Zoosymposia 7: 247–254 (2012)
www.mapress.com/zoosymposia/

ISSN 1178-9905 (print edition)
ZOOSYMPOSIA
ISSN 1178-9913 (online edition)

Estrogen administration to the edible sea urchin *Paracentrotus lividus* (Lamarck, 1816)*

SILVIA MERCURIO[1], MICHELA SUGNI[1,3], DENISE FERNANDES[2] & CINTA PORTE[2] & MARIA DANIELA CANDIA CARNEVALI[1]

[1] *Department of Bioscience, University of Milan, Milano, Italy*
[2] *Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain*
[3] *Corresponding author, E-mail: michela.sugni@unimi.it*

**In*: Kroh, A. & Reich, M. (Eds.) Echinoderm Research 2010: Proceedings of the Seventh European Conference on Echinoderms, Göttingen, Germany, 2–9 October 2010. *Zoosymposia*, 7, xii+316 pp.

Abstract

Despite the extensive use of sea urchins in embryology, the hormonal mechanisms regulating echinoid reproductive processes are scarcely known. This research is focused on the role of estradiol (E2), whose presence and seasonal variations in different echinoderm tissues have been previously reported. Three different concentrations of E2 were administered (*via* peristomial injection, 2/week) to adult specimens of the sea urchin *Paracentrotus lividus* for 2 and 12 weeks. The lowest concentration was close to physiological values, previously measured in field specimens. Despite the increase of circulating E2 in the coelomic fluids, neither short- nor long-term hormonal treatment induced marked variations in the considered reproductive parameters. The Gonad Index appeared to be more influenced by the feed intake than by E2. Similarly, the maturation stage of the gonads was not markedly affected by E2 injection, although some sex-specific differences could be observed: treated females never reached the maximum maturation stage compared to controls, although this was observed in males injected with the lowest E2 concentration. Although further research is needed to confirm our observations, according to the present study E2 does not markedly influence echinoid reproduction and, particularly, it does not promote female maturation, as reported for vertebrates and suggested for asteroid echinoderms.

Key words: Estradiol, Echinoidea, Echinodermata, gonad development, female maturation

Introduction

Despite the extensive use of sea urchins in embryology and developmental biology, the hormonal mechanisms regulating echinoid reproductive processes are scarcely known. The physiological significance of estradiol (E2) in echinoderms has been investigated in a number of experiments by direct hormone administration; most of these studies resulted in appreciable physiological effects on different reproductive parameters (Schoenmakers *et al.* 1981; Barker & Xu 1993; Unuma *et al.* 1999; Wasson *et al.* 2000b). Further evidence of E2 involvement in the regulation of echinoderm reproduction comes from studies on seasonal changes of hormonal levels during the gonadal cycle in several asteroid and echinoid species (Xu & Barker 1990; Hines *et al.* 1992; Wasson *et al.* 2000a; Barbaglio *et al.* 2007). Considering *Paracentrotus lividus*, the experimental model used in the present work, we have previously observed high E2 levels in the ovaries at early maturation stages, suggesting the pos-

Accepted by A. Kroh: 2 August 2011; published 12 Dec. 2012

sible involvement of E2 in the regulation of nutritive phagocyte activity and/or oogonia proliferation. On the contrary, higher levels of E2 were measured in testes at advanced maturation stages, suggesting a role in sperm maturation (unpublished data). In addition, the lower E2 concentrations detected in males than in females (gonads and coelomic fluids) seem to suggest a more important role for this hormone in female individuals (Barbaglio *et al.* 2007; Sugni *et al.* 2010). Nevertheless, the role of E2 in echinoderm reproduction is far from being understood. Thus, to further elucidate the potential function of E2 in the reproductive biology of echinoids, we performed both a short- and a long-term experiment administering the hormone directly to adult specimens of *P. lividus*, a regular sea urchin that is widely distributed in the Mediterranean Sea.

Materials and Methods

Animal health conditions. During the experimental period aquaria physical and chemical parameters were proper and uniform and all the experimental animals appeared in good health conditions.

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. Animals were collected in July 2009 and immediately transferred and maintained in aquaria (10 individuals/tank), filled with artificial sea water, at the laboratory of Milan. Before the E2 treatment, animals were starved for about six weeks in order to reset the reproductive cycle to a resting phase and synchronize all the experimental animals to a comparable starting maturation stage, as described by Spirlet *et al.* (2000). The achievement of this condition was helped by the original maturity condition of the collected field specimens, which are usually in resting phase in July (personal observations). During the administration period, animals were fed in excess with pellets of an artificial diet specifically prepared for sea urchins (Wenger Manufacturing, Inc., Patent n° 085115204). A daily feeding rate (DFR) was calculated as the difference between provided and uneaten pellets (expressed in percentage). During the starvation period temperature was set at a "low" value (15 ± 1°C) in order to minimize possible mortality events (Spirlet *et al.* 2000). Throughout the administration period the temperature was maintained at 20°C and photoperiod was fixed at 10 h : 14 h (dark : light). Animal conditions as well as physical and chemical water parameters (pH, salinity, temperature, nitrites, nitrates) were properly monitored and promptly adjusted, when necessary.

Experimental design. A short-term (T_1: 2 weeks) and a long-term (T_2: 12 weeks) E2 treatment were performed and three different E2 concentrations (2, 20, 200 ng/mL) plus one control solution (0.01 % Acetone in sea water, the same used for the E2 solutions) were tested *via* peristomial injection (2/week). The lowest dose was selected in order to reach in the coelomic fluid an overall and theoretical final E2 concentration of 20 pg/mL [20 pg/mL x 10 mL (mean fluid volume of about 45 mm diameter sea urchins) / 0.1 mL (individual injected dose) = 2 ng/mL]. 20 pg/mL is the mean E2 concentration measured in field specimens (personal observation). A x10 factor was used to select the medium and highest concentrations. T_0 animals were sacrificed just before the first E2 injection. The remaining animals were treated for 2 (T_1) and 12 (T_2) weeks and they were sacrificed 4 days after the last injec-

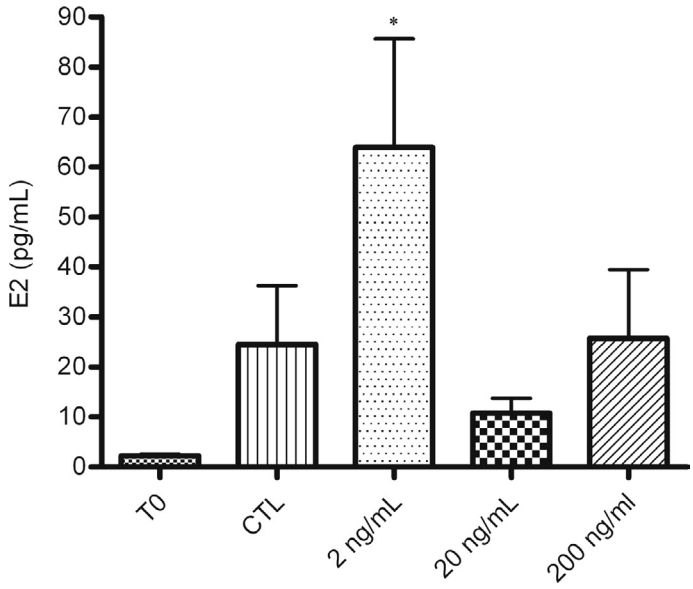

FIGURE 1. E2 levels in coelomic fluids of T_1 experimental groups. Data are expressed as mean ± SEM (n = 9–14). * $p < 0.05$ vs. CTL (Dunn's test).

tion. The coelomic fluids were collected and stored at -80°C for steroid analysis (see below). All five gonads were removed and weighed for Gonad Index (GI) calculation: GI = (gonad fresh weight/animal fresh weight) x 100. One gonad was processed for histological analyses (see below).

Maturation stage evaluation. Reproductive stages were determined by histological analysis. Standard methods for light microscopy (paraffin) were employed as described in previous papers (Candia Carnevali *et al.* 1993). Sections were observed and photographed under a Jenaval light microscope to determine the gonad maturation stage. Five stages were distinguished: *Spent* (immediately after the spawning event), *Recovery* (phagocytosis and nutrient accumulation phase), *Growing*, *Premature* and *Mature* (progressive stages of gametogenesis).

Hormone levels: radioimmunoassay (RIA). Coelomic fluid samples (3–4 mL) were extracted with diethyl ether. The organic extract was transferred into glass vials, evaporated under a nitrogen flow, and stored at -20°C. For RIA analysis, dry extracts were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1 % gelatine, and assayed for estradiol concentration using commercial RIA kits (Beckman Coulter; Marseilles, France) as described in Lavado *et al.* (2006). A standard curve with estradiol dissolved in the same phosphate buffer was produced in every run. The detection limit was 2 pg/mL. The intra-assay coefficient of variation was 3.3 % and the inter-assay coefficient of variation was 3.5 %.

Statistical analysis. Results are presented as mean values ± SEM. Statistical significance was assessed using one-way ANOVA (Tukey's test), whenever normality and homogeneity of variance were verified, or Kruskal-Wallis test (Dunn's multiple comparison test). The maturation stage data were evaluated with X^2 analysis. The relationship between mean GI and DFR values was analysed by linear regression. A *p*-value of less than 0.05 was considered statistically significant. When no significant differences were observed among tanks treated with the same E2 dose, data were pooled. Statistical analysis was performed by the computer program GraphPad Prism 4.

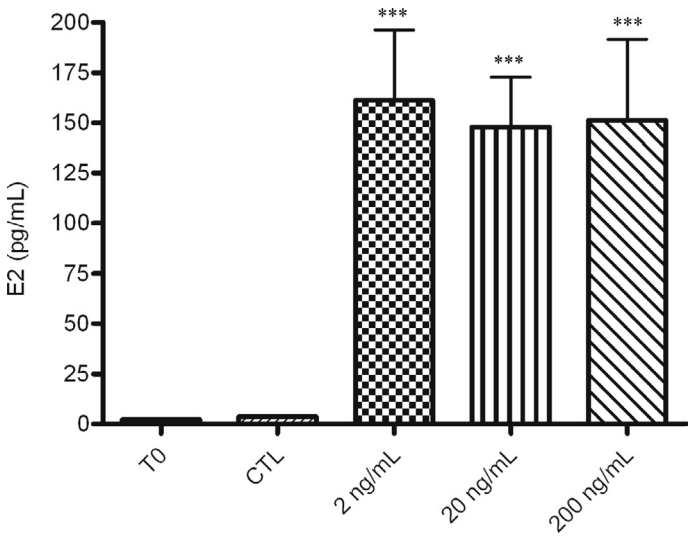

FIGURE 2. E2 levels in in coelomic fluids of T_2 experimental groups. Data are expressed as mean ± SEM (n = 10–20). *** $p < 0.001$ vs CTL (Dunn's test).

Results

Free E2 levels in coelomic fluid. In the T_1 treatment, free E2 concentrations fluctuated markedly in the different experimental groups (Fig. 1): in particular the 2 ng/mL group displayed a significant increase compared with the CTL group ($p < 0.05$, Kruskal-Wallis test, Dunn's multiple comparison test). In T_2 treatment, all three hormonally treated groups displayed a marked (about 50 fold) and significant increase of free E2 levels compared with the CTL group (Kruskal-Wallis test: $p < 0.001$) (Fig. 2). No significant differences were observed between sexes in either T_1 or T_2 groups (Kruskal-Wallis test: $p > 0.05$).

Gonad Index (GI). No significant differences in GI were recorded between the CTL and E2 treated groups (one-way ANOVA: $p > 0.05$) nor between sexes (one-way ANOVA: $p > 0.05$) in either T_1 or T_2 treatments. GI means measured in T_0 (GI = 2) and T_1 CTL groups (GI = 2,6) were significantly lower than those measured at the end of the experimental period (T_2 CTL group GI = 8.8) (one-way ANOVA, Tukey's test: $p < 0.05$). A positive and statistically significant relationship (linear regression: $r^2 = 0.8$; ANOVA: $p = 0.007$) was found between mean GI values and their corresponding feeding rates in T_2 animals (Fig. 3).

Maturation stage. All the T_0 samples were in resting condition (data not shown). In T_1, no differences were observed between control and E2 treated groups regarding the relative frequency of the maturation stages; most of the animals were in resting stages in both control and treated groups (X^2: $p > 0.05$)

In T_2, no marked differences in the relative frequency of reproductive stages were found between the experimental groups (X^2: $p > 0.05$). Although resting stages were still present, all the groups showed a higher percentage (> 60 %) of active gametogenic stages. Taking into account only males, no evident differences were observed among experimental groups, except for the presence of *Mature* specimens in the 2 ng/mL group (Fig. 4). On the contrary, none of the three E2-treated female groups reached the maximum maturation stage (*Mature*), unlike the control group (Fig. 5).

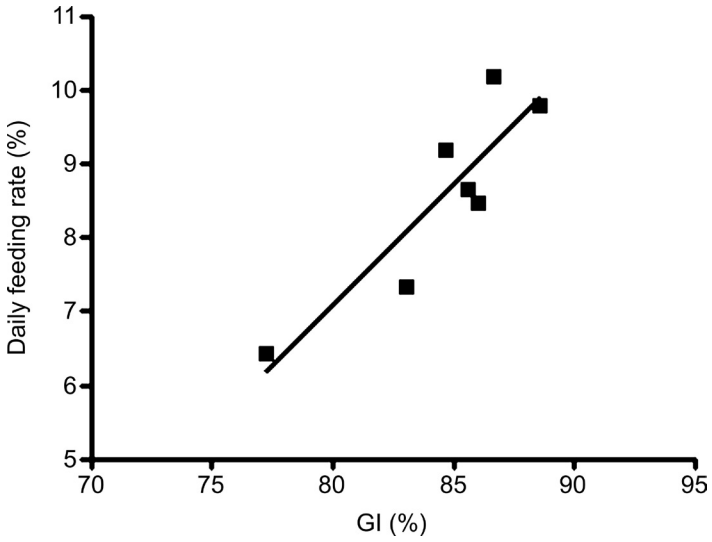

FIGURE 3. Linear regression between mean GI measured in each tank and their corresponding mean daily feeding rates [DFR = (provided food − eaten food) x 100] in the different T_2 experimental groups (linear regression: $r^2 = 0.8$; ANOVA: $p = 0.007$).

Discussion

The actual increase of free E2 in the coelomic fluid following the hormonal injections was determined by RIA in order to reliably relate E2 treatment with any observed effects on the reproductive parameters considered. Regarding T_1 treatment, a significant increase (*vs.* CTL) of circulating E2 was observed in only the lowest dose treated group (2 ng/mL), whereas in the two other treated groups, despite a 10- and 100-fold higher administered dose, coelomic E2 levels were similar to those of the controls. It could be speculated that some metabolic mechanism was activated in specimens injected with medium-high E2 concentrations, in order to eliminate the E2 excess. After 12 weeks of administration (T_2), all the hormonally treated groups displayed a marked (about 50-fold) but similar increase of E2 levels in coelomic fluid compared with controls (Fig. 2). Besides confirming the presence of higher levels of circulating E2 in the treated groups, these results further suggest the presence of protective homeostatic mechanisms to maintain the endogenous E2 levels within a high but still "physiologically accepted" value (about 150–200 pg/mL). These mechanisms might involve E2 esterification and sulfation, that represent well known pathways to eliminate the excess of bioavailable hormone in vertebrates (Hochberg 1998; Strott 1996). The capacity to produce esterified and sulfated steroid is well documented in echinoderms and these conjugated hormones were found to be the major products of steroid metabolism in echinoids (Creage & Szego 1967; Hines *et al.* 1994; Lavado *et al.* 2006). Overall, our results are in conflict with those of Schoenmakers *et al.* (1981), in which E2 administration did not affect endogenous hormone levels in A*sterias rubens* perivisceral fluid. These differences could be related to species-specific (or even class-specific) hormonal mechanisms as well as to the different experimental conditions (administration mode and length, frequency, etc.).

We did not observe any strong effect of E2 treatment on the Gonad Index (GI): in both T_1 and T_2 specimens, similar GI values were measured in control and E2 treated animals. In the lights of these results, we decided to investigate the relationship between gonadal growth (GI) and resource availability (expressed as DFR) and found significant correlation between GI and its corresponding DFR was found in T_2 specimens (Fig. 3). Therefore GI appeared to be more influenced by ingested food

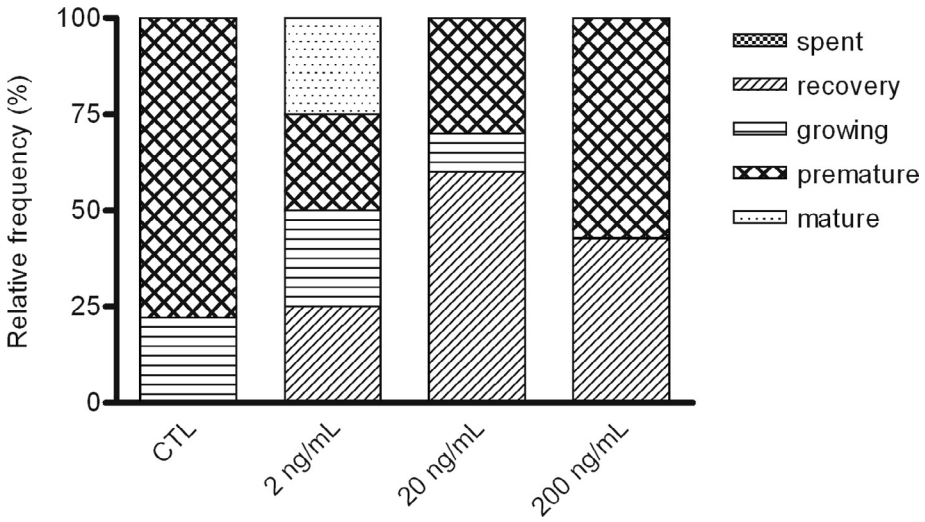

FIGURE 4. Distribution of reproductive stages in males of experimental groups (n = 5–9; χ^2: $p > 0.05$).

than by E2 treatment, suggesting that a long-term availability of food strongly affects gonad size and controls their growth, independently from the hormone. Indeed, it is well documented that quantity and quality of food influence gonad size and the quantity of gametes produced, playing a very important role in the regulation of the reproductive cycle (Pearse 1969; Pearse & Cameron 1991; Lawrence *et al.* 1992; Spirlet *et al.* 1998). Also the maturation stage of the gonads was not markedly affected by E2 administration. Both short- and long-term hormonal treatment did not induce striking variations in the reproductive stage frequency between control and treated groups, although some sex-specific differences could be observed (Figs. 4 and 5). T_2 treated females never reached the maximum maturation stage (*Mature* stage) compared with the controls, in contrast to males injected with the lowest E2 concentration. Therefore E2 did not apparently induce oocyte development, as observed in starfish (Takahashi & Kanatami 1981; Schoenmakers *et al.* 1981; Barker & Xu 1993). The different results could be due to class-specific hormonal mechanisms. This hypothesis is supported by Wasson *et al.* (2000b) who reported inhibited growth of individual oocytes after dietary administration of E2 in the echinoid *Lytechinus variegatus*. Nevertheless, contrasting results have been reported for different echinoid species (Unuma *et al.* 1999; Varaksina & Varaksin 2002). The high variability of response to E2 treatment observed in echinoids could be related to the different experimental conditions: E2 administration type, treatment period, compounds tested, individual differences.

Conclusions

In conclusion, despite the actual increases of circulating E2 in the coelomic fluids, neither short- nor long-term hormonal treatment induced marked variations in the considered reproductive parameters. E2 did not markedly influence echinoid gonad development and, particularly, it did not promote female maturation, as occurs in vertebrates (Lange *et al.* 2003) and asteroid (Schoenmakers *et al.* 1981; Takahashi & Kanatani 1981; Barker & Xu 1993). These results could be explained by the strong activation of some metabolic pathways to reduce the level of biologically active (free) hormones or, less probable, by the existence of a specific windows (in terms of reproductive stage) of E2 sensitivity that was not completely covered in this experiment. According to Schoenmakers *et al.* (1981), there is a threshold oocyte size for E2 effectiveness, as only already developed oocytes

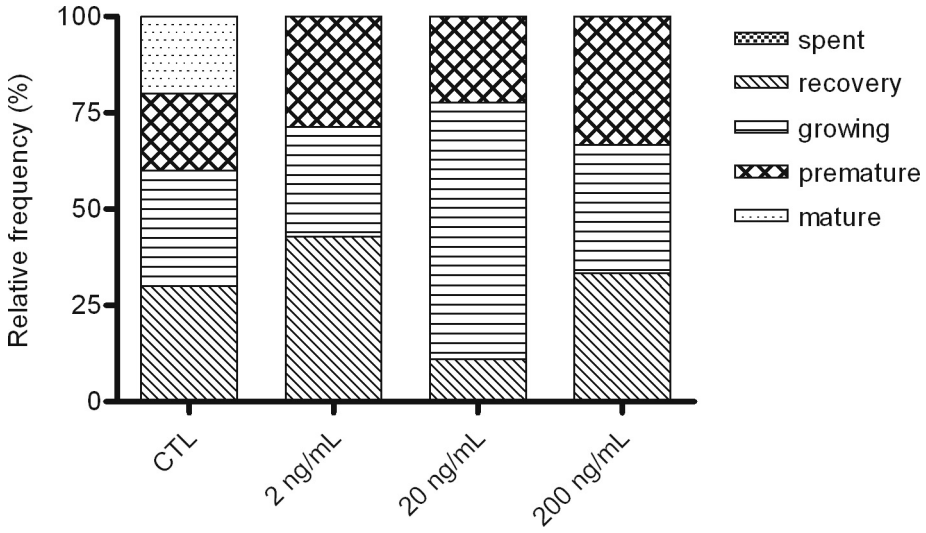

FIGURE 5. Distribution of reproductive stages in females of experimental groups (n = 3–10; χ^2: $p > 0.05$).

appeared to be positively affected by the hormone whereas no effects were observed on small size oocytes. Nevertheless this interpretation is apparently difficult to apply to our results. Although in principle the starting reproductive stage (recovery) of our experimental sea urchins could not have been receptive to steroid (as no oocytes were present), by the end of the administration period most animals had reached advanced maturative stages. This means that throughout the experiment almost all the animals (including those treated) should have "experienced" all the putative E2 sensitive stages and therefore all possible targets (*e.g.*, oocytes at different developmental stage) should have been taken in consideration. Further specific studies will clarify this aspect.

An indirect involvement of E2 in echinoderm reproduction cannot be completely excluded, as this hormone may have a role in the regulation of lipid metabolism and protein synthesis, as suggested by other authors (Barker & Xu 1993; Wasson *et al.* 2000b; Varaksina & Varaksin 2001, 2002). Overall the present research contributes to a better knowledge of basic echinoderm endocrinology. Further specific research on E2 mode of action, physiological function and metabolism is certainly needed, also considering the complexity of this matter.

Acknowledgements

We are grateful to the Protected Marine Area (AMP) "Isola di Bergeggi" (Savona) for the collaboration and permissions of animal collection.

References

Barbaglio, A., Sugni, M., Di Benedetto, C., Bonasoro, F., Schnell, S., Lavado, R., Porte, C. & Candia Carnevali, M.D. (2007) Gametogenesis correlated with steroid levels during the gonadal cycle of the sea-urchin *Paracentrotus lividus* (Echinodermata: Echinoidea). *Comparative Biochemistry and Physiology (Part A: Molecular & Integrative Physiology)*, 147, 466–474.

Barker, M.F. & Xu, R.A. (1993) Effects of estrogens on gametogenesis and steroid levels in the ovaries and pyloric caeca of *Sclerasterias mollis* (Echinodermata: Asteroidea). *Invertebrate Reproduction & Development*, 24(1), 53–58.

Candia Carnevali, M.D., Lucca, E. & Bonasoro, F. (1993) Mechanism of farm regeneration in the feather star *Antedon mediterranea*: healing of wound and early stages of development. *Journal of Experimental Zoology*, 267, 299–317.

Creage, J.E. & Szego, C.M. (1967) Sulphation as a metabolic pathway for oestradiol in the sea urchin *Strongylocentrotus franciscanus*. *Biochemical Journal*, 102, 898–904.
Hines, G.A., Watts, S.A., Sower, S.A. & Walker, C.W. (1992) Sex steroid levels in the testes, ovaries, and pyloric caeca during gametogenesis in the sea star *Asterias vulgaris*. *General and Comparative Endocrinology*, 87(3), 451–460.
Hines, G.A., Watts, S.A. & McClintock, J.B. (1994) Biosynthesis of estrogen derivatives in the echinoid *Lytechinus variegatus* Lamarck. *In*: David, B., Guille, A., Féral, J.-P. & Roux, M. (Eds.), *Echinoderms through Time: Proceedings of the 8th International Echinoderm Conference, Dijon/France/6–10 September 1993*. A.A. Balkema, Rotterdam / Brookfield, pp. 711–715.
Hochberg, R.B. (1998) Biological esterification of steroids. *Endocrinology Review*, 19, 331–348.
Lange, I.G., Hartel, A. & Meyer, H.H.D. (2003) Evolution of oestrogen functions in vertebrates. *Journal of Biochemistry and Molecular Biology*, 83, 219–226.
Lavado, R., Sugni, M., Carnevali, M.D.C. & Porte, C. (2006) Triphenyltin alters androgen metabolism in the sea urchin *Paracentrotus lividus*. *Aquatic Toxicology*, 79, 247–256.
Lawrence, J.M., Fenaux, L., Corre, M.C. & Lawrence, A. (1992) The effect of quantity and quality of prepared diets on production in *Paracentrotus lividus* (Echinodermata: Echinoidea). *In*: Scalera-Liaci, L. & Canicatti, C. (Eds.), *Echinoderm Research 1991: Proceedings of the Third European Conference on Echinoderm/Lecce/Italy, 9–12 September 1991*. A.A. Balkema, Rotterdam / Brookfield, pp. 107–110.
Pearse, J.S. (1969) Reproductive periodicities of Indo-Pacific invertebrates in the Gulf of Suez. II. The echinoid *Echinometra mathaei* (De Blainville). *Bulletin of Marine Science*, 19, 580–613.
Pearse, J.S. & Cameron, A.C. (1991) Echinodermata: Echinoidea. *In*: Giese, A.C., Pearse, J.S. & Pearse, V.B. (Eds.), *Reproduction of Marine Invertebrates, Volume VI*. Boxwood Press, Pacific Groove, CA, pp. 513–661.
Schoenmakers, H.J.N., Van Bohemen, C.H.G. & Dielemman, S.J. (1981) Effects of oestradiol-17β on the ovaries of the starfish *Asterias rubens*. *Development Growth and Differentiation*, 23(2), 125–135.
Spirlet, C., Grosjean, P. & Jangoux, M. (1998) Reproductive cycle of the echinoid *Paracentrotus lividus*: analysis by means of the maturity index. *Invertebrate Reproduction and Development*, 34(1), 69–81.
Spirlet, C., Grosjean, P. & Jangoux, M. (2000) Optimization of gonad growth by manipolation of temperature and photoperiod in cultivated sea urchins, *Paracentrotus lividus* (Lamarck) (Echinodermata). *Aquaculture*, 185, 85–99.
Strott, C.A. (1996) Steroid sulfotransferases. *Endocrinology Review*, 17, 37–49.
Sugni, M., Ciappellano, S.G., Fernandez, D., Porte, C. & Candia Carnevali, M.D. (2010) Dimorfismo sessuale nel riccio di mare *Paracentrotus lividus*: analisi morfologica e biochimica. *Atti del 71 Congresso U.Z.I. Palermo*, 119–120.
Takahashi, N. & Kanatani, H. (1981) Effect of 17β-estradiol on growth of cultured ovarian fragments of the starfish *Asterina pectinifera*. *Development Growth and Differetiation*, 23, 565–569.
Unuma, T., Yamamoto, T. & Akiyama, T. (1999) Effect of steroids on gonadal growth and gametogenesis in the juvenile red sea urchin *Pseudocentrotus depressus*. *Biological Bulletin*, 196, 199–204.
Varaksina, G.S. & Varaksin, A.A. (2001) The effect of estradiol dipropionate on the rate of protein synthesis in the testicle of the sea urchin *Strongylocentrotus nudus*. *Russian Journal of Marine Biology*, 27(2), 94–97.
Varaksina, G.S. & Varaksin, A.A. (2002) Effect of estradiol dipropionate on protein synthesis in oocytes and ovary of the sea urchin *Strongylocentrotus intermedius* at different stages of the reproductive cycle. *Biology Bulletin*, 29(5), 496–500.
Wasson, K.M., Gower, B.A., Hines, G.A.& Watts, S.A. (2000a) Levels of progesterone, testosterone and estradiol, and androstenedione metabolism in the gonads of *Lytechinus variegatus* (Echinodermata: Echinoidea). *Comparative Biochemitry and Physiology (Part C: Toxicology & Pharmacology)*, 126, 153–165.
Wasson, K.M., Gower, B.A. & Watts, S.A. (2000b) Response of ovaries and testes of *Lytechinus variegatus* to dietary administration of estradiol, progesterone and testosterone. *Marine Biology*, 137, 245–255.
Xu, R.A. & Barker, M.F. (1990). Annual changes in the steroid levels in the ovaries and the pyloric caeca of *Sclerasterias mollis* (Echinodermata: Asteroidea) during the reproductive cycle. *Comparative Biochemistry and Physiology (Part A: Physiology)*, 95, 127–133.