spectrometry (GC-MS) (Dieleman and Schoenmakers, 1979; Hines et al., 1992b; Voogt et al., 1992).

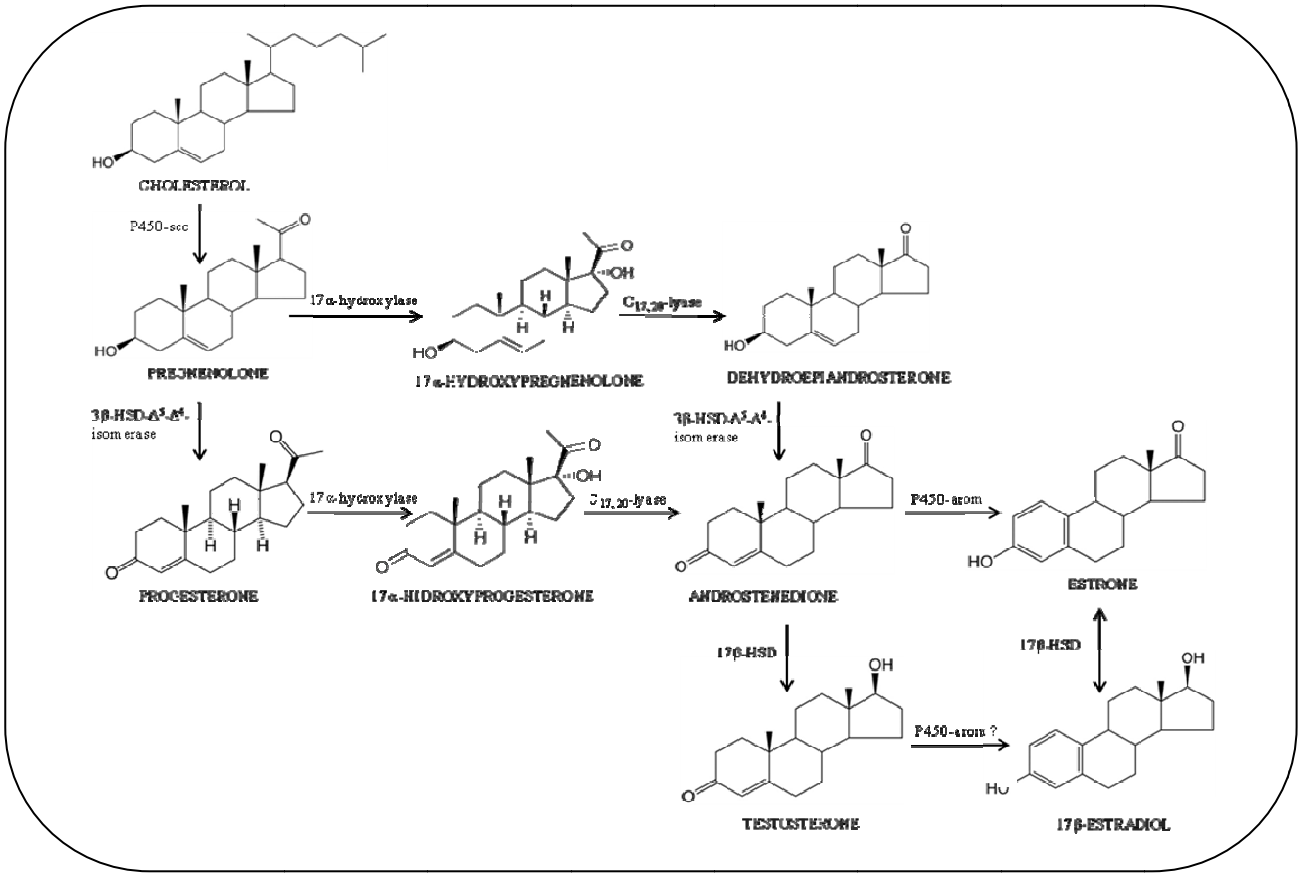

Fig.9. Schematic representation of steroid synthesis and metabolism in echinoderms. P450-scc: P450 side-chain cleavage; P450-arom: P450-aromatase; 17β-HSD: 17β-hydroxysteroid dehydrogenase.

Several studies have also demonstrated the presence of vertebrate-like steroid metabolic pathways in different echinoderm tissues (Schoenmakers, 1979; Schoenmakers and Voogt, 1980; Schoenmakers and Voogt, 1981; Voogt and Van Rheenen, 1986; Hines et al., 1994). The androgen metabolism has been successfully described (Schoenmakers and Voogt, 1981; Voogt and Van Rheenen, 1986; Hines et al., 1992b; Wasson et al., 1998; Janer et al., 2005), whereas the estrogen biosynthesis is still unclear and only few studies have investigated this pathway (Hathaway, 1965; Schoenmakers and Voogt, 1981; Hines et al., 1994) (Fig. 9).

Steroid hormone involvement in the regulation of the echinoderm reproduction has been suggested by several studies on seasonal changes of steroid levels during the gonadal cycle (Voogt and

Dieleman, 1984; Xu and Barker, 1990; Hines et al., 1992a; Wasson et al., 2000a; Barbaglio et al., 2007). Most data refer to asteroids, where the hormone levels appear to vary according to the reproductive cycle and in a sex-specific manner (Voogt and Dieleman, 1984; Xu and Barker, 1990; Hines et al., 1992a). Maximum estrogen levels were registered at the beginning of vitellogenesis in both *Sclerasterias mollis* (Xu and Barker, 1990) and *Asterias rubens* (Schoenmakers and Dieleman, 1981), suggesting that these hormones may affect protein biosynthesis, transport or incorporation into oocytes. Slightly different results were found in *Asterias vulgaris* where E2 increased in the fall in parallel with oogonia/spermatogonia proliferation (Hines et al., 1992a). As far as T is concerned, it seems to be involved in gamete maturation and gonad growth (Voogt and Dieleman, 1984; Xu and Barker, 1990; Hines et al., 1992a). In *A. vulgaris* transient increases in the T levels coincided with spermatogenic column formation and, in the ovaries, T concentrations were high at the onset of oogenesis and during early maturation of oocytes (Hines et al., 1992a), suggesting its involvement in the regulation of early stages of gonad maturation.

Moving to echinoids, the role of steroid hormones in the reproductive processes is still unclear. In both ovaries and testes of *Lytechinus variegatus,* T and E2 concentrations were higher during the period of early gonadal growth. These levels were much lower than those measured in asteroids, probably due to different regulation mechanisms of gamete nutrition in the two echinoderm groups (Wasson et al., 2000a).

Focussing on *P. lividus,* the experimental model employed in this research project, our previous studies did not allow us to derive a clear correlation between T levels and the distribution of reproductive stages through the year. Nevertheless, a relationship between T levels and reproduction was strongly suggested. In testes, T concentrations were significantly lower during spermatogenesis processes than at the end of gametogenesis, suggesting a possible role of T in late sperm maturation and spawning. In ovaries, T levels resulted higher during growing stage, suggesting a T involvement in vitellogenesis (Barbaglio et al., 2007), as also reported in asteroids (Hines et al., 1992a). As far as E2 is concerned, it was clear that E2 concentrations are lower than T levels in both sexes. Furthermore, mean E2 concentration appeared to be lower in testes than in ovaries, possibly reflecting a more important role for this hormone in female individuals. In the ovaries, E2 levels were higher in early maturative stages, indicating a possible E2 involvement in the regulation of nutritive phagocyte activity and/or oogonium proliferation. On the contrary, in the testes, higher levels of E2 were measured in advanced maturative stages, suggesting a role in sperm maturation (Barbaglio et al., 2007). These E2 peaks were “temporally” anticipated in the coelomic fluid, i.e. they were found in the stages immediately before. These findings lead to the hypothesis

that E2, after being synthesizing in digestive tube, can be released in coelomic fluid, through which it reaches gonads, the putative target organs (unpublished data).

In order to elucidate the physiological role of steroids in echinoderms a number of experiments of hormone administration have been performed both *in vivo* and *in vitro* on some asteroid and echinoid species. These researches are summarised in Table 1. In most studies hormonal treatment resulted in appreciable physiological effects on different reproductive parameters.

Species	**Approach**	**Steroid**	**Treatment type**	**Period**	**Effects**	**References**
Asterina pectinifera (A)	in vitro	E2	a; daily	3 d	↑ oocyte diameter and ↑ % of oocyte in advanced maturative stage	(Takahashi and Kanatani, 1981)
Asterias rubens (A)	in vivo	E2	b; daily	16 d	↑ oocyte diameter; ↑ GI ♀; ↑ MI ♀; ↑ E1 levels	(Schoenmakers et al., 1981)
Asterias rubens (A)	in vitro/ in vivo	E2	a & b; 1st & 7th day	8 d	↑ lipid content in pyloric caeca	(Van der Plas et al., 1982)
Luidia clathrata (A)	in vivo	E2	b; every 2 days	16 d	↑ activity of metabolic enzymes (G-6-PDH and 6-PGDH)	(Watts and Lawrence, 1987)

Species	**Approach**	**Steroid**	**Treatment type**	**Period**	**Effects**	**References**
Sclerasterias mollis (A)	in vivo	E2	b, daily	16 d	↑ oocyte area, ↑ ovarian protein, ↑ E1 level	(Barker and Xu, 1993)
Dendraster excentricus and *Strongylocentrotus purpuratus* (E)	in vitro	E2	a	4 & 24 h	Synthesis of novel protein in non-gravid females	(Harrington and Ozaki, 1986)
Pseudocentrotus depressus (E)	in vivo	E2	c, daily	1 m	No effects of E2; E1 ↑ GI ♂ and promote spermatogenesis	(Unuma et al., 1999)
Pseudocentrotus depressus (E)	In vivo	T	C, daily	1 m	No effect of T; Androstenedione ↑ GI ♂	(Unuma et al., 1999)
Lytechinus variegatus (E)	in vivo	E2	c; daily	36 d	↑ ovarian growth; ↑ protein percentage	(Wasson et al., 2000b)
Lytechinus variegatus (E)	in vivo	T	c; daily	36 d	↑ oocyte diameter; ↑ protein percentage	(Wasson et al., 2000b)

Species	Approach	Steroid	Treatment type	Period	Effects	References
Strongylocentrotus purpuratus (E)	in vivo	E2	b; 1/week	8 w	↓embryo sensitivity to E2; ↑ SpSHR2 transcript in the eggs	(Roepke et al., 2005)
Strongylocentrotus nudus (E)	in vivo	E2	b	48 h	↑ protein synthesis	(Varaksina and Varaksin, 2001)
Strongylocentrotus intermedius (E)	in vivo	E2	b	48 h	↑ ovarian protein synthesis; no effect before spawning	(Varaksina and Varaksin, 2002)
Paracentrotus lividus (E)	in vivo	E2	b; 2/week	2 & 12 w	no effect on reproductive parameters	(Mercurio et al., 2012)

Table 1. Experiments of steroid administration in echinoderms. A = asteriod; E = echinoid; a = culture medium; b = injection; c = diet; m = month; w = week; d = day; h = hours; ↓ = decrease; ↑ = increase; GI = Gonad Index; MI = Maturity Index; E1 = estrone; G-6-PDH = glucose-6-phosphate dehydrogenase; 6-PGDH = 6-phosphogluconate dehydrogenase; SpSHR2 = orphan steroid receptor.

In addition, an indirect involvement of steroid hormones in echinoderm reproduction and, particularly, their role in the regulation of lipid metabolism and protein synthesis should be strongly considered, as hypothesized by several other authors (Van der Plas et al., 1982; Barker and Xu, 1993; Wasson et al., 2000b; Varaksina and Varaksin, 2001; Varaksina and Varaksin, 2002).

Previous research suggested that a specific role of E2 in echinoderm reproduction could be related to lipid accumulation during the different reproductive stages (Van der Plas et al., 1982; Wasson et al., 2000b). In the asteroid *A. rubens*, E2 treatment increased the lipid content in the pyloric ceaca (Van der Plas et al., 1982) and, in the sea urchin *L. variegatus*, administration of E2 in combination

with progesterone similarly increased lipid percentage in the gonads (Wasson et al., 2000b). Although species-specific differences can be certainly found, these results indicate a hormonal control of lipid incorporation. This hypothesis is further supported by our previous studies reporting that in ovaries of *P. lividus*, under physiological conditions, higher E2 levels were found right during those reproductive stages characterized by nutrient accumulation and processing (unpublished data).

Studies on the biochemical composition of echinoid gonads have revealed that gametogenesis is typically characterized by increased protein levels; in particular, in *P. lividus*, protein levels were found significantly correlated to the Gonad Index (Fernandez, 1998). As suggested by several studies, steroids could be involved also in protein synthesis: E2 and T administration was demonstrated to enhance the rate of protein synthesis in both asteroid (Barker and Xu, 1993) and echinoid gonads (Varaksina and Varaksin, 2001; Varaksina and Varaksin, 2002) and an E2 induction of protein synthesis was also observed in *Strongylocentrotus purpuratus* and *Dendraster excentricus* coelomocytes (Harrington and Ozaki, 1986). In addition, estrogen could be involved in the expression of the sea urchin Major Yolk Protein (MYP) (Harrington and Ozaki, 1986; Shyu et al., 1987).

Echinoid MYP is a metal-binding glycoprotein of 170-180 kDa, belonging to the transferrin superfamily (Brooks and Wessel, 2002). It was originally identified as the main component of yolk granules in sea urchin eggs and exchanged for a vitellogenin-like protein (Cervello et al., 1994; Unuma et al., 2011). At present, it is well-known that MYP is not homologous to vertebrate vitellogenins: the sequencing of MYP cDNA from *Pseudocentrotus depressus* (Unuma et al., 2001) and other species (Brooks and Wessel, 2002; Noll et al., 2007) has revealed that it has about 25% homology to vertebrate transferrin family, i. e. iron-binding glycoproteins that control the level of free iron in biological fluid (Unuma et al., 2001). MYP has two isoforms with slightly different molecular masses: eggs contain the 170 kDa MYP (EGMYP) and coelomic fluid is rich in the 180 kDa MYP (CFMYP). A detailed analysis of *S. purpuratus* genome confirmed that these isoforms are products of the same gene, being only one gene encoding for MYP in sea urchin genome (Song et al., 2006).

Unlike other oviparous animals, sea urchin yolk protein is not female-specific but MYP is synthesized in both sexes (Shyu et al., 1986). Before gametogenesis, it is produced mainly in the inner epithelium of the digestive tract and in the nutritive phagocytes of ovary and testis (Unuma et al., 1998; Unuma et al., 2009; Unuma et al., 2010). MYP is accumulated in large quantities in the nutritive phagocytes of agametogenic gonads (Unuma et al., 2011) and, as gametogenesis proceeds, the stored protein is degraded to amino acids for the synthesis of new proteins and other nitrogen-

containing substances that constitute eggs and sperms (Unuma et al., 2003). Furthermore, MYP has a zinc-binding capacity which is greater in CFMYP than in EGMYP (Unuma et al., 2007). Although the geometry of MYP iron-binding site differs from other transferrins, it was demonstrated that MYP binds iron, calcium, magnesium, barium, cadmium and manganese, showing a higher affinity for zinc (Brooks and Wessel, 2002; Unuma et al., 2011). It has been proposed that CFMYP may play the role of zinc carrier protein: MYP synthesized in the digestive tract can bind zinc derived from ingested food and transport it through the coelomic fluid to gonads. Here, it can be partially deposited in nutritive phagocyte granules as protein and zinc storage and partially modified to EGMYP with the loss of zinc-binding sites (Unuma et al., 2007; Unuma et al., 2011). MYP seems to play an essential role not only in echinoid reproduction (Unuma et al., 1998; Unuma et al., 2003) but also in embryonic development and immune response. In embryos, MYP serves as a cell adhesion molecule: it is present both in yolk granules and at the surface of plasma membranes and it is involved in cell-to-cell adhesion by mechanisms of calcium binding (Matranga et al., 1986; McClay and Matranga, 1986; Noll et al., 2007). In the coelomic fluid MYP seems to be involved in the clotting phenomenon. Colourless spherule cells, a specific subpopulation of coelomocytes, contain a measurable amount of CFMYP, which is discharged under stress conditions, probably inducing the clotting processes due to the protein adhesive activity (Cervello and Matranga, 1989; Cervello et al., 1994).

Estrogen control of MYP expression has been suggested by several studies (Harrington and Ozaki, 1986; Shyu et al., 1987; Kiyomoto et al., 2008). In vertebrates, estrogens regulate the expression of both vitellogenin and transferrin genes. The hormone first binds the estrogen receptor (ER) and then the resulting complex attaches to short DNA sequences known as estrogen responsive elements (EREs) and located upstream of the modulated genes (Prowse and Byrne, 2012). A palindromic sequence, present in vertebrate EREs and essential for estrogen control, has been found upstream MYP gene, strongly suggesting an estrogen involvement in the protein expression (Shyu et al., 1987).

4. Primary cell cultures from marine invertebrates

Cells under *in vitro* conditions are used in a variety of fields and in many scientific studies and related applications as extremely important experimental tools (Rinkevich, 2005). With respect to marine invertebrates, despite the diversity of species and their potential as *in vitro* models for numerous applications, almost all the efforts to develop proliferative and permanent cell cultures have been unsuccessful (Rinkevich, 2011). At present, several short- and long-term cell cultures from a variety of tissues in an increasing number of species have been developed (Mulford and Austin, 1998; Walton and Smith, 1999; Cao et al., 2003; Odintsova et al., 2005; de Caralt et al., 2007; Sharlaimova et al., 2010; Di Benedetto, 2011) but there are still few established examples of proliferative cell lines from marine invertebrates (Frank et al., 1994; Fraser and Hall, 1999; Rinkevich, 2005; Shashikumar and Desai, 2011).

Continuous cell cultures have been developed from 10 different species of sessile colonial marine cnidarians: primary cultures of various cell types and sizes were obtained from both colony fragments and planula larvae, culturing them in modified Leibovitz L-15 medium with Fetal Calf Serum (FCS). Cell proliferation was observed within 7-20 days after dissociation and cultures were maintained and subcloned for approximately 1 year (Frank et al., 1994).

In crustaceans, proliferative primary cell cultures were derived from the prawn *Penaeus monodon* ovaries at different maturative stages. Cells were maintained for up to 17 months, being subcultured 3 times (Fraser and Hall, 1999). Best results were obtained from the testicular tissues of the crab *Scylla serrata*. Primary cell cultures from both explants and segregated tissues of *S. serrata* testes were shown to be able to proliferate and grow in L-15 crab saline medium supplemented with epidermal growth factors and glucose. These testicular cells were subcultured every 4-6 days and remained healthy for 5 months (Shashikumar and Desai, 2011).

Apart from these few successful research, extensive studies had been performed on many other edible species, above all molluscs and crustaceans, where cells from several tissues were cultured under different conditions and the most effective medium and supplements were evaluated. In most cases, Leibovitz L-15, modified with salts, resulted the best culture medium and cell survival and growth seemed to be improved by the addition of 5-10% heat-inactivated FCS (Moss et al., 1998; Mulford and Austin, 1998; Walton and Smith, 1999; Cao et al., 2003; Rinkevich, 2005). However, all the developed cell cultures could be maintained at least for some months and the proliferation rate was reported to be very low or absent (Mulford and Austin, 1998; Maeda et al., 2003; Odintsova et al., 2005; Rinkevich, 2011).

This failure has been explained in view of the *in vitro* low speed of cell proliferation (Cao et al.,

2003) and the lack of vital information regarding cell physiology and biology and their specific requirements. In addition, it has been recently suggested that marine invertebrate cells enter, 24–72 hours after their isolation, into a quiescent *in vitro* state (Rinkevich, 2011). Thus, cell cultures were mostly developed from tissues with high growth potential, like embryonic, neoplastic, or regenerating tissues (Odintsova et al., 2005).

Echinoderms are well known for their regenerative capabilities (Candia Carnevali, 2006; Candia Carnevali and Burighel, 2010) and, thus, the studies present in literature, related to cell cultures, were mostly performed using cells from tissues involved in the regenerating processes (Odintsova et al., 2005; Sharlaimova et al., 2010; Di Benedetto, 2011). Neurons and neural tissue explants from the starfish *A. rubens* and the brittle star *Ophiura ophiura* were cultured for up to 6 weeks in modified L-15 medium. However, there was limited evidence of increase in cell number and nerve outgrowth, probably due to the lack of specific growth factors. The addition of coelomic fluid, neural tissue extracts and nerve growth factor did not enhance cell conditions, suggesting that neurons require some other specific native conditioning factors (Moss et al., 1998).

On the contrary, long-term cell cultures were successfully developed from regenerating intestine of the sea cucumber *Apostichopus japonicus*, showing that cells from different stages of gut regeneration display different *in vitro* proliferation rates and behaviors (Odintsova et al., 2005). In particular, only primary intestinal cultures, performed 14-16 days after evisceration, were involved in active proliferation and their cell number increased more than twofold by the 20^{th} day of culture. Cultured cells seemed to be capable of mitotic division in suspension as well as in substrate-attached conditions. Although the intensity of cell proliferation depends on both species and regeneration type, this study strongly suggests that regenerating tissues can represent a promising source of cells for long-term cell cultures.

Similar results were obtained in *A. rubens*, where coelomocytes and coelomic epithelium cells were maintained under *in vitro* condition for at least 2 months (Sharlaimova et al., 2010). Coelomocytes isolated 5 hours after injury, displayed a higher functional activity than cells derived from control group: cells from injured animals tended to form large aggregates and network structures in which spread cells were in contact to each other whereas roundish cells were located at the network surfaces or among them. Coelomic epithelium cells formed colony-like aggregates too; in addition, they showed a higher proliferation activity, leading to consider them the most encouraging object for *in vitro* studies on asteroid regeneration processes.

The potential of other tissues and, particularly, gonad tissues, in providing suitable material for cell cultures has been less explored. In echinoderms, only few examples of cultures of ovary and testis fragments were reported. In order to analyze E2 effect on *A. pectinifera* oocyte growth, fragments

of ovary were maintained *in vitro* for only 3 days. Indeed, preliminary experiments had shown that at the end of a culture period of 5 days most of the oocytes, including those cultured in the medium alone, degenerated (Takahashi and Kanatani, 1981). On the other hand, fragments of sea urchin testicular tissues were cultured in serum-supplemented seawater for 5 weeks, showing that male germ-line cells *in vitro* can complete meiosis and the first stages of spermiogenesis (Poccia, 1988).

No study specifically addressed to the development of effective cell cultures from echinoid gonads has been reported in literature. Only recently a novel method for the isolation of oogonia from adult ovaries was developed in the sea urchin *Strongylocentrotus nudus* (Yakovlev et al., 2010). Using a combination of mechanical treatment, size filtration and centrifugation in a Percoll density gradient (Fig. 10), the authors managed to isolate a population of germ cells which contains relatively pure oogonia (70-75% purity). This technique allowed to obtain both differentiated and undifferentiated oogonia, providing important methodological basis for further *in vitro* research in this field.

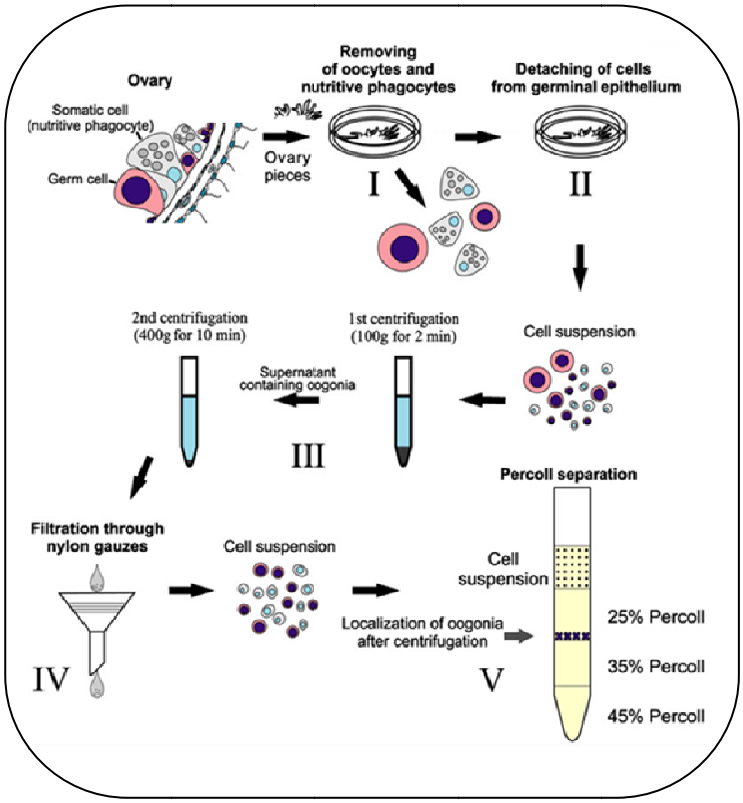

Fig. 10. Representation of oogonia purification procedure from sea urchin ovaries (adapted from Yakovlev et al., 2010).

Chapter III

DEVELOPMENT OF PRIMARY CELL CULTURES FROM SEA URCHIN OVARIES

Publications containing experimental data presented in this chapter:

Mercurio S., Di Benedetto C., Sugni M., Candia Carnevali M. D. (2013a). Development of primary cell cultures from sea urchin gonads. In Springer Netherlands, Proceedings of the symposium "Marine Invertebrate Cell Culture", Cytotechnology, 65, 5, 673-689. (Appendix 1)

Mercurio S., Di Benedetto C., Sugni M. & Candia Carnevali M. D. (2013b).Primary cell cultures from sea urchin ovaries: a new experimental tool. In Vitro Cell. Dev. Biol. – Animal. In press. DOI: 10.1007/s11626-013-9686-1. (Appendix 2)

1. Abstract

In this chapter the development of primary cell cultures from ovaries of the edible sea urchin *Paracentrotus lividus* is described in order to provide a simple and versatile experimental tool for research in echinoderm reproductive biology.

The ovary cell phenotypes, present in culture, were identified and characterized by different microscopic techniques. Although cell cultures could be produced from ovaries at all stages of maturation, the cells appeared healthier and viable, displaying a higher survival rate, when ovaries at early stages of gametogenesis were used. In terms of culture medium, ovarian cells were successfully cultured in modified Leibovitz-15 medium, whereas poor results were obtained in Minimum Essential Medium Eagle and Medium 199. Different substrates were tested, but ovarian cells completely adhered only on poly-L-lysine. To improve *in vitro* conditions and stimulate cell proliferation different serum-supplements were tested. Fetal Calf Serum and an originally developed Pluteus Extract appeared to be detrimental to cell survival, apparently accelerating processes of cell death. In contrast, cells cultured with sea urchin Egg Extract appeared larger and healthier, displaying an increased longevity that allowed to maintain them for up to 1 month.

Overall our study provides new experimental bases and procedures for producing successfully long-term primary cell cultures from sea urchin ovaries offering a good potential to study echinoid oogenesis in a controlled system and to investigate different aspects of echinoderm endocrinology and reproductive biology.

2. Introduction

Despite the traditional use of sea urchin as a favourite model in embryology and developmental biology, the specific mechanisms regulating reproductive processes are still scarcely known in all echinoderms. In echinoids, gametogenesis was demonstrated to be influenced by several environmental factors, such as water temperature and photoperiod (Byrne, 1990; Spirlet et al., 2000; Shpigel et al., 2004; McCarron et al., 2010), food availability (Leoni et al., 2001) and environmental hydrodynamics (Fenaux, 1968). Apart from these exogenous factors, several endogenous molecules, notably hormones and neuropeptides, likely play an important role in regulating reproductive processes (Spirlet et al., 1998; Mita, 2013). Although several studies have been performed in order to identify and to understand the roles of these molecules, their mechanisms of action are still far to be clearly understood. In order to elucidate their complete physiological significance, a simple and adequate model system, such as an appropriate *in vitro* approach, can be certainly helpful, allowing to perform studies under controlled experimental conditions (Odintsova et al., 2005).

The establishment of primary cell cultures from marine invertebrates and, particularly, from echinoderms has been the objective of many previous attempts encountering uncounted obstacles (Rinkevich, 1999). At present, short and long-term cell cultures from a variety of tissues and from numerous species have been developed (Mulford and Austin, 1998; Walton and Smith, 1999; de Caralt et al., 2007; Sharlaimova et al., 2010; Di Benedetto, 2011): however, there are only few rare examples of establishment of proliferative cell lines from marine invertebrates (Rinkevich, 2011; Shashikumar and Desai, 2011). The reasons of these failures have been mostly identified in the *in vitro* low speed of cell proliferation and the lack of vital information regarding cell physiology and biology (Rinkevich, 1999; Cao et al., 2003). Considering all these difficulties, cell cultures were mostly developed from tissues with high growth potential (Odintsova et al., 2005).

In echinoderms, regenerating tissues display high proliferate rates (Candia Carnevali, 2006; Candia Carnevali and Burighel, 2010) and, thus, they represent an optimal source of cells to successfully develop long-term primary cell cultures (Odintsova et al., 2005; Sharlaimova et al., 2010).

The potential of other tissues, such as gonads, in providing an appropriate material for *in vitro* studies have been less investigated. In literature there are only a few examples of cultures of echinoderm ovary and testis fragments. In *Asterias pectinifera,* fragments of ovary were cultured for only 3 days (Takahashi and Kanatani, 1981); on the other hand fragments of sea urchin testicular tissues were cultured in serum-supplemented seawater for 5 weeks, showing that male germ-line cells can complete their maturative processes in *in vitro* conditions (Poccia, 1988). In addition, a novel method for the isolation of oogonia from adult ovaries was developed in the sea urchin *Strongylocentrotus nudus* (Yakovlev et al., 2010). The authors obtained cell populations which contain relatively pure oogonia (70-75% purity), providing the first methodological basis for further *in vitro* research in this field. Nevertheless, no study specifically addressed to the development of effective cell cultures from gonads was previously reported in literature. Taking into account the advantages and the possible applications of the *in vitro* studies, we carried out the first attempt to develop primary cell cultures from ovaries of the common Mediterranean sea urchin *Paracentrotus lividus.* After an accurate characterization of the cell phenotypes present in culture, we focused on culture condition optimization, i.e. to define the suitable medium, substrate and possible serum supplements. Overall, the final aim of this investigation was to set up the experimental basis for producing primary cell cultures from ovaries of this edible and ecologically relevant species. Our results could be useful for improving and expanding the potential employment of echinoderms in experimental research, in particular providing an important tool for *in vitro* studies on echinoid reproductive biology and providing a simple and versatile method for multi-disciplinary applications, such as ecotoxicological and aquaculture applied research.

3. Materials and Methods

3.1. Animals

P. lividus adult specimens were monthly collected in the Protected Marine Areas of Bergeggi and Portofino, on the Ligurian coast of Italy, and immediately transported to the laboratory in cool boxes filled with natural sea water. Animals were kept in aquaria under constant aeration in circulating artificial sea water (Instant Ocean; salinity about 37‰, as in the Mediterranean Sea). Animal conditions as well as all water physical and chemical parameters were daily monitored.

3.2. Cell cultures

P. lividus ovaries were removed from the internal side of the tests: for each specimen one gonad was used for histological analysis and processed for standard methods of light microscopy, whereas the remaining 4 gonads were used to develop primary cell cultures according to the following protocol. Ovaries were washed several times in sterile Ca^{2+} Mg^{2+} Free Sea Water (CMFSW) with antibiotics (40µg/l gentamycin and 100 units/mL penicillin, 100 µg/mL streptomycin) and dissected into small pieces (2-5 mm) using fine-tipped tweezers. Ovary pieces were incubated in 0.5 mg/mL collagenase dissolved in sterile CMFSW and stirred for 1 hour. The resulting cell suspension was filtered through 50 µm nylon gauze (to remove mature oocytes), centrifuged at 300 × g for 6' at 15 °C and the cell pellet was resuspended in modified culture medium. Cells were seeded at a concentration of 3-4 × 10^5 cells/mL in 24-well culture plates, without coating (medium evaluation, see below) or coated with rat collagen (I type, Sigma), gelatin (Merck Millipore) or poly-L-lysine (70-150 kDa, 0.01% solution, Sigma). Three culture media were compared: Leibovitz-15 medium (L-15), Minimum Essential Medium Eagle (MEM) and Medium 199 (M199), all of them modified by the addition of 20.2 g/L NaCl, 0.54 g/L KCl, 0.60 g/L $CaCl_2$, 1 g/L Na_2SO_4, 3.9 g/L $MgCl_2$, 1 g/L glucose, 25 mg/L Taurine, 5 mL/L Insulin (10 mg/mL in solution, SIGMA), 100 mg/L L-Glutamine, 1.75 mg/L vitamin E (1000 mOsm). The pH was adjusted at 7.6. The media were then sterilized by filtration (0.22 µm, Corning-Sigma) and 40 µg/L gentamycin, 100 units/mL penicillin and 100 µg/mL streptomycin were added. All cultures were incubated at 15 °C for at least 20 days. Cell behavior was observed daily for the first two weeks of culture and, then, at each medium replacement using an inverted phase contrast microscope. Replacement of 50% of the medium was carried out every two days.

3.3. Medium and supplement evaluation

As previously anticipated, three different modified culture media were tested: L-15, M199 and MEM. The most effective medium was determined by a detailed analysis based on cell morphology,

using an inverted phase contrast microscope, and on cell viability during a 2 week culture period. Cell viability was estimated in duplicates by direct cell counting, using a "Burker chamber" coupled with Tripan blue exclusion test at 0 (T_0), 2, 7 and 14 (T_n) days. The percentage of viable cells (V_x) at the different time points was calculated as follows:

$$V_x = (\text{viable cell mean n° at } T_n / \text{ viable cell n° at } T_0) \times 100.$$

Once the most effective culture medium was determined, the effects of 2% inactivated Fetal Calf Serum (FCS) and of several concentrations of *P. lividus* "Pluteus Estract" (PE) and "Egg Extract" (EE) were tested. PE was obtained from 48h *P. lividus* larvae. When sea urchin larvae reached the pluteus stage, they were collected, sonicated and centrifuged at 800 × g for 10' at 4 °C. The supernatant was then centrifuged at 17000 × g for 1 h at 4 °C. The same procedure was performed to obtain EE from *P. lividus* mature ovaries. The resulting extracts were protein assayed (BCA protein assay kit, Sigma), sterile filtered, heat-inactivated and stored at -20 °C. Different PE and EE concentrations were tested: 34 ng/mL, 100 ng/mL, 200 ng/mL, 500 ng/mL, 1 µg/mL, 10 µg/mL and 50 µg/mL. Supplement effects on cell morphology and viability was observed and compared during a 2 week culture period. Cell viability was determined at different time points by staining with fluorescent Calcein AM (viable cell marker, Sigma) or by direct cell counting, using a "Burker chamber" coupled with Tripan blue exclusion test (see before).

In order to analyze the cell proliferation activity, the incorporation method employing the thymidine analog 5-bromo-deoxyuridine (BrdU) was used. Cells were exposed to 30 µM BrdU and FdU (10:1) in culture medium for 12 hours. After this period they were fixed in 4% paraformaldehyde for 1 hour, treated with 1.5 N HCl and 0.2% Triton X-100 and incubated with antibodies against BrdU (Cell Proliferation Kit: Amersham, GE Healthcare). Staining with a secondary antibody FITCH conjugated (Anti-mouse IgG FITCH conjugate, SIGMA) was performed for 40 minutes. To determine the BrdU nuclear localization, cells were also stained with DAPI. Samples were examined using a fluorescence phase contrast microscope. The experiments on proliferation activity evaluation were carried out on fresh, one week and two week cell cultures.

3.4. Microscopic and ultramicroscopic analyses

In order to determine ovary reproductive stages and characterize the cellular phenotypes present in fresh cultures, standard methods of light and electron microscopy were employed, as previously described by Barbaglio et al. (2007). Briefly, gonads or cell pellets were fixed with glutaraldeyde 2% in cacodylate buffer 0.1 M and NaCl 1.4%, washed with cacodylate buffer 0.1 M and post fixed with a solution of OsO_4 1% in cacodylate buffer 0.1 M. Then the samples were prestained with uranyl acetate in ethanol 25%, dehydrated through the ethanol series, and finally, after washing in

propylene oxide, embedded in Epon 812–Araldite resin. Semithin (1 μm) and ultrathin (70 nm) sections, were cut with a Reichert–Jung ULTRACUT E using glass knives. Semithin sections, stained with crystal violet and basic fucsin, were observed under a Jenaval light microscope. Ultrathin sections for electron microscopy were mounted on copper grids and stained with uranyl acetate and lead citrate, then observed and photographed in a Jeol 100SX electron microscope.

Five ovary reproductive stages were considered: *Spent* (phase immediately following the spawning event), *Recovery* (phase characterized by phagocytosis and nutrient accumulation), *Growing*, *Premature* and *Mature* (all phases characterized by gametogenesis in progress) (Barbaglio et al., 2007).

3.5. Scanning electron microscopy

Cells adhesion to the substrates was investigated by scanning electron microscopy. 48h cell cultures were fixed in glutaraldeyde 2% in artificial sea water (ASW) for 2 hours at 4 °C. After overnight wash in filtered ASW at 4 °C, samples were post fixed with a solution of OsO_4 1% in ASW and glucose for 2 hours, washed in distilled water and dehydrated through the ethanol series. Absolute ethanol was gradually substituted with Hexamethyldisilazane (HMDS; Sigma). Samples were left to dry, mounted on stabs, covered by thin pure gold layer (Sputter Coater Nanotech) and observed with a scanning electron microscope (LEO-1430).

3.6. Electrophoresis

Sodium dodecylsulfate-polyacrilamide gel electrophoresis (SDS-PAGE) was performed using 6% slab gel (Laemmli, 1970) in order to verify and compare the protein content in PE and EE supplements. Prior to electrophoresis samples were dialyzed, diluted with sample buffer (SDS reducing buffer) and boiled for 5 minutes. Gels were run at 100 V at room temperature. Protein bands were visualized with Coomassie brilliant blue R-250. SDS-PAGE standards (StoS Protein Marker, Genespin s.r.l.) were also run for molecular weight calibration.

3.7. Statistical analysis

Data are presented as mean values±SEM. Statistical significance was assessed using Paired t test and one-way ANOVA (Tukey's post-hoc test). A *p*-value of less than 0.05 was considered statistically significant. Statistical analysis was performed by the computer program GraphPad Prism 4.

4. Results

4.1. Development of primary cell cultures

Ovarian cells were successfully cultured and maintained for up to 1 month, but no proliferation activity was observed. Performing a detailed histological analysis the cellular phenotypes present in fresh cultures were characterized: only germinal cells at different stages of maturation and active nutritive phagocytes were observed (Fig.12 a and d).

Generally, germinal cells were small roundish cells displaying a large nucleus and a big nucleolus; this latter was excentric in the oogonia, whereas it located found in the nucleus centre in the early vitellogenic oocytes. Nutritive phagocytes were characterized by large heterogeneous cytoplasmatic inclusions, particularly huge phagosomes, often containing gametes in progressive digestion. Mature oocytes/eggs were successfully removed by the filtration procedure during cell culture development. Cellular phenotypes were well recognized using phase contrast microscopy: oogonia and oocytes appeared as roundish cells, ranging in size from 8 to 50 µm (Fig. 12 c); nutritive phagocytes showed a granular surface, ranging in size from 20 to 50 µm, and often formed a loose network with their long filopodia variously branched and connected together (Fig. 11; Fig. 12 f). This trend was more evident in cell cultures obtained from ovaries at early stages of gametogenesis. In fact, although primary cell cultures could be obtained from ovaries at all stages of maturation, cells appeared obviously healthier and viable, displaying a higher survival rate, when ovaries at *Growing* stage were used.

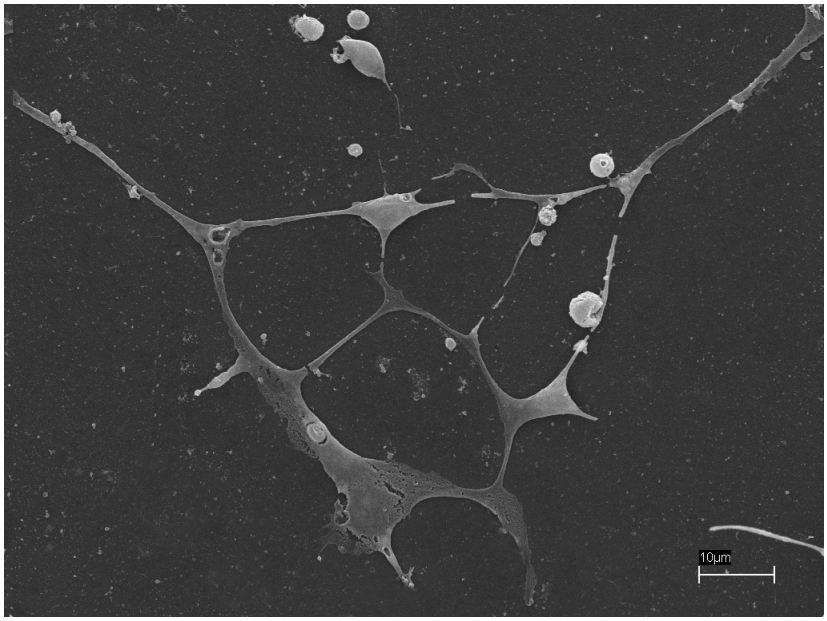

Fig. 11. SEM: nutritive phagocytes forming a network with their filipodia.

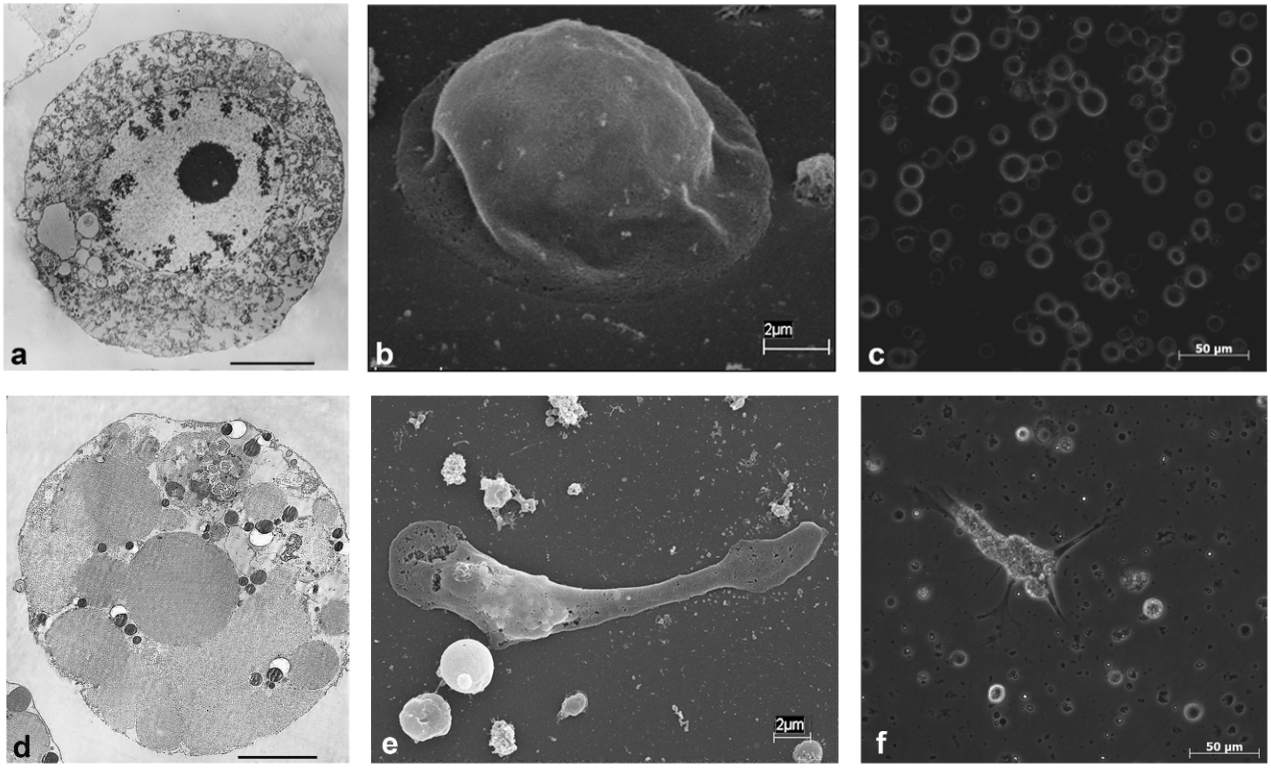

Fig. 12. Cellular phenotype characterization in *P. lividus* ovary fresh cell cultures. **a)** TEM: oocyte. Bar = 5 µm. **b)** SEM: oogonium adhering to poly-L-lysine substrate. **c)** Phase Contrast Microscopy: oocytes at different developmental stages. **d)** TEM: nutritive phagocyte full of heterogeneous inclusions, including relict material from phagocytosed gametes. Bar = 10 µm**. e)** SEM: nutritive phagocyte adhering to poly-L-lysine substrate with large lamellipodia. **f)** Phase Contrast Microscopy: nutritive phagocyte with long lamellipodia

4.2. Medium evaluation

The general morphology of the ovary cells cultured in the 3 different modified media employed (L-15, MEM and M199 media) was observed and compared using a phase contrast microscope. 12-hour cell cultures appeared comparable, regardless of the used medium. During the following 2 weeks, cell culture appearance markedly changed depending on the medium, displaying remarkable differences in terms of cell size, shape and number.

After 2 weeks in modified L-15 medium, cells were still numerous and most of them appeared healthy, the phagocytes being still attached to the plate bottom and forming a network of long filopodia. In modified MEM and M199 media, ovarian cells were smaller with irregular and elongated shapes and first signs of cell degeneration were observed by the end of the first week of culture (Fig. 13).

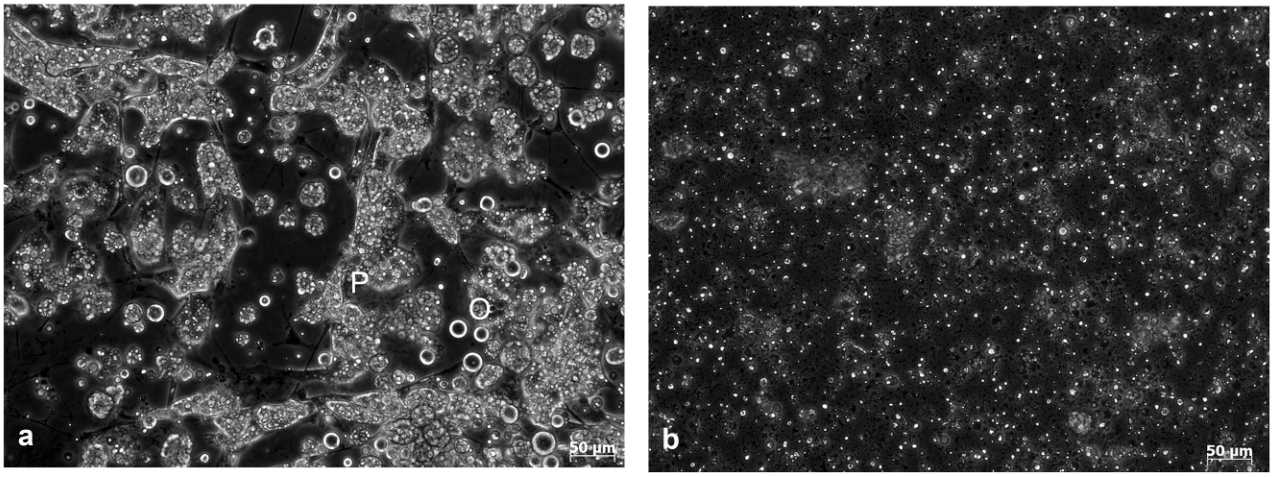

Fig. 13. Phase Contrast Microscopy. Primary cell cultures from *P. lividus* ovaries after 2 weeks in **a)** modified L-15 medium and **b)** modified MEM. After 2 weeks of culture in modified L-15 medium, cells are still numerous and the 2 phenotypes can be easily distinguished: oocytes with a rounded regular shape and nutritive phagocytes with a granular surface, forming a network with long lamellipodia. In modified MEM, the few cells alive are smaller, with irregular elongated shapes. Cell debris can be observed all over the plate. O = oocytes; P = nutritive phagocytes.

Cell viability analysis confirmed the morphological observation. Comparing mean V_x values in the three different modified culture media, cell viability resulted significantly higher (One-way ANOVA: $P< 0.05$) in L-15 cell cultures at all the considered time points (Fig. 14).

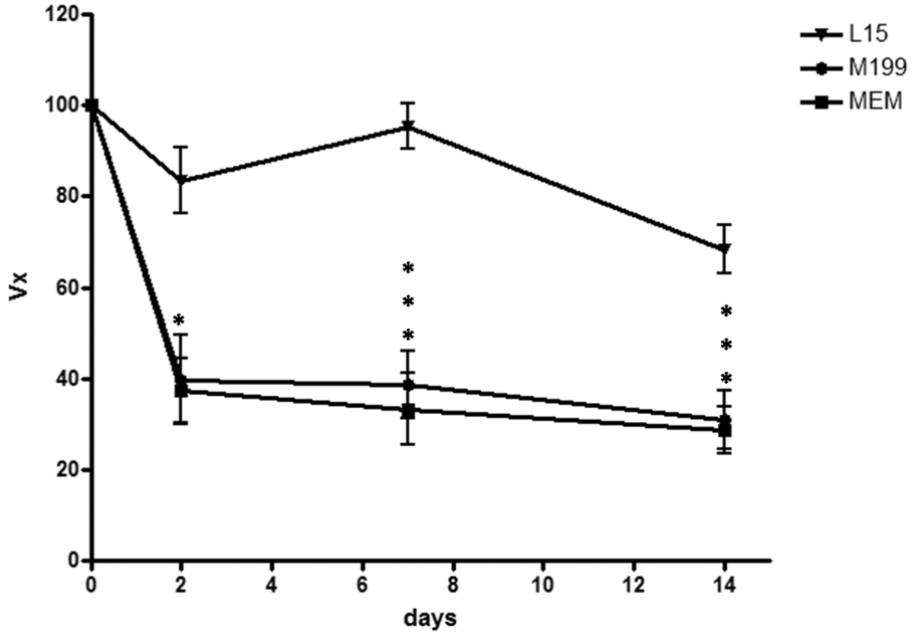

Fig. 14. Percentage of viable cells (V_x) at the considered time points (2, 7 and 14 days) in the three tested modified culture media. * = $P<0.05$; *** = $P<0.001$.

Considering all these results, modified L-15 medium appeared to be the most effective for ovarian cell growth and survival and was therefore used for all the following analyses.

4.3. Substrates

Cell adhesion was investigated by both phase contrast microscopy and scanning electron microscopy (SEM). Cell behaviour changed depending on both the tested substrates and the cell phenotypes. Germinal cells never adhered on untreated multi-well plastic, rat collagen and gelatin; nutritive phagocytes adhered only partially to these substrates.

Both oocytes and phagocytes completely adhered on poly-L-lysine substrate (Fig. 12 b and e; Fig 15).

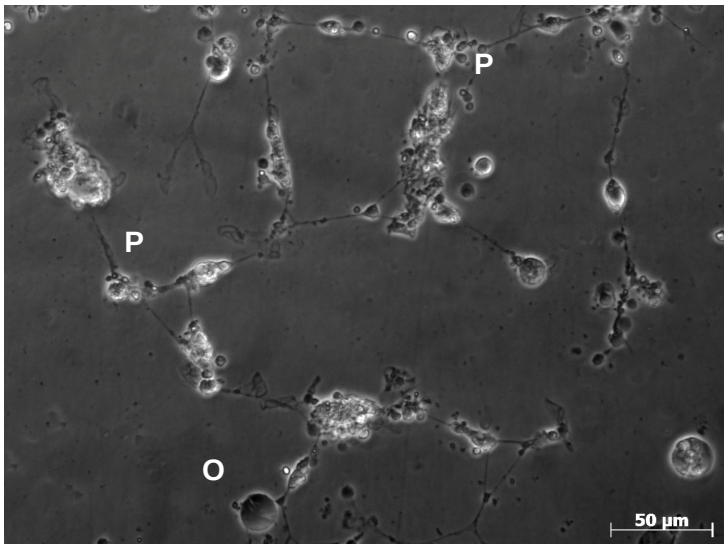

Fig. 15. Phase Contrast Microscopy: *P. lividus* ovarian cells cultured in modified L-15 2 days after their isolation.
P = nutritive phagocytes; O = oocyte

4.4. Effects of medium supplements

To stimulate cell growth and survival different supplements were added to modified L-15 medium and their effects on cell morphology and viability were investigated. Control cells were cultured in the unsupplemented medium.

The addition of 2% (v/v) inactivated Fetal Calf Serum (FCS) to modified L-15 medium did not improve cell health conditions and, after 1 week of culture, signs of cell degeneration were observed. Furthermore 2% (v/v) FCS cell cultures displayed a significantly lower viability (Paired t test $P< 0.05$) than unsupplemented L-15 cultures at all the considered time points (Fig. 16).

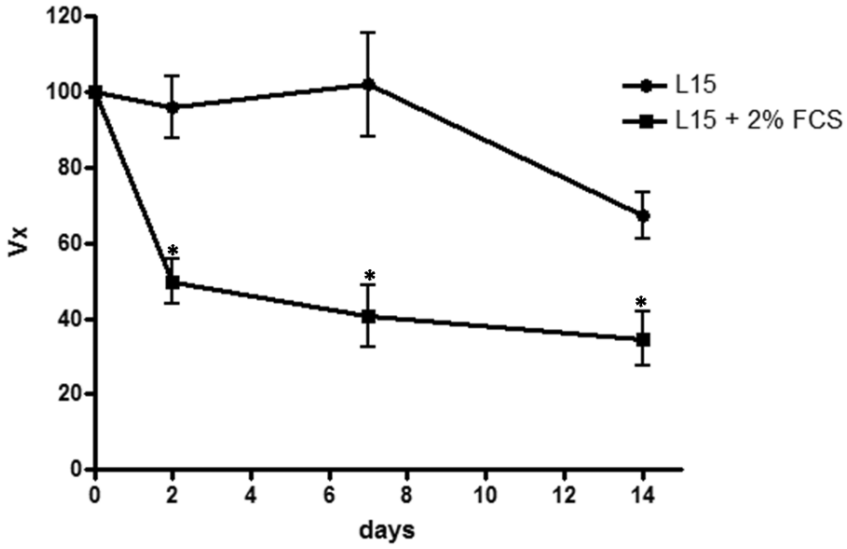

Fig.16. Percentage of viable cells (V_x) at the considered time points (2, 7 and 14 days) in unsupplemented L-15 medium and L-15 medium supplemented with 2 % FCS . * = $P<0.05$.

P. lividus "Pluteus Estract" (PE) did not enhance cell growth as well (Fig. 17). Using the Calcein AM method it was evident that all the PE concentrations tested (34 ng/mL, 100 ng/mL, 200 ng/mL, 500 ng/mL, 1 µg/mL, 10 µg/mL and 50 µg/mL) were detrimental to cell survival, apparently accelerating processes of cell death even at the lowest concentration.

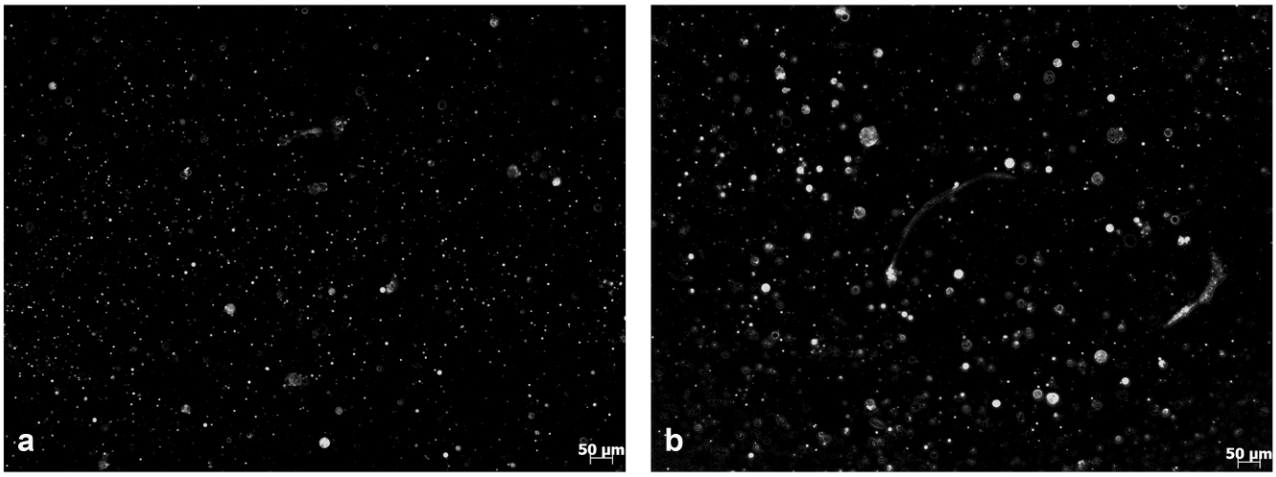

Fig. 17. Fluorescence Microscopy. Calcein AM method: viable cells appear fluorescent. Primary cell cultures from *P. lividus* ovary after 1 week in modified L-15 medium: (**a**) with 1 µg/mL PE and (**b**) without PE.

P. lividus "Egg Extract" (EE) seemed to slightly improve cell conditions (Fig. 18). Cells cultured in L-15 medium supplemented with 100 ng/mL EE appeared larger and healthier than the controls for all the considered culture period. Furthermore, EE appeared to increase cell longevity, allowing to maintain them for up to 1 month.

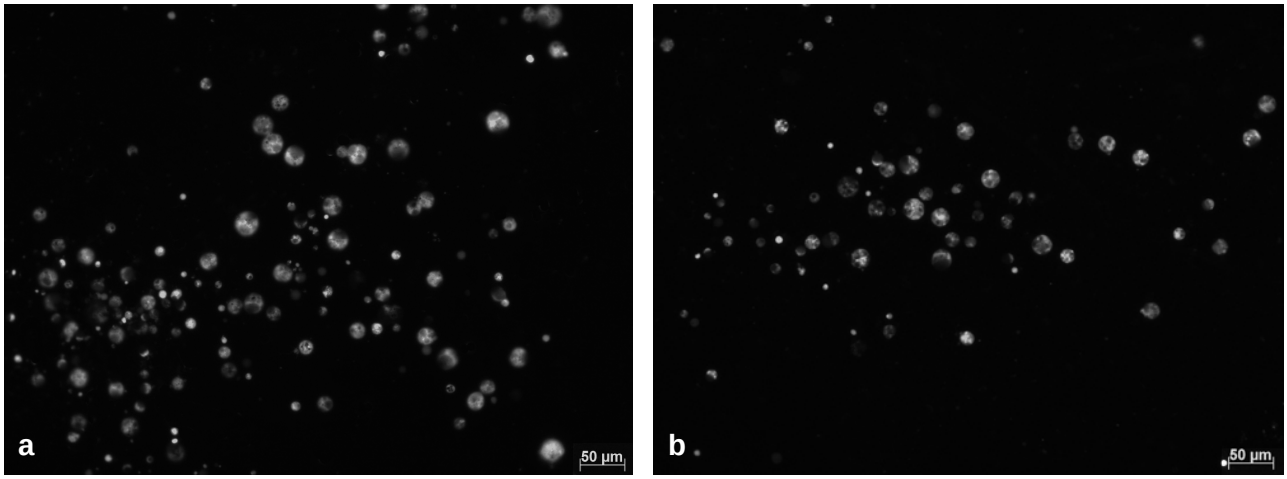

Fig. 18. Fluorescence Microscopy. Calcein AM method: viable cells appear fluorescent. Primary cell cultures from *P. lividus* ovary after 1 week in modified L-15 medium: (**a**) with 100 ng/mL EE and (**b**) without EE.

4.5. Protein analysis

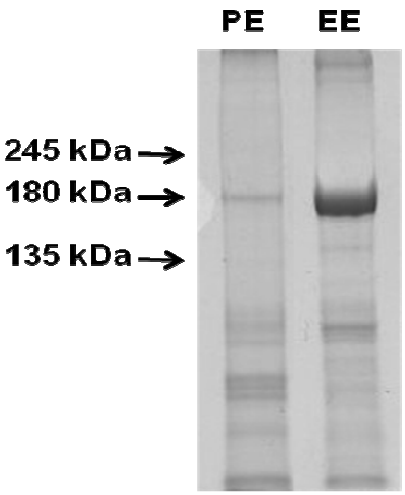

Fig. 19. 6% SDS-PAGE analysis of *P. lividus* PE and EE

We used SDS-PAGE to test *P. lividus* PE and EE for the presence of the Major Yolk Protein (MYP) (Fig. 19).

A 180 kDa protein appeared to be present in PE, whereas a protein band with a slightly lower molecular weight (170 kDa) was predominant in EE. Considering the molecular weights and the current knowledge (Unuma et al., 2011; Prowse and Byrne, 2012) we concluded that in PE the band probably corresponded to CFMYP and that EGMYP was present in EE.

In addition, the extracts showed further differences in the protein profiles, that could not be analyzed in detail due to the scarce information available about sea urchin proteome.

5. Discussion

The *in vitro* approach represents a valuable experimental tool for different research fields and applications. In particular, primary cell cultures from echinoid gonads can provide a simple model system for investigating different aspects of reproductive physiology and endocrinology, allowing studies under controlled experimental conditions (Odintsova et al., 2005). In the present work cells from *P. lividus* ovaries were successfully cultured for up to one month and their *in vitro* behaviour was well-characterized. Two different cell phenotypes, previously described in the *in vivo* model, were observed in cell cultures: nutritive phagocytes and germ cells at different maturative stages. Considering the importance of nutritive phagocytes in supporting, protecting and providing nutrients to the growing germ cells (Chia and Bickell, 1983; Walker et al., 2000), heterogeneous cell cultures, composed by both cell phenotypes, were preferable to mono-phenotype cultures. Only semi-mature oocytes and eggs were avoided. However, in phase contrast microscopy the cellular phenotypes were easily recognized thanks to their evident differences in morphology (phagocytes display granular surface) and attitude (nutritive phagocytes tend to form networks with their long filopodia). Oogonia and oocytes appeared as small roundish smooth cells, usually located among phagocytes or on their network surface (Fig. 15). In particular, when primary cell cultures were obtained from ovaries at early stages of gametogenesis an enhanced nutritive phagocyte ability to form network structures as well as a more active and healthier cellular aspect were observed. In fact, at these stages nutritive phagocytes are voluminous, full of inclusions containing relict material from both phagocyted gametes and nutritive material, acting as storages, transfer sites and active

synthesizing places (Chatlynne, 1969; Houk and Hinegardner, 1980; Chia and Bickell, 1983; Spirlet et al., 1998). This starting condition probably affects cell cultures. Nutritive phagocytes indeed appeared more active and an improvement in cell viability was observed, probably thanks to the presence of nutritive substances and molecular factors required for oocyte growth and maturation.

Comparing cell morphology and viability in the three tested modified culture media, L-15 medium appeared to be the most suitable for *P. lividus* ovary cells growth and survival. Our results are in agreement with previous studies (Moss et al., 1998; Odintsova et al., 2005; Shashikumar and Desai, 2011), suggesting that the composition of this medium is one of the most complete and effective for culturing marine invertebrate cells. On the contrary, modified M199 and MEM media resulted in cell degeneration during the whole culture period. M199 and MEM media were not effective probably because they revealed pH instability: as also reported by Mulford and Austin (1998), these culture media showed variable pH shift (7.6-8.5) that could influence cell health conditions.

Testing different cell substrates showed that nutritive phagocytes and germinal cells are differentially sensitive to substrate in terms of cell adherence and migration. Oocytes adhered only on poly-L-lysine substrate, whereas nutritive phagocytes adhered partially on the different tested substrates, probably depending on their functional state, and completely on poly-L-lysine. The effectiveness of this substrate has already been reported for echinoderm embryonic cells (Odintsova et al., 1994) and for cells from holothurian regenerating gut (Odintsova et al., 2005). In both studies, poly-L-lysine was reported to be one of the best substrates for echinoderm cells, improving cell survival and proliferation. This is in agreement with our results and suggests that echinoderm primary cell cultures probably display similar requirements for achieving cell adhesion.

Although echinoid ovary cells could be maintained in culture under certain conditions, no improvement in cell condition and viability was obtained using different embryo extracts. Fetal Calf Serum (FCS) is a widely used serum-supplement in marine invertebrate cell cultures (Mulford and Austin, 1998; Walton and Smith, 1999; Odintsova et al., 2005). Although FCS was reported to enhance cell growth and survival in different echinoderm species (Odintsova et al., 2005; Sharlaimova et al., 2010), this serum was detrimental to ovarian cell survival, apparently accelerating processes of cell death even at low concentration. Similar poor results were observed with a specifically developed *P. lividus* PE. PE did not improve cell health conditions at all the tested concentrations. Maybe further manipulation and purification of this extract could improve its effects: at present state of knowledge, no conclusions can be drawn about PE effectiveness. Considering that ovary cells probably require more tissue-specific factors and nutrients, a *P. lividus* egg extract (EE) was also tested. The addition of 100 ng/mL EE to modified L-15 medium seemed to slightly improve cell *in vitro* conditions, allowing to maintain them for up to one month. During

oogenesis several substances are accumulated in eggs (Kanatani, 1983) in order to allow embryo development, and it is likely that similar nutrients and/or molecules are required for growth and maintenance of different types of cells. Furthermore, SDS-PAGE analysis demonstrated the presence of EGMYP, the most abundant ovarian protein (Unuma et al., 1998; Brooks and Wessel, 2002), in EE. Although its functions are still not completely understood (Cervello and Matranga, 1989; Unuma et al., 2009), there is no doubt about its importance in sea urchin biology and physiology. The different results obtained with the addition of PE and EE could be also related to the MYP isoforms contained in the extracts. In fact, EGMYP was not found in PE that seems to contain the coelomic fluid isoform, CFMYP, as already suggested by previous authors (Noll et al., 2007; Unuma et al., 2009). It could be speculated that PE detrimental effects on ovarian cells were induced by the presence of CFMYP that is usually abundant in the coelomic fluid and is modified in EGMYP before being accumulated in nutritive phagocytes (Unuma et al., 2007). However, SDS-PAGE analysis demonstrated the existence of other important differences in extract protein profiles, suggesting that the results observed could be also related to the different PE and EE protein compositions.

In conclusion, we developed primary cell cultures from sea urchin ovaries, providing a new useful approach for successful investigations of echinoid reproductive biology, especially, for focusing on events occurring at the beginning and during oogenesis. Furthermore, our model system can represent a new simple and versatile experimental tool for a wide range of applied researches (in both ecotoxicology and aquaculture field), finally allowing to unravel the mechanisms regulating sea urchin gametogenesis.

Chapter IV

SEX-STEROIDS IN ECHINOID REPRODUCTION: AN *IN VIVO* & *IN VITRO* APPROACH

1. Abstract

In this chapter we deeply described *in vivo* and *in vitro* experiments specifically addressed to determine possible sex-steroid involvement in echinoid reproduction. Indeed, echinoid reproductive cycle has been studied in several species but the mechanisms regulating gametogenesis processes are still scarcely understood. Apart from environmental factors, different studies have suggested a steroid role in gonad maturation and growth. To finally clarify possible E2 and T involvement in echinoid reproductive biology, first of all, a long-term experiment of steroid dietary administration was performed in adult specimens of the common Mediterranean sea urchin *Paracentrotus lividus*. We analysed and compared different reproductive parameters (Gonad Index, Maturative Index and maturative stage distribution) in 4 experimental groups: control group (CTL), E2 and T groups fed with pellets containing respectively 17β-estradiol and testosterone, and E2-4 weeks group fed with control pellets for the first 4 weeks and then treated with 17β-estradiol. In particular, this latter was chosen in order to verify the existence of a specific E2-sensitive gametogenic stage, as proposed in different asteroid species. Subsequently, possible steroid effects on *P. lividus* female reproduction was investigated with an *in vitro* approach. Cells, isolated by ovaries in the same maturative conditions considered in the *in vivo* experiment, were cultured in presence of E2 and T physiological concentrations for 2 weeks. Possible hormone effects on ovarian cell morphology and behaviour were investigated. In addition, steroid regulation of the sea urchin Major Yolk Protein (MYP) expression was analyzed 24 and 48 hours after E2 and T exposure.

According to our results, E2 and T do not markedly influence echinoid gonad maturation, particularly not promoting gamete maturation. Hormonal dietary administration did not induce striking variations in the considered reproductive parameters and no effect was observed also when males and females were analyzed separately. In addition, no specific maturative stage sensitive to E2 was found, suggesting the existence of different hormonal mechanisms in asteroids and echinoids. Similar considerations could be inferred taking into account the *in vitro* experiments. E2 and T exposure did not affect ovarian cell size and behaviour nor MYP expression.

Overall, the present research provided new information on sex-steroid implications in echinoid reproductive processes. Indeed, the obtained results suggest that these hormones are not directly involved in either gamete maturation, as occurs in vertebrates, or in vitellogenesis processes, as reported for several asteroid species. However a steroid involvement in echinoid physiology can not be completely excluded and their role in the regulation of lipid metabolism and protein synthesis during the different reproductive stages should be strongly considered as a future interesting field of investigation.

2. Introduction

Although echinoid reproductive cycle has been extensively described in different species (Himmelman, 1978; Byrne, 1990; Spirlet et al., 1998; Kelly, 2000) the mechanisms regulating their reproductive processes are still scarcely known. Environmental factors, such as temperature, photoperiod and food availability, surely play an important role (Byrne, 1990; Pearse and Cameron, 1991; Spirlet et al., 2000; Leoni et al., 2001; Shpigel et al., 2004). In addition, further endogenous factors, notably hormones, are probably involved in regulating and synchronizing gonad maturation individually (Spirlet et al., 1998).

The involvement of sex-steroid hormones, in particular 17β-estradiol (E2) and testosterone (T), in echinoderm reproduction has been suggested by several studies on seasonal changes of steroid levels during the gonadal cycle (Voogt and Dieleman, 1984; Xu and Barker, 1990; Hines et al., 1992a; Wasson et al., 2000a; Barbaglio et al., 2007).

In asteroids, the hormone levels appear to vary according to the reproductive cycle and in a sex-specific manner (Voogt and Dieleman, 1984; Xu and Barker, 1990; Hines et al., 1992a). Estrogens seem to affect vitellogenesis processes and, particularly, protein biosynthesis, transport and/or incorporation into oocytes (Schoenmakers and Dieleman, 1981; Xu and Barker, 1990). Furthermore, the existence of a specific window (in terms of reproductive stages) of E2 sensitivity has been hypothesized (Schoenmakers et al., 1981; Takahashi and Kanatani, 1981). According to Schoenmakers et al. (1981), in *Asterias rubens* there is a threshold oocyte size for E2 effectiveness, as only already developed oocytes appeared to be positively affected by the hormone whereas no effect was observed on small oocytes. Similar results were also reported in *Asterina pectinifera* in which E2 was found to promote oocyte growth only during vitellogenesis stages (Takahashi and Kanatani, 1981). As far as T is concerned, this hormone seemed to be involved in gamete maturation and gonad growth in both females and males (Xu and Barker, 1990; Hines et al., 1992a).

On the other hand, in echinoids a relationship between steroid levels and reproduction has not been clearly proved yet, probably due to the lower number of studies performed and the big variability of results obtained (Wasson et al., 2000a; Barbaglio et al., 2007). During the annual reproductive cycle of *Lytechinus variegatus* T and E2 concentrations were found to be higher during the period of early gonadal growth in both males and females (Wasson et al., 2000a), suggesting a steroid role in gamete nutrition. In *Paracentrotus lividus*, the experimental model used in this research, T levels appeared to vary according to the reproductive stages, but no significant correlation was found along the year. Concerning E2, higher hormone levels were observed in ovary at early maturative stages, supporting a possible E2 involvement in the regulation of nutritive fagocytes activity and/or

oogonia proliferation. On the contrary, in the testis, higher levels of E2 were measured in advanced maturative stages, suggesting a role in sperm maturation. Furthermore, mean E2 concentrations appeared to be lower in testes than in ovaries, possibly reflecting a more important role for this hormone in female individuals (Barbaglio et al., 2007).

In order to elucidate the physiological significance of sex-steroid in echinoids several experiments of hormone administration have been performed; however there were really too variables in terms of species, life stage, administration period and type and therefore results were not comparable. In juveniles of *Pseudocentrotus depressus*, estrone administration induced testis growth and promoted spermatogenesis whereas E2 treatment did not result in any significant effects (Unuma et al., 1999). Also in *P. lividus* E2 direct administration did not affect any of the considered reproductive parameters (Mercurio et al., 2012). On the contrary, in *L. variegatus*, E2 and T dietary administration resulted in appreciable effects on several reproductive parameters: E2 appeared to enhance ovarian growth but no effect was observed on oocytes diameter, which was instead increased by T treatment. In addition, both the hormones seemed to increase protein concentration in the gonads, suggesting a steroid influence in protein accumulation (Wasson et al., 2000b). Similar results were reported in *Strongylocentrotus nudus* (Varaksina and Varaksin, 2001) and in *Strongylocentrotus intermedius* (Varaksina and Varaksin, 2002). In these species estradiol dipropionate injections stimulated protein synthesis in both testes and ovaries, leading to the hypothesis of a steroid control of gonad protein expression. This idea has been further suggested by studies concerning sea urchin Major Yolk Protein (MYP) (Shyu et al., 1987; Prowse and Byrne, 2012). MYP is a glycoprotein belonging to the transferrin-like superfamily, which is present in almost all echinoid tissues and represents the most abundant protein of sea urchin eggs (Shyu et al., 1986; Unuma et al., 1998; Unuma et al., 2001; Unuma et al., 2003). This protein seems to play different roles and its importance in echinoid reproduction is well known (Cervello et al., 1994; Noll et al., 2007; Unuma et al., 2007). The sequencing of MYP cDNA has revealed the presence of a putative estrogen responsive element (ERE) upstream the gene. In particular, a palindromic sequence, similar to that present in vertebrate EREs and essential for estrogen control, has been found, strongly suggesting an estrogen involvement in the regulation of MYP expression (Shyu et al., 1987).

Considering this background of knowledge, it is clear that the role of steroid hormones in echinoid reproduction is far to be well understood. To overcome this gap of knowledge, in this work, we investigated T and E2 involvement in the reproductive biology of the common Mediterranean sea urchin *Paracentrotus lividus*, applying both an *in vivo* and *in vitro* approaches. Firstly, a long-term experiment of T and E2 dietary administration in adult specimens was performed and different

reproductive parameters were analysed. Then ovarian cells, isolated from ovaries at the same reproductive stages considered in the *in vivo* study, were cultured and exposed to the same steroids. The results, obtained from the two different approaches, were compared and analysed to figure out final general considerations.

3. Materials and Methods

3.1. In vivo experiment

3.1.1. Experimental animals and maintenance

P. lividus adult specimens were collected in the Protected Marine Area of Bergeggi (44°14'N; 8°26'E), on the Ligurian coast of Italy (Tyrrhenian Sea), at 3-5 meters of depth. 84 animals were collected at the end of July and immediately transferred to the laboratory, at the University of Milan, in cool boxes filled with natural sea water.

After their arrival in the laboratory, animals were randomly distributed in 9 aquaria (10 animals in each aquarium, except for an aquarium in which 4 animals were placed). The 50 l glass aquaria were filled with artificial sea water (Instant Ocean; salinity about 37‰, as in the Mediterranean Sea) and provided with circulation system as well as mechanical, chemical and biological filters. Animal conditions as well as all physical and chemical parameters were daily (temperature and salinity) or weekly (pH, KH, Ca^{2+}, Mg^{2+}, PO_4, NO_2, NO_3) monitored throughout the experimental period (including the pre-administration phase, see below). At the beginning of each week filters were cleaned and 10-20% of the sea water was renewed.

Before the hormonal treatment, animals were starved for six weeks (Fig. 20). According to Spirlet et al. (2000) an appropriate starvation period should reset the reproductive cycle to a resting phase in which gonads are almost devoid of sexual cells. In this way all the experimental animals are supposed to be synchronized in the same starting maturative condition. The achievement of this condition was helped by the real maturative state of the collected field specimens, most of which were naturally in resting phase in July (Barbaglio et al., 2007). Throughout the starvation period temperature was set at 16±1 °C and photoperiod was fixed at 16h:8h (dark:light), thus simulating winter condition. This condition should prevent mortality during the starvation period, as the animal metabolism is supposed to slow down in winter. At the beginning of the feeding period these parameters were gradually increased up to 20 °C and 10h:14h (dark:light) respectively, as in summer time, and remained fixed for the whole hormonal administration period. Indeed, it is reported that the changes in water temperature and daylight length (winter→summer) experienced by field animals stimulate the onset of gametogenesis (Pearse and Cameron, 1991; Kelly, 2001;

Shpigel et al., 2004): in particular 20 °C results as the optimal temperature for promoting gonad maturation in cultivated sea urchins (Spirlet et al., 2000). Feeding re-started one week before the beginning of hormonal dietary administration (Fig. 20), in order to partially restore the animals after the starvation stress.

3.1.2. Experimental design

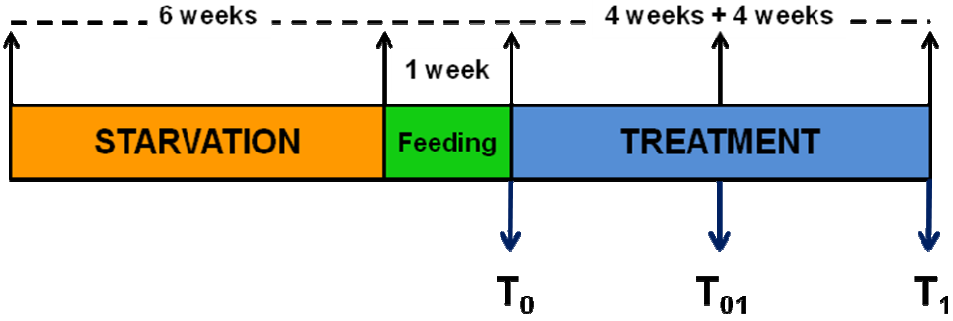

Fig. 20. Schematic representation of the *in vivo* experimental design.

To evaluate the starting reproductive conditions of the animals and to confirm the synchrony within the experimental population, 10 specimens were sacrificed just before the beginning of the hormonal dietary administration (T_0): animals were weighted, opened in two halves and their five gonads were removed from the internal side of the tests for Gonad Index (GI) calculation. In each specimen one gonad was processed for standard methods of light microscopy.

All the remaining animals (74) were distributed in 9 aquaria, each containing 8 animals, except for one containing 10 control animals. In order to reduce competition and control daily feeding rates each aquarium was divided in 4 compartments, containing only 2 individuals, by using plastic grids (Fig. 21). 4 experimental groups were designed:

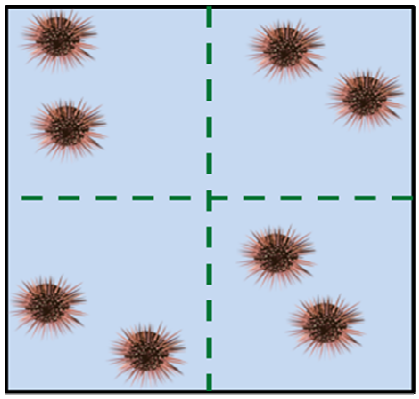

Fig. 21. T_1 animal distribution in aquarium.

- CTL group: 26 animals fed with control pellets;
- E2 group: 16 animals fed with pellets containing 17β-estradiol;
- E2-4weeks group: 16 animals fed with control pellets for the first 4 weeks and, then, with pellets containing 17β-estradiol until the end of the experimental period;
- T group: 16 animals fed with pellets containing testosterone.

After 4 weeks of hormone dietary administration 10 control animals were sacrificed (T_{01}): after being weighted, animal were opened and gonad were removed for GI calculation and histological analysis. T_{01} animals were required to confirm the onset of the reproductive processes and determine the maturative conditions in which E2-4weeks group was at the beginning of the E2

treatment. This was particularly important because E2-4weeks was specifically designed to investigate the presence of a specific stage (*Growing* stage) sensible to E2, as reported in asteroids.
At the end of the steroid administration period (8 weeks) all the experimental animals were weighted, sacrificed and gonads were processed as described above for GI calculation and histological analysis.

3.1.3. Hormonal dietary administration

Hormone dietary administration occurred daily for 8 weeks. For all the treatment, feed pellets were prepared by mixing 30% pulverized sea urchin formulated feed (Wenger Manufacturing, Inc.) with boiled distilled water containing 3% agar. The mixture was allowed to solidify at room temperature in specific moulds in order to obtain pellets of about 0.2 g. Taking into account that steroid hormones can be inactivated by heat, pellets containing E2 and T were prepared following the same described procedure with some modifications: E2/T (Sigma) powder was firstly accurately mixed with the pulverized sea urchin feed and, then, distilled water with agar was added only when its temperature decreased till 40 °C. Fresh pellets were prepared every week and stored at -20 °C.
Each animal was fed daily with a 0.2 g pellet. Each T pellet contained 10 µg of testosterone whereas each E2 pellet contained 1 µg of 17β-estradiol. Steroid administered doses were carefully chosen taking into account: the physiological T and E2 concentrations in sea urchin gonads, the relevant metabolic activity in sea urchin digestive tube (Lavado et al., 2006; Barbaglio et al., 2007) and previous indications from other authors (Unuma et al., 1999; Wasson et al., 2000b).

3.2. In vitro experiments

3.2.1. Experimental design and animal maintenance

The experimental design performed for the *in vivo* study was adapted for the *in vitro* experiments (Fig. 22). 16 adult specimens, collected in the Protected Marine Area of Bergeggi (on the Ligurian coast of Italy) at the end of July were immediately transferred and maintained in aquaria at the laboratory of Milan. Animal conditions as well as all water physical and chemical parameters were properly monitored, as described before (see 3.1.1. section).
Animals were starved for six weeks. Temperature was set at 16±1 °C and photoperiod was fixed at 16h:8h (dark:light), simulating winter condition. At the beginning of the feeding period these parameters were gradually increased up to 20 °C and 10h:14h (dark:light) respectively, as in summer time.
At the end of the starvation period, 8 animals were sacrificed and primary cell culture were developed only from gonads of female individuals (C_0). Ovaries were removed from the internal

side of the test: one gonad was processed for standard methods of light microscopy, whereas the remaining 4 gonads were used to obtain primary cell cultures and test steroid exposure.

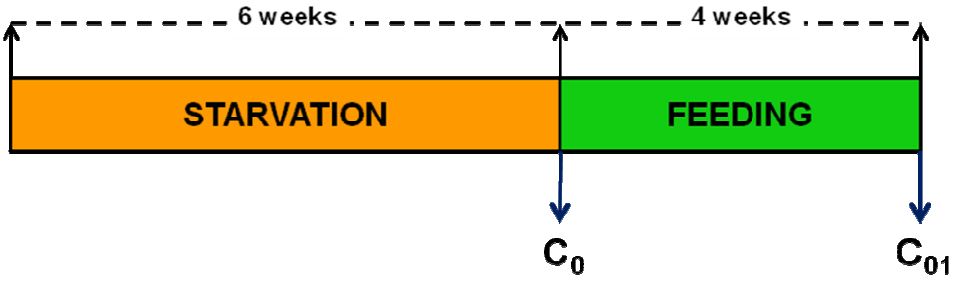

Fig. 22. Schematic representation of the *in vitro* experimental design.

The remaining 8 animals were arranged in an aquarium as described for T_1 animals (Fig. 21) and daily fed with control pellets. After 4 weeks of feeding (C_{01}) the animals were sacrificed and ovaries were used for cell culture development and histological analysis, as performed for C_0 animals.

3.2.2. Cell cultures

Primary cell cultures from *P. lividus* ovaries were developed, as previously described in Chapter 3 with some modifications. Briefly, ovaries were washed in sterile Ca^{2+} Mg^{2+} Free Sea Water (CMFSW) with antibiotics (40 µg/L gentamycin, 100 units/mL penicillin and 100 µg/mL streptomycin). Ovary pieces were incubated in 0.5 mg/mL collagenase dissolved in sterile CMFSW and stirred for 1 hour. The resulting cell suspension was centrifuged at 300 × g for 6' at 15 °C and the cell pellet was resuspended in modified Leibovitz-15 medium without supplement addition.

Ovarian cells, isolated from each female, were divided in 3 different experimental groups:

- CTL group: cells maintained in culture medium without hormones;
- E2 group: cells cultured in presence of 20 pg/mL 17β-estradiol;
- T group: cells cultured in presence of 200 pg/mL testosterone.

In order to analyze the E2 and T effects on cell morphology and viability, cells were seeded at a concentration of 3-4 × 10^5 cells/mL in 24-well culture plates, coated with poly-L-lysine (70-150 kDa, 0.01% solution, Sigma). Cultures were developed in replicates and incubated at 15 °C. Replacement of 50% of the medium was carried out every two days. Cell behavior was observed daily using an inverted phase contrast microscope . For the biochemical analyses, cells were seeded at a concentration of 3 × 10^6 cells/mL in 6-well culture plates, coated with poly-L-lysine, and maintained at 15 °C for 24 h and 48 h.

3.2.3. Chemicals and solution preparation

All chemicals were of reagent grade. 17-β estradiol and testosterone were purchased from Sigma. E2 and T final exposure concentrations were determined on the basis of our previous original results about sex-steroid physiological levels (unpublished data). Culture medium containing E2 was prepared as follows: 2 mg E2 were dissolved in 10 mL acetone (Merck), then 10 µL of this solution was dissolved in 10 mL autoclaved artificial sea water. 10µL of this latter solution were mixed with 10 mL of modified Leibovitz-15 medium obtaining a stock solution which was subdivided in aliquots and maintained at -20 °C in the dark, in order to prevent steroid degradation. Finally, to reach the final concentration of 20 pg/mL, 500 µL of the stock solution were dissolved in 4.5 mL of medium. Final acetone concentration was considered negligible (0.00001%). To prepare the culture medium containing 200 pg/mL T, the same procedure was followed but 20 mg T were used. Fresh culture medium with hormones was prepared every time. Stock solutions were prepared every week.

3.3. Determination of reproductive stages

Reproductive stages were determined by histological analysis. Standard methods for light microscopy (paraffin and/or resin) were employed.

3.3.1. Microscopic analysis

Gonads were fixed with Bouin (picric acid, formaldehyde, acetic acid, 75:25:5) for at least 24 h and washed several times in tap water until all the fixative solution was completely removed. Samples were then dehydrated with an ethanol series (70%, 90%, 95% and 100%), washed with xilene and left overnight in a solution of xilene and paraffin 56–58 °C (1:1). Gonads were then washed three times in paraffin and finally embedded. Longitudinal sections (5-7 µm) were cut with a Reichert OmE sledge microtome and stained with Milligan's Trichrome. Before staining, the sections were washed in xilene and ethanol (100% and 95%) and placed into a solution of potassium dichromate and hydrochloric acid. They were first stained with acid fuchsin, fixed with phosphomolybdic acid (1%), stained with orange G and fast green FCF, and then treated with a solution of acetic acid 1%. Finally, sections were cleared in xilene and dehydrated with ethanol (95% and 100%) before mounting.

Both T_0 and C_0 samples were embedded in resin and processed for semithin sections (which provide better quality and higher resolution) because difficulties in determining animal sex were expected after the starvation period. Briefly, gonads were prefixed with 2% glutaraldehyde in 0.1 M cacodylate buffer and NaCl 1.4% for 2 h and, after overnight washing in the same buffer, postfixed with 1% solution of OsO_4 in 0.1 M cacodylate buffer (2 h). After standard dehydration in an ethanol

series (25%, 70%, 90% and 100%), the samples were washed in propylene oxide, left in propylene oxide:resin (1:1) for 2 h. After overnight washing in resin they were embedded in Epon-Araldite 812 resin. The semithin (about 1 μm) sections were cut with a Reichert-Jung ULTRACUT E using glass knives and stained with crystal violet and basic fuchsin.

Both resin and paraffin sections were observed and photographed under a Jenaval light microscope to determine the gonad maturative stage. Five stages were considered: *Spent* (immediately after the spawning event), *Recovery* (phagocitosis and nutrient accumulation phase), *Growing*, *Premature* and *Mature* (progressive stages of gametogenesis).

3.3.2. Maturity Index and Gonad Index

Maturity Index (MI) was calculated as the mean value of the numerical maturative stages of each experimental group based on histological analysis (*Spent*=0, *Recovery*=1, *Growing*=2, *Premature*=3, *Mature*=4). The numerical maturative stage of each animal was corrected with +0,25 or -0,25 when in advanced or precocious conditions, respectively.

Gonad Index (GI) was determined as a percentage of the ratio between gonads wet weight (GW) and total wet weight (TW):

$$GI = (GW/TW) \times 100$$

3.4. Electrophoresis

3.4.1. Sample preparation

The ovarian cells, cultured in 6-well culture plates, were processed for electrophoresis analysis after 24 and 48 hours of *in vitro* steroid exposure.

To prepared samples for running on gel, cells were gently scraped off the dish, using an ice-cold plastic cell scraper. Cell suspension was transferred into a pre-cooled tube and centrifuged at 300 × g for 6’ at 4 °C; the resulting cell pellet was resuspended in 100 μL lysis buffer. Cells were lysed in ice-cold 20 mM Tris-HCl (pH 7.5) with protease inhibitors (5 mM EDTA, 0.2 mM PMSF and 4 mM NEM), maintaining them in constant agitation for 30 minutes. Then, samples were centrifuge at 12000 × g for 20’ at 4 °C and the supernatant was transferred into a fresh tube, kept on ice. Finally, samples were dialyzed, protein assayed (BCA protein assay kit, Sigma) and stored at -20 °C until electrophoresis was performed.

3.4.2. SDS-PAGE

Sodium dodecylsulfate-polyacrilamide gel electrophoresis (SDS-PAGE) was performed using 6% slab gel (Laemmli, 1970) in order to verify and compare the sample protein content in C_0 and C_{01}

experimental groups (CTL, E2 and T). Samples containing 10 µg of total proteins were diluted with sample buffer (SDS reducing buffer), boiled for 5 minutes and, then, applied to each lane. Gel were run at 100 V at room temperature. Protein bands were visualized with Coomassie brilliant blue R-250. SDS-PAGE standards (StoS Protein Marker, Genespin s.r.l.) were also run for molecular weight calibration.

3.5. Statistical analysis

Results are presented as mean values±SEM. Statistical significance was assessed using one-way ANOVA (Tukey's test) and χ^2 test. A *p*-value of less than 0.05 was considered statistically significant. Statistical analysis was performed by the computer program GraphPad Prism 4.

4. Results

4.1. Animal health conditions

During both the *in vivo* and the *in vitro* experimental periods, aquaria physical and chemical parameters were properly monitored and promptly adjusted, when necessary. All the animals appeared healthy and no mortality event was observed throughout the experiments. As far as the steroid dietary administration is concerned, animal daily feeding rate, calculated as mean percentage of the eaten pellets, was close to 100% for all the experimental groups (CTL: 99.4%; E2: 99.6%; T: 99.7%; E2-4weeks: 100%), ensuring that daily E2 and T administration correctly occurred.

4.2. In vivo experiment

4.2.1. Sex ratio

Comparing sex ratio values (Fig. 23), no statistically significant difference was found among all the experimental groups (χ^2 test: P>0.05). T_0, T_{01} and all the hormonally treated groups displayed similar frequencies: males were ∽40% (T_0: 50%; T_{01}: 40%; E2: 37.5%; T: 43.75%; E2-4weeks: 31.25%) and females were ∽60% (T_0: 50%; T_{01}: 60%; E2: 62.5%; T: 56.25%; E2-4weeks: 68.75%) in each group. Only in CTL group, the relative frequencies of males and females were quite different from the others, showing 68.75% males and 31.25% females.

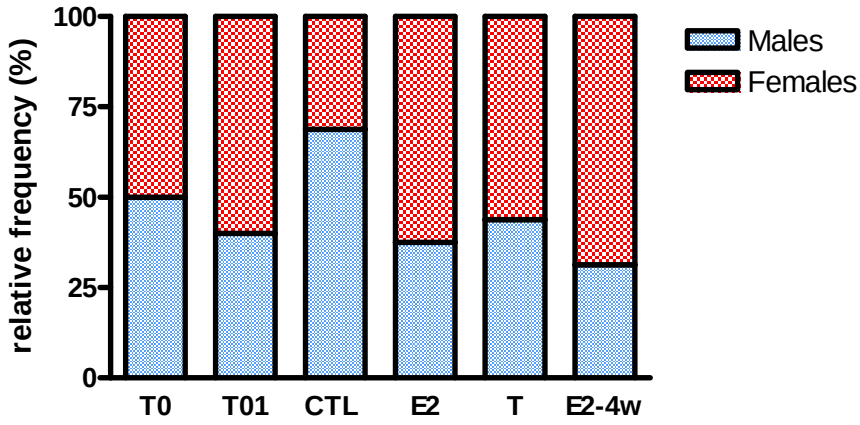

Fig. 23. Relative frequencies of male and female animals in the different T_1 experimental groups.

4.2.2. Gonad Index (GI)

Mean GI values (Fig. 24) measured in T_0 and T_{01} groups were significantly lower than that measured in T_1 CTL group (one-way ANOVA, Tukey's test: P<0.001). A slight and progressive increase of GI was observed from T_0 (3.8±0.5) to T_{01} (5.2±0.4) (P>0.05). The highest mean GI was registered in T_1 CTL (8.5±0.5).

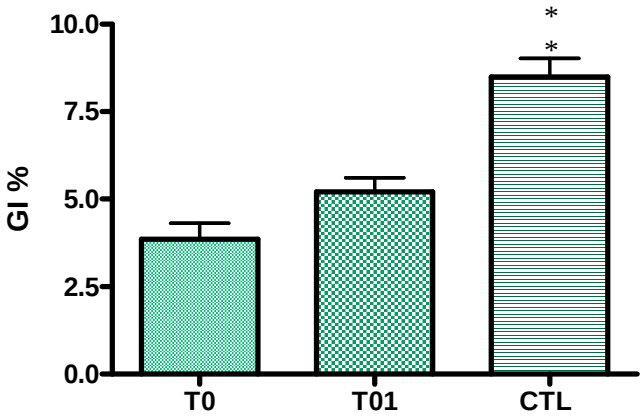

Fig. 24. GI (%) of untreated groups (T_0, T_{01}, T_1) during the experimental period. Data are expressed as mean±SEM. ** = P<0.001 (N= 10-16).

Considering T_1 animals no significant difference in GI was recorded between CTL and hormonally treated groups (one-way ANOVA: P>0.05): GI were almost the same in the different experimental groups, varying between 8.5±0.5 and 9.6± 0.7. Statistically significant differences were not found also considering males and females separately (one-way ANOVA: P>0.05) (Fig. 25).

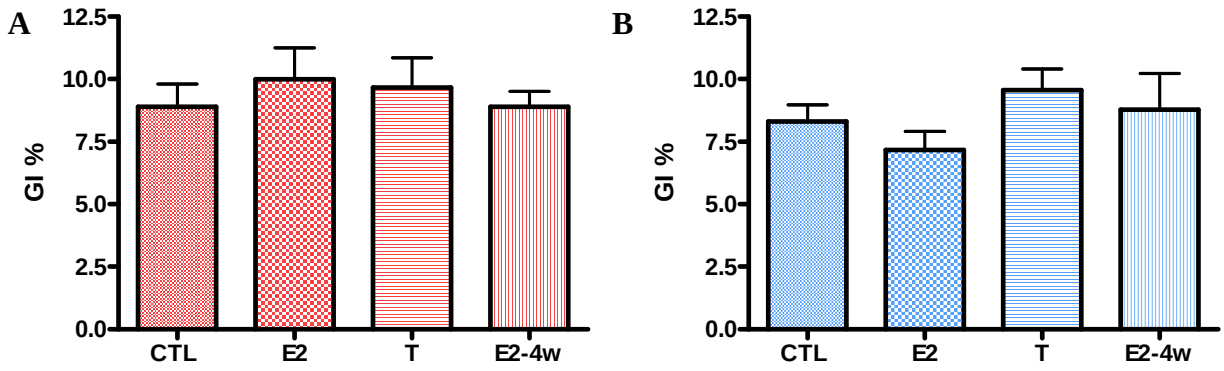

Fig. 25. GI (%) values in females (**A**) and males (**B**) of T_1 groups. Data are expressed as mean±SEM (N = 5-11).

In females (Fig. 25 A), mean GI values oscillated between 8.9±0.9, registered in CTL specimens, and 9.9±1.2, measured in E2 group. In males (Fig. 25 B), GI values were less uniform. The lowest GI was recorded in E2 group (7.2±0.7) whereas the highest GI was found in T samples (9.6±0.8). Male CTL and E2-4weeks groups showed pretty similar mean values (CTL: 8.3±0.7; E2-4weeks: 8.8±1.4).

4.2.3. Maturity Index (MI)

Mean MI values of T_0 and T_{01} animals were significantly lower than that measured at the end of the experimental period in CTL T_1 (one-way ANOVA, Tukey's test: T_0 *versus* CTLT_1: P<0.001; T_{01} *versus* T_1: P<0.05). MI gradually increased from T_0 (1.4±0.2) to T_{01} groups (2.3±0.1) (P>0.05) and, finally, reached the highest mean value in T_1 samples (2.8±0.3) (Fig. 26).

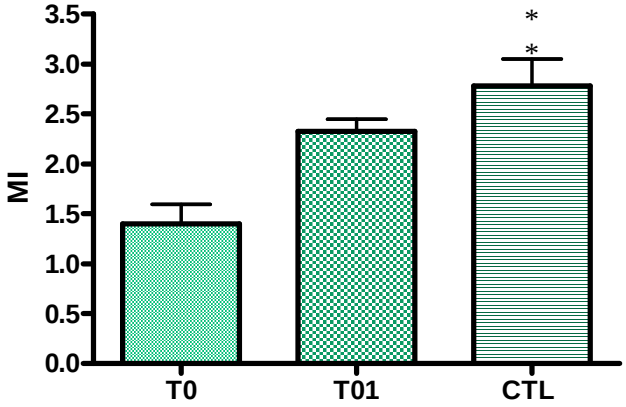

Fig. 26. MI (%) of T_0, T_{01}, T_1 CTL groups. Data are expressed as mean±SEM. ** = P<0.001 (N = 10-16).

Considering MI values of T_1 experimental set, no significant difference was recorded between CTL and hormonally treated groups (one-way ANOVA: P>0.05). A similar result was found when considering males and females separately (Fig. 27) (one-way ANOVA: P>0.05). In males, mean MI values were pretty similar (∽2.5) in all the experimental groups (Fig. 27 B), whereas, in females, treated groups showed slightly lower values (P>0.05) compared to CTL (Fig. 27 A).

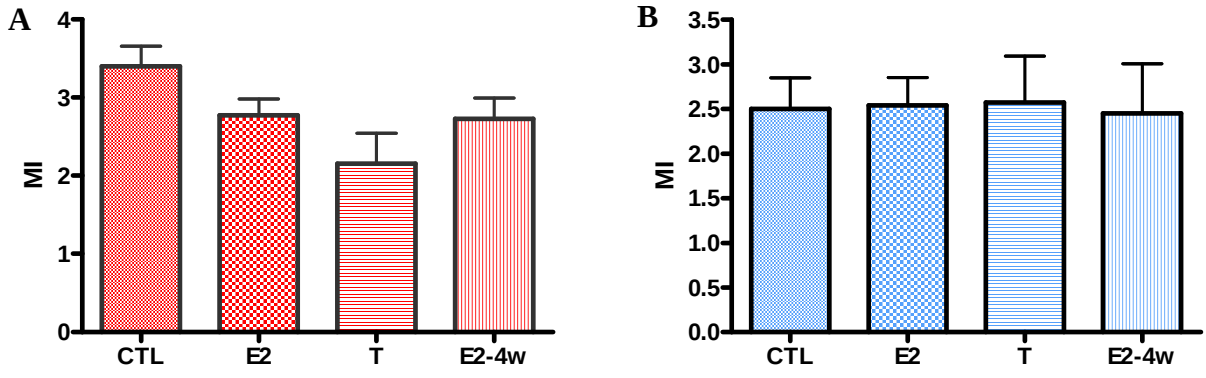

Fig**. 27.** MI values in females (**A**) and males (**B**) of T_1 groups. Data are expressed as mean±SEM (N = 5-11).

4.2.4. Reproductive stages

Analysing the distribution of the reproductive stage frequencies further considerations could be reported (Fig. 28 and 29). At the end of the starvation period, almost all the samples were in

Recovery stage, regardless the sex. After 4 weeks of feeding, in T_{01} specimens, the reproductive processes began: all females (Fig. 28) were in *Growing* stage whereas males (Fig. 29) presented a higher percentage of samples in *Premature* stage (67%).

Focussing on T_1 sample no striking difference was observed between control and hormonally treated groups in the relative frequencies of the maturative stages, also considering males and females separately.

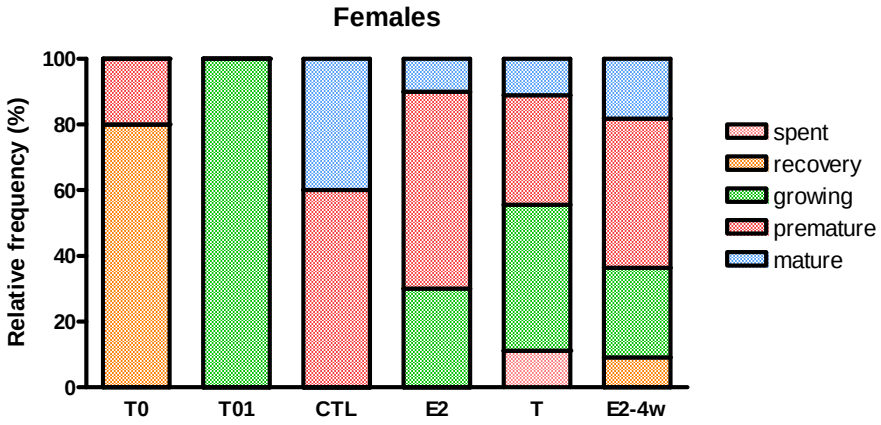

Fig. 28. Distribution of reproductive stages in females of the experimental groups (N=1-6).

In T_1 females (Fig. 28), although active gametogenic stages (*Growing*, *Premature* and *Mature* stages) were present in each experimental groups, hormonally treated groups showed a slight delay in the gametogenesis processes in comparison with CTL. Control animals were all found in *Premature* (60%) and *Mature* (40%) stages whereas in treated groups a low percentage of samples still in *Growing* stage was observed (E2: 30%; T: 45% and E2-4weeks: 27%). Resting non-gametogenic stages (*Spent* and *Recovery* stages) were occasionally found in E2-4weeks and T groups.

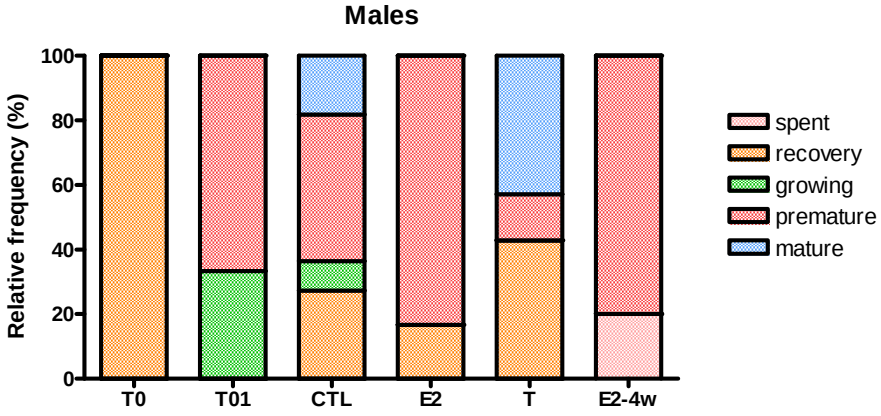

Fig. 29. Distribution of reproductive stages in males of the experimental groups (N=1-5).

Taking into account only T_1 male samples (Fig. 29), a higher variability could be noticed. Both resting and active gametogenic stages were present in each experimental group and no evident difference was observed between control and hormonally treated groups.

4.3. In vitro experiments

4.3.1. Cell cultures

C_0 and C_{01} cell cultures were obtained from ovaries at the same maturative stages displayed by T_0 and T_{01} animals (*in vivo* experiment). In particular, all C_0 individuals were in *Recovery* stage whereas C_{01} animals were in *Growing* stage.

Ovarian cells, cultured in presence of 20 pg/mL E2 and 200 pg/mL T, were observed daily under a phase contrast microscope in order to identify possible hormone effects on cell morphology and behaviour.

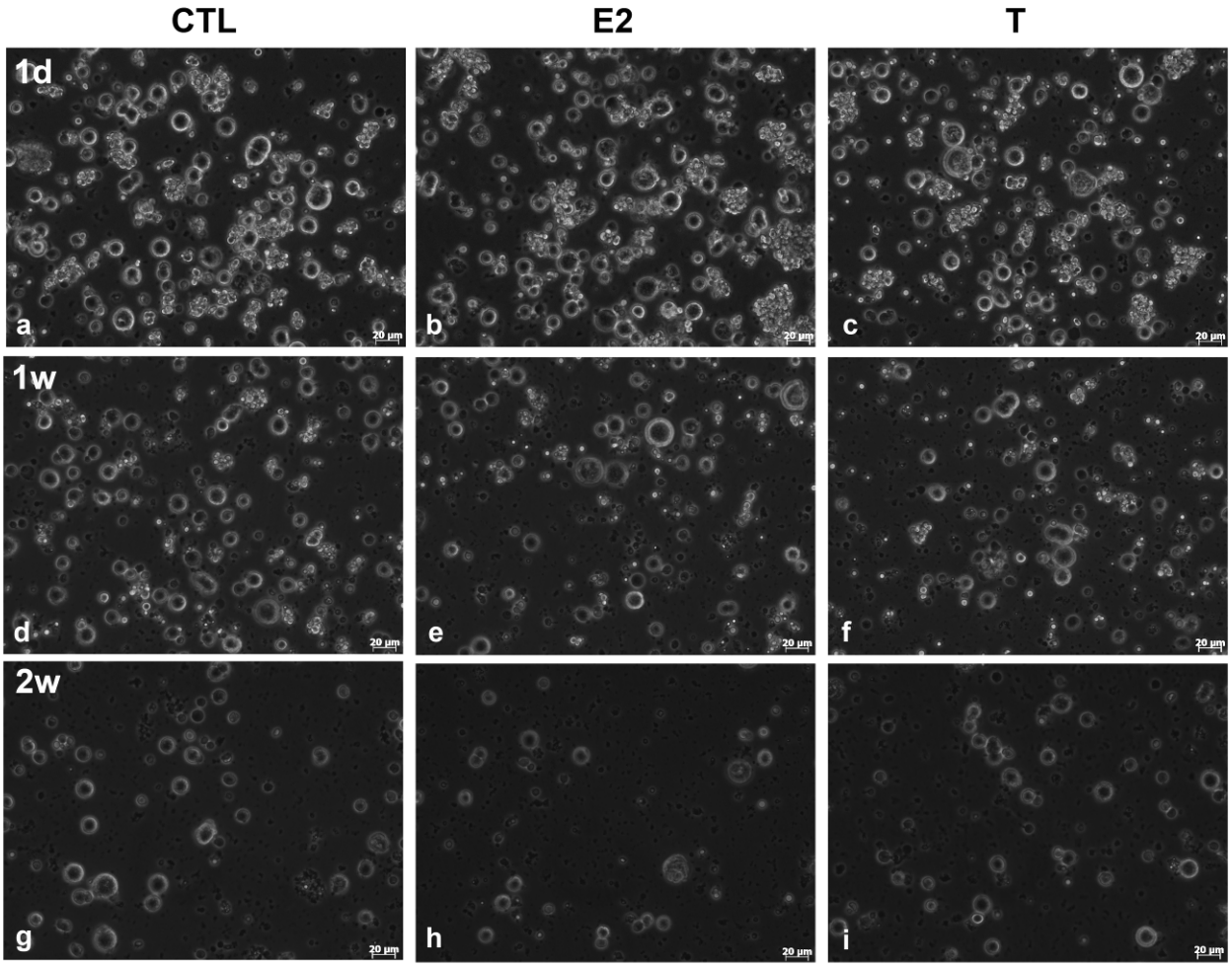

Fig. 30. Phase contrast microscopy. C_0 ovarian cells cultured with: no steroid hormones (**a**, **d**, **g**), 20 pg/mL E2 (**b**, **e**, **h**) and 200 pg/mL (**c**, **f**, **i**), during the experimental period. 1d = 1 day after cell isolation (**a**, **b**, **c**); 1w = 1 week cell cultures (**d**, **e**, **f**); 2w = 2 week cell cultures (**g**, **h**, **i**).

Considering C_0 cell cultures (Fig. 30), no marked difference was found between control and steroid exposed cells. E2 and T did not affect cell morphology and behaviour during the considered culture period. In particular, no difference in size of both nutritive phagocytes and oocytes was observed between the experimental groups.

Cell viability appeared similar in all the groups: cell remained alive and healthy for the first week of culture but the viability gradually fell down during the following period (Fig. 30 g, h and i).

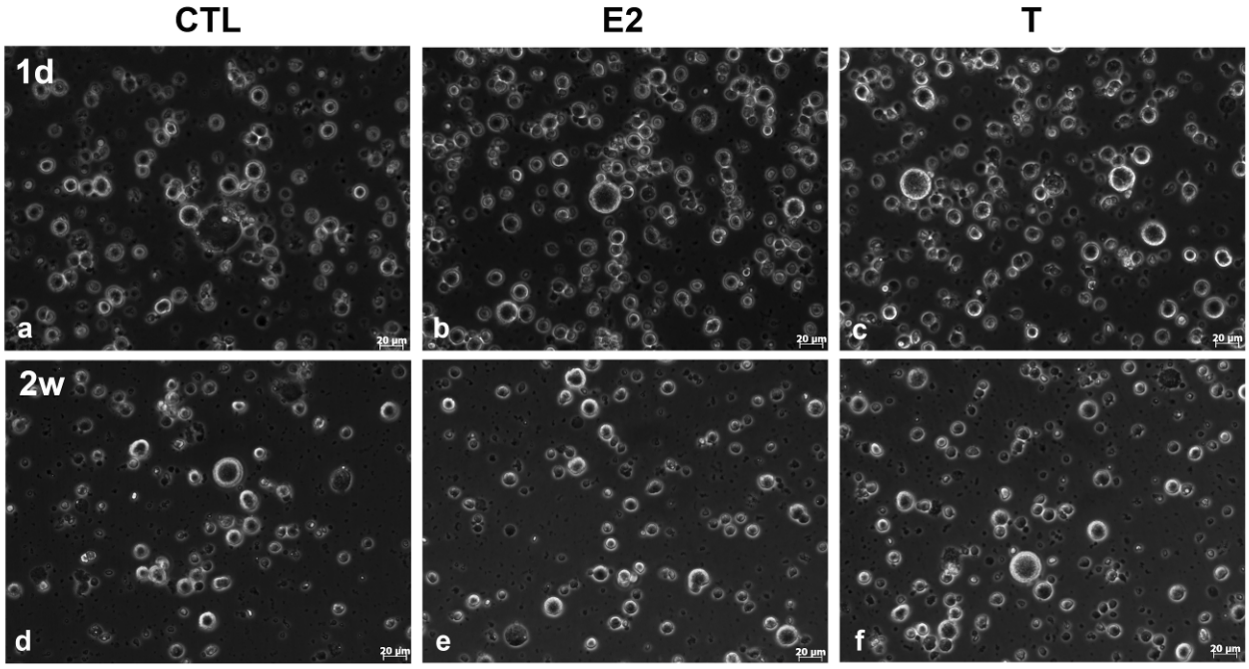

Fig. 31. Phase contrast microscopy. C_1 ovarian cells cultured with: no steroid hormones (**a** and **d**), 20 pg/mL E2 (**b** and **e**) and 200 pg/mL (**c** and **f**), during the experimental period. 1d = 1 day after cell isolation (**a**, **b**, **c**); 2w = 2 week cell cultures (**d**, **e**, **f**).

Similar results were obtained in C_{01} cell cultures. Ovary cells, cultured in presence of sex-steroids, appeared comparable with the controls in terms of both size and behaviour (Fig. 31).

Cell viability was similar in all the experimental groups as well. As in C_0 samples, during the second week of culture a decrease in cell number was noticed. However, in C_{01} cell cultures the viability decline was less marked and slower than that observed in C_0.

4.3.2. Electrophoresis

E2 and T effects on Major Yolk Protein (MYP) expression was investigated performing SDS-PAGE analysis. MYP content of C_0 and C_{01} samples was analyzed and compared 24 and 48 h after the *in vitro* steroid exposure (Fig. 32).

Firstly, a 180 kDa protein appeared to be present in all the samples. Considering the molecular weights and the current knowledge (Unuma et al., 2011; Prowse and Byrne, 2012) we concluded that the band corresponded to EGMYP. In addition, other 2 protein bands with slightly different

molecular weights were found. The 170 kDa protein band could correspond to CFMYP (Unuma et al., 2011).

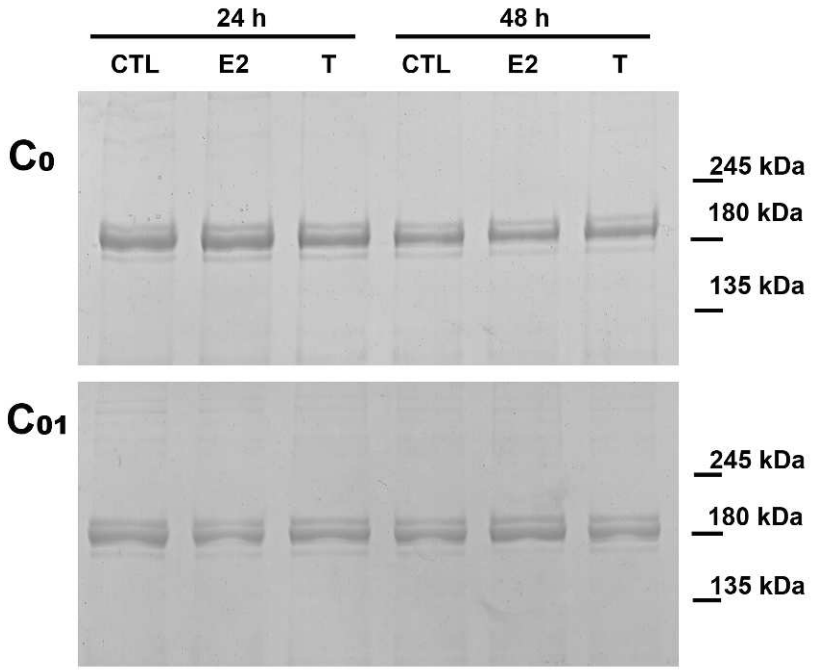

Fig. 32. 6% SDS-PAGE analysis of C_0 and C_{01} cell extracts.

Comparing C_0 experimental groups, no difference in EGMYP expression was observed in cells exposed for both 24 and 48 hours. Steroids did not affect EGMYP content in C_{01} ovary cells as well: in E2 and T samples the intensity of MYP band was comparable to the control (Fig. 32).

5. Discussion

In this study E2 and T involvement in echinoid reproduction was explored applying both an *in vivo* and *in vitro* approach.

Concerning the *in vivo* study, a long-term experiment of steroid dietary administration was performed in adult specimens of *P. lividus* and different reproductive parameters were investigated. The experiment was successfully designed in order to synchronize the starting maturative conditions of the animals and reduce as much as possible the variability present in field population. Animals were divided in 4 groups: CTL, E2, T and E2-4 weeks. In particular, the latter was chosen in order to verify the existence of a specific maturative stage sensitive to E2, as proposed in different asteroid species. In fact, in both *A. rubens* and *A. pectnifera* E2 seemed to enhance oocyte growth only during vitellogenesis, suggesting the presence of a threshold oocyte size for E2 effectiveness (Schoenmakers et al., 1981; Takahashi and Kanatani, 1981). To investigate the existence of similar mechanisms in echinoids, E2-4weeks specimens were fed with control pellets

for 4 weeks and then treated with E2, i.e. after they reached the *Growing* stage during which gametogenesis processes began.

The nutritional conditions of the animals resulted to be optimal: in fact a progressive increase of GI mean values was observed from starved animals (T_{00}) to sea urchins well-fed for 4 (T_{01}) and 8 weeks (T_1). It is well documented that quantity and quality of food influence gonad size and the amount of gametes produced, thus playing a very important role in the regulation of the reproductive cycle (Pearse and Cameron, 1991; Spirlet et al., 1998). MI values of control groups (T_0, T_{01} and CTL) displayed a marked and progressive increase throughout the experimental period as well. Therefore, despite the stress caused by the maintenance conditions, after starvation gametogenesis processes restarted and sea urchins progressively reached higher maturative stages. These results underlined the success of the experimental design and confirmed the presence of active gametogenesis processes during the hormonal treatment.

E2 and T dietary administration did not affect animal health conditions and the tested steroid concentrations did not cause acute toxicity effects. No mortality event occurred and all sea urchins appeared healthy throughout the experimental period.

The sex ratio was close to 1.5:1 (females:males) in most of the experimental groups. This value, although slightly different from data reported for field specimens (Pearse and Cameron, 1991), was completely in agreement with our previous observations in the population of *P. lividus* considered in this work (unpublished data). Focussing on E2 and T treatment, steroid dietary administration did not influence sex ratio: males and females frequencies found in controls and treated groups were pretty similar with the only exception being representing by the CTL animals that displayed a higher percentage of male individuals. This result was probably due to natural variability and did not seem to be related to hormonal treatment. Indeed, in all the other control groups (T_0 and T_{01}) sex ratio values were similar to those observed in T_1 treated groups.

In vertebrates, exposure/administration of E2 and T induces sex-reversal phenomena during early stages (i.e. during sexual differentiation), even in those classes (such as fish, amphibians, birds, etc.) with a genetic sex determination mechanism. Sex-reversal of adult specimens is very rare and it is reported only for those species, mainly fishes, which are naturally "programmed" to change sex during their life (hermaphroditic species) (Gutzke and Bull, 1986; Baroiller et al., 1999; Nakamura, 2010). The lack of T and E2 influence on sex ratio on our experimental animals was therefore quite expected (although not excluded *a priori*) since in the present research we employed adult sea urchins which had already undergone sex differentiation. As almost all sea urchins, *P. lividus* is a gonochoristic species without marked sexual dimorphism (Swann, 1954). Sex-determination

mechanisms are scarcely understood, although some authors suggest a genotypic basis (Delavault, 1966; Lipani et al., 1996).

Considering the analyzed reproductive parameters, E2 and T administration did not affect gonad growth, expressed as GI, in both males and females. Sex-steroid exogenous administration led to different and contrasting results on this parameter in both asteroids and echinoids (Schoenmakers et al., 1981; Van der Plas et al., 1982; Barker and Xu, 1993; Unuma et al., 1999; Wasson et al., 2000b). Particularly, E2 seemed to stimulate ovarian growth in the starfish *A. rubens* and in the sea urchin *L. variegatus* whereas in the other studied species no effect was reported. In asteroids, estrogens appeared to be more involved in oocyte maturation and growth: an E2 induced increase of oocyte diameter in several species and a hormone involvement in vitellogenesis processes was strongly suggested (Schoenmakers et al., 1981; Takahashi and Kanatani, 1981; Barker and Xu, 1993). As far as T is concerned, in echinoids no effect on GI was observed in several species. In *P. depresuss* an increase of testis size was obtained only after the administration of another androgen, androstenedione, whereas T was found to promote oocyte growth in *L. variegatus* (Unuma et al., 1999; Wasson et al., 2000b). These heterogeneous results could be explained in the light of the existence of species-specific hormonal mechanisms; on the other hand the different experimental conditions (treatment length, steroid concentrations, etc.) could have affected the experimental results and therefore have to be carefully considered.

Analysing the mean maturative state, i.e. the Maturity Index (MI) reached by the experimental groups of the present study, neither E2 nor T treatment affected gonad maturation, even when considering males and females separately. To better investigate this reproductive aspect, the relative frequencies of male and female reproductive stages were analyzed. Also in this case, no striking variations were observed, although all hormonally treated female animals showed a slight delay in the gametogenesis processes, that, however, seemed to be less affected by hormonal treatment than by the low number of samples per group (*P. lividus* specimens have no evident sexual dimorphism and so it is not possible to equally distribute males and females in each experimental group). Thus, E2 did not apparently induce oocytes maturation and no temporal window of E2 sensitivity was found, as, on the contrary, was observed in starfishes (Schoenmakers et al., 1981; Takahashi and Kanatani, 1981; Barker and Xu, 1993). It has been proposed that this different results could be due to class-specific mechanisms: echinoids and asteroids could simply have a different hormonal regulation of oocytes development and growth. This hypothesis was supported by Wasson et al. (2000b) who reported an inhibited growth of individual oocytes after dietary administration of E2 in the echinoid *L. variegatus*. This findings however disagreed with our results: neither E2 nor T affected reproductive parameters. Furthermore, contrasting results were reported for other echinoid

species. In *S. intermedius* and *S. nudus* the administration of estradiol dipropionate stimulated gonad development and gamete growth (Varaksina and Varaksin, 2001; Varaksina and Varaksin, 2002), whereas no response to E2 and T was observed in *P. depressus* yearling females (Unuma et al., 1999). The high variability of responses to steroid treatment observed in echinoids could be reasonably related to the different experimental conditions. In particular, steroid hormone administration (dietary or injections), treatment period (days or weeks), hormone doses and individual differences (age, species, reproductive state of gonads) could in fact strongly influenced the results. Our experiment was designed in order to reduce as much as possible individual variability and, most of all, to synchronize animal reproductive conditions. In this way the results should be less affected by the heterogeneity present in field population. The administered steroid concentrations were carefully chosen taking into account the physiological T and E2 concentrations in sea urchin gonads, the high metabolic activity present in sea urchin digestive tube (Lavado et al., 2006; Barbaglio et al., 2007) and also previous results obtained by other authors (Unuma et al., 1999; Wasson et al., 2000b). In view of these considerations, our results can be considered realistic and reliable.

However, in order to further confirm these findings and deepen the possible E2 and T involvement in echinoid reproduction we additionally performed *in vitro* exposure experiments. Although the *in vitro* conditions are obviously different and far from the *in vivo* model, cell cultures allow studies under controlled experimental conditions without most of the metabolic interferences that can occur in animals. Ovarian cells were cultured for 2 weeks in presence of physiological levels of E2 and T in order to investigate possible morpho-functional effects. Primary cell cultures were developed from gonads at two different starting maturative conditions (C_0: *Recovery* stage; C_{01}: *Growing* stages) in order to make the results comparable with those obtained in the experiments of steroid dietary administration.

Analysing cell morphology and behaviour no variation was observed between controls and hormonally exposed groups in either C_0 or C_{01} cultures. The size of nutritive phagocytes and oocytes was not affected by E2 and T exposure throughout the experimental period as, on the contrary, was reported in many asteroid and echinoid species (Schoenmakers et al., 1981; Takahashi and Kanatani, 1981; Wasson et al., 2000b). Nevertheless, most of the studies were performed with an *in vivo* approach and only one study based on *in vitro* E2 exposure work was attempted. In *A. pectinifera*, fragments of ovary were cultured for 3 days in medium containing 10^{-6} M E2 and a significant increase of mean oocyte diameter was observed (Takahashi and Kanatani, 1981). These contrasting results could be due to the different type of culture performed (organ cultures *vs* and

primary cell cultures) or, as previously proposed, to the existence of specie-specific differences, the employment of different steroid concentrations and diverse experimental designs.

To better understand steroid possible involvement in sea urchin physiology and reproductive biology further investigations were performed i*n vitro* concerning E2 and T role in the regulation of the Major Yolk Protein (MYP) expression. Indeed, estrogen control of MYP expression was firstly proposed by Shyu et al., (1987), who found a palindromic sequence similar to the estrogen-responsive element upstream MYP gene. MYP is a metal-binding glycoprotein belonging to the transferrin superfamily (Noll et al., 2007; Unuma et al., 2011) and in vertebrates transferrin genes are regulated by estrogens (Prowse and Byrne, 2012). Considering echinoderm phylogenetic position (close to chordates, including vertebrates) the existence of common hormonal mechanisms can reasonably be proposed.

SDS-PAGE analysis of cell extracts was performed in control and hormonally exposed samples but no difference was observed between the experimental groups. Our results were in agreement with those reported in *S. nudus* larvae and juveniles: E2 did not affect MYP expression in all the considered embryo stages. On the contrary, estrone (E1) seemed to be more involved in the protein expression, playing a suppressive role during specific developmental stages (Kiyomoto et al., 2008). Nevertheless, these results were obtained in larvae and post-metamorphosis juveniles, i. e. stages very different from the adult condition in which sexual development and reproductive activities are not yet established. Furthermore, our data could be influenced by the *in vitro* condition and no analysis was performed on either MYP mRNA levels or culture medium protein content. In the sea cucumber *Apostichopus japonicus*, although the intensity of MYP band in SDS-PAGE gel remained stable throughout ovarian development, a clear increase of MYP transcription was observed. The inconsistency of the levels of MYP mRNA and MYP protein was proposed to be due to MYP delivery from the ovary to other animal regions, such as coelomic fluids, or to its rapid consumption (Fujiwara et al., 2010). Taking into account all these considerations further research are surely needed to confirm our results: steroid involvement in protein expression should be deeply investigated expanding the target organs (MYP is mainly synthesized in both gonads and digestive tube) and employing different technical and methodological approaches. This is further supported by previous studies, reporting steroid involvement in protein synthesis: in fact E2 and T administration was demonstrated to enhance the rate of protein synthesis in both asteroid (Barker and Xu, 1993) and echinoid gonads (Varaksina and Varaksin, 2001; Varaksina and Varaksin, 2002) and E2 stimulation on protein synthesis was also observed in *Strongylocentrotus purpuratus* and *Dendraster excentricus* coelomocytes (Harrington and Ozaki, 1986).

In conclusion, E2 and T did appear to not markedly affect *P. lividus* reproductive biology either *in vivo* or *in vitro*. Hormonal dietary administration did not induce striking variations in the considered reproductive parameters and no effect was observed in ovarian cell cultures exposed to hormone physiological concentrations.

Overall, the present research provided new information on sex-steroid implications in echinoderm reproductive processes. Indeed, according to the obtained results, E2 and T do not markedly influence echinoid gonad maturation and, particularly, they do not promote gamete maturation, as on the contrary reported in vertebrates (Lange et al., 2002). In addition, no specific maturative stage sensitive to E2 was found, suggesting the existence of different hormonal mechanisms controlling these processes in asteroids and echinoids. However, an indirect involvement of steroids and, in particular, of E2 in echinoderm reproduction can not be completely excluded and its role in the regulation of lipid metabolism and protein synthesis during the different reproductive stages should be strongly considered, as suggested by several other authors (Wasson et al., 2000b; Varaksina and Varaksin, 2001; Varaksina and Varaksin, 2002).

Chapter V

GENERAL DISCUSSION

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FUTURE PROSPECTIVES

The present research project was designed in order to explore different aspects of sex-steroid involvement in echinoid reproductive biology by performing both *in vivo* and in *vitro* experiments. The employment of these different and, at the same time, complementary approaches allowed us to provide a better understanding of sea urchin endocrinology and help to elucidate E2 and T role in echinoderm reproduction.

Furthermore, we set up new and important experimental tools. Indeed, at our knowledge, we were the first to develop effective primary cell cultures from sea urchin ovaries and apply them for *in vitro* exposure tests. On the other hand, in the *in vivo* model, we optimized an innovative experimental design that gave the opportunity to study the effects induced by exogenous administration without risking that individual variability affected our final results.

Thus, in this research, we provided not only new information on echinoid reproduction but also new and successful methodological approaches, that could be useful for expanding the potential employment of echinoderms in experimental research and, in particular, for investigating further endogenous factors involved in the regulation of echinoderm reproductive processes.

1. Development of primary cell cultures

Considering the importance and possible applications of the *in vitro* studies, we developed primary cell cultures from sea urchin ovaries. Heterogeneous cultures, composed by both nutritive phagocytes and germ cells were preferred as nutritive phagocytes play important and multiple roles, providing support, protection and nutriments to the growing germinal cells (Chia and Bickell, 1983; Walker et al., 2000). Considering the lack of knowledge about germ cell requirements and the need to maintain cells in healthy conditions, heterogeneous cultures appeared more suitable, at least in this starting phase of the research. However, the two cellular phenotypes present in culture were well recognizable thanks to their overall morphology and behaviour and, thus, this heterogeneity did not cause difficulties in the *in vitro* analyses.

Contamination is a central aspect of the *in vitro* studies and it has often represented an important obstacle in the development of effective cell cultures and even cell lines from a variety of marine organisms (Rinkevich, 1999). In the present research, contamination occurred at a very low rate and, in most of the cases, bacteria and moulds were the main responsibles. This was probably due to the combination of two main aspects: the addition of antibiotics in culture medium and the presence of natural antibacterial compounds in sea urchin gonads/gametes. In fact, in different echinoderm species both seminal plasma and eggs were found to exert an antibacterial activity and, particularly, a lysozyme-like activity seemed to be involved (Stabili and Canicattì, 1994; Stabili and Pagliara, 1994). This specific gonad feature, together with antibiotic and antimycotic (gentamycin,

streptomycin and penicillin) addition, surely helped to maintain bacterial contaminations at a very low level and to drastically reduce mould occurrence.

Ovarian cell culture were produced from ovaries at all stages of maturation but a higher survival rate and a more lively behaviour was observed when ovaries at early stages of gametogenesis (*Growing* and *Premature* stages) were used. This attitude was noticed also during the sex-steroid exposure experiments (Chapter IV). C_0 cell cultures, obtained from ovary in resting non-gametogenic stages, displayed a cell viability decrease during the second week of culture that was less evident and slower in C_{01} cells, isolated from ovaries at *Growing* stage. It has to be underlined that in the *in vitro* E2 and T exposure experiments no supplements were added to the culture medium in order not to affect biochemical analyses. Since the exposure period was only 2 weeks the lack of supplements was not considered determinant and a general decrease in cell viability, during the second week, was expected. What is really interesting is the different viability showed by C_0 and C_{01} cell cultures, probably due to the maturative stage of the ovaries from which they were derived. In fact, during gametogenic stages nutritive phagocytes appear voluminous and full of inclusions, containing nutritive substances and, probably, molecular factors required for oocyte growth and maturation (Chatlynne, 1969; Houk and Hinegardner, 1980; Chia and Bickell, 1983; Spirlet et al., 1998). Thus, gonad starting conditions affected primary cell cultures in terms of both viability and behaviour and gametogenic ovaries appeared to be more suitable, containing probably specific growth factors and nutrients required for cell health.

Investigations on the proper culture medium showed that modified Leibovitz-15 medium is the most suitable for sea urchin ovarian cells. The effectiveness of this medium has been previously reported for other echinoderm and marine invertebrate species (Moss et al., 1998; Odintsova et al., 2005; Shashikumar and Desai, 2011), suggesting that its composition is particularly appropriate for marine invertebrate cells since it probably provides most of the nutrients and molecules required for cell growth and survival.

Moving to cell-substrate interactions, nutritive phagocytes and germinal cells appeared differentially sensitive to the tested substrates. Nutritive phagocytes partially adhered on different substrates and even on uncoated culture plates, tending to produce long filopodia and forming networks. Their attitude in migrating and contacting each other was probably related to their functional state: it appeared more evident when cultures were obtained from gonads in advanced maturative stages. Oocytes and oogonia did not adhered on any tested substrates with the only exception of poly-L-lysine. In particular, poly-L-lysine was found to be the only analyzed substrate

allowing complete adherence of both nutritive phagocytes and oocytes. Indeed, poly-L-lysine was reported to be one of the best substrates for echinoderm embryonic cells (Odintsova et al., 1994) and for cells isolated from holothurian gut (Odintsova et al., 2005). Although poly-L-lysine effectiveness was determined and previously suggested for echinoderm cells, no improvement in cell survival and growth was observed. To enhance cell migration and viability other possible substrates could be investigated: laminin and fibronectin seem to be the most valid candidates (Spiegel et al., 1983; Venkatasubramanian and Solursh, 1984; Odintsova et al., 1994; Moss et al., 1998). In addition, echinoderm fibrillar collagen, directly extracted from sea urchin tissues, could represent another promising solution (work in progress).

To improve cell conditions and stimulate proliferation different embryo extracts were tested. Considering the poor results obtained with the addition of FCS, a widely used serum-supplement in primary cell cultures from other echinoderms (Odintsova et al., 2005; Sharlaimova et al., 2010), we moved to more species-specific extracts. *P. lividus* PE and EE were specifically developed and their addition to the culture medium was deeply investigated at different concentrations. PE did not enhance cell growth and survival at all the tested concentrations and it even seemed to accelerate processes of cell death. Although poor results were observed with this extract, it is possible that further manipulations and purifications may be necessary before conclusions can be drawn about its effectiveness. On the other hand, EE appeared to slightly improve cell health conditions, finally allowing to maintain ovarian cultures for up to one month. This marked difference between PE and EE effectiveness could be related to the different MYP isoforms contained in the extracts: EGMYP was present in EE whereas CFMYP was found in PE. The egg isoform was reported to play a main role in reproduction functioning as a nutritive supply for both gametogenesis and developing embryos (Unuma et al., 2007; Unuma et al., 2010). On the contrary, CFMYP was observed to play multiple functions and, particularly, PE detrimental effects on ovarian cells could be due to its involvement in sea urchin immune response (Cervello et al., 1994). However, SDS-PAGE analyses showed that PE and EE deeply differed in their protein profiles, suggesting that their different effects could be simply due to their protein compositions.

Although we set up the basis for the successful development of primary cell cultures from sea urchin ovaries, other research are strongly recommended to optimize cell growth conditions and promote cell proliferation. In particular, other supplements and growth factors should be tested. The testicular cells isolated from the crab *Scylla serrata* were successfully cultured in L-15 but the addition of epidermal growth factor (EGF) was required to promote good proliferation and extend cell survivability (Shashikumar and Desai, 2011). The results exhibited by this study further underlined the necessity to test even factors known to be active in vertebrate systems, as EGF or

other specific hormones and neuropeptides. However, the lack of effect by this kind of factors is not uncommon in invertebrate studies (Moss et al., 1998) and the employment of species-specific or even tissue-specific extracts could represent a promising alternative. In echinoderms, different studies have demonstrated the higher mitotic activity displayed by cell cultures produced from regenerating tissues (Odintsova et al., 2005; Sharlaimova et al., 2010), suggesting the presence of molecules and factors specifically involved in proliferation induction. The regenerating conditions could be used to improve other kinds of primary cell cultures. For examples, in our case, ovarian cell proliferation could be stimulated by the addition of inactivated cell-free coelomic fluid, previously collected from regenerating individuals.

Overall, primary cell cultures from sea urchin ovaries were successfully developed and maintained for up to one month. However, further studies are surely necessary and strongly suggested. Future research should be firstly addressed to identify supplements and growth factors able to optimize cell culture conditions, improving cell growth and stimulating cell proliferation. In addition, to achieve the optimal *in vitro* conditions other substrates could be investigated and, particularly, fibrillin and echinoderm collagen should be taken in consideration. Finally, mono-phenotype cell cultures from sea urchin ovaries could be developed, employing both size filtration and a density gradient techniques, as already described for oogonia isolation in the sea urchin *Strongylocentrotus nudus* (Yakovlev et al., 2010).

2. Sex-steroid involvement in echinoid reproduction

Sex-steroid involvement in echinoderm reproductive biology has been proposed in several species. Most of the studies were performed in asteroids, where these hormones seemed to control vitellogenesis and gamete maturation (Schoenmakers et al., 1981; Xu and Barker, 1990; Hines et al., 1992a). In echinoids, only few studies have been performed, reporting different and, sometimes, even contrasting results (Unuma et al., 1999; Wasson et al., 2000b; Barbaglio et al., 2007). Considering the poor information about the mechanisms regulating sea urchin reproductive processes, the role of E2 and T in echinoid reproduction was firstly investigated performing a long-term experiment of sex-hormone dietary administration in the common sea urchin *P. lividus*. Analyzing our experimental results, neither E2 nor T administration induced marked variations in the considered reproductive parameters, suggesting that these hormones are not involved in sea urchin gametogenesis processes. Similar results were also obtained in the *in vitro* exposure experiments: *P. lividus* ovarian cells were exposed to physiological concentrations of E2 and T for 2 weeks but no effect on cell morphology or behavior was observed. These findings strongly contrast with what has been reported in several asteroid species (Schoenmakers et al., 1981; Takahashi and

Kanatani, 1981; Voogt et al., 1991; Hines et al., 1992b; Voogt et al., 1992; Barker and Xu, 1993) and suggest the existence of different class-specific hormonal mechanisms in these marine organisms. This idea is further supported by studies on echinoderm yolk protein characterization and evolution.

Within the phylum of Echinodermata, MYP was demonstrated to be present in the Echinozoa (echinoids and holoturoids) (Shyu et al., 1986; Brooks and Wessel, 2002; Noll et al., 2007; Fujiwara et al., 2010; Unuma et al., 2010) whereas only few data have suggested its presence in the Asterozoa (asteroids and ophiuroids) (Reimer and Crawford, 1995; Reunov et al., 2010). MYP is the most abundant protein present in sea urchin eggs and for this reason it was originally exchanged for a vitellogenin-like protein. At the present several investigations have revealed that MYP is a metal-binding glycoprotein from the transferrin superfamily (Brooks and Wessel, 2002; Noll et al., 2007; Unuma et al., 2007), completely different from vitellogenin (vtg) that belongs to the large lipid transfer protein (LLTP) superfamily. Recently, asteroid yolk protein has been characterized and it was demonstrated to be a *vtg*. Vitelligenin was reported to be produced and cleaved to form abundant yolk protein in eggs of two asteroid species with completely different developmental models, indicating that the protein function is evolutionary conserved within this group. On the contrary, in echinoids no vitellogenin-like molecule has been found until now and, although sea urchin genome contains a predicted *vtg*, this is probably a pseudogene as its several atypical features have suggested (Prowse and Byrne, 2012). Taking into account that gender and tissue expression of sea urchin MYP and starfish *vtg* are strikingly similar, it has been proposed that during evolution MYP progressively assumed *vtg* reproductive functions in Echinozoa, leading to the current differences between these sister clades. All these considerations strongly underline the diversity present between the two classes, suggesting that not only yolk proteins but probably several other aspects, including hormonal mechanisms, in sea urchin reproductive biology could be differentially evolved. Indeed, although in vertebrates estrogens modulates the expression of both *vtg* and transferrin genes (Prowse and Byrne, 2012), this might not occur in echinoderms. According to our *in vitro* experiments, in *P. lividus*, MYP expression was not influenced by E2 exposure, whereas estrogen treatment affected vitellogenesis and protein incorporation into oocytes in different asteroid species (Schoenmakers et al., 1981; Takahashi and Kanatani, 1981; Van der Plas et al., 1982). However, further research are certainly needed to clarify these aspects: at the moment, these interpretations are still highly speculative, because are based on a fragmentary knowledge and not on a comprehensive view of the complex integrated biological system. Studies on the nature of yolk protein genes in other echinoderm classes are surely necessary to verify the interesting yolk protein evolution hypothesis proposed by Prowse and Byrne (2012). In addition, the

mechanisms regulating *vtg* expression in asteroids should be deeply investigated and, in particular, the estrogen involvement in this protein expression and synthesis has to be seriously taken into consideration.

Nevertheless, sex-steroid involvement in sea urchin physiology and, particularly, a possible E2 function in the regulation of lipid metabolism and protein synthesis should be strongly considered, as suggested by several previous studies (Van der Plas et al., 1982; Barker and Xu, 1993; Wasson et al., 2000b; Varaksina and Varaksin, 2001; Varaksina and Varaksin, 2002).

Indeed, in echinoderm reproduction, a specific role of estrogens in lipid accumulation during the different reproductive stages has been suggested in both asteroids and echinoids (Van der Plas et al., 1982; Wasson et al., 2000b). In *A. rubens*, E2 treatment increased the lipid content in the pyloric ceaca (Van der Plas et al., 1982) and, in the sea urchin *L. variegatus*, E2 administration in combination with progesterone similarly increased lipid percentage in the gonads (Wasson et al., 2000b). Apart from class-specific differences, these results suggest a hormonal control of lipid incorporation. This hypothesis is further supported by our previous studies reporting that in ovaries of *P. lividus*, under physiological conditions, higher E2 levels were found right during those reproductive stages characterized by nutrient accumulation and processing (unpublished data). Studies on the biochemical composition of echinoid gonads have revealed that gametogenesis is typically characterized by increased protein concentrations: particularly, in *P. lividus*, protein levels were found significantly correlated to the Gonad Index (Fernandez, 1998). Furthermore, several studies have reported a possible steroids involvement in protein synthesis: E2 and T administration was demonstrated to enhance the rate of protein synthesis in both asteroid (Barker and Xu, 1993) and echinoid gonads (Varaksina and Varaksin, 2001; Varaksina and Varaksin, 2002) and an E2 induction of protein synthesis was also observed in *Strongylocentrotus purpuratus* and *Dendraster excentricus* coelomocytes (Harrington and Ozaki, 1986). However, the actual nature and identity of these proteins are still unknown. Previous research have suggested a possible steroid involvement in MYP expression (Shyu et al., 1987; Prowse and Byrne, 2012) but, in the light of our results and also considering the importance and the ubiquity of this glycoprotein in echinoid biology and physiology, this hypothesis does not seem persuasive.

To elucidate sex-steroid control of protein synthesis and expression and to finally clarify their involvement in echinoid biology and physiology, proteomic-mass spectrometry based technique appears to be the most exhaustive approach. Nowadays, proteomic techniques, from the two-dimensional SDS electrophoresis (2-DE) to the mass spectrometry analysis, are widespread and applied in a variety of different scientific research. During my PhD, I had the opportunity to learn this technique and to personally experience the potential of these analyses (LLP - Erasmus Student

Placements, 2012-2013). In particular, functional proteomic, that allows the quantification and identification of the differentially expressed proteins among distinct conditions, might represent the successful approach to finally solve the present question. 2-DE allows to separate, visualize, quantify several thousands of proteins in a single gel from a complex biological sample, providing a large-scale analysis of protein expression differences (Depagne and Chevalier, 2012). Then, the resulted protein compositions can be compared between different biological situations, just calculating the ratio between the spot intensities at the same spot position. Mass Spectrometry (MS) is a set of powerful analytical techniques that finally allows the identification of the proteins, giving the mass of a molecule if the charge is known. Further information about the studied samples can also be obtained performing the tandem mass spectrometry (MS/MS). Taking into account the described potential of proteomic analysis and the complexity of the scientific problem faced in the present research, the proteomic-mass spectrometry based approach seems to be an optimal and practical solution, also considering that protein expression pattern of sea urchin mature ovary has been already successfully identified (Sewell et al., 2008).

Furthermore, in echinoderms steroid mode of action is still unknown. In vertebrates, sex-steroids act at a genomic level, generally binding an intracellular receptor and forming the typical hormone-receptor complex that contacts the hormone responsive element and promotes specific gene transcription (Guerriero, 2009). Recent research have also shown that steroids can also exert non-genomic effects via membrane receptors or novel interactions with classical steroid receptors (Hau, 2007). In echinoderms, there are evidences for a receptor-mediated signal transduction (Köhler et al., 2007). In *A. rubens* the presence of a specific E2 binding protein in pyloric caeca of the female starfish was demonstrated (Waal et al., 1982) and, more recently, the existence of specific cytosolic androgen and estrogen binding sites in the echinoid *P. lividus* and the crinoid *Antedon mediterranea* has been determined by radioreceptor assay (Köhler et al., 2007). Studies on endocrine disrupting compounds (EDCs) have provided further interesting information. E2 and other xenoestrogen compounds were found to cause developmental toxicity in *S. purpuratus* and *Lythechinus anamesus* by a mechanism sensitive to tamoxifen, i. e. a partial Estrogen Receptor (ER) antagonist, but insensitive to complete ER antagonist (Roepke et al., 2005). In addition, in *S. purpuratus* maternal exposure to E2 and other EDCs altered the sensitivity of the developing sea urchin embryos to the same compounds. Maternal exposure to E2, regardless of concentration, was shown to have the most significant effect on embryo sensitivity and to alter the orphan steroid receptor SpSHR2 expression, inducing a dose-independent up regulation (Roepke et al., 2006).

However no definitive evidence was obtained on the existence of classical steroid receptors in echinoids. In particular, no typical vertebrate Estrogen Receptor (ER) has been found on the sea

urchin genome, where only an Estrogen Receptor-related Receptor (ERR) is present (Goldstone et al., 2006) and three orphan members of the steroid nuclear receptor superfamily have been characterized (Kontrogianni-Konstantopoulos et al., 1996; Kontrogianni-Konstantopoulos et al., 1998; Kontrogianni-Konstantopoulos and Flytzanis, 2001). Summarizing the current knowledge, there is probably a sex-steroid receptor-mediated signalling cascade in echinoderms, but it remains to be demonstrated whether this mechanism involves a nuclear-receptor, a membrane-associated receptor, or a completely different way of signalling (Roepke et al., 2005).

In conclusion, although the present research gives a significant contribution to a better knowledge of basic echinoderm endocrinology. However, specific research on steroid hormone mode of action, physiological function and metabolism are certainly needed. In particular, functional proteomic appears to be one of the most suitable approaches to finally clarify steroid involvement in echinoid biology and physiology. In addition, many efforts should be directed towards the identification of the specific target organs/tissues of these hormones. This can be reliably obtained only by localizing the specific receptors. Preliminary screening of the Sea Urchin Genome (*S. purpuratus*), released by The Sea Urchin Genome Sequencing Consortium (Consortium et al., 2006), could not reveal the presence of classical estrogen and androgen receptors (ERs and ARs). Nevertheless, since sex-steroids are present in echinoderms and they are endogenously synthesized, some kind of receptor/transducer is likely to be present, although this is probably structurally different from that of vertebrates, thus preventing the application of classical genomic methodologies.

Finally, investigations on sex-steroid role in reproduction of other echinoderm classes (at first, holoturoids) might help to understand the different results obtained in asteroids and echinoids, providing a more complete picture of the echinoderm reproductive endocrinology.

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APPENDIX 1

Mercurio S., Di Benedetto C., Sugni M., Candia Carnevali M. D. (2013a). Development of primary cell cultures from sea urchin gonads. In Springer Netherlands, Proceedings of the symposium “Marine Invertebrate Cell Culture”, Cytotechnology, 65, 5, 673-689.

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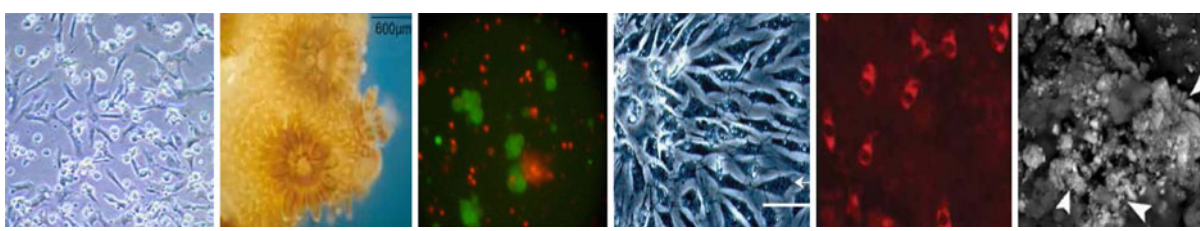

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Isabelle Domart-Coulon and Stéphanie Auzoux-Bordenave
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Preface

The symposium '**Marine Invertebrate Cell Culture'** was held at the 'Marine Biology Station of the French Muséum national d'Histoire naturelle, in Concarneau (France) on August 30 and 31 2012. In the context of the expanding field of marine biotechnology, this symposium aimed at promoting scientific exchanges and transfer of knowledge on marine invertebrate cell cultures. In the past 50 years, the progress in vertebrate cell culture models demonstrated the value of in vitro systems as biological models targeting specific organs or cell types. These models enabled research on fundamental biological questions such as cell lineage differentiation processes, symbiosis, biomineralization, as well as the development of in vitro models of human or other vertebrate diseases and assays for pathology and ecotoxicology. However, in contrast to vertebrate cells, the development of marine invertebrate cell cultures has progressed slowly, and in the last five decades, all attempts to produce continuously proliferating cell cultures have failed. To date no marine invertebrate cell line exists and we are still using primary cell cultures which cannot be propagated more than a few times after their establishment from the organism.

Twenty years ago, a symposium entitled 'Marine invertebrate cell culture: breaking the barriers' was held in Anaheim, California, by the National Oceanographic and Atmospheric Administration. Since then, there have been a few isolated communications reporting technological improvements or applications of marine invertebrate cell cultures, mostly in meetings of the Society for In Vitro Biology and Marine Biotechnology Conferences. This research field is very fragmented, both in space (involving a handful of scientists in academic institutions from different countries) and in time (as many scientist stop exploring this technology when they face persistent difficulties and failure). Marine invertebrate cell culture is a field requiring substantial funding (for cell culture equipment, consumables and reagents) for a slow return on investment, in terms of success and publications. Most failures are not reported, so the same mistakes are often repeated.

The present MICC symposium gathered 52 participants, senior and early-stage researchers, from 12 countries, to exchange their experiences and discuss recent advances and current challenges in the field. The main topics covered in this symposium included cell lineages and proliferation, 3D culture and tissue regeneration, cell-microorganisms interactions and various applications of primary cell cultures. Communications were distributed within five sessions of oral presentations, each introduced by a keynote presentation, and two poster sessions. The communications highlighted the great diversity of the cellular models originated from sponges, cnidarians, molluscs, crustaceans and echinoderms, including commercially important species. The symposium concluded with two round table discussions on 'Technical issues' and 'Stem cell cultures'.

This Special Issue of the journal *Cytotechnology* contains the abstracts of all the contributions presented at the symposium and 7 manuscripts peer-reviewed by external referees according to the review procedure of the journal. We wish to thank all authors who submitted abstracts and/or manuscripts for publication and referees for their care in the review process.

The symposium committee acknowledges the Muséum national d'Histoire naturelle (MNHN), the Centre National de la Recherche Scientifique (CNRS), the GIS Europôle Mer and the City of Concarneau for their support. The committee also wishes to thank the director and staff of the Station de Biologie Marine of the MNHN in Concarneau who hosted the symposium, for their help in organizing convivial and friendly breaks between sessions, thus contributing to successful informal exchanges between participants.

A consensus was reached between participants to organize regular gathering of the scientific community involved in marine invertebrate cell cultures, at few year intervals, to strengthen the development of this research field.

The symposium committee:

Dr. Stéphanie Auzoux-Bordenave (MNHN-UPMC, Concarneau, France)
Dr. Isabelle Domart-Coulon (MNHN, Paris, France)
Pr. Dominique Doumenc (MNHN, Paris, France)
Pr. Yves LeGal (MNHN, Concarneau, France)
Pr. Werner Müller (Univ Mainz, Germany)
Dr. Christine Paillard (LEMAR, Brest, France)
Pr. Shirley Pomponi (Florida Univ., USA)
Pr. Baruch Rinkevich (NIO, Haifa, Israël)

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Session 1: Cell lineages and proliferation

Cell cultures from marine invertebrates: past failures and future promises

Baruch Rinkevich

Israel Oceanographic and Limnological Research, National Institute of Oceanography, Tel- Shikmona, P.O. Box 8030, Haifa 31080, Israel

Corresponding author: Baruch Rinvevich, buki@ocean.org.il

Despite decades of extensive research efforts, and notwithstanding all attempts, immortalized cell lines from marine invertebrates are not yet available, in part, because of the wide variety of cell types in marine invertebrates from different phyla, limited knowledge on the nutritional needs, growth factors and other unique conditions that support proliferation of marine invertebrate cells in vitro. While the biological reasons of these failures are still elusive, it is customary to document that marine invertebrate cells stop dividing in vitro within 24–72 h after their isolation, starting cellular quiescence. The limited achievement in marine invertebrate cell cultures is also associated with the fact that scientific journals usually avoid publishing failed experiments, so much of the unsuccessful attempts are not presented to the scientific community. Summarising past failures in the development of cell cultures from marine invertebrates, novel biological methodologies, recently developed, bring new approaches and new hopes for successful development of cell cultures from these organisms. For example, evaluating the list of cell lines developed from insects and mammals elucidates that a significant portion of these new cell lines represents transformed cells, immortal cells acquired from naturally developed tumors, hybridomas, induced mutagenesis or plasmid transfected cells, use of adult stem cells, employment of induced pluripotent stem (iPS) cells, and additional new approaches. Also, the fast application of genomic and proteomic methodologies in marine biology, may enable researchers to survey globally the alterations at messenger RNA and protein levels for advancing the knowledge on in vitro cellular quiescence versus cell proliferation. The present talk will summary current trends and scientific approaches in the research discipline of invertebrate cell cultures and will focus on novel promising avenues in the research. It is claimed that recapturing cellular immortality that has failed when using the traditional approaches for cell cultures can be successfully achieved by employing modern molecular and cellular tools, developed for mammalian systems.

Keywords: Marine invertebrates, Cell division, Quiescence, Stem cells, Immortalization

Spicule formation and pigment cell differentiation in primary cell cultures of sea urchin embryos. Cryopreservation of the cultures

Nelly Odintsova[1,2], Natalia Ageenko[2], Andrey Boroda[1,2], Yulia Kiprushina[1,2]

[1]Far Eastern Federal University, 690950, Sukhanova Str. 8, Vladivostok, Russia; [2]A.V. Zhirmunsky Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences, 690059, Palchevsky Str. 17, Vladivostok, Russia.

Corresponding author: Nelly Odintsova, nelodin54@yahoo.com

Marine organisms passed through the long path of evolution and adaptations; this is duly reflected in the peculiarities of their biosynthesis and metabolism. The purpose of the study is to reveal exogenous factors that influence the implementation of the spicule- and pigment-formation program in a culture of sea urchin embryonic cells and to estimate the effect of these factors on cell differentiation. As shown by Okazaki (1975), isolated sea urchin micromeres can under certain conditions differentiate into cells capable of forming spicules. We have found that the process of spicule formation depends on the substrate type and the medium composition. The maximal number of spicules was detected in cells cultivated on fibronectin. For the first time, we have shown that the serum required for spicule formation in vitro can be replaced by a complex of factors, including insulin, transferrin, and lectins. Recently, we have characterized the expression of a new gene, *Si-VEGF2*, which is a member of the vascular endothelial growth factor family in the sea urchin *Strongylocentrotus intermedius*. Based on the RT-PCR and in situ hybridization results, we assume that Si-VEGF2 can play an essential role in skeleton formation. In normal development, the nonskeletogenic mesoderm gives rise to several differentiated cell types, one of which is the pigment cell type. Pigment cells could provide a source of pharmacologically important quinone pigments that would help to reduce the impact on the adult sea urchin population. We have previously shown that some foreign genes, such as the yeast transcriptional activator *gal4* gene, can be incorporated into the genome of sea urchin embryos inducing abnormal embryo development. After 20 days of cultivation of the transformed embryos, they dissociated into single pigment cells. Here we continued the studies of the pigment differentiation of sea urchin cells in culture and developed conditions for committed differentiation of pigment cells without transfection of sea urchin embryos with foreign genes. After 2–3 days of cultivation, the cells of a blastula-derived culture were transferred into new dishes with fibronectin coated coverslips. Shikimic acid, the precursor of naphthoquinone pigments, has been found to affect the expression of some pigment cell-specific genes in the cell culture. The clearest effect was detected with sea water medium and the coelomic fluid of injured sea urchins. We failed to develop a potential permanent

cultured black pearl industry. Sperm freezing requires the control of different steps: preparation of breeders, sperm collection, evaluation of sperm quality and the freezing process itself.

The objective of this study is to estimate the quality of cryopreserved spermatozoa immediately after thawing. Therefore, different criteria need to be evaluated such as the ultrastructure, concentration, movement characteristics of the sperm before and after cryopreservation. Sperm was manually collected after natural "shedding" from the gonopore. After appropriate dilution in swimming media, spermatozoa movement characteristics were estimated under light microscopy using CASA image analysis. Ultrathin sections were prepared for TEM examination. The presence of parvalbumin-like protein (indicator of spermatozoa maturity) was immunodetected after electrophoresis.

Concerning the freezing process, sperm was diluted in a cryoprotectant then drawn into semen straws. After equilibration at room temperature then in liquid nitrogen steam, the straws were immersed in the liquid nitrogen at least for 2 h and finally thawed at room temperature.

Our results showed that, spermatozoa can be reactivated in alkaline media and are able to restore motility after cryopreservation.

Keywords: Black-lip pearl oyster, *Pinctada margaritifera*, Cryopreservation, sperm quality

Haemocyte primary-culture from three mollusc species and its application in ecotoxicology

Charles Le Pabic*, Antoine Mottier*, Katherine Costil, Noussithé Koueta, Jean-Marc Lebel, Antoine Serpentini

CNRS-INEE FRE 3484 BioMEA (Biologie de Mollusques Marins et des Ecosystèmes Associés), University of Caen Basse-Normandie, Esplanade de la Paix, 14032 Caen Cedex, France. * These authors contributed equally to the work.

Corresponding author: Charles Le Pabic, charles.lepabic@unicaen.fr

Haemocytes play a fundamental role in invertebrate immune system and are responsible for different types of immune responses such as phagocytosis, pathogen hydrolysis or phenoloxydase cascade. These cells could potentially be affected by contaminants such as pesticides and metals. To better understand impacts of contaminants on mollusc immune system, in vitro primary-culture of haemocytes could be a useful tool (Mottin et al. 2010; Latire et al. 2012). The aims of this work were to improve culture parameters for haemocytes from three species of marine molluscs (*Crassostrea gigas*, *Sepia officinalis* and *Haliotis tuberculata*) in order to use those cells for ecotoxicity assays.

To assess our cellular culture conditions, MTT reduction assay or Water Soluble Tetrazolium salts (WST-1) assays were performed after different times of culture. After the validation of culture parameters, viability assays and other biomarkers were conducted to assess the effects of different types of contaminants.

Experiments on *Crassostrea gigas* haemocytes showed difficulty to maintain these cells in culture. Different cell concentrations and different kinds of culture media were tested but a high decrease of cell viability was observed on the first 48 h of culture. In *Sepia officinalis,* haemocyte primary-culture is poorly documented and different parameters were thus tested. Combination of modified L-15, Hank's 199 and DMEM media, and two temperatures were assessed. Results showed that the combination of L-15 medium and the temperature of 15 °C was the best one for cuttlefish haemocyte primary-culture. First experiments carried out on cuttlefish haemocytes under zinc contamination showed a negative effect on lysosomal system after 48 h of exposure.

Parameters for *Haliotis tuberculata* haemocyte primary-culture are well known and those cells could be maintained for 10 days in modified Hank's 199 medium without any decrease of viability. Effects of an herbicide (glyphosate) and a metal (zinc) were tested after 10 days of exposures. Glyphosate appeared to have no effect on haemocyte viability even at very high doses (e.g. 100,000 μg L^{-1}) whereas an EC_{50} of 6,300 μg L^{-1} could be calculated for zinc.

This approach will allow us to compare haemocyte responses from three species of molluscs which differ phylogenetically and ecologically.

Keywords: Haemocytes, primary culture, Molluscs, ecotoxicology

Development of primary cell cultures from sea urchin gonads

Silvia Mercurio, Cristiano Di Benedetto, Michela Sugni, M. Daniela Candia Carnevali

University of Milan, Department of Life Sciences, Via Celoria, 26 - 20133 Milano, Italy.

Corresponding author: Silvia Mercurio: silvia.mercurio@unimi.it

The Mediterranean sea urchin *Paracentrotus lividus* is one of the favorite and most used experimental model in developmental biology. Taking into account the possible advantages and applied implications of in vitro studies (Rinkevich 1999), first attempts to develop primary cell cultures from gonads of this species were carried out. Gonads were collected, dissected into small pieces and incubated in sterile Ca^{2+} Mg^{2+} Free Sea Water with 0.5 mg/mL collagenase for 1 h. The resulting cell suspensions were filtered through 50 μm nylon gauze, then centrifuged (330 g × 6 min) and the cell pellets were resuspended in culture medium. The obtained cell phenotypes (germ cells and nutritive phagocytes) were determined by detailed histological analysis. Three different modified culture media were tested: Leibovitz-15 (L-15), Medium 199 (M199) and Minimum Essential Medium Eagle (MEM). According to cell morphology and viability tests (direct cell counting using "Burker chamber" coupled with Trypan blue exclusion test), L-15 appeared to be the most suitable medium for cell growth and survival. Particularly, cell viability resulted significantly higher in L-15 cell cultures (One-way ANOVA: $P < 0.001$) at all considered time points (2 days, 1 and 2 weeks). M199 and MEM media were not effective probably because they revealed pH instability, as also reported by Mulford and Austin (1998). Various substrates were tested. Gonad cells adhered only on poly-L-lysine substrate, whereas we did not find any

improvements in terms of cell adhesion using mammalian collagen, gelatin and sea urchin insoluble collagen substrates. To stimulate cell growth and survival L-15 medium was supplemented with: (1) inactivated standard Fetal Calf Serum (FCS) or (2) a "sea urchin Pluteus Estract" (PE) specifically developed in our lab. In FCS-supplemented cell cultures signs of degeneration on were observed early and cell viability was significantly lower than in unsupplemented cultures (One-way ANOVA: $P < 0.05$), suggesting that for sea urchin gonad cells this extract does not improve cell conditions, as, on the contrary, was reported for other marine invertebrates (Mulford and Austin 1998; Walton and Smith 1999; Odintsova et al. 2005). Different concentrations (up to 50 μg/mL) of PE were tested but no improvement in cell conditions was obtained as well. PE apparently increased and accelerated processes of cell death even at low concentration. This preliminary study suggests that it is possible to develop primary cell cultures from sea urchin gonads and maintain these cells under in vitro conditions for more than 1 month. Overall, our findings represent an important starting point for the establishment of proliferative primary cell culture from *P. lividus* gonads.

Keywords: Sea urchin, Gonads, Primary cell culture, Medium, Viability

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Characterization of abalone *Haliotis tuberculata–Vibrio harveyi* interactions in gill primary cultures

Delphine Pichon[1], Benoit Cudennec[2], Sylvain Huchette[3], Christine Paillard[4], Stéphanie Auzoux-Bordenave[1,5]

[1]Station de Biologie Marine, Muséum National d'Histoire Naturelle, DMPA, UMR BOREA 7208 CNRS/MNHN/IRD/UPMC, 29900 Concarneau;[2] Laboratoire ProBioGEM, Université Lille1, Villeneuve d'Asq, France, [3] France-Haliotis 29880 Plouguerneau, [4] LEMAR Université de Bretagne occidentale, Brest, France, [5] Université Pierre et Marie Curie Paris VI, 4 place Jussieu, 75005 Paris, France

Corresponding author: Delphine Pichon, pichon@mnhn.fr

The decline of European abalone *Haliotis tuberculata* populations have been associated with various parasites among them the bacteria of the genus *Vibrio*. Following the summer mortalities of 1998 and 2000 in France, *Vibrio harveyi* strains were isolated from both farmed and wild abalones, allowing in vivo and in vitro studies on the interactions between abalone *H. tuberculata* and *Vibrio harveyi*.

This work reports the development of primary cell culture from abalone gill tissue, a target tissue for bacterial infection, and their use for in vitro study of host cell—*Vibrio harveyi* interactions. Gill cells originated from 4-day-old explant primary cultures were successfully sub-cultured in multi-well plates and maintained in vitro for up to 24 days. Cytological parameters, cell morphology and viability were monitored over time using flow cytometry analysis and semi-quantitative assay (XTT). Then, gill cell cultures were used to investigate in vitro the mode of action of *V. harveyi*. The effects of two bacterial strains were evaluated on gill cells: a pathogen bacterial strain ORM4 which is responsible of abalone mortalities and LMG7890 a non-pathogenic strain. Cellular responses of gill cells exposed to increasing concentrations of bacteria were evaluated by measuring mitochondrial activity (XTT assay) and phenoloxydase activity, an enzyme which is strongly involved in immune response. The abiltity of gill cells to phagocyte *V. harveyi* was studied by flow cytometry and gill cells-*Vibrio harveyi* interactions were characterized using fluorescent microscopy and transmission electron microscopy.

During phagocytosis process we evidenced that *Vibrio harveyi* bacteria induced significant changes in gill cells metabolism and immune response. Together, the results showed that primary cell cultures from abalone gills are suitable for in vitro study of host-pathogen interactions, providing complementary assays to in vivo experiments.

Keywords: *Haliotis tuberculata*, *Vibrio harveyi*, Gills cell culture, Pathogenicity

Establishment of a primary cell culture from *Crangon crangon* and their characteristics

Frauke Symanowski, Bianka Grunow

Fraunhofer Research Institution for Marine Biotechnology, Paul-Ehrlich-Straße 1-3, 23562 Lübeck, Germany

Corresponding author:
frauke.symanowski@emb.fraunhofer.de

The present study reports a culture technique for hepatopancreatal tissues of brown shrimp (*Crangon crangon*). This commercially important species of shrimp is widely distributed along the European coast from the White Sea to Morocco within the Atlantic and North Sea as well as throughout the Mediterranean and Black Sea. Hepatopancreatal tissues were isolated and digested by a trypsin–EDTA treatment and a mechanical dissection with scissor, resulting in a slowly but continuously proliferating cell culture. The cells were stable in the process of long-term cultivation over 6 months. Light and electron microscopically studies as well as red oil staining were carried out in order to characterize the cells and the isolated tissue of the brown shrimp. Two types of cells were recognized: dark pigmented cells with highly different shape and colorless spherule cells with vacuoles containing lipid droplets.

These results contribute to the development of invertebrate cell culture and provide an important tool for research of *Crangon crangon* populations regarding to virus infection and other immunological studies.

Keywords: Cell culture, Crangon, Shrimp, Marine invertebrates

APPENDIX 2

Mercurio S., Di Benedetto C., Sugni M. & Candia Carnevali M. D. (2013b).
Primary cell cultures from sea urchin ovaries: a new experimental tool.
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Primary cell cultures from sea urchin ovaries: a new experimental tool

Silvia Mercurio · Cristiano Di Benedetto · Michela Sugni · M. Daniela Candia Carnevali

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Abstract In the present work, primary cell cultures from ovaries of the edible sea urchin *Paracentrotus lividus* were developed in order to provide a simple and versatile experimental tool for researches in echinoderm reproductive biology. Ovary cell phenotypes were identified and characterized by different microscopic techniques. Although cell cultures could be produced from ovaries at all stages of maturation, the cells appeared healthier and viable, displaying a higher survival rate, when ovaries at early stages of gametogenesis were used. In terms of culture medium, ovarian cells were successfully cultured in modified Leibovitz-15 medium, whereas poor results were obtained in minimum essential medium Eagle and medium 199. Different substrates were tested, but ovarian cells completely adhered only on poly-L-lysine. To improve in vitro conditions and stimulate cell proliferation, different serum-supplements were tested. Fetal calf serum and an originally developed pluteus extract were detrimental to cell survival, apparently accelerating processes of cell death. In contrast, cells cultured with sea urchin egg extract appeared larger and healthier, displaying an increased longevity that allowed maintaining them for up to 1 month. Overall, our study provides new experimental bases and procedures for producing successfully long-term primary cell cultures from sea urchin ovaries offering a good potential to study echinoid oogenesis in a controlled system and to investigate different aspects of echinoderm endocrinology and reproductive biology.

Keywords Primary cell culture · Ovary · Sea urchin · Microscopic characterization

S. Mercurio (✉) · C. Di Benedetto · M. Sugni · M. D. Candia Carnevali
Department of Biosciences, University of Milan, via Celoria, 26-20133 Milan, Italy
e-mail: silvia.mercurio@unimi.it

Introduction

Despite the traditional use of sea urchin as favorite model in embryology and developmental biology, the specific mechanisms regulating echinoderm reproductive processes are still scarcely known. In echinoids, gametogenesis was demonstrated to be influenced by several environmental factors, such as water temperature and photoperiod (Byrne 1990; Spirlet et al. 2000; Shpigel et al. 2004; McCarron et al. 2010), food availability (Leoni et al. 2001), and environmental hydrodynamics (Fenaux 1968). Apart from these exogenous factors, several endogenous molecules, notably hormones and neuropeptides, likely play an important role in regulating reproductive processes (Spirlet et al. 1998; Mita 2013). Although several studies have been performed in order to identify and to understand the roles of these molecules, their mechanisms of action are still far to be clearly understood. In order to elucidate their complete physiological significance, a simple and adequate model system, as an appropriate in vitro approach, can be certainly helpful, allowing studies under controlled experimental conditions (Odintsova et al. 2005).

The establishment of primary cell cultures from marine invertebrates and, particularly, from echinoderms has been the objective of many previous attempts encountering uncounted obstacles (Rinkevich 1999). At present, short- and long-term cell cultures from a variety of tissues and from an increasing number of species have been developed (Mulford and Austin 1998; Walton and Smith 1999; Cao et al. 2003; Odintsova et al. 2005; de Caralt et al. 2007; Sharlaimova et al. 2010); however, there are only few rare examples of establishment of proliferative cell lines from marine invertebrates (Rinkevich 2011; Shashikumar and Desai 2011). This failure has been explained in view of the in vitro low speed of cell proliferation and the lack of vital information regarding cell physiology and biology (Rinkevich 1999; Cao et al. 2003). In addition, it has been suggested that marine invertebrate cells

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enter into a quiescent state and stop cell cycle activities 24–72 h after their isolation (Rinkevich 2011). Considering all these difficulties, cell cultures were mostly developed from tissues with high growth potential, like embryonic, neoplastic, or regenerating tissues (Odintsova et al. 2005).

Echinoderms are well known for their regenerative capabilities (Candia Carnevali 2006), and, thus, the studies present in literature, related to cell cultures, were mostly performed using cells from tissues involved in the regenerating processes (Odintsova et al. 2005; Sharlaimova et al. 2010).

The potential of other tissues, particularly of gonad tissues, in providing suitable material for cell cultures have been less explored. In echinoderms, only a few examples of cultures of ovary and testis fragments were reported. In *Asterias pectinifera*, fragments of ovary were cultured for only 3 d (Takahashi and Kanatani 1981); on the other hand, fragments of sea urchin testicular tissues were cultured in serum-supplemented seawater for 5 wk, showing that male germ line cells can complete meiosis and the first stages of spermiogenesis in in vitro conditions (Poccia 1988). Nevertheless, no study specifically addressed to the development of effective cell cultures from gonads was previously reported in literature. Only recently, a novel method for the isolation of oogonia from adult ovaries was developed in the sea urchin *Strongylocentrotus nudus* (Yakovlev et al. 2010). The authors obtained cell populations which contain relatively pure oogonia (70–75% purity), providing the first methodological basis for further in vitro research in this field.

Taking into account the advantages and the high potential of possible applications of the in vitro studies, we carried out a first attempt to develop primary cell cultures from ovaries of the common Mediterranean sea urchin *Paracentrotus lividus*. After an accurate characterization of the cell phenotypes present in culture, our priorities were to optimize the culture conditions, i.e., to define the suitable medium, substrate, and possible serum supplements. Overall, the final aim of this investigation was to set up the experimental basis for producing primary cell cultures from ovaries of this edible and ecologically relevant species. Our results could be useful for improving and expanding the potential employment of echinoderms in experimental research, providing in particular an important tool for in vitro studies on echinoid reproductive biology specifically addressed to (1) the elucidation of the mechanisms involved in gonad maturation and (2) the identification of the role of endogenous factors (such as hormones and neuropeptides) in the regulation of echinoderm reproductive processes. Furthermore, the in vitro approach might represent a valuable tool for ecotoxicological and aquaculture applied studies, being a simple and versatile method for multidisciplinary applications.

Materials and Methods

Animals. *P. lividus* adult specimens were monthly collected in the Protected Marine Areas of Bergeggi and Portofino, on the Ligurian coast of Italy, and immediately transported to the laboratory in cool boxes filled with natural seawater. Animals were kept in aquaria under constant aeration in circulating artificial seawater (Instant Ocean; salinity about 37‰, as in the Mediterranean Sea). Animal conditions as well as all water physical and chemical parameters were properly monitored.

Cell cultures. *P. lividus* ovaries were removed from the internal side of the tests; one gonad was used for histological analysis and processed for standard methods of light microscopy, whereas the remaining gonads were used to develop primary cell cultures according to the following protocol.

Ovaries were washed several times in sterile Ca^{2+} Mg^{2+}-free seawater (CMFSW) with antibiotics (40 μg/l gentamicin, 100 U/mL penicillin, and 100 μg/mL streptomycin). The coelomic epithelium was removed, and ovaries were dissected into small pieces (2–5 mm) using fine-tipped tweezers. Ovary pieces were incubated in 0.5 mg/mL collagenase dissolved in sterile CMFSW and stirred for 1 h. The resulting cell suspension was filtered through 50 μm nylon gauze (to remove mature oocytes), centrifuged at 300×*g* for 6' at 15°C, and the cell pellet was resuspended in modified culture medium. Cells were seeded at a concentration of $3–4\times10^5$ cells/mL in 24-well culture plates, without coating (medium evaluation, see below) or coated with rat collagen (I type, Sigma, St. Louis, MO), gelatin (Merck Millipore, San Diego, CA), or poly-L-lysine (70–150 kDa, 0.01% solution, Sigma). Three culture media were compared as follows: Leibovitz-15 medium (L-15), minimum essential medium Eagle (MEM), and medium 199 (M199), all of them modified by the addition of 20.2 g/L NaCl, 0.54 g/L KCl, 0.60 g/L $CaCl_2$, 1 g/L Na_2SO_4, 3.9 g/L $MgCl_2$, 1 g/L glucose, 25 mg/L taurine, 5 mL/L insulin (10 mg/mL in solution, Sigma), 100 mg/L L-glutamine, and 1.75 mg/L vitamin E (1,000 mOsm). The pH was adjusted at 7.6. The media were then sterilized by filtration (0.22 μm, Corning-Sigma), and 40 μg/L gentamicin, 100 U/mL penicillin, and 100 μg/mL streptomycin were added. All cultures were incubated at 15°C for at least 20 d. Cell behavior was observed daily for the first 2 wk of culture and, then, at each medium replacement using an inverted phase contrast microscope. Replacement of 50% of the medium was carried out every 2 d.

Medium and supplement evaluation. As described above, three different modified culture media were tested: L-15, M199, and MEM. The most effective medium was determined by a detailed analysis based on cell morphology, using an inverted-phase contrast microscope, and on cell viability during a 2-wk culture period. Cell viability was

estimated in duplicates by direct cell counting, using a "Burker chamber" coupled with Trypan blue exclusion test at 0 (T_0), 2, 7, and 14 (T_n) days. The percentage of viable cells (V_x) at the different time points was calculated as follows: V_x=(viable cell mean n° at T_n/ viable cell n° at T_0) × 100.

Once the most effective culture medium was determined, the effects of 2% inactivated fetal calf serum (FCS) and of several concentrations of *P. lividus* "pluteus extract" (PE) and "egg extract" (EE) were tested. PE was obtained from 48 h *P. lividus* larvae. When sea urchin larvae reached the pluteus stage, they were collected, sonicated, and centrifuged at 800×*g* for 10' at 4°C. The supernatant was then centrifuged at 17,000×*g* for 1 h at 4°C. The same procedure was performed to obtain EE from *P. lividus* mature ovaries. The resulting extracts were protein assayed (BCA protein assay kit, Sigma), sterile filtered, and stored at −20°C. Different PE and EE concentrations were tested as follows: 34 ng/mL, 100 ng/mL, 200 ng/mL, 500 ng/mL, 1 μg/mL, 10 μg/mL, and 50 μg/mL. Supplement effects on cell morphology and viability was observed and compared during a 2-wk culture period. Cell viability was determined at different time points by staining with fluorescent Calcein AM (viable cell marker, Sigma) or by direct cell counting, using a "Burker chamber" coupled with Trypan blue exclusion test (see before).

In order to analyze the cell proliferation activity, the incorporation method of a thymidine analog, i.e., 5-bromodeoxyuridine (BrdU), was used. Cells were exposed to 30 μM BrdU and FdU (10:1) in culture medium for 12 h. After this period, they were fixed in 4% paraformaldehyde for 1 h, treated with 1.5 N HCl and with 0.2% of Triton X-100, and incubated with antibodies against BrdU (Cell Proliferation Kit: Amersham, GE Healthcare, Piscataway, NJ). Staining with a secondary antibody FITCH conjugated (anti-mouse IgG FITCH conjugate, Sigma) was performed for 40 min. To determine the BrdU nuclear localization, cells were also stained with DAPI. Samples were examined using a fluorescence-phase contrast microscope. The experiments on proliferative activity evaluation were carried out on fresh, 1-wk and 2-wk cell cultures.

Microscopic and ultramicroscopic analyses. In order to determine ovary reproductive stages and to characterize the cellular phenotypes present in fresh cultures, standard methods of light and electron microscopy were employed, as previously described (Barbaglio et al. 2007). Briefly, gonads or cell pellets were fixed with glutaraldehyde 2% in cacodylate buffer 0.1 M and NaCl 1.4%, washed with cacodylate buffer 0.1 M, and post fixed with a solution of OsO_4 1% in cacodylate buffer 0.1 M. Then the samples were prestained with uranyl acetate in ethanol 25%, dehydrated through the ethanol series, and finally, after washing in propylene oxide, embedded in Epon 812–Araldite resin. Semithin (1 μm) and ultrathin (70 nm) sections were cut with a Reichert–Jung ULTRACUT E using glass knives. Semi-thin sections stained with crystal violet, and basic fuchsin were observed under a Jenaval light microscope. Ultrathin section were mounted on copper grids and stained with uranyl acetate and lead citrate for electron microscopy, then observed and photographed in a Jeol 100SX electron microscope.

Five ovary reproductive stages were considered as follows: *Spent* (phase immediately following the spawning event), *Recovery* (phase characterized by phagocytosis and nutrient accumulation), *Growing*, *Premature*, and *Mature* (phase characterized by gametogenesis in progress) (Barbaglio et al. 2007).

Scanning electron microscopy. Cells adhesion to the substrates was investigated by scanning electron microscopy (SEM). Forty-eight-hour cell cultures were fixed in glutaraldehyde 2% in artificial seawater (ASW) for 2 h at 4°C. After overnight wash in filtered ASW at 4°C, samples were post fixed with a solution of OsO_4 1% in ASW and glucose for 2 h, washed in distilled water, and dehydrated through the ethanol series. Absolute ethanol was gradually substituted with hexamethyldisilazane (Sigma). Samples were left to dry, mounted on stabs, covered by thin pure gold layer (Sputter Coater Nanotech), and observed with a scanning electron microscope (LEO-1430).

Statistical analysis. Data are presented as mean values± SEM. Statistical significance was assessed using paired *t* test and one-way ANOVA (Tukey's post hoc test). A *P* value of less than 0.05 was considered statistically significant. Statistical analysis was performed by the computer program GraphPad Prism 4.

Results

Development of primary cell cultures. Ovarian cells were successfully cultured and maintained for up to 1 mo, but no proliferation activity was observed. Performing a detailed histological analysis, the cellular phenotypes present in fresh cultures were characterized; only germinal cells at different stages of maturation and active nutritive phagocytes were observed (Fig. 1*a*, *d*). Generally, germinal cells were small roundish cells displaying a large nucleus and a big nucleolus, which was in contact with the inner nuclear membrane in the oogonia, whereas it was found in the nucleus center in the early vitellogenic oocytes. Nutritive phagocytes were characterized by large heterogeneous cytoplasmatic inclusions, and, particularly, huge phagosomes containing gametes in digestion were noticed. Mature oocytes/eggs were successfully removed by the filtration procedure during cell culture development. Cellular phenotypes were well recognized using

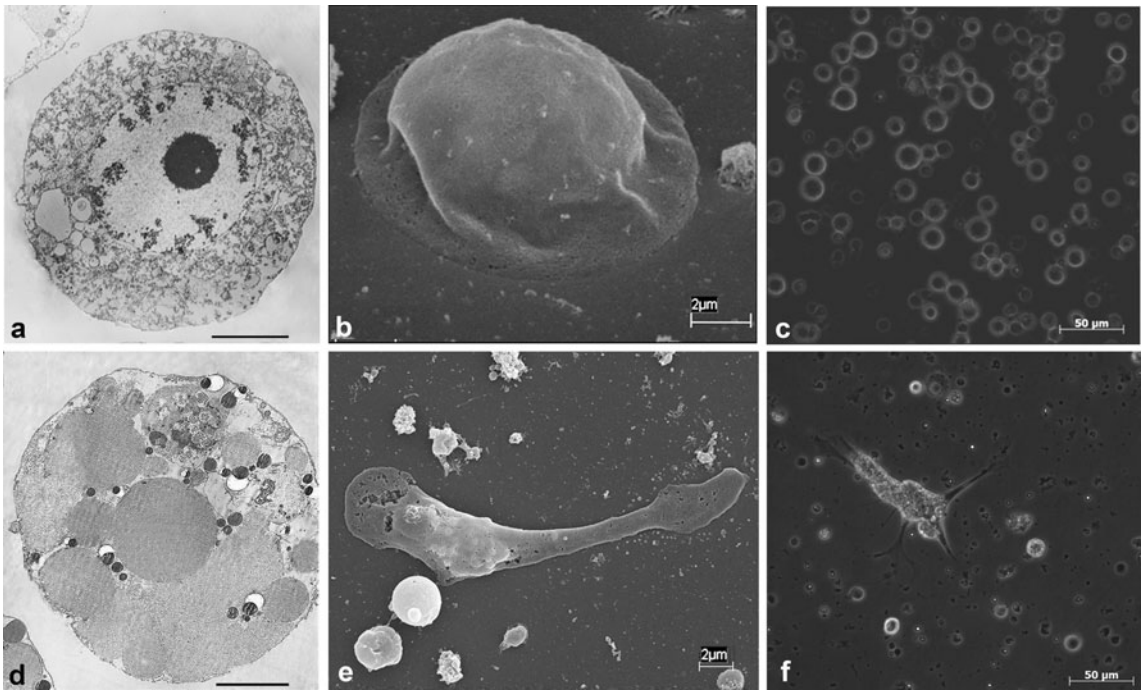

Figure 1. Cellular phenotype characterization in *P. lividus* ovary fresh cell cultures. (*a*) TEM: oocyte. *Bar* = 5 µm. (*b*) SEM: oogonium adhering to poly-L-lysine substrate. (*c*) Phase contrast microscopy: oocytes at different developmental stages. (*d*) TEM: nutritive phagocyte full of heterogeneous inclusions, including relict material from phagocytosed gametes. *Bar* = 10 µm. (*e*) SEM: nutritive phagocyte adhering to poly-L-lysine substrate with large lamellipodia. (*f*) Phase contrast microscopy: nutritive phagocyte with long lamellipodia.

phase contrast microscopy; oogonia and oocytes appeared as roundish cells, ranging in size from 8 to 50 µm, whereas nutritive phagocytes showed a granular surface, ranging in size from 20 to 50 µm, and often formed a loose network with their long filopodia variously branched and connected together (Fig. 1*c*, *f*). This trend was more evident in cell cultures obtained from ovaries at early stages of gametogenesis. In fact, although primary cell cultures could be obtained from ovaries at all stages of maturation, the cells appeared obviously healthier and viable, displaying a higher survival rate, when ovaries at *Growing* stage were used.

Medium evaluation. The general morphology of the ovary cells cultured in the three different modified media employed (L-15, MEM, and M199 media) was observed and compared using a phase contrast microscope. Twelve-hour cell cultures appeared comparable, regardless of the used medium. During the following 2 wk, cell culture appearance markedly changed depending on the medium, displaying remarkable differences in terms of size, shape, and number. After 2 wk in modified L-15 medium, cells were still numerous and most of them appeared healthy; the phagocytes being still attached to the plate bottom and forming a network of long filopodia. In modified MEM and M199 media, ovarian cells were smaller with irregular and elongated shapes, and first signs of cell degeneration were observed by the end of the first wk of culture (Fig. 2).

Cell viability analysis confirmed the morphological observation. Comparing mean V_x values in the three different modified culture media, cell viability resulted significantly higher (one-way ANOVA: $P < 0.05$) in L-15 cell cultures at all the considered time points (Fig. 3).

Considering all these results, modified L-15 medium appeared to be the most effective for ovarian cell growth and survival and was, therefore, used for all the following analyses.

Substrates. Cell adhesion was investigated by both phase contrast microscopy and by scanning electron microscopy. Cell behavior changed depending on both the tested substrates and the cell phenotypes. Germinal cells never adhered on untreated multi-well plastic, rat collagen, and gelatin, whereas nutritive phagocytes adhered only partially to these substrates. Both oocytes and phagocytes completely adhered on poly-L-lysine substrate (Fig. 1*b*, *e*).

Effects of medium supplements. To stimulate cell growth and survival, different supplements were added to L-15 medium, and their effects on cell morphology and viability were investigated. Control cells were cultured in the unsupplemented medium.

The addition of 2% (v/v) of inactivated FCS to modified L-15 medium did not improve cell health conditions, and after 1 wk of culture, signs of cell degeneration were observed.

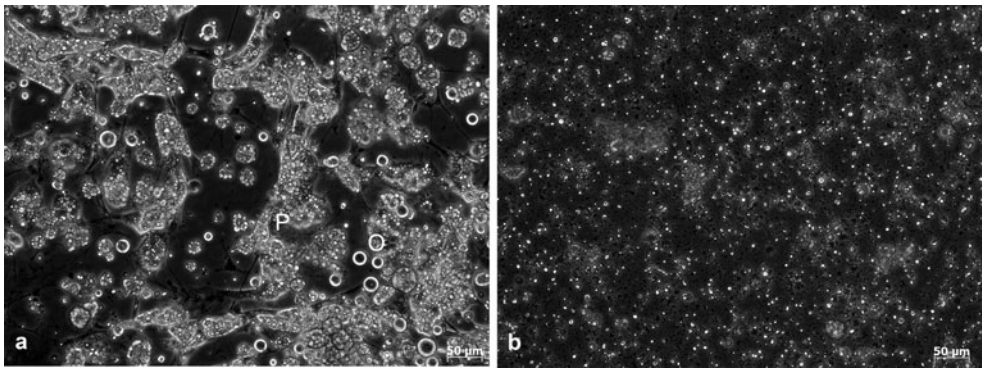

Figure 2. Phase contrast microscopy. Primary cell cultures from *P. lividus* ovaries after 2 wk in modified (*a*) L-15 medium and (*b*) MEM. After 2 wk of culture in modified L-15 medium, cells are still numerous, and the two phenotypes can be easily distinguished: oocytes with a rounded regular shape and nutritive phagocytes with a granular surface, forming a network with long lamellipodia. In modified MEM, the few cells alive are smaller, with irregular elongated shapes. Cell debris can be observed all over the plate. *O*=oocytes; *P*=nutritive phagocytes.

Furthermore, 2% (v/v) FCS cell cultures displayed a significantly lower viability (paired *t* test $P<0.05$) than unsupplemented L-15 cultures at all the considered time points (Fig. 4).

P. lividus PE did not enhance cell growth as well (Fig. 5). Using the Calcein AM method, it was evident that all the PE concentrations tested (34 ng/mL, 100 ng/mL, 200 ng/mL, 500 ng/mL, 1 µg/mL, 10 µg/mL, and 50 µg/mL) were detrimental to cell survival, apparently accelerating processes of cell death even at the lowest concentration.

P. lividus EE seemed to slightly improve cell conditions (data not shown). Cells cultured in L-15 medium supplemented with 100 ng/mL EE appeared bigger and healthier than the control for all the culture period. Furthermore, EE appeared to increase cell longevity, allowing maintaining them for up to 1 mo.

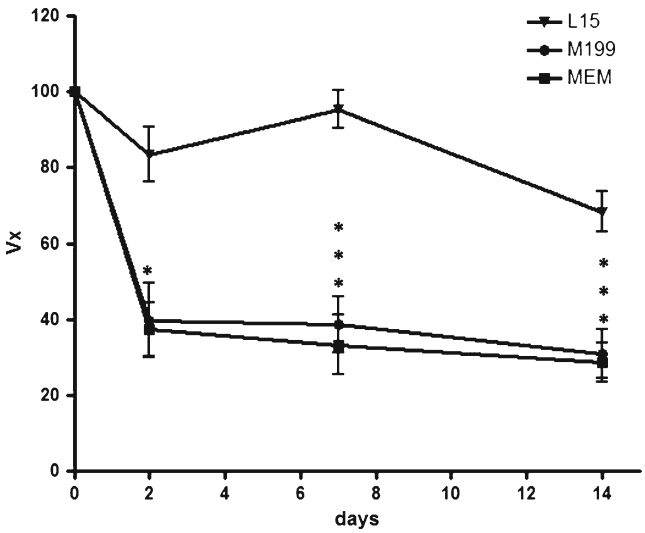

Figure 3. Percentage of viable cells (V_x) at the considered time points (2, 7, and 14 days) in the three tested modified culture media. *$P<0.05$; ***$P<0.001$.

Discussion

The in vitro approach represents a valuable experimental tool for different research fields and applications. In particular, primary cell culture from echinoid gonads can provide a simple model system for investigating different aspects of reproductive physiology and endocrinology, allowing studies under controlled experimental conditions (Odintsova et al. 2005). In the present work, cells from *P. lividus* ovaries were successfully cultured for up to 1 mo, and their in vitro behavior was well-characterized. Two different cell phenotypes, previously described in the in vivo model, were observed in cell cultures; nutritive phagocytes and germ cells at different maturative stages. Considering the importance of nutritive phagocytes in supporting, protecting, and providing nutrients to the growing germ cells (Chia and Bickell 1983; Walker et al. 2000), heterogeneous cell cultures, composed by both cell phenotypes, were preferable to mono-phenotype cultures. Only semi-mature oocytes and eggs were avoided. However, in phase contrast microscopy, the cellular phenotypes were easily recognized thanks to their overall morphology (phagocytes display granular surface) and attitude to form networks with their long filopodia (phagocytes). In particular, this behavior as well as a more active and healthier cellular aspect were observed, when primary cell cultures were obtained from ovaries at early stages of gametogenesis. In fact, at these stages, nutritive phagocytes are voluminous, full of inclusions containing relict material from both phagocyted gametes and nutritive material, acting as storages, transfer sites, and active synthesizing places (Chatlynne 1969; Houk and Hinegardner 1980; Chia and Bickell 1983; Spirlet et al. 1998). This starting condition probably affects cell cultures. Nutritive phagocytes appeared more active, and an improvement in cell viability was observed, probably thanks to the presence of nutritive substances and molecular factors required for oocyte growth and maturation.

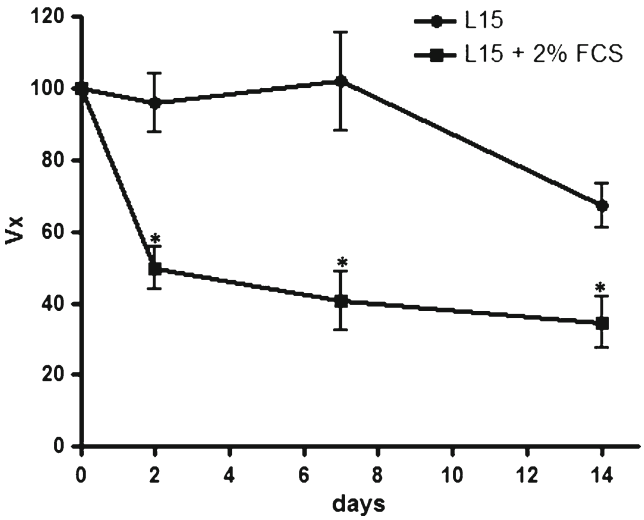

Figure 4. Percentage of viable cells (V_x) at the considered time points (2, 7, and 14 d) in unsupplemented L-15 medium and L-15 medium supplemented with 2% FCS. *$P<0.05$.

Comparing cell morphology and viability in the three tested modified culture media, L-15 medium appeared to be the most suitable for *P. lividus* ovary cells growth and survival. Our results are in agreement with previous studies (Moss et al. 1998; Odintsova et al. 2005; Shashikumar and Desai 2011), suggesting that its composition is one of the most complete and effective for culturing marine invertebrate cells. On the contrary, modified M199 and MEM media resulted in cell degeneration during the whole culture period. M199 and MEM media were not effective probably because they revealed pH instability; as also reported by Mulford and Austin (1998), these culture media showed variable pH shift (7.6–8.5) that could influence cell health conditions.

The testing of different cell substrates showed that nutritive phagocytes and germinal cells are differentially sensitive to substrate in terms of cell adherence and migration. Oocytes adhere only on poly-L-lysine substrate, whereas nutritive phagocytes adhere partially on the different tested substrates, probably depending on their functional state and completely on poly-L-lysine. The effectiveness of this substrate has already been reported for echinoderm embryonic cells (Odintsova et al. 1994), confirming our results and suggesting that echinoderm primary cell cultures display similar requirements for achieving cell adhesion.

Although echinoid ovary cells could be maintained in culture under certain conditions, no improvement in cell condition and viability was obtained using different embryo extracts. FCS is a widely used serum supplement in marine invertebrate cell cultures (Mulford and Austin 1998; Walton and Smith 1999; Odintsova et al. 2005). Although FCS was reported to enhance cell growth and survival in different echinoderm species (Odintsova et al. 2005; Sharlaimova et al. 2010), this serum was detrimental to ovarian cell survival, apparently accelerating processes of cell death even at low concentration. Similar poor results were observed with a specifically developed *P. lividus* PE. PE did not improve cell health conditions at all the concentration tested. Maybe further manipulation and purification of this extract could improve its effects; at present state of knowledge, no conclusions can be drawn about PE effectiveness. Considering that ovary cells probably require more tissue-specific factors and nutrients, a *P. lividus* EE was also tested. One of the most abundant egg (and therefore ovarian) protein is the major yolk protein (MYP) (Brooks and Wessel 2002; Unuma et al. 1998). MYP is also present in all sea urchin tissues (Giga and Ikaia 1985; Noll et al. 2007). Its functions are still not completely clear (Cervello and Matranga 1989; Unuma et al. 2009), but there is no doubts about its importance in sea urchin biology and physiology. The addition of 100 ng/mL EE to modified L-15 medium seems to slightly improve cell in vitro conditions, allowing maintaining them for up to 1 month. During oogenesis, several substances are accumulated in eggs (Kanatani and Nagahama 1983) in order to allow embryo development, and it is likely that similar nutrients and/or molecules are required for growth and maintenance of different type of cells.

In conclusion, we developed primary cell cultures from sea urchin ovaries, providing a new useful approach for the successfully investigation of echinoid reproductive biology, especially, for focusing on events occurring at the beginning and during oogenesis. Furthermore, our model system can

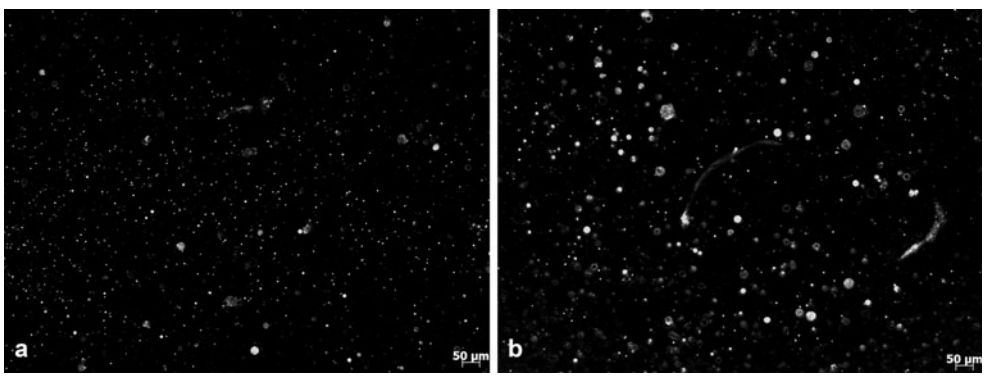

Figure 5. Fluorescence microscopy. Calcein AM method; viable cells appear fluorescent. Primary cell culture from *P. lividus* ovary after 1 wk in modified L-15 medium with (*a*) and without (*b*) 1 µg/mL of PE.

represent a new simple and versatile experimental tool for a wide range of applied researches (in both ecotoxicology and aquaculture field), finally allowing to unravel the mechanisms regulating sea urchin gametogenesis.

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