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Unraveling estradiol metabolism and involvement in the reproductive cycle of non-vertebrate animals: The sea urchin model

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ABSTRACT

Estradiol (E2) is a well-known hormone in vertebrates whereas in invertebrates its unambiguous presence was verified only in some species. Weather this presence is also associated to similarly conserved roles in animal phylogeny is similarly uncertain. Due to their phylogenetic position, echinoderms represent ideal experimental models to provide evolutionary insights into estrogen appearance and function. Therefore, in this research, we investigated if E2 is truly present and has a role in the reproductive biology of the sea urchin *Paracentrotus lividus*. Presence of 17β estradiol in body fluids was confirmed by liquid chromatography–mass spectrometry. By immunological methods (RIA) we evaluated the physiological circulating E2 levels of adult specimens and, on the basis of these, we directly administered E2 to study its metabolism and its putative effects on gonad development at physiological doses. Although different E2 tested concentrations, a correspondent dose-dependent increase of hormone levels was not found in both body fluids and gonads, suggesting the presence of potent homeostatic/detoxification mechanisms. These latter do not involve enzymes such as aromatase-like, sulfotransferase-like and acyltransferase-like, whose activities were not affected by E2 administration. Despite the increase of endogenous E2, the treatment did not induce significant variations in none of the considered reproductive parameters. Overall, this research (1) provides definitive evidence of E2 presence in sea urchin tissues and (2) demonstrate that, differently from vertebrates and starfish, E2 does not play a key role in sea urchins reproductive processes. Intra-phylum differences suggest the existence of class-specific hormonal mechanisms and highlight the risk of Phylum generalization.

1. Introduction

Estrogens are involved in many physiological processes of vertebrates, having an essential role in their reproduction, metabolism, development and behavior [18]. As for all sex steroids, they were originally considered vertebrate-specific hormones but in the last decades this perspective partially changed. Indeed estrogen-like compounds have been found in almost all invertebrate groups [17]. Nevertheless, only in a limited number of cases the unambiguous presence of estrogens (mainly 17β estradiol, E2) was clearly demonstrated by direct methods (e.g. gas chromatography–mass spectrometry), including mollusks (for a review see [29]), tunicates [5] and echinoderms [47]. Besides their presence, it is still under debate also whether estrogens are endogenously synthesized and have a conserved physiological role in animal phylogeny [21,29,30]. As basal deuterostomes, echinoderms occupy a key-phylogenetic position [4,40], which can provide a relevant perspective on evolutionary insights related to estrogen appearance and function in metazoans [7].

In echinoderms, estrogen-like compounds has been detected in different tissues, including gonads, but mainly by means of indirect methods [1,2,13,19,48] and only in asteroids (i.e. starfish) their presence was confirmed by GS–MS analyzes [47]. The biosynthesis of estrogens in echinoderms has been – and is still-similarly under debate [12,21]. A P450 aromatase-like activity was measured in sea urchin digestive tube, suggesting this tissue might be the main putative biosynthesis site of estrogens [1,19]. Nevertheless, the molecular structure of this putative estrogen biosynthetic enzyme is likely to be different from the vertebrate aromatase (Cyp 19) since an homologous gene was not found in the completely sequenced sea urchin genome [33]. This is in agreement with

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Markov et al. [21] who proposed an independent evolution of steroidogenic enzymes in vertebrates and invertebrates, which may have led to a functional evolutionary convergence in structurally different proteins. Even so, echinoderm P450 aromatase-like activity was affected by triphenyltin (TPT), a well-known inhibitor of vertebrate aromatase [19].

Echinoderm tissues can also efficiently metabolize exogenously administered estrogens. In sea urchins, estrogens were rapidly converted to estrogen-conjugated: aqueous-soluble, mainly estrogen-sulfates, and lipophilic compounds [6,12]. In the mussel *Mytilus galloprovincialis* estradiol is mainly transformed to esterified-estrogens [15], indeed esterification renders steroids to an apolar form, which is retained in the lipid matrices of the body and therefore may act as a long-term hormone storage. Accumulation of these compounds indicates that they are major estrogen metabolites also in sea urchins, although their biological significance has still to be elucidated.

Besides their presence, synthesis and metabolism, the involvement of estrogen-like compounds in the regulation of echinoderm reproduction was suggested by several authors since their physiological levels varied according to the reproductive cycle and in a sex-specific manner [1,2,13,46,48,51]. Additionally, in the last decades a number of experiments of direct hormone administration have been performed both *in vivo* and *in vitro* on asteroid and echinoid species, in order to elucidate the physiological significance of estrogens in these invertebrates (see Table 1). In most studies, E2 treatment apparently resulted in appreciable physiological effects on different parameters, although a clear and positive influence on ovary/oocyte maturation and development could be inferred only for asteroids, similarly to what described for vertebrates and other invertebrates. Conversely, different and non-conclusive results were reported for sea urchins, moving from absence of effects [39,42] to positive regulation of ovarian growth [49].

The specific mechanism of action of E2 in echinoderms is also unknown as, similarly to the biosynthetic enzymes, no classic estrogen receptor (ER) gene was found in the sea urchin genome [33] and nothing is known for starfish.

Despite these discrepancies within the same Phylum and the so-far presented "still open questions", in the literature echinoderm reproduction is usually reported as estrogen-sensitive, a fact that may lead to dangerous generalizations. Additionally, the increasing use of echinoderms in research addressed to endocrine disruption assessment [37,38] necessarily require a better understanding of their baseline endocrinology to really understand their potential susceptibility to these environmental contaminants.

On the basis of this, the aim of this work was to (1) confirm the presence of estrogens (17β estradiol and estrone) in the sea urchins *Paracentrotus lividus* by direct methods (i.e. chromatography coupled to mass spectrometry) and provide a validation of the routinely used (and more practical) immunological analyzes (Radioimmunoassays, RIA); (2) verify the putative involvement of E2 in sea urchin reproduction by assessing the effects of E2 administration at physiological doses. This was done by looking at (a) estradiol biosynthesis and metabolism and (b) gonad development; particular attention was paid to the experimental design (doses, reproductive cycle resetting, triggering environmental cues, feeding rates) in order to reduce the individual variability, possible source of misinterpreted results, and synchronously activate sea urchin gametogenesis.

2. Materials and methods

2.1. Animal sampling and maintenance

For GS–MS confirmation of estrogen presence, 15 adult specimens of *P. lividus*, were maintained in laboratory conditions (artificial sea water) and fed with an artificial diet for about one month. Body (coelomic) fluids were collected, immediately frozen in liquid nitrogen and stored at −40 °C until chromatographic analyzes.

For evaluation of physiological circulating E2 levels by RIA, 102 adult specimens of *P. lividus* were monthly collected for a whole year in the Protected Marine Area "Isola di Bergeggi" (44°14′N; 8°26′E; Tyrrhenian Sea). After their arrival to the laboratory, animals were immediately sacrificed; body fluids (coelomic fluids) were collected with a syringe, frozen in liquid nitrogen and stored at −80 °C until RIA analyzes. One gonad was processed for sex and reproductive stage evaluation.

For the experiment of direct E2 administration, 146 *P. lividus* adult specimens (diameter about 45 mm) were collected in the same location on July 2009, immediately transported to the

Table 1
Summary of the main experiments of E2 administration in echinoderms found in the literature.

Species	Exp. approach	E2 administr. type and freq.	Exp. period	Effects	References
Starfish					
Asterina pectinifera	*in vitro*	a; daily	3 d	↑ Oocyte diameter and ↑ % of oocyte in advanced reprod stage	[41]
Asterias rubens	*in vivo*	b; daily	16 d	↑ Oocyte diameter; ↑ GI ♀; ↑ MI ♀; ↑ E1 levels	[28]
Asterias rubens	*in vitro/ in vivo*	a & b; 1st & 7th day	8 d	↑ Lipid content in pyloric caeca	[43]
Luidia clathrata	*in vivo*	b; every 2 days	16 d	↑ Activity of metabolic enzymes (G-6-PDH and 6-PGDH)	[50]
Sclerasterias mollis	*in vivo*	b; daily	16 d	↑ Oocyte area, ↑ ovarian protein, ↑ E1 level	[3]
Sea urchin					
Dendraster excentricus and *Strongylocentrotus purpuratus*	*in vitro*	a	4 h & 24 h	Synthesis of novel protein in non-gravid females	[10]
Pseudocentrotus depressus	*in vivo*	c; daily	1 m	No effects	[42]
Lytechinus variegatus	*in vivo*	c; daily	36 d	↑ GI ♀; ↑ protein percentage	[49]
Strongylocentrotus. purpuratus	*in vivo*	b; 1/week	8 w	↓Embryo sensitivity to E2; ↑ embryo sensitivity to TBT and DDD; ↑ SpSHR2 transcript in the eggs	[27]
S. nudus	*in vivo*	c	48 h	↑ Protein synthesis	[44]
S. intermedius	*in vivo*	c	48 h	↑ Ovarian protein synthesis; no effect before spawning	[45]
P. lividus	*in vivo*	b; 2/week	2 w & 12 w	No effect	[39]

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; TBT and DDD = endocrine disrupting compounds, tributyltin and dichlorodiphenyldichloroethane, respectively; SpSHR2 = orphan steroid receptor.

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