Estradiol and Endocrine Disrupting Compounds Effects on Echinoderm Reproduction and Development: Developmental Sensitivities and Defense Mechanisms

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## ABSTRACT

Marine invertebrates are exposed to endocrine disrupting compounds (EDCs) and must utilize defense mechanisms to survive and reproduce; however, little is known about the effects of EDCs on marine invertebrates. Using the sea urchin developmental model, we ascertained that embryos were sensitive at environmentally relevant concentrations to estradiol, ethynylestradiol, 4-octylphenol, tributyltin and *o,p*-DDD in a stage-specific manner with early embryos being the most sensitive. Incubation with tamoxifen, a selective estrogen receptor modulator, 'protected' the embryos from estrogenic toxicity suggesting a receptor-mediated mechanism of toxicity. Estradiol is produced during gastrulation and binds to a low-capacity, low-affinity receptor. Maternal exposure to EDCs altered embryo sensitivity and the expression of mRNA of a steroid receptor in the oocyte in a treatment and dose-dependent manner demonstrating that maternal EDC exposure in invertebrates may directly effect the embryos. A 'first-line-of-defense' mechanism present in many marine invertebrates is the multidrug resistance (MDR) transporter proteins which transport hydrophobic and conjugated compounds out of the cell. In sea stars, MDR transporters are expressed during oogenesis prior to oocyte maturation and an upregulation of MDR transport activity occurs during oocyte maturation. An increase in transport activity occurs during oocyte maturation and involves both permeability glycoprotein (P-gp) and multidrug resistance-associated (MRP) transporters. The upregulation during maturation is initially due to a translocation of stored proteins to the oocyte cell membrane followed by translation of stored transporter mRNA. Expression of the transporters is not upregulated at fertilization in the sea star but there are changes in expression of the transporters after fertilization and

during blastula formation and gastrulation. The inhibition of first cleavage in fertilized eggs by vinblastine is potentiated by specific inhibitors which also reduce first cleavage when used alone. The inhibition of cleavage suggests that MDR transporters may serve an important role in cell mitosis during early development. Several transcripts encoding MRP transporters were identified using gene sequencing and cDNA macroarray technology including transcripts with homology to vertebrate MRP1, 5 and 8. In conclusion, these data demonstrate that marine invertebrates are sensitive to estrogenic compounds but do utilize defense mechanisms to ensure proper gametogenesis and embryonic development.

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# INTRODUCTION

Endocrine disruption in wildlife populations has been a research focus for over a decade across the globe. Numerous chemicals called endocrine disrupting compounds (EDCs) including polychlorinated biphenyls (PCBs), dioxin, DDT, insecticides, fungicides, and alkylphenols, and interfere with vertebrate endocrine systems (Soto et al., 1994; White et al., 1994; Nimrod and Benson, 1996). A major research interest of mine has been the effects of physical and chemical stressors on the reproduction and development of marine organisms. After an inspirational course on reproductive toxicology taught by Dr. Marion Miller, I decided to focus my dissertation on the effects of EDCs, specifically estrogenic compounds, on marine invertebrate reproduction.

While studies have shown that estrogenic compounds can affect adult invertebrate physiology, the effects of these compounds on gametogenesis and early development in invertebrates has not been thoroughly examined. The invertebrate phyla Echinodermata includes two well-characterized model systems for gametogenesis and early development. In sea stars, oocyte maturation occurs after a hormonal cue from the surrounding follicular cells. The maturation-inducing hormone in the sea star is 1-methyladenine, which interacts with a G-protein-coupled receptor to initiate a series of signal transduction pathways leading to oocyte maturation (reviewed in Meijer & Mordret, 1994; Kishimoto, 1998). In the sea urchin the early events of development and embryogenesis have been a model system for developmental biology for over a century. The cellular physiology and biochemistry of fertilization and early embryo development in the sea urchin are well understood, and as an invertebrate deuterostome sea urchins exhibit a developmental pattern similar to chordates, providing a valuable tool for comparative studies of development.

In aquatic environments, the source of steroidal estrogens and estrogenic EDCs is mainly point-source pollution from human sewage and industrial activities (Atkinson et al., 2003; Tashiro et al., 2003) and can reach concentrations ranging up to ng/ml (Desbrow et al., 1998; Atkinson et al., 2003). In marine invertebrate populations, a well-known example of endocrine disruption in a wild population is the induction of imposex (masculinaztion of females) in gastropods by exposure to the anti-fouling agent tributyltin (TBT) in coastal marine environments (Gibbs et al., 1988). The androgenic/anti-estrogenic mechanism(s) by which TBT exerts its toxicity is still unclear but may involve inhibition of steroidogenesis, alteration of cell signaling molecules or interactions with nuclear receptors (Morcillo et al., 1998; Cima and Billarin, 2000; Nishikawa et al., 2004). Other EDCs can act as estrogens by interacting with the estrogen receptor (ER), the nuclear steroid receptor through which estrogens predominantly exert their effects on cells (Jobling et al., 2003).

Vertebrate groups impacted by estrogenic EDCs include birds, fish, amphibians, and reptiles. Documented impacts occur in invertebrate species such as molluscs, polychaetes and crustaceans from exposure to specific estrogenic EDCs (reviewed in Tyler et al., 1998; Depledge and Billinghurst, 1999; Hutchinson, 2002; Segner et al., 2003). While most invertebrate EDC research focuses on the effects of these compounds on adult survival and reproductive capacity, studies have shown vertebrate and invertebrate embryos are sensitive to estradiol and estrogenic compounds at environmentally relevant concentrations (Hutchinson, 2000; Westerlund et al., 2000; Kim et al., 2001; Yoshida et al., 2002; Segner et al., 2003; Brion et al., 2004).

My original goal for this dissertation was to determine which EDCs affected sea urchin development and inhibited sea star oocyte maturation at environmentally relevant concentrations. While I had reasonable success with the sea urchin development portion of my dissertation, experiments with the *in vitro* sea star oocyte maturation assay proved to be fruitless. No EDC, estrogenic or otherwise, inhibited the *in vitro* maturation of the oocyte except for a partial inhibition by TBT. While one obvious answer to the insensitivity of oocyte maturation to these compounds was the lack of cellular targets involved in the signal transduction pathways before and during oocyte maturation, another explanation was that the oocytes possessed a cellular defense mechanism that prevented EDCs from entering the oocytes, i.e. a "first-line-of-defense". Therefore, I investigated the expression of multidrug resistance (MDR) transporter proteins that are known to decrease bioaccumulation of xenobiotics in many cell types across phyla.

Invertebrate embryos possess several cellular defense mechanisms to deal with exposure to EDCs and other toxicants and these include expression of stress proteins, detoxification enzymes, and metal binding proteins. Another immediate defense or tolerance mechanism that most cells possess is the expression of promiscuous transporters in the plasma membrane removing toxins and their metabolic endproducts from the cell. These MDR transporters proteins are an evolutionarily conserved family of efflux transporters belonging to the transmembrane ATP-Binding Cassette (ABC) superfamily of transporters, which function by utilizing the hydrolysis of ATP to pump substrates across the plasma membrane (Borst et al., 2000; Dean et al., 2001).

The MDR transporters known to transport endogenous waste and, as a part of the generalized cellular stress response, xenobiotic compounds, include the permeability

glycoproteins (P-gp)/MDR (ABCB) transporters and multidrug resistance associated proteins (MRP)/CFTR (ABCC) transporters (Borst et al., 2000; Dean et al., 2001). Substrates for these MDR transporters are typically moderately hydrophobic compounds, lipids, conjugated organic anions, hormones and xenobiotics (reviewed in Krishna & Mayer, 2000 and Kruh & Belinsky, 2003). Furthermore, MDR transporters are regulated by endogenous hormones, including estradiol (Hagen et al., 2000; Ee et al., 2003), suggesting a mechanism by which exposure to environmental hormones could alter the MDR efflux capacity of invertebrates as well as their embryos.

MDR transporters are expressed in an assortment of developmental cell types including human trophoblast cells and other placental tissues (Atkinson et al., 2003; Utoguchi et al., 2000), mouse and porcine oocytes (Elbling et al., 1993; Takebayashi et al., 2001) and in amphibian embryos (Bonfanti et al., 1998). In invertebrates, embryos from diverse phyla express MDR transporters including nematodes, molluscs, annelids and echinoderms (Broeks et al., 1996; Hamdoun et al., 2002, 2004; McFadzen et al., 2000; Minier et al., 2002). In all these systems, a protective role for these transporters has been suggested including defense from heavy metals, microbial toxins and hydrocarbon biodegradation products and other xenotoxins.

The two seemingly disparate subjects of my dissertation are related when viewed as an examination of mechanisms of toxicity and tolerance involved in exposure to endocrine disrupting compounds. A mechanism of toxicity typically involved in estrogenic EDC exposure is often a receptor-mediated gene regulation pathway. The alteration of gene transcription by these compounds may also increase the expression of cellular defense mechanisms. A prime example of this response to toxic exposure is the

increase in the expression of detoxification enzymes following exposure to dioxin functioning through the aryl hydrocarbon receptor (Mandal, 2005). Organisms exposed to a physical or chemical stressor exhibit a suite of defensive mechanisms to protect against the stressor and its downstream effects. These defense mechanisms are often inducible by stressors and can be used as a detection tool for stressor exposure. Understanding the mechanisms of stressor "toxicity" and stressor-induced "tolerance" can inform ecotoxicological research with the basic biological tools to assess the real world issues surrounding the field of environmental endocrine disruption.

The four specific aims for the portion on the effects of estradiol and estrogenic EDCs on sea urchin development were divided in to two chapters (Ch. 1 and 2). Chapter 1 examines the developmental sensitivities of sea urchin embryos to steroids and EDCs using a standard developmental bioassay and the stage-specific sensitivities and steroid receptor-mediated mechanisms of teratogenicity by modification of the developmental bioassay. Chapter 2 studies the effects of maternal exposure to EDCs on embryo sensitivities to EDCs and the expression of a steroid hormone receptor in oocytes. This chapter also assesses the endogenous production of estradiol in the embryo and the binding characteristics of estradiol to embryo homogenates. The last two chapters of my dissertation focus on the expression of MDR transporters in sea stars. Chapter 3 reports the storage and translocation of MDR transporters before and during oocyte maturation while Chapter 4 examines the expression and activity of the transporters during fertilization and embryonic development.

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# CHAPTER 1

## Estradiol and endocrine disrupting compounds adversely affect development of sea urchin embryos at environmentally relevant concentrations

**Abstract**

Environmental endocrine disrupting compounds (EDCs) are a wide variety of chemicals that typically exert effects, either directly or indirectly, through receptor-mediated processes, thus mimicking endogenous hormones and/or inhibiting normal hormone activities and metabolism. Little is known about the effects of EDCs on echinoderm physiology, reproduction and development. We exposed developing sea urchin embryos (*Strongylocentrotus purpuratus* and *Lytechinus anamesus*) to two known EDCs (4-octylphenol (OCT), bisphenol A (BisA)) and to natural and synthetic reproductive hormones (17β-estradiol ($E_2$), estrone ($E_1$), estriol ($E_3$), progesterone ($P_4$) and 17α-ethynylestradiol ($EE_2$)). In addition we studied two non-estrogenic EDCs, tributyltin (TBT) and *o,p*-DDD. Successful development to the pluteus larval stage (96 hours post-fertilization) was used to define EDC concentration-response relationships. The order of compound potency based on $EC_{50}$ values for a reduction in normal development was as follows: $TBT_{L.\ anamesus} > OCT > TBT_{S.\ purpuratus} >> E_2 > EE_2 > DDD >> BisA > P_4 > E_1 >> E_3$. The effect of TBT was pronounced even at concentrations substantially lower than those commonly reported in heavily contaminated areas, but the response was significantly different in the two model species. Sea urchin embryos were generally more sensitive to estrogenic EDCs and TBT than most other invertebrate larvae. Stage-specific exposure experiments were conducted to determine the most sensitive developmental periods using blastula, gastrula and post-gastrula (pluteus) stages. The stage most sensitive to $E_2$, OCT and TBT was the blastula stage with less overall sensitivity in the gastrula stage, regardless of concentration. Selective estrogen receptor modulators (SERMs) were added to the experiments individually and in combination with estrogenic

EDCs to interfere with potential receptor-mediated actions. Tamoxifen, a partial ER agonist, alone inhibited development at concentrations as low as 0.02 ng/ml and was effective at this concentration in decreasing the sensitivities of the embryos to estradiol and estrogenic EDCs. The complete antagonist ICI 182,780 inhibited development at concentrations as low as 0.03 ng/ml but increased embryo sensitivity to estradiol and estrogenic EDCs. Estradiol and estrogenic EDCs all cause developmental toxicity in sea urchins through a TAM-sensitive but an ICI-insensitive mechanism. It remains to be demonstrated whether this mechanism involves an estrogen-responsive nuclear receptor (NR), a membrane receptor (NR or non-NR-related) or a completely different mechanism of toxicity. However, early embryo sensitivity and the differential response to SERM co-incubation further suggests more than one mode of EDC action in the developing sea urchin embryo.

keywords: estrogens, sea urchin, embryos, endocrine disruptors, SERMs

## Introduction

Environmental endocrine disrupting compounds (EDCs) are chemicals that can either directly or indirectly affect endocrine systems. EDCs can mimic endogenous hormones or inhibit normal hormonal activity of endocrine and neuroendocrine systems. Numerous chemicals including polychlorinated biphenyls (PCBs), dioxin, DDT, insecticides, fungicides, and alkylphenols can interfere with vertebrate endocrine systems (Soto et al., 1994; White et al., 1994; Nimrod and Benson, 1996). Some of these chemicals can act as estrogens (estrogenic EDCs) and can cause feminization, thus limiting the reproductive capacities of exposed species (Raloff, 1994; Jobling et al., 2003). Organisms impacted by estrogenic EDCs include humans, birds, fish, amphibians, and reptiles. Invertebrate species including mollusks and crustaceans are also impacted by specific estrogenic EDCs (reviewed in Tyler et al., 1998; Depledge and Billinghurst, 1999; Hutchinson, 2002; Segner et al., 2003). In aquatic environments, the source of steroidal estrogens and estrogenic EDCs is mainly human sewage and industrial activities (Atkinson et al., 2003; Tashiro et al., 2003).

The actions of estradiol and other estrogenic EDCs are predominantly mediated by specific intracellular receptors (estrogen receptors (ERs)) that regulate transcription of mRNA for a number of proteins. ERs in vertebrate systems act as transcription factors in regulating cell homeostasis, proliferation and differentiation and are also involved in the development of neuronal, cardiovascular and reproductive tissue (Singleton and Khan, 2003). ERs have low-level activity in the absence of ligand, constitutively controlling gene transcription (Tzukerman et al., 1990), but can also be associated with cell

membrane functions and cell signaling pathways, potentially adding alternative mechanisms of toxicity for estrogenic EDCs (Das and Thomas, 1999; Singleton and Khan, 2003). Since ERs appear prior to maternal estrogen exposure during early mammalian pre-implantation development, estradiol and estrogenic EDCs may also activate embryonic receptors (Hou and Gorski, 1993; Hiroi et al., 1999).

Recent publications have examined the effects of EDCs on vertebrate embryos. For example, murine embryos reached the blastocyst stage at a higher frequency when exposed to the weak estrogen, bisphenol A (<100 ng/ml) in a tamoxifen-sensitive manner (Takai et al., 2000). The sensitivities of vertebrate embryos to estrogenic EDCs can involve an altered expression of the mRNA coding for the ER. *Xenopus* embryos were sensitive to concentrations of estradiol and diethylstilbestrol (DES) in the low μM (~250-500 ng/ml) range (Nishimura et al., 1997). Zebrafish embryos were sensitive to estradiol and hydroxylated PCBs following maternal exposure to concentrations as low as nmol/kg (~0.2 ng/ml; Westerlund et al., 2000). Both of these latter studies reported changes in the expression of ER mRNA and protein associated with exposure to estrogenic EDCs. ER mRNA is known to respond negatively or positively to steroid hormone exposure in a tissue/cell type dependent manner (Pillai et al., 2002; Marin-Castano et al., 2003).

The sea urchin embryo provides a useful developmental model system since the physiology and biochemistry of fertilization and early embryo development are well understood. As one of two invertebrate deuterostome phyla, echinoderms exhibit a developmental pattern that is similar to chordates, thus providing a valuable tool for studies of development. Also, the sea urchin is a prime candidate for developmental toxicology research because extensive knowledge of its molecular physiology can be

coupled with ongoing genome characterization (NIH Sea Urchin Genome Project; Hood et al., 2001).

Until recently when a putative estrogen receptor was reported in a mollusc (*Aplysia*), no estrogen receptor had been identified in any invertebrate (Thornton et al., 2003). Likewise, little is known about the role of steroid hormones or their receptors in echinoderm reproduction and development. Limited evidence suggests that steroid hormones probably do function in echinoderm reproduction. Schoenmakers et al. (1981) reported an increase in the ovarian content and the growth of oocytes after injection of estradiol into sea stars while Barker and Xu (1993) reported alterations in progesterone levels after injection of estradiol. Sea stars are also known to metabolize the androgen, androstenedione, to other androgens in both somatic and gonadal tissue (Hines et al., 1992).

To further the understanding of estradiol's role in development and the potential for EDCs to adversely affect normal development, we exposed sea urchin embryos to a range of concentrations of several reproductive hormones and estrogenic EDCs. For comparison, we also exposed embryos to two non-estrogenic EDCs to illustrate differences in mechanisms of teratogenicity. We also investigated developmental stage specificity and receptor selectivity. The aims of this research were to test whether (1) reproductive hormones affect normal sea urchin development at environmentally relevant concentrations and (2) determine if estrogenic and non-estrogenic EDCs can impede normal development, including the formation of spicules that form the internal skeleton of the sea urchin larvae, through similar mechanisms.

## Materials and Methods

### 2.1. Reagents and chemicals

17β-estradiol, estrone, estriol, progesterone, 17α-ethynylestradiol, 4-octylphenol, bisphenol A, tributyltin, 1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane (*o,p*-DDD; mitotane), tamoxifen and calcein were purchased in powdered form from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). ICI 182, 780 was purchased from Tocris Cookson (Ellsville, MO, USA). Stock solutions of each chemical were made up in DMSO (Sigma) except TBT, which was diluted in filtered seawater (FSW). All stocks were stored at –20 °C until use. Glutaraldehyde for embryo fixing was purchased from Polysciences, Inc. (Warrington, PA, USA) and made into a 5% solution with FSW.

### 2.2. Animals

Due to seasonal reproductive cycles, two species of sea urchins were used in this study, *Strongylocentrotus purpuratus* and *Lytechinus anamesus*. *S. purpuratus* is reproductive from November to April while *L. anamesus* is reproductive during the summer and early fall months in its native Southern Californian waters. *L. anamesus* can be maintained in a reproductive state year-round in the colder waters off Northern California if they are well fed and allowed sufficient recovery time after spawning. Experimental animals were maintained at the Bodega Marine Laboratory (BML) in

continuous flow-through, sand-filtered sea water (13°C +/- 2°) with weekly feedings of locally-collected kelp.

2.3. Developmental Exposures

Developmental toxicity (teratogenicity) caused by exposure to compounds was assessed in the embryos by determining the morphology of larvae at the pluteus stage of development (see Hamdoun et al., 2002). Gametes were collected (and larvae subsequently produced) from adult sea urchins from both species during the reproductive season for each species. The adults were washed with FSW and induced to spawn by injection of < 0.5 mL of 0.5M KCl into the body cavity via the peristomial membrane. For each bioassay, one male and 2-3 females were spawned. Eggs were collected into chilled 0.7 μm FSW and kept on ice until use. Motility and fertilization assessments were conducted to ensure viable gametes with 80-90% fertilization. The eggs were washed three times in FSW to remove the extracellular matrix coating and then fertilized in a large batch. Thirty minutes after the elevation of the fertilization envelope, the fertilized eggs were washed to remove sperm and concentrated by settling and aspirating the upper portion of FSW to a density of 1000 eggs per ml. Approximately 350 eggs were transferred to the experimental vials (30 ml acid-washed, borosilicate glass shell vials) containing 20 ml of 0.22 μm FSW. The compounds were added to the vials to the appropriate concentrations and mixed. Seawater (no compound added) and solvent controls were also prepared. All treatments were run in triplicate for each experiment. The embryos were incubated at 17 °C to 96 hours post-fertilization (hpf) (See Fig. 1).

At the early pluteus stage ≅ 96 hpf, larvae were fixed with 100 μL of 5% glutaraldehyde (<0.05% final) and allowed to settle in the vials. The first 100 embryos observed were assessed for normal development in each vial using an inverted phase contrast microscope. Larvae were separated into five categories and all were compared to the standard morphology of normal pluteus larvae in seawater controls. The categories were designated normal, delayed, abnormal, elongated and hatched. Delayed embryos/larvae were embryos that appeared normal in their development but never reached the same stage as the controls i.e., early prism larva at 96 hpf rather than full pluteus larva. Delayed embryos indicate a inhibition of the rate of growth by increases in metabolic costs or some other growth-associated factor. Abnormal embryos/larvae are defective typically exhibiting misshapen spicules, missing arms, incomplete guts, etc. Elongated larvae, a special type of abnormality, had flattened or thin bodies but normal arms. The hatched category denotes unhatched blastula or hatched, ciliated blastula that did not develop beyond this stage. Photographs of *S. purpuratus* embryos were taken at specific concentrations at 4 time points: 18 hpf (Blastula); 36 hpf (Gastrula); 48 hpf (Prism) and 96 hpf (Pluteus) using an Olympus BX50WI fixed stage microscope equipped with Differential Interference Contrast (DIC) optics and a Roper Scientific CoolSnap HQ cooled CCD camera and analyzed with Metamorph© (Universal Imaging Corp., Downington, PA, USA).

2.4. Concentration-Response Relationships

The concentration ranges for each of the hormones and EDCs were centered on environmentally relevant concentrations (Ko et al., 1995; Guillette et al., 1996; Desbrow et al., 1998). Final concentrations were in the ng/ml range. The sea urchin embryos were exposed across a range ($10^6$) of treatment concentrations in triplicate for each batch, and when necessary, concentrations were log transformed in graphs for complete visualization of the response curve. The exposure experiments were repeated at least three times. Batches were only accepted when controls had at least 70% normal development. Because the availability of viable gametes from each species was seasonal, we tested both *S. purpuratus* and *L. anamesus* for the concentration-response relationships over the course of the year. The response was similar in both species except where noted. Data shown are from *S. purpuratus* unless otherwise noted.

## 2.5. Stage Specificity

To identify the most sensitive developmental stage, embryos were exposed during three developmental stages: 1) fertilization to pre-hatched blastula, 2) hatched blastula to gastrula and 3) post-gastrulation to early pluteus. For the first stage, called 'Blastula', chemicals were added at 1hpf and washed out (via settling of unhatched embryos) before hatching had occurred at 18 hpf. For the second stage, called 'Gastrula', the treatments were added at 19 hpf when most embryos were hatched and swimming and washed out at 36 hpf using a 40 μm cell strainer to rinse swimming embryos. In the final category, 'Pluteus', chemicals were added at 39 hpf with no removal for a total exposure of 51 hr (see Fig. 1).

*S. purpuratus* embryos and larvae were exposed to three different concentrations of toxicant noted as 'Low', 'Mid' and 'Hi'. For $E_2$, the concentrations were 0.5, 10 and 250 ng/ml. For OCT, the concentrations were $1x10^{-3}$, 0.1 and 5 ng/ml. TBT concentrations were $1x10^{-4}$, 0.1 and 1 ng/ml. The concentrations of DDD used were 5, 50 and 100 ng/ml. After 90 hpf, larvae were fixed and the first 100 larvae were counted for normal development only. The concentrations and controls were run in triplicate and repeated at least three times with different batches.

The length of exposure time for the 'Pluteus' stage is 3 times as long in hours as the other stages. This longer exposure time may have confounded the results. To address this issue, larvae from the post-gastrulation stage, `Pluteus', were exposed during a preliminary experiment for only 20 hr or for the full 51 hr of the Pluteus stage. For the shorter exposure time, larvae were removed by cell strainer at 59 hpf, washed three times with FSW and transferred to fresh FSW for the remainder of the incubation. Counts of normal larvae were not significantly different between the short and the long exposure period (data not shown).

## 2.6. Selective Estrogen Receptor Modulators (SERMs)

To provide insight into the mechanism of teratogenicity through which estrogenic EDCs may exert their effects, selective estrogen receptor modulators (SERMs) were employed in the experiments. Embryos from *S. purpuratus* were exposed to the SERMs, tamoxifen (TAM) and ICI 182,780 (ICI) using standard experimental protocol to determine the lowest effective concentration (LEC). The LEC (0.019 ng/ml for TAM

and 0.03 ng/ml for ICI) for each modulator was subsequently used in co-incubation experiments with the estrogenic EDCs. The four concentrations of each estrogenic compound were chosen centered around the respective $EC_{50}$ value as determined by this study (see Table 1). Embryos were exposed to the estrogenic EDCs both in the presence and absence of the SERMs. Experiments were fixed at 96 hpf and counted for normal development only. The all concentrations and controls were run in triplicate and repeated at least three times with different sets of gametes.

## 2.7. Spicule Development using Fluorescence Microscopy

Sea urchin spicules are calcareous structures secreted by the primary mesenchyme cells (PMCs) forming the 'skeletal' structure of the early embryo. A toxicant may interfere with normal calcium secretion and alter the shape, length and strength of the spicule. Defects in spicule development can be illuminated by incubation with the fluorescent calcium-sensitive dye, calcein. Because spicule formation is more prominent in *L. anamesus*, embryos from that species was used for these experiments. To determine the qualitative effects on spicule development larvae were exposed $E_2$, OCT, TBT or DDD as described above. To stain spicules during formation, 10 μM calcein was added at 40 hpf. At 96 hpf, larvae were fixed in 95% ethanol and rinsed with FSW three times to decrease the background calcein fluorescence. Fixed larvae were photographed using epifluorescence microscopy with an Olympus BX50WI (equipped with a xenon source and excitation/emission filter wheels) with excitation at 490 nm and emission at 530 nm and a Photometrics Coolsnap HQ cooled CCD camera (Roper Scientific).

## 2.8. Isotopic Dilution

The solubility of estrogens in seawater may play a significant role in the teratogenicity of these compounds to developing marine embryos. Estrogens in particular are only slightly soluble in seawater and may also adhere to the glass of the vials decreasing the actual free estradiol concentration. To measure the concentrations available to embryos and larvae, isotopic dilution of $^{3}$[H]-estradiol was employed. Radiolabeled estradiol (500 nCi ~ 2ng/ml) was added to FSW-filled shell vials containing approximately 500 embryos (from *L. anamesus* due to availability) in triplicate. Embryos were incubated in these vials to the early pluteus stage at 96 hpf. At the end of the incubation, the embryos were separated from the FSW containing the radiolabeled estradiol, washed three times and transferred to vials containing scintillation fluor. Ten (10) µl of the radiolabel-containing FSW and of a 2 ml ethanol wash of the vial were also transferred to scintillation vials and counted using a Beckman LS 7000 scintillation spectrometer. By comparing the CPM/µl of larvae, FSW and ethanol wash, the partitioning of estrogen in the experimental vials was determined.

The amount of radiolabeled ligand associated with the washed larvae was 5.8% of the total added. Slightly more than 1% of the total radiolabeled estradiol was detected in the solvent wash of the experimental vials with the rest of the radiolabeled estradiol (93.1%) remaining in solution. Isotopic dilution demonstrated that at this concentration most of the estradiol was in the seawater and not adhered to the glass vial. Thus, the embryos were exposed to levels of estradiol close to the nominal concentrations. The

amount of radiolabeled estradiol measured in the washed larvae may represent the binding of the hormone (and its endproducts) to the exterior of the larvae or soluble particulates associated with the them.

### 2.9. Data Analysis

Concentration response data for each hormone, toxicant and SERM were analyzed by means of Tidepool Scientific Toxcalc© (Arcata, CA, USA) program. Results are presented as mean ± standard deviation (SD). Significant differences were evaluated by means of one-way ANOVA followed by the Tukey-Kramer test for the concentration-response curves. Stage exposure experiments were analyzed using two-way ANOVA with Tukey Test for pairwise multiple comparison for comparisons within a stage amongst the three concentrations and one-way ANOVA for comparisons between the stages within a given toxicant using the Student-Newman-Keuls Method of pairwise multiple comparisons. Comparisons were also made between a single concentration across stages using a Tukey Test. The probability level adopted as significant was $P<0.05$.

## Results

### 3.1. Concentration-Response Relationships

Estradiol and all EDCs inhibited normal development. $EC_{50}$ values illustrate the relative effectiveness of the toxicants in disturbing normal development (Table 1). The sensitivity of sea urchin embryos from both species to estradiol and other reproductive hormones range from low ng/ml for estradiol and ethynylestradiol to $1.5 \times 10^4$ ng/ml for estriol. Figure 2 presents the effects of the same compounds after concentrations were log transformed for complete visualization of all curves. Except for TBT, responses of species to toxicants were not significantly different (data not shown). For the sake of continuity, only *S. purpuratus* results are presented in the graphs unless otherwise noted.

Octylphenol, a linear structure, was 100 times more potent at inhibiting normal development than $E_2$, typically the most effective estrogenic compound (Table 1; Fig. 3A). In contrast, $E_2$ was about 15 times more potent than the estrogenic compound, bisphenol A (Table 1; Fig. 3B). TBT elicited an unusual response in the embryos and larvae according to species (Fig. 3C). In *S. purpuratus*, concentrations below $1x\ 10^{-3}$ ng/ml (log = -3) inhibited normal development 10 to 20% more than those above $1 \times 10^{-3}$ ng/ml, thus creating a biphasic curve. Only after 0.5 ng/ml did normal development drop steeply. Conversely, in *L. anamesus*, the TBT concentration-response is not 'biphasic' but a typical response curve with an $EC_{50}$ value of 0.037 ng/ml, about 24 times more sensitive than *S. purpuratus*. DDD had a slightly lower potency than estradiol (Fig. 3D).

Similar slopes between response curves are indicative, although not definitive, of similar mechanisms. By adding a trendline to individually-graphed curves without log transformation, we compared the slope of the curve for all concentration responses. Estrone, estradiol and estriol has slopes ranging from –0.17 for estrone to –0.28 for estradiol. The slopes for octylphenol (-0.24) and bisphenol A (-0.12) were within a

similar range. The slope for ethynylestradiol (-0.41) was lower than the other estrogenic EDCs. The slope for DDD (-0.42) was similar to ethynylestradiol. Progesterone had a much lower slope (-0.73). The slope for TBT-exposed *L. anamesus* was -0.87 and for S. *purpuratus*, the slope was -0.59 using only the response above 0.1 ng/ml.

Differential counting of fixed larvae illustrated the different responses of the embryos to EDC exposures. As the concentration of estradiol increased, the percentage of abnormally developing embryos increased until 500 ng/ml, at which point the majority of these embryos did not hatch or develop beyond ciliated blastula (Fig. 4A). Delayed larvae were 50% of those exposed at 1 to 5 ng/ml octylphenol (Fig. 4B). *S. purpuratus* larvae were 25-30% delayed when exposed to low TBT levels, while the delay was decreased at higher concentrations (Fig. 4C). At concentrations over 1 ng/ml, an increase in abnormal development and/or unhatched or ciliated blastula was observed. Both delayed larvae and morphologically abnormal larvae percentages increased with higher DDD levels. At 160 ng/ml DDD, the unhatched embryos constituted 30% of the total number counted (Fig. 4D). The differential counts for $E_1$, $E_3$ and $EE_2$ were similar to estradiol while the differential count for BisA and $P_4$ was similar to OCT (data not shown). The major type of abnormality as observed at the highest concentration used (when normal development approached zero) differed by toxicant. For example, ciliated blastula exposed to 10 ng/ml TBT accounted for nearly 90% of the total counted; whereas, in OCT, 50% of the larvae were of the delayed variety similar to the prism stage. $E_2$ and DDD both had higher percentages of abnormal larvae or ciliated blastula.

## 3.2 Stage Specificity

To determine which developmental stage was more sensitive to estradiol and the EDCs, stage-specific exposures were conducted with $E_2$, OCT, TBT and DDD (Fig. 5) using *S. purpuratus*. Embryos were exposed during the blastula stage until hatching (Blastula), during gastrulation (Gastrula) or during the late embryo stages up to the larval stage, early pluteus (Pluteus). Embryo responses were normalized to controls and analyzed for significant differences either between stages, within stages, or across stages at each concentrations and are shown as bar graphs in Figure 5. All responses were significantly different from controls.

Significant differences within stages are noted in Figure 5 with either an (a) or a (b) indicating significant difference when compared to the low concentration (a) or when compared to both the low and mid concentrations (b). For example, the high concentration of $E_2$ (Fig. 5A) was significantly different only from the low concentration in the Blastula stage but significantly different from both low and mid concentrations in the Gastrula and Pluteus stages. Estradiol did have a mild effect on early embryo development before hatching; the number of hatched embryos decreased slightly but significantly when exposed to $E_2$ compared to the control (data not shown). Most embryos exposed to the $EC_{50}$ concentration still hatched and continued to develop to gastrulation.

Sensitivities between stages differed for the four compounds. The most sensitive stage was the Blastula stage ($P<0.05$) for both $E_2$ and OCT (Fig. 5A&B). For TBT (Fig. 5C), the Blastula and Pluteus stage were equal and for DDD (Fig. 5D), the Pluteus stage was the most sensitive. For all chemicals, the Gastrula stage was the least sensitive

($P<0.05$). Except for $E_2$, embryos exposed during the Gastrula stage were not affected by an increase in concentration. Embryos exposed to DDD were less sensitive in the Hatch stage with a higher percentage of normal development at the mid and high concentrations (Fig. 5D).

Responses also differed for a single concentration across the three stages. The responses of each individual stage to the mid (white bar) concentration of $E_2$ (Fig. 5A) were significantly different from each other ($P<0.05$) while the responses at the high (gray bar) concentration were similar than the Blastula and Pluteus stage but significantly different than the Gastrula stage ($P<0.05$). All responses at the high concentration of OCT (Fig. 5B) were significantly different ($P<0.05$) but at the mid concentration only the Blastula and Gastrula stage were significantly different ($P<0.05$). Embryos exposed to non-estrogenic EDCs did not respond across the stages similarly to the estrogenic EDCs.

### 3.3. Selective Estrogen Receptor Modulators

The differences in sensitivity of the sea urchin embryos to the estrogens, $E_1$, $E_2$ and $E_3$, are similar to their respective affinities for vertebrate ERs (Petersen et al., 1998; Hawkins and Thomas, 2004). This may indicate the involvement of an ER-like receptor in the effects of the estrogens and estrogenic EDCs in the sea urchin embryo and larvae. In order to provide support for such a mechanism, selective estrogen receptor modulators (SERMs) were added to the embryos alone and in co-incubation experiments with the estrogenic EDCs. Tamoxifen (TAM), a partial agonist of mammalian ER, and ICI 182,

780 (ICI), a complete ER antagonist in mammals, were chosen because of availability and specificity.

First, each SERM was added to the experiments without toxicants. Both of the SERMs inhibited normal development within the same concentration range (1x $10^{-3}$– 0.15 ng/ml). $EC_{50}$ values were 0.05 ng/ml for TAM and 0.095 ng/ml for ICI (Table 1) and the slopes were –0.37 for TAM and –0.42 for ICI. The LEC (~20% abnormal development) was chosen as the experimental concentration for all SERM co-incubation experiments with the estrogenic EDCs. Preliminary experiments with SERM concentrations less than the LEC did not result in any alteration of the slope of the concentration-response curve or change in potency (data not shown).

Tamoxifen interfered with effects of the estrogenic EDCs on development (Fig. 6). The teratogenicity of $E_2$ was diminished as illustrated by the shallowness of the slope of the $E_2$ + TAM curve compared to the slope of the line for $E_2$ alone. The overall trends for the pair of lines were significantly different ($P<0.05$). However, in vials without estradiol, TAM significantly decreased normal development compared to controls. TAM similarly inhibited the teratogenicity of $EE_2$, OCT and BisA (Fig. 6C, D&E). The decreased efficacy of estrogenic EDCs in the presence of TAM suggests the involvement of a receptor in at least a portion of the effects associated with estrogen exposure in the sea urchin embryo. Conversely, the receptor antagonist ICI increased the potency of all estrogenic EDCs significantly ($P<0.05$) (Fig. 7). Co-incubation with ICI increased developmental abnormalities by 10 to 20% for all compounds. The increase in teratogenicity suggests either an ICI-insensitive mechanism or some other non-receptor mediated toxicity.

3.4. Morphology of Embryo Development

Both DIC and fluorescence microscopy showed developmental abnormalities in the embryos and larvae exposed to estradiol and the EDCs. These included delayed growth and alterations in normal spicule development (Fig. 8 & 9). The DIC micrographs reveal the non-specific delay in growth (OCT+Pluteus (96 hpf) plate – no arms) and abnormal development ($E_2$+Pluteus (96 hpf) plate – misshapen gut and delayed growth) of embryos from *S. purpuratus* exposed to $E_2$ and OCT. A unique deformation can be seen in the $E_2$+ and OCT+Blastula (18 hpf) plates (Fig. 8). Some of the early embryos appeared to have split between the animal and vegetal poles forming a smaller blastula-like embryo associated with a loose congregation of cells, possibly PMCs. These split blastulae were found qualitatively in increasing numbers in a concentration-dependent manner (data not shown). The animal portion of the split embryo may have continued to develop without PMCs or formation of an archenteron (Fig. 8: $E_2$+ and OCT+Gastrula (36 hpf) plates).

Normal spicule development was also examined using the calcium sensitive dye, calcein, which incorporates into calcified structures (Fig. 9). The larvae from *L. anamesus* were clearly affected by toxicant exposure but only exhibited common non-specific defects in spicule development when exposed to EDCs. The spicules were shortened or bent (Fig. 9C&E) and some larvae had a change in the angle of the spicule forming an 'A-frame' shape (Fig. 9B&D). It appears that spiculogenesis is a secondary target of estrogens and EDCs and not a primary one.

## Discussion

### 4.1. Embryonic sensitivity to estradiol and estrogenic EDCs occur within relevant environmental ranges

This study tested the hypothesis that estradiol and estrogenic EDCs will adversely affect sea urchin embryos at environmentally relevant concentrations through similar mechanisms. Comparisons to other marine species are discussed for estradiol, octylphenol and bisphenol A as well as a discussion of possible teratogenic mechanisms. The slope of the curves and the differential counts from the developmental assays are evaluated to further describe the mechanisms of teratogenicity. The reproductive hormone, estradiol and its synthetic counterpart, ethynylestradiol, inhibited normal embryonic development in the sea urchin embryo and larvae at concentrations relevant to those present in the environment ($1 \times 10^{-3}$ ng/ml to 10 ng/ml) (Desbrow et al., 1998; Atkinson et al., 2003; Takahashi et al., 2003; Brion et al., 2004). Estradiol and ethynylestradiol inhibited normal development at low ng/ml levels with significant inhibition (15-20% abnormal) around 0.1 ng/ml. Sea urchin embryos had a similar sensitivity to estradiol and ethynylestradiol when compared to mollusk embryos but were more sensitive than other invertebrate embryos (crustaceans and other arthropods) (Hutchinson, 2002; Segner et al., 2003).

Estradiol functions through at least two types of receptors by which teratogenic effects may occur from exposure to significant concentrations of estradiol (or estrogenic

EDCs). Predominantly, estradiol functions genomically through specific intracellular receptors called steroid nuclear receptors. These receptors regulate expression of a number of transcription factors involved in regulating cell homeostasis, proliferation and differentiation. Estradiol in vertebrates also functions through an ER localized to the plasma membrane affecting the cell through nongenomic pathways. The membrane-associated ER can activate mitogen-activated protein kinase (Singh et al., 2000) and phosphatidylinositide 3-kinase signaling pathways (Singleton and Khan, 2003). Other nongenomic actions include the alteration of electrical activity in neurons (Singleton and Khan, 2003) and calcium mobilization in both granulosa cells and osteoblasts (Morley et al., 1992; Leiberherr et al., 1993). Estradiol (and estrogenic EDCs) may alter the normal functions of signaling pathways and calcium regulation in the developing sea urchin embryo and larvae accounting for a portion of the teratogenicity reported in this study. As such, the teratogenicity of estrogen exposure may be due to perturbations of both genomic and nongenomic cellular processes.

The levels of octylphenol, an alkylphenolic compound, ($EC_{50}$ = 0.174 ng/ml) effective at significantly inhibiting normal embryonic development were below those levels reported in some coastal environments, which range from 1-100 ng/ml (Blackburn et al., 1999; Fenet et al., 2003). Octylphenol has been shown to be estrogenic in a number of systems (White et al., 1994; Nagel et al., 1997). Octylphenol inhibits development in eelpout embryos *in ovario* at 25 μg/l (ng/ml) (Rasmussen et al., 2002) and causes mortality in *Xenopus laevis* embryos at 1μM (~200 ng/ml) (Bevan et al., 2003). The affinity of octylphenol for vertebrate ERs is much lower than estradiol (Loomis and Thomas, 2000; Matthews et al., 2000). In the present study, octylphenol was

approximately 100 times more potent as a sea urchin developmental toxicant than estradiol, suggesting that the effects of octylphenol on embryos and larvae may also occur through different, non-ER-dependent mechanisms. The higher sensitivity of the embryos to octylphenol compared to estradiol may involve such mechanisms as the inhibition of L-type $Ca^{2+}$ channels (Ruehlmann et al., 1998) or increases in reactive oxygen species (Seike et al., 2003).

The effective concentrations of bisphenol A (~ 125 ng/ml) are above levels that are environmentally relevant and 10 to 15 times higher than estradiol when comparing $EC_{50}$ values. Bisphenol A levels are <0.1 to 10 ng/ml near U.S. manufacturing sites (Staples et al., 2000), although higher (100 ng/ml) in Japanese river water (Takahashi et al., 2003). In comparison to other aquatic species, sea urchin embryos showed similar sensitivity. Bisphenol A has been shown to have no effect on the hatching rate of zebrafish (Segner et al., 2003), but does negatively impact the development of medaka embryos and larvae exposed chronically to 200 ng/ml (Pastva et al., 2001). In some vertebrate systems, estradiol and octylphenol are more potent than bisphenol A (reviewed in Safe et al., 2001) and have greater affinity for the vertebrate ER isoforms than bisphenol A (Loomis and Thomas, 2000; Matthews et al., 2000). In the developing sea urchin embryo system, estradiol and octylphenol are both more potent than bisphenol A.

The different responses to these compounds, which are all known to interact with vertebrate ERs (Loomis and Thomas, 2000; Matthews et al., 2000; Hawkins and Thomas, 2004), indicate the action of two different teratogenic mechanisms. While not conclusive, the slopes of the response curves can be split into two clusters suggesting at least two teratogenic mechanisms involved during exposure to the reproductive estrogens

and estrogenic EDCs. The first cluster ranges from –0.12 to –0.28 and includes estradiol, estrone, estriol, octylphenol and bisphenol A. The second cluster of slopes ranges from –0.37 to –0.42 and includes ethynylestradiol and both SERMs. The two slope clusters were significantly different by a paired t-test comparison ($P<0.001$). The notion of two teratogenic mechanisms involved in the inhibition of normal sea urchin development is reinforced by the two groups of outcomes in the differential counts (Fig. 4). In the first group, all the estrogens including ethynylestradiol showed more abnormal larvae and dead ciliated blastula at the higher concentrations, while the second group (octylphenol, bisphenol A and both SERMs) displayed more delayed (prism-like) larvae at the higher concentrations. Delayed embryos represent an inhibition of the growth rate while abnormal embryos are morphologically defective and dead blastula represents a complete cessation of development. The clusters in the slopes analysis and the groups in the differential count analysis do not have identical members but nevertheless illustrate the two-mechanism hypothesis. The two mechanisms possibly involve a NR (cytosolic or membrane-associated) and/or interactions with different signaling molecules involved in growth and differentiation.

4.2. Non-estrogenic EDCs elicit different responses than estrogenic EDCs in sea urchin embryos and larvae

Embryos were also exposed to non-estrogenic EDCs, TBT and DDD, for a comparison of mechanisms of teratogenicity with estradiol and the estrogenic EDCs. Sea urchin embryos are sensitive to TBT at environmentally relevant concentrations (< 1

ng/ml). Environmental levels of TBT are dependent on proximity to shipping and boat yards and can range from 1 μg/g of sediment (~1 x $10^3$ ng/ml) in large harbors (Ko et al., 1995) to estimated oceanic water concentrations of 0.4-0.8 ng/L (pg/ml) (Yamada et al., 1997). The concentrations of TBT causing abnormal development in both experimental species in the study were similar to those causing developmental effects in other species of sea urchins (Marin et al., 2000). However, *S. purpuratus* embryos responded in a biphasic manner while *L. anamesus* embryos did not. In *L. anamesus*, the $EC_{50}$ was below ng/ml, similar to *Paracentrotus lividus*, while the $EC_{50}$ for *S. purpuratus* was 30 times higher than that of both species (Marin et al., 2000). Sea urchin embryos are more sensitive to TBT than other invertebrate embryos and larvae including polychaetes, bivalves and crustaceans (Hagger et al., 2002). Embryos from these invertebrate species when exposed to TBT have $LC_{50}$ values in the low ng/ml range, about 10 to 100 times greater than the $EC_{50}$ values for sea urchin embryos from this study.

TBT toxicity is not typically associated with a pathway mediated by a steroid nuclear receptor. TBT is an aromatase inhibitor in gastropods (anti-estrogenic) and effective at altering sexual characteristics, steroid hormone synthesis and reproduction (Oehlmann and Bettin, 1996; Gibbs et al., 1988; Morcillo et al., 1998). The toxicity of TBT can also be associated with the inhibition of microtubule polymerization as well as oxidative phosphorylation (Cima et al., 1996). Relatively high concentrations (> 148 ng/ml) of TBT have recently been shown to alter cellular calcium homeostasis in ascidian phagocytes by interfering with calmodulin regulation of $Ca^{2+}$-ATPase (Cima and Ballarin, 2000) and in trout hepatocytes, by stimulation of calcium release from the endoplasmic reticulum (Reader et al., 1999). Calcium is a key signaling molecule in many

developmental processes, and the potential alteration of calcium signaling by TBT may be an important cause of developmental toxicity in the sea urchin embryo and larvae. The atypical concentration response of *S. purpuratus* may be due to subtle differences in calcium signaling between species.

Sea urchin embryos were sensitive to DDD, one of the DDT metabolites, at levels 10 to 100 times of those found in the environment (~1 x $10^{-3}$ ng/ml). The $EC_{50}$ for DDD (67.6 ng/ml) was within the range of experimental levels of DDT known to inhibit normal embryonic development for *S. purpuratus* and *S. droebachiensis* (Dinnel et al., 1989). DDT and its metabolites have estrogenic activity as demonstrated by the proliferation of MCF-7 cells but may have other endocrine disrupting capabilities in a taxa-dependent manner (Guillette et al., 1996). DDD is not typically considered estrogenic but, due to anti-androgenic activity, can appear estrogenic in some systems. Anti-androgenic activity is attributed to DDD in a mammalian cell line (Maness et al., 1998). There is evidence of estrogenic activity in fish and reptiles (Loomis and Thomas, 2000; Guillette, personal communication). The pathway of teratogenicity for this pesticide may not be identical to the estrogenic EDCs because DDT and its derivatives all have very low affinities for vertebrate ERs ($EC_{50}$ and $IC_{50}$ values ~ $10^{-4}$ to $10^{-6}$ M (μg/ml) (Loomis and Thomas, 2000; Matthews et al., 2000).

4.3. Teratogenicity of estradiol and all EDCs is different between developmental stages of the sea urchin embryo

Sea urchin embryos are more sensitive at specific stages of development to certain molecules (Pillai et al., 2003). The stage-specific sensitivity may indicate when particular mechanisms are available or functioning. The sensitivity of the Blastula stage to $E_2$ is suggestive of nongenomic activities of estrogens, since limited new transcription occurs from early embryogenesis until blastula formation (Davidson, 1999). Thus, teratogenicity prior to hatching may be associated with the activation of signaling pathways by the estrogenic EDCs (Singleton and Khan, 2003), or perhaps by some unknown mechanisms. Beyond hatching, embryonic transcription is fully functional. The relative insensitivity to the treatments during the Gastrula stage is possibly due to the depletion of maternal RNAs of the estrogen-responsive receptor during blastula development. Another explanation for Gastrula insensitivity is a change in expression/activity of critical molecules that could be perturbed during early blastula formation, but not after hatching by the treatments used in this study. However, there may also be a delay in receptor availability until the end of gastrulation.

The Blastula and Pluteus stages were not as sensitive to TBT and DDD as they were to $E_2$ and OCT. The lower sensitivities imply the involvement of other pathways of teratogenicity for TBT and DDD. Both of TBT and DDD may exhibit non-NR mediated effects that may be at least partially responsible for the deleterious effects on development. It is interesting to note that Blastula embryos were less sensitive to higher DDD concentration than lower concentrations. If a receptor-mediated mechanism is involved in DDD teratogenicity, receptor down-regulation after over-exposure may occur during this 18 hr exposure. The regulation of other receptors occurs at this level of

development in the sea urchin embryo (Hung et al., 1995; McCoon et al., 1996) and may function in a similar manner as vertebrate NR regulation (Reid et al., 2002).

4.4. Co-incubation with SERMs alters the teratogenicity of estradiol and estrogenic EDCs in sea urchin embryos and larvae

The effect on normal development of the three natural estrogens was in order of their respective affinity for the vertebrate ER (Petersen et al., 1998; Hawkins and Thomas, 2004). The similarity to affinities for ER suggests a receptor-mediated process functioning through an ER-like receptor. The involvement of receptor-mediated processes is also supported by the "protective" nature of tamoxifen when co-incubated with estradiol and the estrogenic EDCs. Tamoxifen is a partial agonist of the mammalian ER but in the presence of a full agonist like estradiol, tamoxifen acts as an antagonist. Therefore, the co-incubation of estradiol with tamoxifen while mediating the teratogenicity of estradiol may also allow for some " protective" receptor activity. It should be noted that tamoxifen teratogenicity was not additive during co-incubation with the higher concentrations of estradiol and the estrogenic EDCs, supporting the hypothesis that this SERM was interfering with normal ligand-receptor binding.

The teratogenicity of tamoxifen used alone could be partially explained by its calcium antagonism and inhibition of calcium-calmodulin complex formation at higher concentrations (London and Mailhes, 2001), or by the generation of reactive oxygen species (Pagano et al., 2001). The affinity of tamoxifen for various vertebrate ERs is 10 to 100 times less than estradiol (Matthews et al., 2000; Hawkins and Thomas, 2004). The

tamoxifen $EC_{50}$ value (competitor concentration displacing 50% of estradiol) for a testicular membrane ER in the Atlantic croaker was 100 times higher than the LEC (20% abnormal development) used in this study (Loomis and Thomas, 2000), potentially meaning a higher receptor affinity in the sea urchin embryo for tamoxifen. However, more direct evidence for such a receptor(s) is necessary before we can conclude that a receptor belonging to the nuclear steroid superfamily is mediating steroid signaling during sea urchin development.

In mammalian systems, ICI is a complete antagonist effectively blocking estradiol from binding to the ER not allowing genomic activation. If teratogenicity were through a classical receptor-mediated mechanism, then the ICI response should be similar to the co-incubation experiments with tamoxifen. However, the embryo response to co-incubation with ICI did not elicit a "protective" effect during exposure to estradiol and estrogenic EDCs. The different responses between Figures 6 and 7 indicate nongenomic mechanisms of teratogenicity (Singleton and Khan, 2003) or an ICI-insensitive receptor as reported in mammalian cell lines (Larsen et al., 1997; Singh et al., 2000). The affinity of ICI for vertebrate ERs also depends on the isoforms with ICI having greater relative affinity for ERα and ERβb in the Atlantic croaker than estradiol (Hawkins and Thomas, 2004). The affinity of ICI for a putative sea urchin receptor may be lower than the LEC used in this study. The teratogenicity could also be due to both non-receptor mediated effects by ICI (Morley et al., 1992; Leiberherr et al., 1993; Salerno et al., 1999, Pagano et al., 2001) and interactions with cell surface receptors (Das and Thomas, 1999; Loomis & Thomas, 2000). Identification of an estradiol/steroid receptor in the sea urchin embryo and larvae is required to directly address these issues.

## 4.5. Conclusions

We have demonstrated that both natural and anthropogenic estrogens can inhibit normal sea urchin embryonic development at concentrations relevant to aquatic ecosystems. The sea urchin embryos were more sensitive than most invertebrate larvae to several of the EDCs including ethynylestradiol, octylphenol and tributyltin. Estradiol and the estrogenic EDCs all cause developmental abnormalities in sea urchins such as a delay in growth through a TAM-sensitive but an ICI-insensitive mechanism. It is uncertain at this time whether the teratogenic mechanisms involve an ER-like receptor, a membrane receptor, or a completely different mechanism of toxicity. The comparison of the response curve slopes and the differential counts between the estrogens and the estrogenic EDCs indicate at least two teratogenic mechanisms. Finally, the embryos exhibited stage-specific sensitivities to estradiol and estrogenic EDCs that may entail more than one mechanism of teratogenicity involving either genomic or nongenomic processes.

The response of the developing sea urchin embryo to estradiol and estrogenic EDCs exposure share both similarities and differences with estrogenicity in vertebrate systems. The relative potencies of steroidal estrogens are in a similar order to the relative potencies in most vertebrate systems and for the affinities for the several subtypes of the ER. Estradiol is more potent than either estrone or estriol and only slightly more potent than ethynylestradiol. The estrogenic compound, bisphenol A, is generally less potent than estradiol in most vertebrate system and is also in the sea urchin embryo. Another

similarity is the attenuation of teratogenicity by co-incubation with tamoxifen. A major difference between the sea urchin embryo and estrogenicity in vertebrates is the relatively greater potency of octylphenol and the similar potencies of DDD and estradiol. Unlike tamoxifen, ICI 182, 780 does not positively alter the teratogenicity during exposure to estradiol or estrogenic EDCs but significantly increases abnormal development during co-incubation.

A BLAST search of the sea urchin genome using amino acid sequences from three mammalian ERs and one molluscan ER returned several hits with significant E-values associated with the DNA-binding domain of the ER (data not shown). For future studies, these sequences may be useful for the characterization of steroid receptors within the sea urchin. Identification of steroid receptor(s) may also lead to the development of a molecular marker for EDC exposure using the sea urchin as a model species (Rotchell and Ostrander, 2003). Once the key components of the estrogen pathway(s) have been identified, the mechanisms of teratogenicity employed by estrogenic EDCs or endogenous steroids involved during sea urchin development will be understood establishing an effective model for the investigation of environmental estrogens and their effects on development.

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TABLE 1. $EC_{50}$ values with 95% fiducial limits for all hormones and toxicants.

| | Treatment | $EC_{50}$ (ng/ml) | Lower limit | Upper limit |
|---|---|---|---|---|
| **Hormones** | **$E_1$** | 604.4 | 492.6 | 706.6 |
| | **$E_2$** | 14.2 | 0.7 | 77.3 |
| | **$E_3$** | 1515.4 | 361.4 | 3731.3 |
| | **$P_4$** | 546.6 | 363.1 | 745.9 |
| | **$EE_2$** | 30.3 | 2.1 | 90.6 |
| **EDCs** | **OCT** | 0.174 | 0.035 | 0.489 |
| | **BisA** | 226.5 | 121.6 | 323.5 |
| | **TBT (*S. purpuratus*)** | 0.893 | 0.731 | 1.689 |
| | **TBT (*L. anamesus*)** | 0.037 | 0.004 | 0.099 |
| | **DDD** | 67.6 | 52.1 | 81.6 |
| **SERMs** | **TAM** | 0.05 | 0.031 | 0.062 |
| | **ICI** | 0.058 | 0.04 | 0.07 |

Table 1. $EC_{50}$ values with 95% fiducial limits for all toxicants and hormones. Reproductive hormones are abbreviated as estrone ($E_1$), 17β-estradiol ($E_2$), estriol ($E_3$), progesterone ($P_4$) and 17α-ethynylestradiol ($EE_2$). EDCs are abbreviated as 4-octylphenol (OCT), bisphenol A (BisA), tributyltin (TBT) and *o,p*-DDD (DDD). SERMs are abbreviated as tamoxifen (TAM) and ICI 182, 780 (ICI). The units for all treatments are ng/ml. The $EC_{50}$ value for TBT: *S. purpuratus* was calculated from the final slope of the curve from 0.50 ng/ml to 10 ng/ml. $EC_{50}$ values were calculated using TidePool Scientific ToxCalc Software© and were calculated from *S. purpuratus* experiments unless otherwise noted.

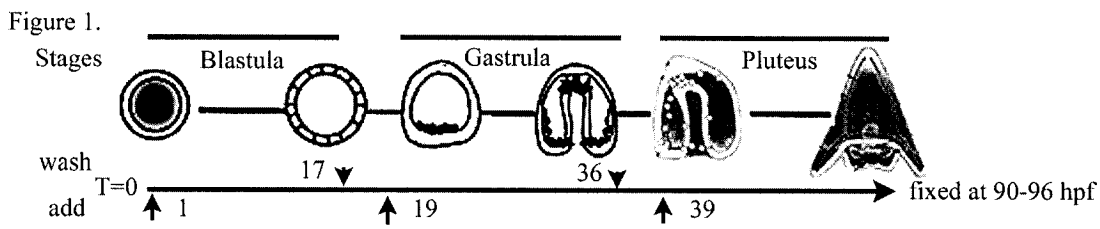



Figure 1. Diagram illustrating the timing of the developmental and stage specific exposure experiments. For the developmental experiments, embryos were exposed from 1 hpf to 96 hpf. For the stage specific experiments, stages were defined as Blastula, Gastrula and Pluteus. Blastula exposure period was from 1 hpf (add treatment) to 17 hpf (wash). Gastrula exposure period was from 19 hpf (add treatment) to 36 hpf (wash) and Pluteus was from 39 hpf (add treatment) to 90 hpf (no washing, fixed at early pluteus larval stage).

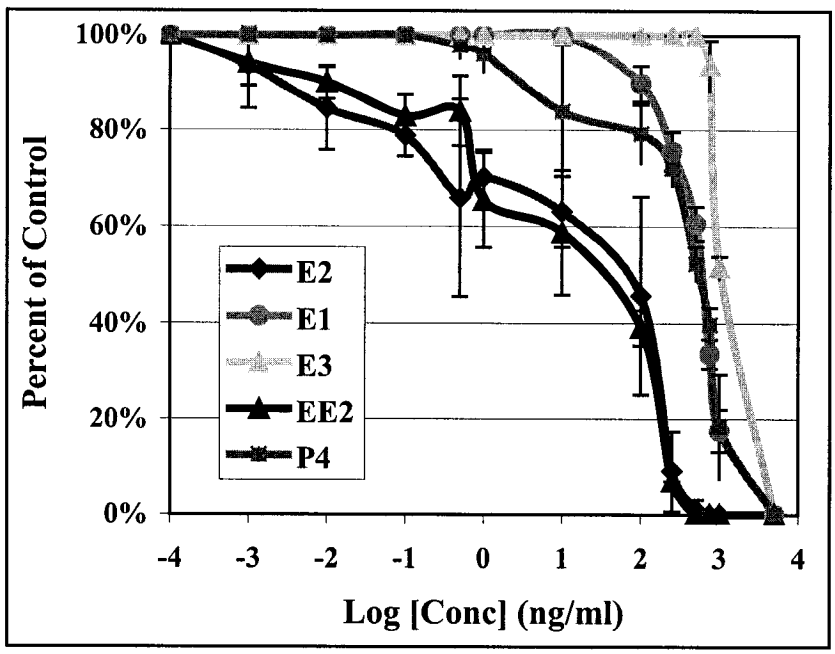
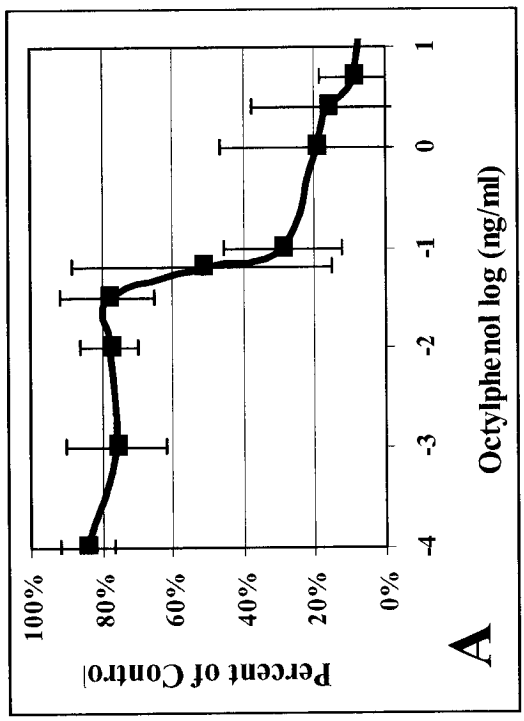
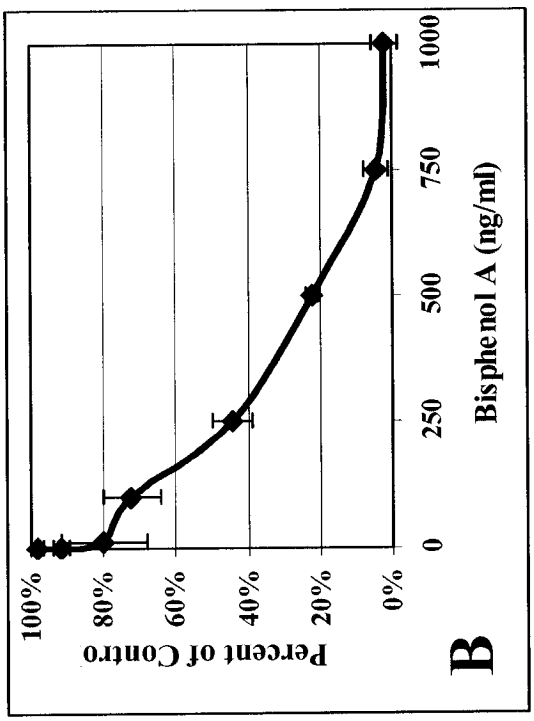
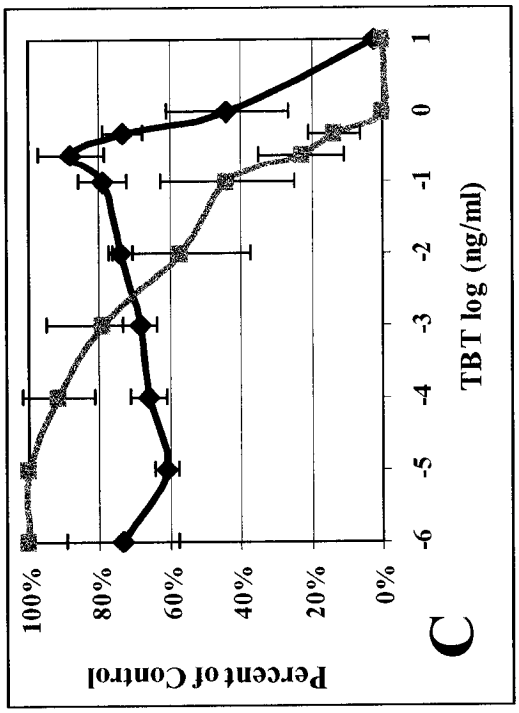



Figure 2. Percentage of normal sea urchin embryo development following exposure to natural and synthetic reproductive hormones. Chemical abbreviations are given in Table 1. Error bars represent 1 standard deviation from the mean of three separate experiments. The concentrations of the reproductive hormones were **log** transformed for observation of low concentration effects. The responses with $E_2$ and $EE_2$ are significantly different from the other hormones ($P < 0.05$). Data from experiments using *S. purpuratus*.

Figure 3. Percentage of normal sea urchin embryo development following exposure to A: octylphenol, B: bisphenol A, C: tributyltin and D: DDD. Error bars represent 1 standard deviation from the mean of three separate experiments. The ng/ml concentrations of octylphenol and TBT are **log** transformed for observation of low concentration effects. Data from experiments using *S. purpuratus* except for TBT. The response to TBT between the two species of sea urchins (*S. purpuratus* = black diamonds and *L. anamesus* = gray squares) was significantly different ($P < 0.05$).



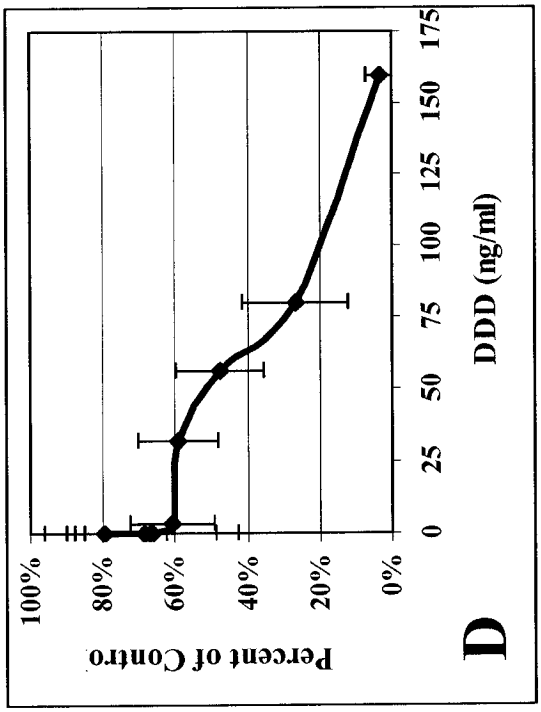
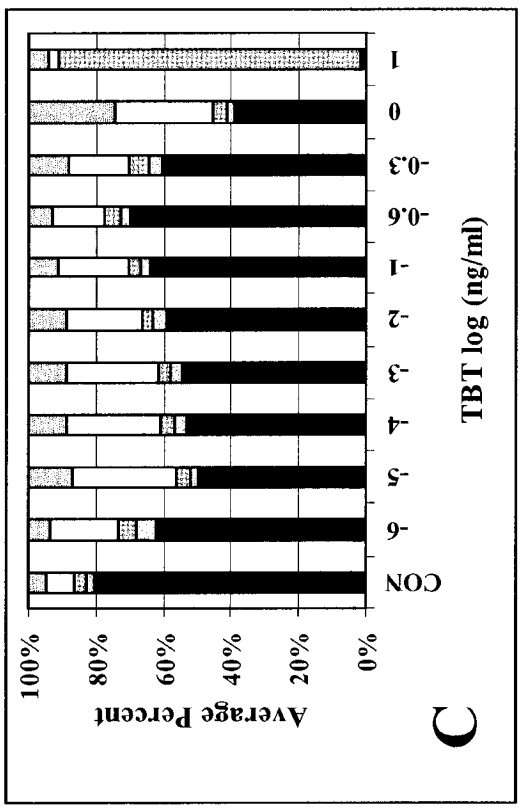
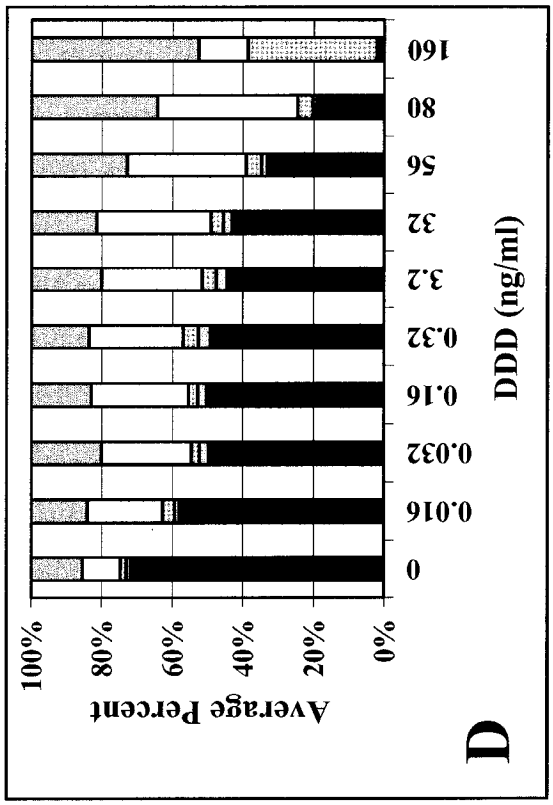
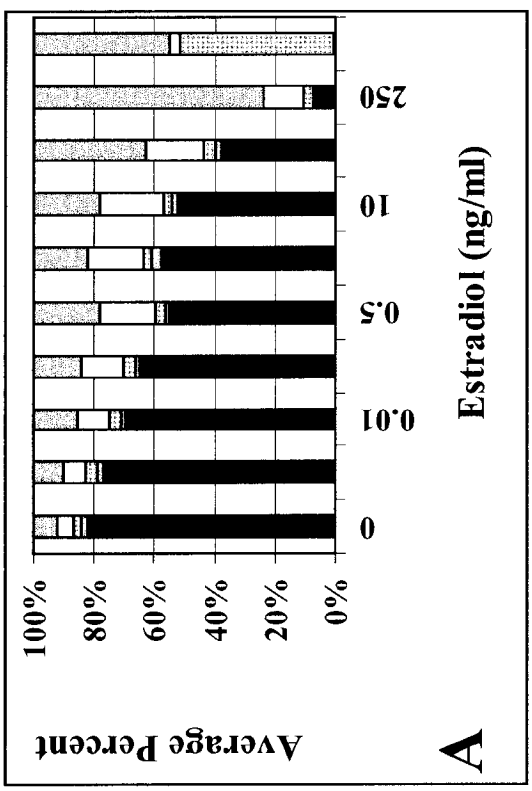



Figure 4. Differential counts of sea urchin embryos exposed to A: estradiol, B: octylphenol, C: tributyltin and D: DDD. The black portion  represents normal development. The gray denotes  the elongated embryos. The checkered portion  represents the percentage of ciliated blastulae or embryos did not develop beyond unhatched blastula. The white bar  denotes the percentage of delayed development. The hatched gray bar  represents abnormal development. Graph C: TBT was taken from the differential counts from *S. purpuratus* concentration response data with concentrations of TBT expressed as **log** (ng/ml). Each bar represents the average of three separate experiments.



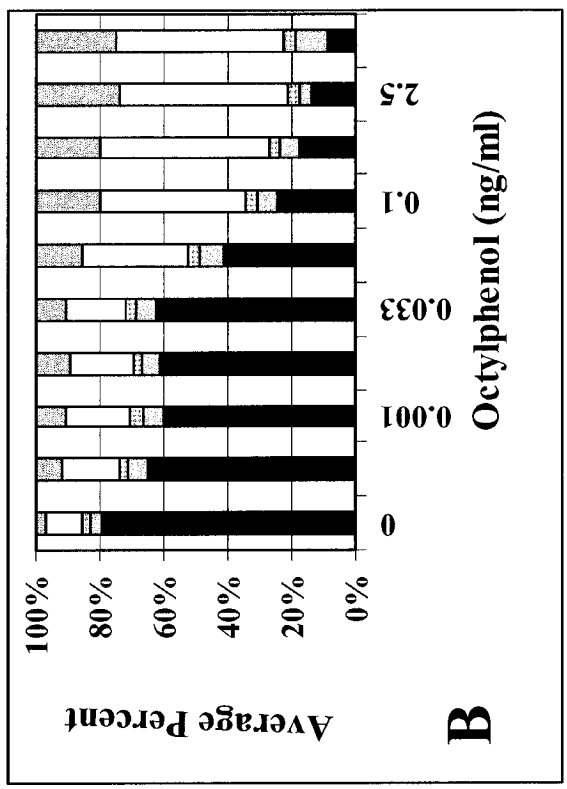



Figure 5. Percent of normal sea urchin embryo development from *S. purpuratus* after stage-specific exposure to A: estradiol, B: octylphenol, C: tributyltin and D: DDD. Black represents the low concentrations, white the mid concentrations and gray the high concentrations. For $E_2$, the low, mid and high concentrations were 0.5, 10 and 250 ng/ml; for OCT, $1x10^{-3}$, 0.1 and 5 ng/ml; for TBT, $1x10^{-4}$, 0.1 and 1 ng/ml and for DDD, 5, 50 and 100 ng/ml. Error bars represent 1 standard deviation from the mean of three separate experiments. All responses were significantly different from controls. (a) denotes a significant difference compared to the low (black bar) concentration only ($P<0.05$) using Tukey Test. (b) denotes a significant difference compared to both low and mid (white bar) concentrations. Gastrula as a group had significantly higher percent normal development than other stages for each toxicant ($P<0.05$) using the Student-Newman-Keuls Method of pairwise multiple comparisons.

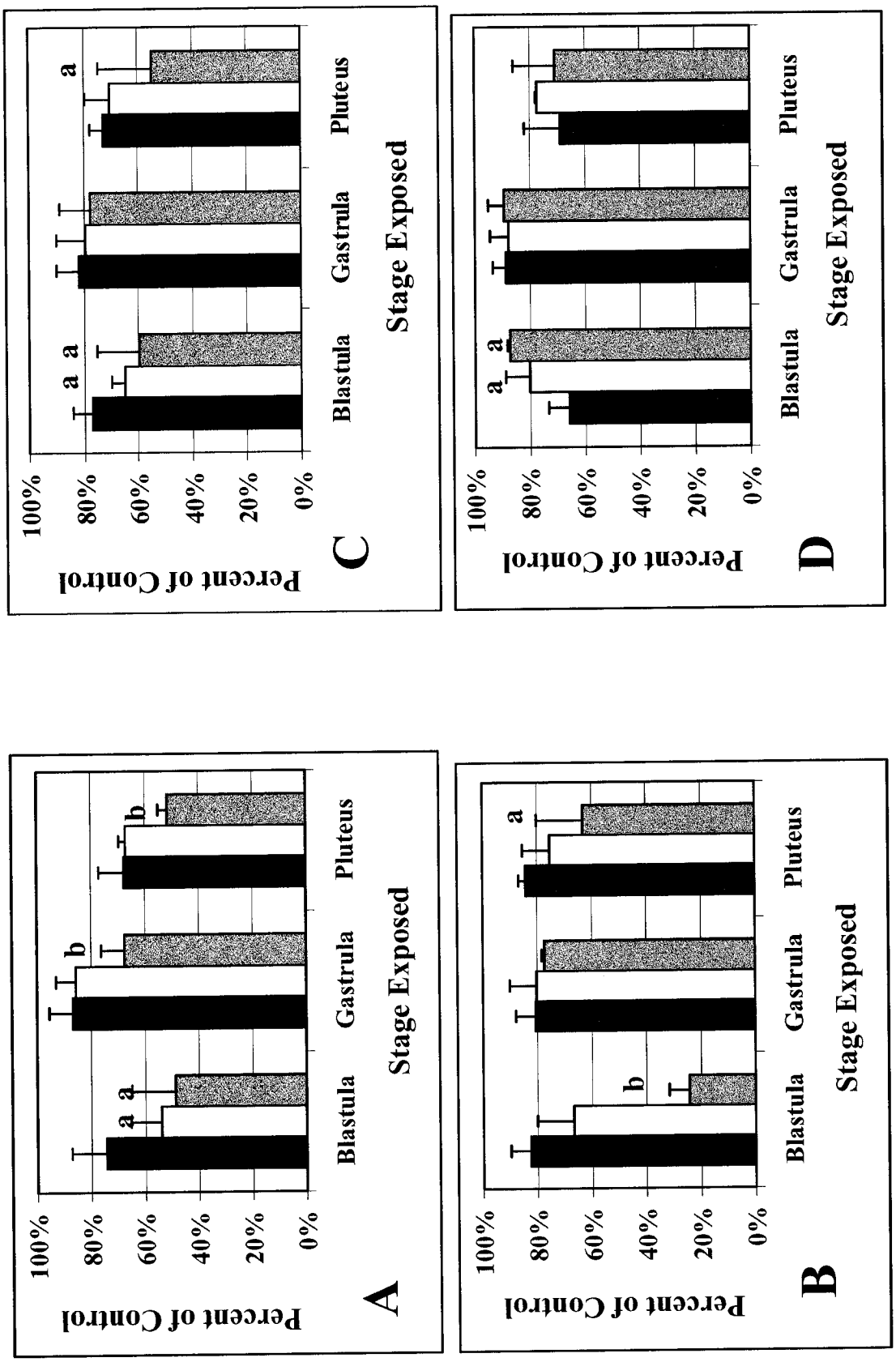


Figure 6. Percent of normal sea urchin development using *S. purpuratus* embryos following exposure to estrogens and estrogenic compounds with or without tamoxifen (TAM). A: Percent of normal development after exposure to estradiol with (black squares) or without (gray diamonds) the LEC (0.019 ng/ml) of TAM. B: ethynylestradiol, C: octylphenol and D: bisphenol A. The protective response from co-incubation with TAM was seen with all estrogenic compounds. Error bars represent 1 standard deviation from the mean of three separate experiments. The overall trends for each are significantly different ($P<0.05$) using one-way ANOVA.

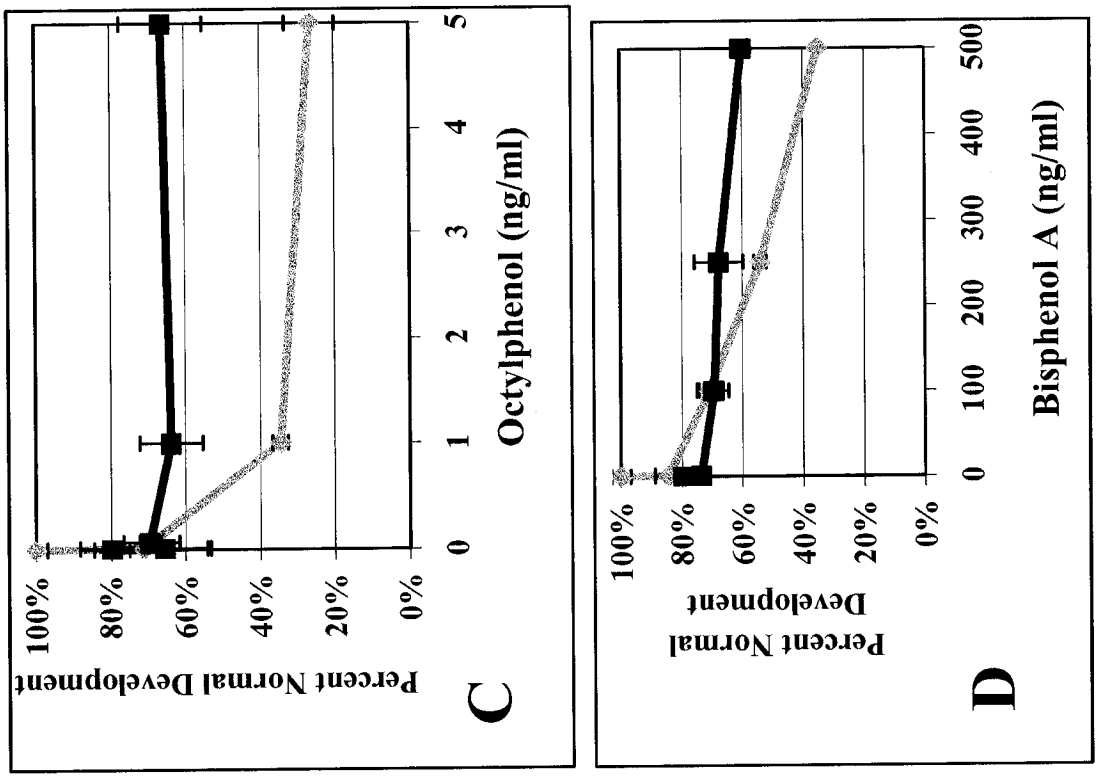
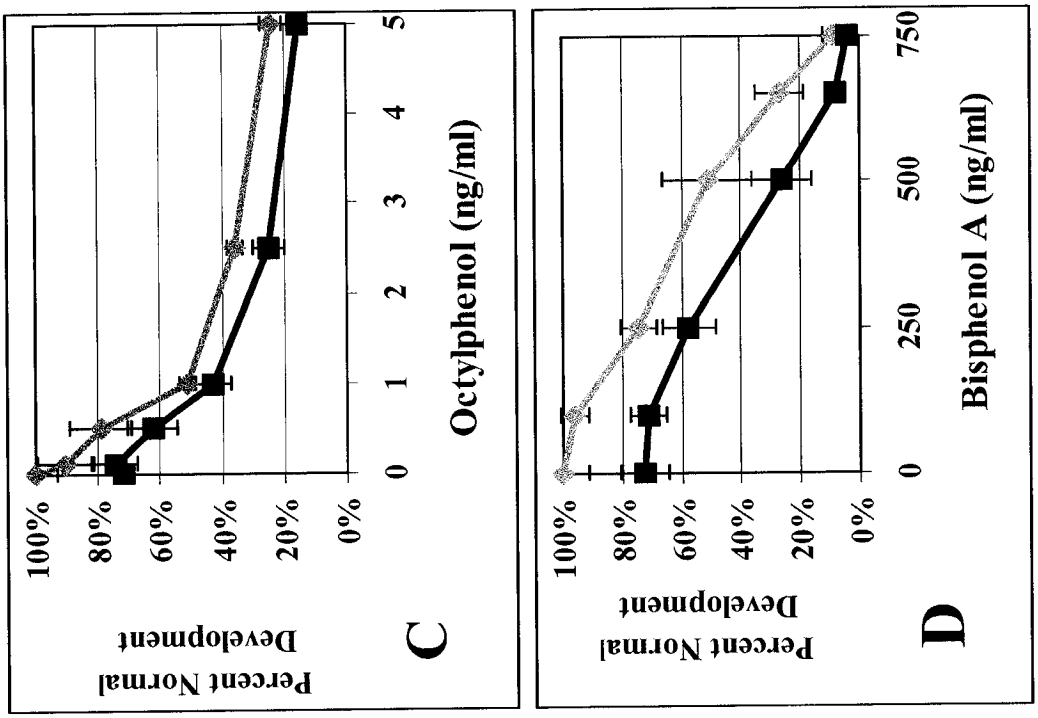


Figure 7. Percent of normal sea urchin development using *S. purpuratus* embryos following exposure to estrogens and estrogenic compounds with or without ICI 182, 780 (ICI). A: Percent of normal development after exposure to estradiol with (black squares) or without (gray diamonds) the LEC (0.03 ng/ml) of ICI. B: ethynylestradiol, C: octylphenol and D: bisphenol A. ICI did not 'protect' embryos during co-incubation with all estrogenic compounds. Error bars represent 1 standard deviation from the mean of three separate experiments. The overall trends for each are significantly different ($P<0.05$) using one-way ANOVA.



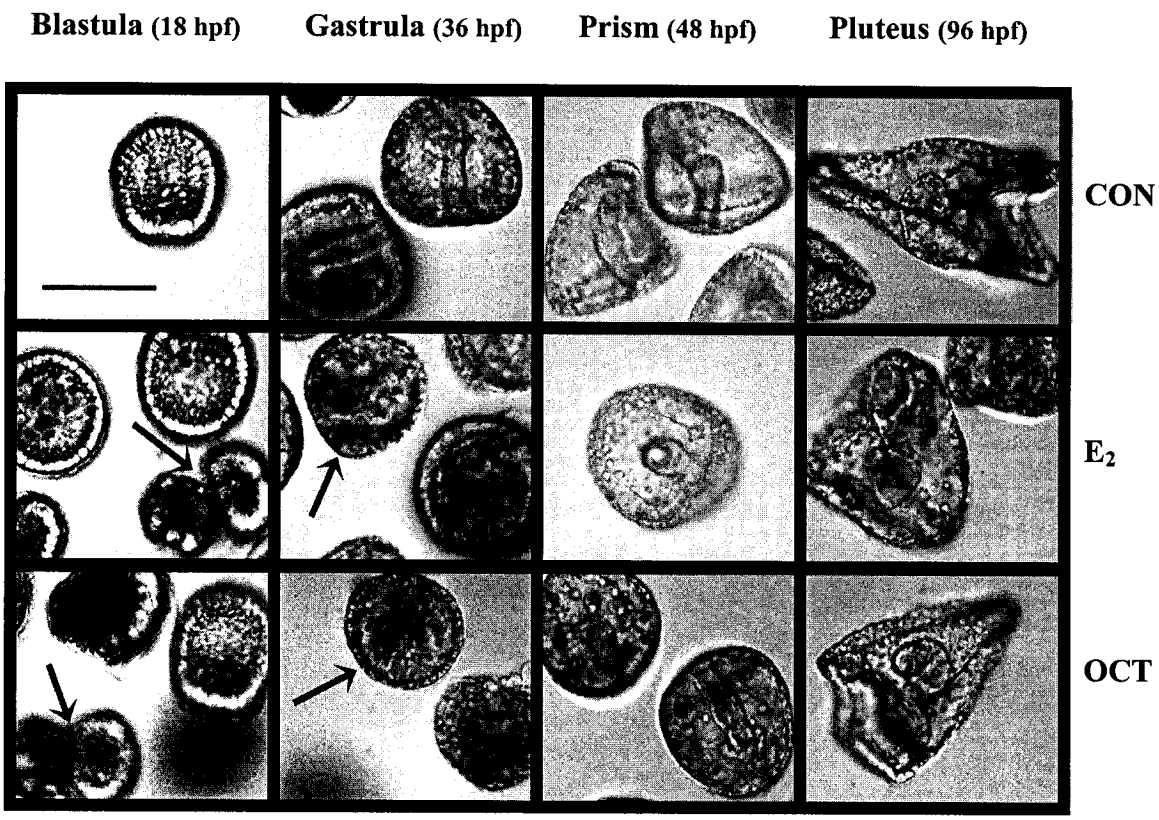



Figure 8. Comparison of developmental morphologies between hormone and EDC exposed embryos. Photomicrographs of embryos exposed to the toxicants were taken at specific concentrations at specific developmental time points using an fixed stage microscope equipped with DIC optics and a CCD camera and analyzed using the imaging program, Metamorph©. The embryos were fixed at 4 time points: 18 h post-fertilization (Blastula); 36 hpf (Gastrula); 48 hpf (Prism) and 96 hpf (Pluteus) using *S. purpuratus* embryos. Toxicant concentrations for estradiol and octylphenol were 50 ng/ml and 0.5 ng/ml, respectively. Arrows point to 'split' blastula and 'empty' gastrula embryos. Black bar equals 50 nm for all plates.

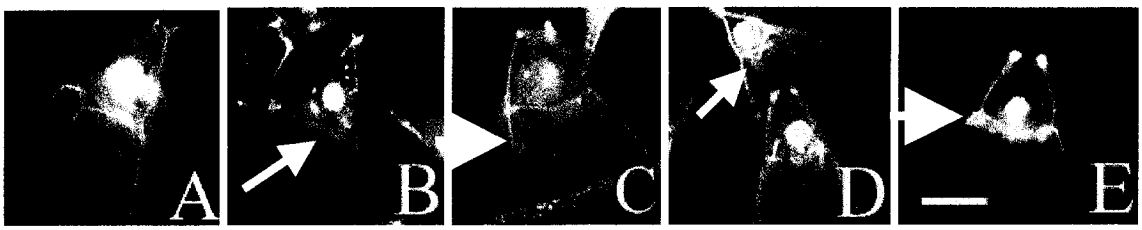



Figure 9. Comparison of spicule development between hormone and EDC exposed embryos using fluorescent calcein dye. The fluorescent calcium-sensitive dye, calcein, was added to the *L. anamesus* embryos in culture after gastrulation was complete. The embryos were exposed to A: no treatment control; B: 50 ng/ml $E_2$; C: 0.1 ng/ml OCT; D: 0.01 ng/ml TBT and E: 80 ng/ml DDD for 96 h, fixed and photographed using an epifluorescence microscope with excitation at 490 nm and emission at 530 nm. White bar equals 50 nm for all plates. Arrow points to 'A-frame' embryos and arrowhead points to short or bent spicule arms.

# CHAPTER 2

## Maternal exposure to estradiol and endocrine disrupting compounds alters the sensitivities of sea urchin embryos and the expression of an orphan steroid receptor

**Abstract**

Endocrine disrupting compounds (EDCs) are known to affect reproduction and development in marine invertebrates. In previous work, we have shown that developing sea urchin embryos were sensitive to estradiol and estrogenic EDCs at environmentally relevant concentrations in a tamoxifen-sensitive manner (Roepke et al., 2005). In this study, we report the effects of maternal exposure to EDCs on embryo sensitivity and regulation of a putative receptor in sea urchin eggs. Maternal exposures were conducted by injecting female *Strongylocentrotus purpuratus* sea urchins initiating oogenesis with two concentrations of estradiol, octylphenol, tributyltin and *o,p*-DDD for 8 weeks with an induced spawning before and after the injection cycle. Developing embryos were less sensitive to estradiol following maternal exposure to estradiol, octylphenol and DDD. The steroidogenesis inhibitor, spironolactone, and the aromatase inhibitor, formestane, affected normal sea urchin development with $EC_{50}$ values of 18 μM and 2 μM, respectively. Specific binding of estradiol was demonstrated in homogenates of sea urchin embryos by filtration centrifugation and column chromatography, but saturation was not reached until 4-6 hr and was highly variable. Analysis of eggs from pre- and post-injection spawns using real-time Q-PCR for the mRNA of an orphan steroid receptor, SpSHR2 shows that receptor mRNA increased in eggs with estradiol, octylphenol and tributyltin but decreased with DDD. RIA showed that estradiol is present during gastrulation. In summary, maternal exposure to estradiol and EDCs alters embryo sensitivity and regulates the expression of an orphan steroid receptor in the egg,

however this may not be the low-capacity, low-affinity receptor for estrogenic compounds that is present in the eggs and embryos.

keywords: estradiol, EDCs, sea urchin, embryos

## Introduction

Estradiol and endocrine disrupting compounds (EDCs) are known to inhibit normal development in the sea urchin embryo utilizing a tamoxifen-sensitive mechanism (Roepke et al., 2005) suggestive of an endogenous role for estrogens during sea urchin development. However, the role of estradiol in the reproductive biology of mature female sea urchins and the development of their embryos is unclear because the basic mechanisms of sea urchin reproductive endocrinology are poorly understood. In vertebrate systems, estradiol and estrogenic EDCs predominantly function through a steroid nuclear receptor-mediated process and subsequently regulate the transcription of mRNA for a number of proteins. Expression of the nuclear estrogen receptor (ER) mRNA is differentially regulated by steroid and steroidal compound exposure (Pillai et al., 2002; Cappelletti et al., 2003; Marin-Castano et al., 2003). Since the level of new transcription occurring during early development of the sea urchin is low (Davidson, 1999), exposure of females to estradiol and EDCs may alter or affect sea urchin embryos by changing the expression of receptors and signaling molecules in the maternal pool of mRNA deposited in the eggs. Any alteration in the expression of receptors during early development may alter embryonic sensitivities to estrogens and estrogenic compounds.

The effects of maternal exposure to environmental estrogens on embryos and neonates has been reported in teleost and mammalian embryos. Zebrafish embryos maternally-exposed to estradiol and hydroxylated PCBs had significantly lower hatching rates than controls and caused the induction of ER mRNA in male embryos (Westerlund et al., 2000). The estrogenic compound, 4-*tert*-octylphenol, decreased embryonic growth

through changes in maternal-fetal trophic mechanisms, induced embryonic vitellogenin mRNA and protein expression and altered gonadal development in male embryos in the viviparous eelpout (Rasmussen et al., 2002). In mammalian systems, maternal exposure to dioxin alters the expression of ER mRNA in fetal male swine testes and decreases ER mRNA in the epididymis and gubernaculums (Barthold et al., 1999) while exposure to octylphenol can affect the onset of puberty in the F1 generation and reduced litter size in the pig (Bogh et al., 2001). In rats, estrogenic compounds can alter uro-genital tract development and ER mRNA expression (Yoshida et al., 2002) and increase pregnancy failures, pre- and post-implantation loss and fetal developmental delays (Kim et al., 2001).

In vertebrates, the mechanism of teratogenicity as a result of maternal exposure to estradiol and EDCs functions through the various forms of the ER. However, in the sea urchin, an estrogen-responsive nuclear receptor has not been identified although there is evidence of high-affinity estradiol-binding receptor in the sea star (De Waal et al., '82). Three orphan members of the steroid nuclear receptor superfamily have been characterized in sea urchins and have been named SpCOUP-TF, SpSHR2 and SpSHR3 (Kontrogianni-Konstantopoulos et al., 1996; Kontrogianni-Konstantopoulos et al., 1998; Kontrogianni-Konstantopoulos and Flytzanis, 2001). Of the three receptors, the mRNA transcript for SpSHR2 is highly expressed in maternal pool of mRNA in the egg and the transcript and protein are both present during early embryogenesis (Kontrogianni-Konstantopoulos and Flytzanis, 2001). The estradiol binding characteristics of these receptors have not been investigated, nor are they known to be activated by steroidal ligands of any type. Nonetheless, these receptors are transcription factors which may be regulated by negative or positive feedbacks much like estrogen receptors (ER). Exposure

to estradiol and/or estrogenic compounds may alter expression of mRNA for this nuclear receptor during oogenesis in the adult female sea urchin.

**Materials and Methods**

2.1. Reagents and chemicals

17β-estradiol, 4-octylphenol, tributyltin, 1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane (*o,p*-DDD; mitotane), spironolactone, formestane and 2,4 $^{3}$H-17β-estradiol were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). 1,2,6,7 $^{3}$H-17β-estradiol was purchased from Perkin-Elmer/NEN (Boston, MA, USA). Stock solutions of each chemical were made up in DMSO (Sigma) except TBT, which was diluted in filtered seawater (FSW). All stocks were stored at –20 °C until use. Glutaraldehyde for embryo fixing was purchased from Polysciences, Inc. (Warrington, PA, USA) and made into a 5% solution with FSW.

2.2. Maternal exposure to EDCs by injection

*Strongylocentrotus purpuratus* adults are reproductive from November to April in the coastal oceans off Northern California. Animals were collected in November and were maintained at the Bodega Marine Laboratory (BML) in continuous flow-through, sand-filtered sea water (13°C +/- 2°) for three weeks with biweekly feedings of locally-collected kelp (see Fig. 1). Females were separated into ten groups of four females each:

two controls - no injection control and carrier control (DMSO) and two concentrations of each toxicant. After the initial recovery period, females were spawned by 0.5 M KCl injection. The eggs were washed and separated into two subsamples. The first subsample was pooled with equal amounts of eggs from each individual in each group for developmental exposure experiments. The second subsample from individual females were packed (~500 μl) and frozen in liquid nitrogen for real-time Q-PCR. The females were separated into individual, labeled sections of a seawater trough with constant high-level flow. The females were allowed to recover for 3 weeks with constant feeding of freshly collected kelp. Females were then injected with treatments once per week for eight weeks with constant kelp feeding and weekly cleanings. The two injection concentrations for estradiol, octylphenol and DDD were 0.5 and 50 mg/kg. The two concentrations for tributyltin were 0.5 and 50 μg/kg. Injection concentrations were ascertained by a preliminary experiment conducted with a range of concentrations to determine the appropriate sublethal injection concentrations. After the injection cycle, females were allowed to recover for three weeks with kelp feeding. Finally, females were spawned by 0.5 M KCl injection and the eggs were collected and prepared for exposure experiments and real-time Q-PCR as above.

## 2.3. Developmental Exposures

Developmental toxicity (teratogenicity) caused by exposure to compounds was assessed in the embryos by determining the morphology of larvae at the pluteus stage of development (see Roepke et al., 2005). Female sea urchins were washed with FSW and

induced to spawn by injection of < 0.5 mL of 0.5 M KCl into the body cavity via the peristomial membrane. Eggs were collected from each female sea urchin into chilled 0.7 μm FSW, washed and prepared for fertilization. For each exposure experiment, one male was also spawned and the sperm was kept on ice until use. Motility and fertilization assessments were conducted to ensure viable gametes with 80-90% fertilization. Eggs pooled from each female within each group were fertilized and, thirty minutes after the elevation of the fertilization envelope, the fertilized eggs were washed to remove sperm and concentrated by settling and aspirating the upper portion of FSW to a density of 1000 eggs per ml. Approximately 500 eggs were transferred to the experimental vials (30 ml acid-washed, borosilicate glass shell vials) containing 20 ml of 0.22 μm FSW. The compounds were added to the vials to the appropriate concentrations and mixed. Seawater (no compound added) and solvent controls were also prepared. All treatments were run in triplicate for each experiment. The embryos were incubated at 17 °C to 96 hours post-fertilization (hpf). At the early pluteus stage ≅ 96 hpf, larvae were fixed with 100 μL of 5% glutaraldehyde (<0.05% final) and allowed to settle in the vials. The first 100 embryos observed were assessed for normal development in each vial using an inverted phase contrast microscope. The concentration ranges for estradiol and EDCs were centered on the $EC_{50}$ values determined from previous research (Roepke et al., 2005).

2.4. Real-time Q-PCR of SpSHR2

Oocyte samples from individual females were washed, pelleted and frozen in liquid nitrogen immediately following each spawn. Frozen samples were lysed in 1x ABI lysis buffer (Applied Biosystems, Foster City, CA). Before RNA extraction, the lysates were transferred into 96 deep-well plates, ground using two stainless steel beads for 2 min at 1500 rpm (GenoGrinder 2000, SpexCertiprep, Metuchen, NJ), digested with Proteinase K (Invitrogen, Carlsbad, CA) for 30 min at 37 °C and cooled for 30 min at –20 °C. Total RNA was extracted from the lysates using a 6700 automated nucleic acid (ANA) workstation (Applied Biosystems) according to the manufacturer's instructions. Total RNA was eluted in 100 µl of elution buffer.

TaqMan PCR systems were evaluated according to Leutenegger et al. (1999). Genomic DNA contamination in the total RNA preparation was digested using RNase-free DNase I (Invitrogen, Carlsbad, CA) at 37 °C for 15 min. Genomic DNA background was tested on digested tRNA samples using the 18S specific TaqMan PCR system. Complementary DNA (cDNA) was synthesized using 100 units of SuperScript III (Invitrogen), 300 ng random hexadeoxyribonucleotide ($pd(N)_6$) primers (random hexamer primer), 10 U RNaseOut (RNase inhibitor) and 1 mM dNTPs (all Life Technologies) in a final volume of 40 µl. The reverse transcription reaction proceeded for 120 min at 50 °C. After addition of 60 µl of water, the reaction was terminated by heating for 5 min to 95 °C and cooled on ice.

Each PCR reaction contained 400 nM of each primer (primers were determined from sequences available using BLAST searching; see Table 1 for primer sequences), 80 nM of the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3),

50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 µl of the diluted cDNA sample in a final volume of 25 µl. The samples were placed in 96 well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C.

Final relative quantitation was done using the comparative CT method (User Bulletin #2, Applied Biosystems) and is reported as relative transcription or the n-fold difference relative to a calibrator cDNA (i.e. lowest target gene transcription or controls). In brief, the housekeeping gene 18S rRNA was used to normalize the TaqMan signals for the target genes. The ΔCT values were calibrated against the control ΔCT values for each target gene (ΔΔCT). The relative linear quantity of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta CT}$. Therefore, all transcription data is expressed as an *n*-fold difference relative to the calibrator. The post-injection calibrated level of transcript was then divided by the pre-injection calibrated level of the transcript for each individual female and then divided by the average change in transcript in the carrier control group. The *n*-fold change in transcript was averaged between the females in each injection treatment.

## 2.5. Estradiol Radioimmunoassay

A standard radioimmunoassay protocol using an estradiol-specific antibody was conducted on homogenates of sea urchin egg, blastula and gastrula embryos and prism

and pluteus larvae (Pinaud et al., 1991). The antiserum (Niswender number 244), characterized by England et al. (1974), was highly specific for estradiol and exhibits 3% crossreactivity to estrone and estriol. Briefly, homogenates were extracted with ether which was then allowed to evaporate. 300 µl of phosphate-buffered saline with 1% gelatin (PBS/G) was added to all samples and then incubated with the antibody (1:50,000) overnight at 4 °C. 100 µl of 1,2,6,7 $^{3}$H-17β-estradiol (~26,000 DPM/100 µl) was added to all tubes and incubated for 6 hr at 4 °C. At the end of the hot incubation, Dextran-coated charcoal was added and incubated for 20 min at 4 °C. The samples were centrifuged for 10 min at 2500 rpm. The supernatant was decanted into scintillation vials containing fluor and counted using a Beckman LS 7000 scintillation spectrometer. The limit of the sensitivity of the assay was 10 pg/ml.

2.6. Ligand-receptor binding assays

To initially determine the specific binding of estradiol, homogenates (500 µl) of sea urchin eggs, embryos and pluteus larvae were incubated with 2,4 $^{3}$H-17β-estradiol (~33,000 DPM), with or without 1000-fold excess unlabeled estradiol for one hour at 15 °C. The homogenates were then transferred to individual Amicon YM-3 filtration units (3000 kDa pore size) and centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge for 2 hr at 7000 rpm at 4 °C. After centrifugation, the volumes of the retentate were equalized using standard buffer between labeled and labeled+unlabeled homogenate pairs. Ten µl aliquots from both the retentate and filtrate were transferred in triplicate to scintillation vials containing scintillation fluor and counted using a Beckman LS 7000

scintillation spectrometer. The average CPM in the retentate of the labeled was compared to the average CPM in the labeled+unlabeled retentate for each developmental stage. Experiments were repeated three times and data were expressed as the average of the ratio of labeled/labeled+unlabeled for each stage.

Column chromatography was performed with Sephadex G-100 superfine (2.0 x 6.5 cm bed) with phosphate-buffered saline buffer as the eluent. The exiting eluent was monitored at 280 nm for protein using a Pfizer-Pharmacia spectrophotometer and in 4 min fractions were collected for all treatments. One ml of prism embryos and pluteus larvae homogenates were incubated with 2,4 $^{3}$H-17β-estradiol (~330,000 DPM) with or without 1000-fold excess unlabeled estradiol for 2 hr at 15 °C. After incubation, samples were individually applied to the column and fractions were collected for four to five hours. 500 µl of each fraction were transferred to scintillation vials containing fluor and counted using a Beckman LS 7000 scintillation spectrometer. Protein standards (myosin and β-galactosidase) were also applied to the column to approximate the molecular weight of any specific binding.

The rate of association ($k_1$) was measured by incubating tubes containing 57 nM of $^{3}$H-17β-estradiol in homogenates of pluteus larvae with and without 1000-fold excess unlabeled estradiol. After various incubation times, bound hormone was separated from free hormone by centrifugation using Amicon YM-30 (30,000 kDa pore size) filtration units. Ten µl aliquots from the retentate were transferred in triplicate to scintillation vials containing scintillation fluor and counted using a Beckman LS 7000 scintillation spectrometer. The rate of dissociation ($k_{-1}$) was measured by first incubating homogenates with 57 nM of $^{3}$H-17β-estradiol for 4 hr. 1000-fold excess unlabeled

estradiol was then added to the individual incubation mixtures and, after various time periods, bound hormone was separated from free hormone by centrifugation using Amicon YM-30 filtration units. Ten μl aliquots from the retentate was transferred in triplicate to scintillation vials containing scintillation fluor and counted using a Beckman LS 7000 scintillation spectrometer.

**Results**

3.1. Maternal exposure to EDCs alters embryo sensitivity to toxicants

Maternal exposure to both injection concentrations of estradiol (0.5 and 50 mg/kg) and the high concentrations of octylphenol (50 mg/kg) all decreased embryo sensitivity (less abnormal development) to estradiol (Table 2). The low concentration of octylphenol (0.5 mg/kg) did not elicit a change in embryo sensitivity. The estrogenic compounds did not significantly alter sensitivity to octylphenol except for the high injection concentration of octylphenol which increased (more abnormal development) embryo sensitivity to octylphenol. The effects of maternal exposure to estrogenic compounds increased sensitivity to tributyltin and DDD except for the low injection concentration of octylphenol that had no affect on sensitivity.

The effects of maternal exposure to non-estrogenic EDCs, tributyltin and DDD, on embryo sensitivity were highly variable (Table 2). Exposure to both injection concentrations of tributyltin (0.5 and 50 μg/kg) had no affect on estradiol sensitivity but increased sensitivity to octylphenol. Conversely, injections of DDD (0.5 and 50 mg/kg)

regardless of concentration decreased sensitivity to estradiol. The lower injection concentration of DDD did not affect octylphenol sensitivity but the higher concentration did decrease sensitivity to octylphenol. The lower injection concentration of tributyltin had no effect on either tributyltin or DDD sensitivity but the higher concentration increased sensitivity to tributyltin and had a partial increased effect on DDD sensitivity. Both injection concentrations of DDD increased sensitivity to tributyltin, but decreased the sensitivity to DDD.

3.2. Orphan steroid receptor regulation in egg after maternal exposure to EDCs

The transcript for the sea urchin orphan steroid receptor, SpSHR2, was present in detectable levels in the maternal pool of mRNA in the egg. Maternal exposure to both injection concentrations of estradiol significantly increased by 1- to 2-fold (a 0-fold increase or decrease represents no difference from the control value) the amount of transcript in the egg (Fig. 2). The lower concentration of octylphenol did not have a significant affect on transcript expression in the egg but the higher concentration doubled the amount of SpSHR2 transcript. Maternal exposure to the two injection concentrations of tributyltin had widely different affects on the expression of SpSHR2 in the egg. The lower concentration (0.5 μg/kg) increased the expression of the receptor more than any other compound regardless of concentration ~ 3-fold higher in treated oocytes. Conversely, the higher concentration of tributyltin (50 μg/kg) decreased by almost 50% the transcript in the egg. Both injection concentrations of DDD decreased the transcript

level in the egg by 50-60%. Another sea urchin orphan steroid receptor, SpCOUP-TF, was not regulated by estradiol or octylphenol exposure during preliminary experiments.

3.3. Estradiol production during embryogenesis and specific binding of estradiol to sea urchin homogenates

Using a radioimmunoassay for estradiol, the production of estradiol was detected in post-hatched blastula and late gastrula embryos (Fig. 3) as the measured amount of estradiol was significantly above the detection limit for the assay (10 pg/ml). Unfertilized eggs and prism and pluteus larval stages did not produce statistically significant amounts of estradiol above the detection limit. Before homogenization, eggs were washed three times with 500 ml of filtered seawater to dilute any estradiol contributed by the female ovary. Embryos and larvae for each stage were concentrated through a 40 μm cell strainer from batch cultures of 4 liters or more of filtered seawater; hence, the presence of estradiol in the homogenates was not from contamination of the cultures.

Developing embryos were also exposed to a range of concentration of an inhibitor of steroidogenesis, spironolactone (Piorier et al., 2001) and an inhibitor of aromatase, formestane (Miller et al., 2003), separately. The embryos were sensitive in the high nanomolar and low micromolar range of the both inhibitors with $EC_{50}$ values of 18 μM and 2 μM, respectively (data not shown). Significant inhibition of normal development (>20%) occurred at concentration near 100 nM. The major type of teratogenicity found in the exposed embryos was a delay in growth or development wherein embryos were

appeared normal but did not reach a similar stage of larval development compared to controls.

To initially determine stage-specific specific binding of estradiol, $^{3}$H-17β-estradiol was incubated with homogenates of egg, blastula, gastrula, prism and pluteus stages and separated using Amicon YM-3 filtration units. A ratio of labeled/labeled+unlabeled statistically greater than one (1) was considered significant evidence of specific binding. After three trials, specific binding was measured in blastula and prism embryonic stages and pluteus larval stage. Eggs and gastrula stage embryos did not bind estradiol specifically (Fig. 4). The strongest signal for specific binding was in the prism and pluteus stages. Further binding experiments used prism and pluteus stages since the signal for specific binding appears to be the highest in these stages.

Specific binding of estradiol to homogenates of prism and pluteus stages incubated with $^{3}$H-17β-estradiol was also conducted using gel permeation column chromatography with Sephadex G-100 (Fig. 5). Specific binding of estradiol occurred in both pluteus and prism homogenates illustrated by the loss of the peak around fraction 10 when homogenates were incubated with 1000-fold excess of unlabeled estradiol (see arrows). The size of the protein specifically binding estradiol can be determined with column chromatography by correlating the protein peaks from the spectrophotometer with standard proteins of known molecular weight. The fraction 10 peak was slightly before the peak for the standard β-galactosidase indicating that the molecular weight of the estradiol-binding protein was > 116 kDa.

Estradiol binding was further examined by determining the estradiol association and dissociation curve in pluteus larval homogenates (data not shown). Saturation was

reached within 6 hr but was highly variable. Likewise, dissociation was also highly variable and was not fully attained until 4-6 hr. Due to the apparent slow rates of association and dissociation, rate constants ($k_1$ and $k_{-1}$) were not determined.

**Discussion**

Embryos from various invertebrates species are sensitive to estradiol and endocrine disrupting compounds, and these include molluscs, crustaceans and echinoderms (Hutchinson, 2002; Segner et al., 2003; Roepke et al., 2005). This study reports an alteration in the sensitivity of the developing sea urchin embryo to estradiol and EDCs following maternal exposure to the same compounds during oogenesis. To the best of our knowledge, this study presents some of the first evidence of maternal exposure effects on embryo sensitivity to EDCs in any invertebrate and the first to report the regulation of a receptor belonging to the steroid nuclear receptor superfamily by EDC exposure. Maternal exposure to estradiol, regardless of concentration, had the most significant effect on embryo sensitivity. The change in embryo sensitivity in the sea urchin may involve a change in the expression of a steroid nuclear receptor or some other unknown receptor-mediated pathway. However, no estrogen-responsive receptor (ER-like) has been identified in the sea urchin genome.

In vertebrate systems where basic reproductive endocrinology and steroid biochemistry is well characterized, maternal-exposure effects on embryos have been reported and do involve changes in ER expression. In fish, estradiol, ethynylestradiol, octylphenol and hydroxylated PCBs are all known to significantly affect embryos after

maternal exposure (Westerlund et al., 2000; Foran et al., 2002; Rasmussen et al., 2002). Similar results are found in rats and pigs exposed maternally to dioxin, octylphenol and bisphenol A (Barthold et al., 1999; Bogh et al., 2001; Kim et al., 2001; Yoshida et al., 2002). There is also evidence of paternal-exposure effects in fish to estradiol and bisphenol A (Shioda and Wakabayashi, 2000).

One possible mechanism of teratogenicity in the developing sea urchin embryos is the regulation of steroid nuclear receptors by exposure to estradiol and EDCs. Estradiol, estrogenic compounds and other endocrine disruptors are known to alter the expression and function of various nuclear receptors including ERs, retinoid X receptors (RXR) and peroxisome proliferator-activated receptor γ (PPAR) (Cappelletti et al., 2003; Nishikawa et al., 2004; Kanayama et al., 2005; Nishizawa et al., *in press*). In the sea urchin, only three orphan receptors from the steroid nuclear receptor superfamily have been identified (Kontrogianni-Konstantopoulos et al., 1996; Kontrogianni-Konstantopoulos et al., 1998; Kontrogianni-Konstantopoulos and Flytzanis, 2001). Messenger RNA for one of these receptors, SpSHR2, is expressed and stored in the maternal pool of mRNA in the oocyte and persists to the blastula stage. Maternally-derived protein is translated in the oocyte and is present throughout all early embryonic stages (Kontrogianni-Konstantopoulos et al., 1998). SpSHR2 has 50% amino acid identity to the orphan human testis receptor 2-11 with 79% and 62% amino acid identity in the DNA-binding and ligand-binding domains, respectively (Kontrogianni-Konstantopoulos et al., 1998).

As with other steroid nuclear receptors in vertebrate systems, the expression of SpSHR2 mRNA in sea urchin oocytes from females exposed to estradiol, octylphenol, TBT and DDD is differentially modified. This maternal-exposure effect of EDC exposure

has not been previously reported before in the invertebrate literature. The effects of maternal exposure to estradiol and EDCs on embryonic development and receptor-expression may be a form of gene imprinting as discussed by McLachlan et al. (2001). Fetal and neonatal exposure to estrogenic compounds in mammals can alter gene transcription producing long-term changes as a result of estrogenic compound exposure through mechanisms that change the pattern of DNA methylation and involve other cell signaling pathways.

A 1-fold increase in receptor expression was considered not only statistically significant but biologically significant as well. Estradiol, octylphenol and the lower concentration of TBT (0.5 μg/kg) increased the expression of SpSHR2 while the higher concentration of TBT (50 μg/kg) and both concentrations of DDD decreased expression. Surprisingly, the most effective injection treatment at altering the expression of receptor mRNA was the lower concentration of TBT. TBT has recently been reported to increase differentiation in adipocytes through a PPAR/RXR pathway (Kanayama et al., 2005) and is apparently able to enhance the transcription of androgen receptor (AR) responsive genes in prostate cancer cells (Yamabe et al., 2000).

It should be noted that the evidence presented here does not necessarily suggest SpSHR2 is the receptor through which estradiol and EDCs are exerting their teratogenicity nor does it imply that regulation of the SpSHR2 gene has to be controlled by steroid receptor transcription factors such as ER and AR. Results of this study showed that there is no obvious correlation between the changes in embryo sensitivity and the alterations in SpSHR2 mRNA expression.

While data regarding sea urchin reproductive endocrinology is limited, many of the key elements to the production and function of estrogens have been elucidated. Both estradiol and testosterone have been measured in gonadal tissue using radioimmunoassays and the presence of steroidogenic enzymes including 17β–hydroxysteroid dehydrogenase, 5α-reductase and 3α/β-hydroxysteroid dehydrogenase have been determined using radiotracers. However, aromatase enzyme activity and the production of estrogens has not determined in any study although the conversion of estradiol to estrone and estradiol esters has been reported (reviewed in Wasson and Watts, 2001). Androgen and progesterone metabolism has been demonstrated during the annual reproductive cycle of sea urchins (reviewed in Wasson and Watts, 2001). In the sea urchin embryo, inhibitors of steroidogenesis and aromatase activity significantly inhibit normal development in the low micromolar concentrations suggesting either a role for these enzymes in normal development or the inhibitors are functioning through some unknown mechanism of teratogenicity.

The production of estradiol and its binding characteristics in the developing sea urchin embryos has not been previously investigated. In this study, the majority of estradiol production during sea urchin development occurs during gastrulation. Specific binding of estradiol does not occur in that stage. Conversely, definite specific binding of estradiol occurs during late embryonic and early larval stages without significant estradiol production. The time lag between estradiol production and estradiol specific binding suggests that the developmental role of estradiol is not functional until later in development after gastrulation. The lack of specific binding in the gastrula may account for the lower sensitivity of this development stage to estradiol and estrogenic compounds

(Roepke et al., 2005). Conversely, the production of estradiol during gastrulation may decrease the specific binding of exogenous labeled estradiol in homogenates of gastrula embryos and may account for the lack of specific binding in the filtration centrifugation assay.

The only stage to exhibit both estradiol production and estradiol-specific binding was the blastula stage prior to hatching. Early embryos prior to hatching are also more sensitive to estradiol exposure (Roepke et al., 2005). Therefore, the blastula sensitivity is supported by these data and may be an indicator of maternal-effect receptors in the early embryo. Because no aromatase enzyme has activity has been identified in the sea urchin (Wasson and Watts, 2001), the production of estradiol could be the result of contamination of samples or the binding of the antibody to other estrogens or phytoestrogens. It has been suggested that estrogenic compounds from natural sources such as phytoestrogens from kelp, the main food source for *S. purpuratus*, may provide an exogenous mechanism for regulation of reproduction in adult sea urchins (Wasson and Watts, 2001).

One hypothesis is that specific binding of estradiol may occur in blastula stage embryos due to maternally-derived receptors (translation of stored maternal mRNA transcripts) and then occurs in late embryonic and early larval stages due to expression (transcription and translation) of embryonic receptors. Steroid receptors are expressed in vertebrate pre-implantation embryos although the role of these receptors is unknown (Hou and Gorski, 1993; Hiroi et al., 1999). The molecular weight of the specific binding protein in the column chromatography experiments is > 116 kDa which is higher than the molecular weight of the monomeric form of other steroid nuclear receptors (Carson-Jurica et al., 1990; Luisi et al., 1994). The potential binding protein in the sea urchin

homogenates may be in a dimeric form with associated proteins accounting for the higher molecular weight.

The kinetics of estradiol association and dissociation is slow and highly variable indicating a low capacity, low affinity receptor compared to binding in sea star pyloric caeca which has a low-capacity, high affinity estradiol binding protein (De Waal et al., 1982). In invertebrates, ER have been found in molluscs (Thornton et al., 2003) and crustaceans (Paolucci et al., 2002) but not in tunicates (Thornton, personal communication) or during a search of the Sea Urchin Genome Project database. Estrogenic compounds are known to have different affinities for the vertebrate ER in a species-dependent manner due to variation in the amino acid sequence within the ER ligand-binding domain (Matthew et al., 2000) that may explain the low affinity of the sea urchin estradiol binding protein. The low affinity binding of estradiol to the sea urchin homogenates suggests that the sensitivity of the embryos to estradiol is not functioning solely through this receptor since equal or lower concentrations (<50 nM) of estradiol (and other estrogenic compounds) significantly inhibit normal development.

In summary, the present study suggests that maternal exposure to endocrine disrupting compounds alters the sensitivity of embryos to teratogenic chemicals in invertebrates species and this is similar to vertebrates embryos. This alteration of sensitivity may involve receptors belonging to the nuclear steroid receptor superfamily or some other unknown receptor-mediated pathway (membrane receptors). The sensitivity changes and receptor regulation combined may be utilized as a functional and molecular marker of endocrine disruption in invertebrates (Rotchell and Ostrander, 2003). However, an estradiol-binding receptor in the sea urchin embryo was not clearly

identified even though there is evidence of estradiol production during gastrulation and low-capacity, low-affinity binding in the prism embryonic and pluteus larval stages.

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Table 1. Oligonucleotide sequences of primers used for PCR and TaqMan probes for analysis of SpSHR2

| Target | Primer | Sequence (5'->3') | Length | Probe | Probe sequence (5'->3') |
|---|---|---|---|---|---|
| **Sp rRNA** | Sp18S -232f | GGGATCAGATACGGCCCTAGT | 77 | Sp18S-258p | CCATAAACGATGCCGACTGACGATCC |
| **(18S rRNA)** | Sp18S-308r | GCCGCGTCATGGGAGTAA | | | |
| **SpSHR2** | SpSRH2-402f | CAATCGTAACCGCTGCCAGTA | 68 | SpSRH2-424p | TGCCGCCTGCAGAAGTGCCTC |
| | SpSRH2-469r | CAGAGTCCGACTTCATGCCCAT | | | |

Table 2. Comparisons of Embryo Sensitivity to Estradiol and EDCs after Maternal Exposure.

| **Injection Treatment** | **Control** | **E2** | **E2** | **OCT** | **OCT** | **TBT** | **TBT** | **DDD** | **DDD** |
|---|---|---|---|---|---|---|---|---|---|
| **Embryo Exposure Treatment** | | **(0.5 mg/kg)** | **(50 mg/kg)** | **(0.5 mg/kg)** | **(50 mg/kg)** | **(0.5 μg/kg)** | **(50 μg/kg)** | **(0.5 mg/kg)** | **(50 mg/kg)** |
| **Estradiol** | no Δ | decrease | decrease | no Δ | decrease | no Δ | no Δ | decrease | decrease |
| **Octylphenol** | no Δ | no Δ | +/- | no Δ | increase | increase | increase | no Δ | decrease |
| **Tributyltin** | no Δ | increase | increase | no Δ | increase | no Δ | increase | increase | increase |
| **DDD** | no Δ | increase | increase | increase | increase | no Δ | +/- | +/- | decrease |

no Δ = no significant change in abnormal development
increase = more sensitive (more abnormal development) after maternal exposure
decrease = less sensitive (less abnormal development) after maternal exposure
(+/-) = portions of dose response are significantly different compared to pre-exposure

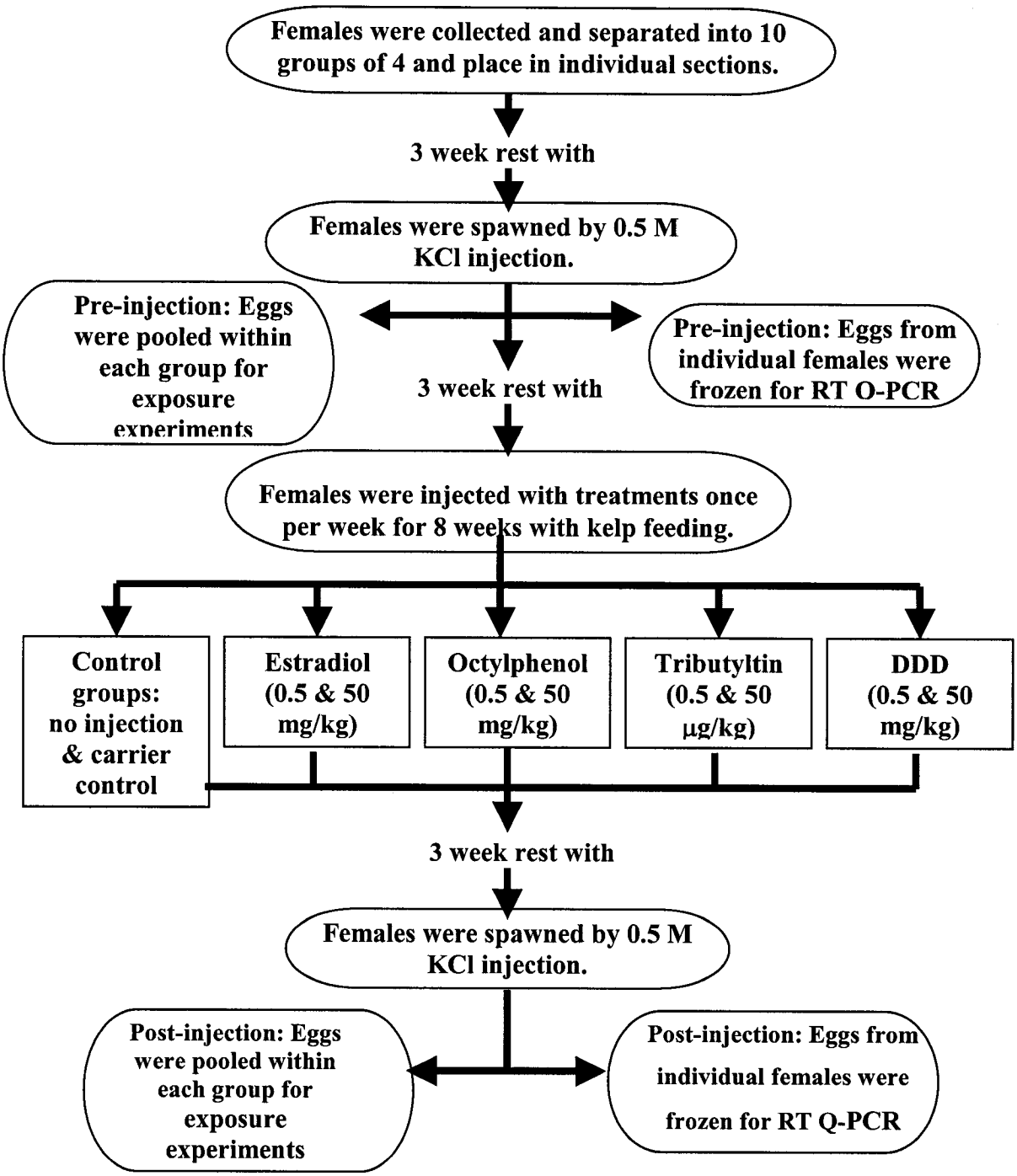


Figure 1. The experimental timeline for maternal exposure to estradiol and EDCs. See Methods section: Maternal exposure to EDCs by injection for details.

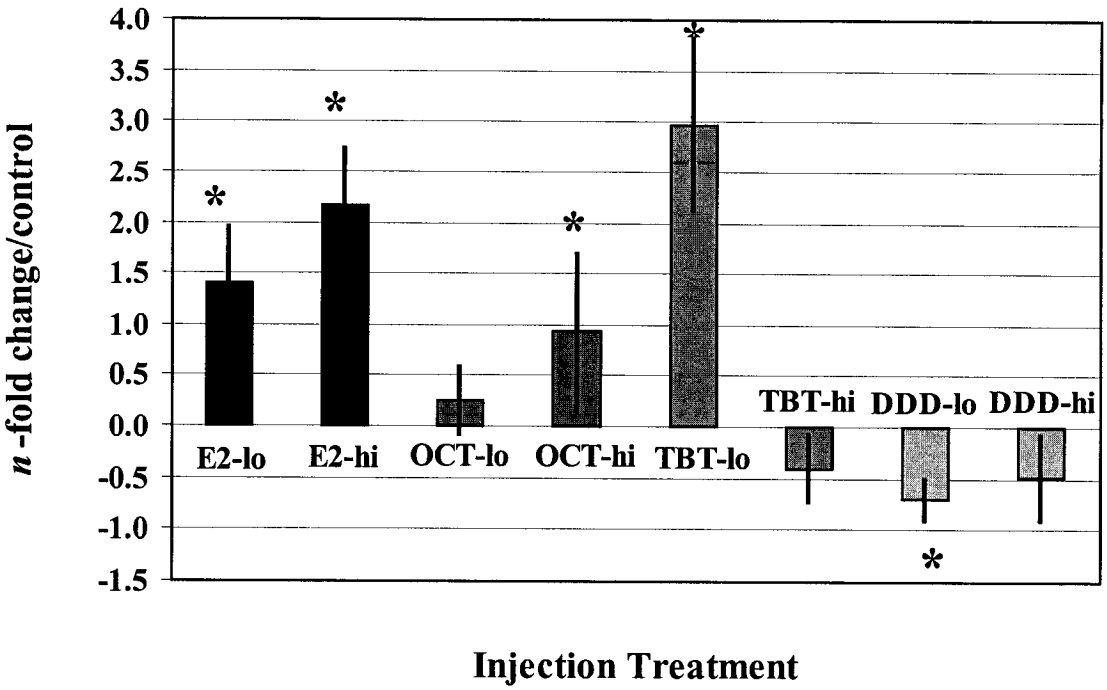


Figure 2. *n*-fold change in the expression of SpSHR2 mRNA in eggs from pre-maternal exposure and post-maternal exposure by real-time Q-PCR analysis. Data are expressed as the change in transcript in eggs from pre-exposure spawn to post-exposure spawn for each female in each treatment divided by the average change in transcript from pre- to post-exposure in the carrier control. Fold change was averaged for each injection treatment. Significant changes in transcript expression are noted by (*) ($P<0.05$). Injections treatment are estradiol (E2), octylphenol (OCT), tributyltin (TBT) and DDD (*o,p*-DDD) and are labeled either lo (0.5 mg/kg or 0.5 μg/kg for TBT) or hi (50 mg/kg or 50 μg/kg for TBT).

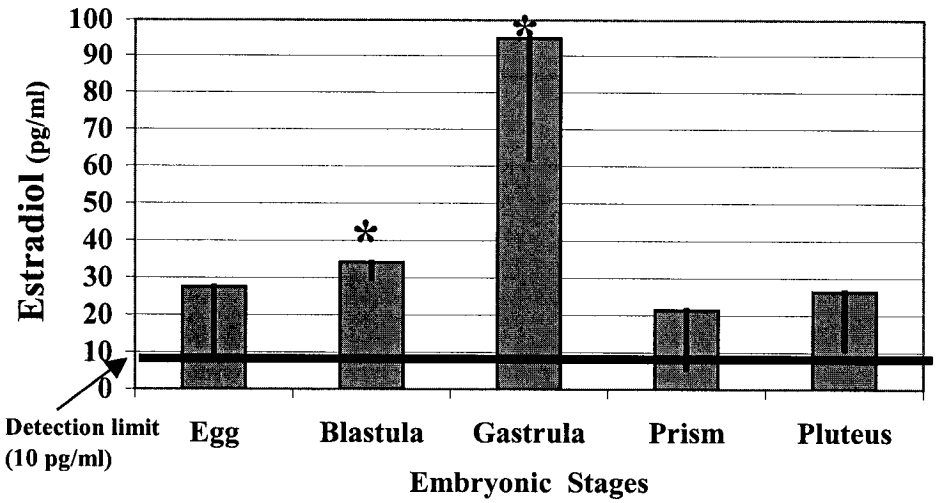


Figure 3. Amount of estradiol measured in homogenates of sea urchin eggs, embryos and larvae by radioimmunoassay using a highly-specific estradiol-binding antibody purchased from Niswender. Significant production of estradiol is considered above 10 pg/ml, the detection limit of the method and is noted by (*).

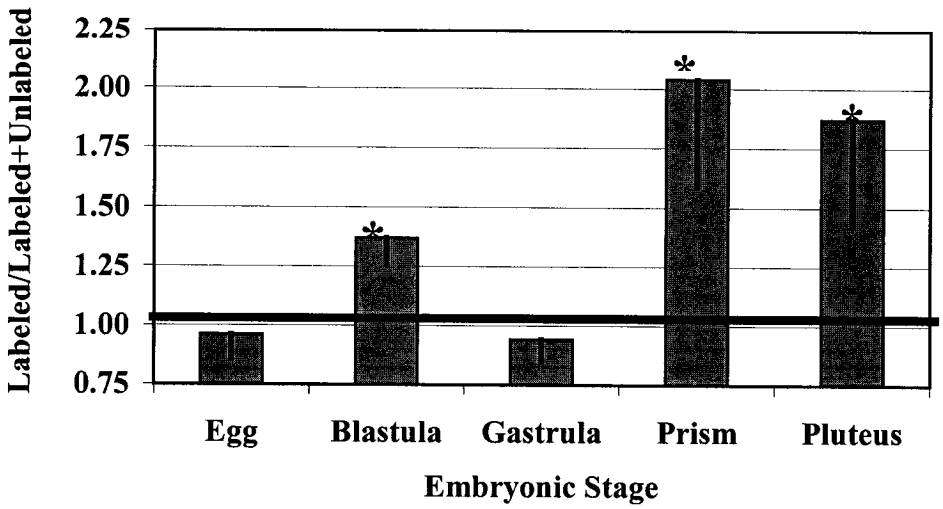


Figure 4. Counts per minute (CPM) of radiolabeled estradiol in the retentate from sea urchin developmental homogenates containing radiolabeled estradiol divided by the CPM of radiolabeled estradiol in the retentate from radiolabeled+1000-fold excess unlabeled estradiol using Amicon 3000 MW filtration units. Ratio data are averaged from three experiments. An average ratio greater than 1 (± std, $P<0.05$) is considered significant and evidence for specific binding in the respective homogenate.

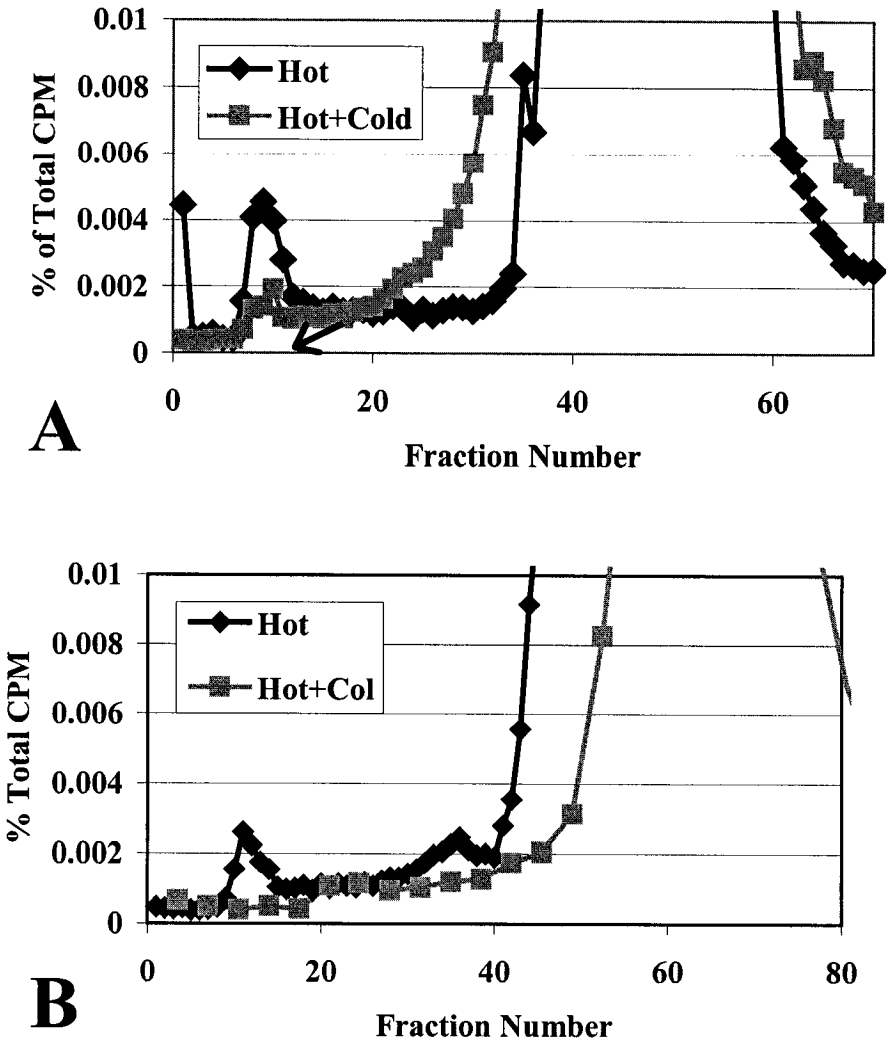


Figure 5. Sephadex G-100 column chromatography of sea urchin pluteus larval (A) and prism embryo (B) homogenates incubated with radiolabeled estradiol (hot, black diamonds) or radiolabeled estradiol+1000-fold excess unlabeled estradiol (hot+cold, gray squares). Data are expressed as percentage of the total CPM of each sample. Two protein standards, myosin and β-galactosidase, were also applied to the column separately to estimate the molecular weight of relevant peaks.

# CHAPTER 3

## An increase in multidrug transport activity is associated with oocyte maturation in sea stars

**Abstract**

Multidrug resistance (MDR) transporters are transmembrane proteins present in organisms as diverse as bacteria and mammals. In immature sea star oocytes, we observed mdr activity before and after oocyte maturation, induced by 1-methyladenine, using the fluorescent dye, calcein-AM. MDR activity increased by 2-3-fold 4-8 hours post-maturation (hpm). Kinetic experiments demonstrate changes in dye transport activity during the maturation process. Dye accumulation experiments using specific and non-specific dyes and inhibitors demonstrate a change in the transporter population occurs involving both P-gp and MRP-like transporters. Analysis using a monoclonal antibody to the mammalian mdr P-glycoprotein (P-gp) showed a 45 kDa protein increasing in intensity 5-7 hpm. Analysis using a polyclonal antibody to conserved peptides of sea urchin multidrug resistance-associated protein (MRP) shows three bands in the immature oocytes but only two in mature eggs (5 hpm). Dye accumulation experiments using transcriptional, translational and microtubule inhibitors suggest that translation and microtubule function are important in transport activity increases. Immunolabeling revealed a cytoplasmic storage of the P-gp and MRP protein prior to maturation, with the translocation of both transporters to the cell membrane 1 hpm. These mdr transporters may serve a protective role in oocytes and eggs as demonstrated by experiments using the maturation inhibitor vinblastine, which was far more effective in the presence of specific mdr transport inhibitors.

keywords: MDR, P-gp, MRP, sea star, oocyte maturation, translocation

## Introduction

Multidrug resistance (MDR) is an evolutionarily conserved efflux activity present in many cell types, and is mediated by the family of transmembrane ATP-Binding Cassette (ABC) superfamily transporters. MDR transporters export moderately hydrophobic compounds, lipids, conjugated organic anions, hormones and xenobiotics (reviewed in Krishna and Mayer, 2000 & Kruh and Belinsky, 2003). MDR proteins transport hydrophobic compounds from exocrine and endocrine glands (Sarkadi et al., 1994), epithelial microvillar transport function (reviewed in Lange and Gartzke, 2001) and the developmental physiology of plants (*Arabidopsis*) and molds (*Dictyostelium*) (Friml et al., 2003; Gaedeke et al., 2001; Good and Kuspa 2000). Relevant MDR efflux transporters are the permeability glycoproteins (P-gp) and multidrug resistance associated proteins (MRP) (Borst et al., 2000; Dean et al., 2001).

MDR transport is utilized by freshwater and marine species including fish, molluscs, sponges, sea urchins and echiuroid worms (Epel, 1998; Hamdoun et al., 2004; Kurelec, 1992; Smital and Kurelec, 1998; Smital et al., 2004) and may have a protective function by transporting cellular products and xenobiotics across the plasma membrane (Epel, 1998; Hamdoun et al., 2002; Kurelec, 1997; Toomey and Epel, 1993). Transporter expression in aquatic model systems is induced by exposure to various environmental toxicants (Minier et al., 1993; Smital and Kurelec, 1998). In invertebrate systems, P-gp-mediated efflux is the main focus of most research on efflux transporters although recent

evidence confirms the vital role of MRP-mediated efflux in a several species (Hamdoun et al., 2004; reviewed in Leslie et al., 2001).

In both vertebrates and invertebrates, MDR transporters are expressed in a variety of reproductive and developmental cell types. In humans, both MRP and P-gp are expressed in trophoblast cells and other placental tissues (Atkinson et al., 2003; Utoguchi et al., 2000). Murine oocytes and early embryos express P-gp which is thought to function as a protectant against toxicants (Elbling et al., 1993). Porcine oocytes express mRNA alone for both P-gp (*MDR1*) and MRP (*MRP1*) transporters (Takebayashi et al., 2001). Amphibian embryos express P-gp proteins and exhibit active P-gp mediated dye efflux (Bonfanti et al., 1998). In invertebrates, embryos from diverse phyla express MDR transporters including nematode, mollusc and echinoderm (Broeks et al., 1996; Hamdoun et al., 2004; McFadzen et al., 2000; Minier et al., 2002). Embryos of the echiuroid worm, *Urechis caupo*, which live in habitats naturally rich in environmental toxins, express P-gp transporter proteins (Hamdoun et al., 2002; Toomey and Epel, 1993).

In general, little is known regarding the specific functions of MDR transport activity in reproduction and development. However, a recent study demonstrated that a dramatic upregulation of MDR activity occurs in sea urchin eggs at fertilization, and this increase is post-translationally regulated (Hamdoun et al., 2004). MRP- and P-gp-like transporters are present in sea urchin eggs and embryos, with the majority of transport activity being associated with MRP (Hamdoun et al., 2004). While MDR transporters may have an as yet unknown regulatory role in early development, their transport activity

has been clearly linked to protection from exogenous compounds (Epel, 1998; Hamdoun et al., 2002, 2004; Toomey and Epel, 1993).

Sea star oocyte maturation is an event analogous to fertilization in that it involves a reorganization of the oocyte cytoplasm (Jaffe and Terasaki, 1993; 1994). Sea star oocytes are arrested in prophase of meiosis I in the ovaries and are induced to mature by 1-methyladenine released from the surrounding follicular cells at spawning. The primary characteristics of oocyte maturation are germinal vesicle breakdown, completion of meiosis I and II and female pronuclear formation. Sea star oocyte maturation is mediated by signaling molecules including cAMP, kinases, phospholipases, and phosphatases (reviewed in Meijer & Mordret, 1994; Kishimoto, 1998) and results in reorganization of the cytoplasmic contents including endoplasmic reticulum, ribosomes (Terasaki, 1994) and cortical granules (Santella et al., 1999) utilizing F-actin (Heil-Chapdelaine and Otto, 1996) and microtubules (Ookata et al., 1993). Oocyte maturation as determined by germinal vesicle breakdown is completed within an hour of 1-methyladenine addition. Injection of a higher concentration of 1-methyladenine into female sea stars will induce spawning within 1-3 hours during which the mature eggs separate from the supportive follicular cells and are released (Meijer and Guerrier, 1984). The purpose of this study was to determine the MDR transport activity of sea star oocytes and eggs, and to determine if there is an increase in MDR-mediated efflux associated with oocyte maturation.

## Materials and Methods

### 2.1. Reagents and chemicals

1-methyladenine, actinomycin D, cycloheximide, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), emetine, colchicine, 17β-estradiol, 17α-ethynylestradiol, 4-octylphenol, tributyltin, *o,p*-DDD, reversin (R-205), rhodamine B, 1-benzoyl-5-methoxy-2-methylindole-3-acetic acid (BMMA), Nonidet P-40, horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody and HRP-conjugated rabbit anti-goat antibody were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Calcein-acetoxymethylester (c-am), 5 (and 6)-carboxy-2',7'-dichlorofluorescein diacetate (CDFDA), Alexa Fluor® 488 goat anti-mouse IgM and Alexa Fluor® 488 rabbit anti-goat IgM was purchased from Molecular Probes (Eugene, OR, USA). The MRP inhibitor, MK571, was purchased from Cayman Chemical (Ann Arbor, MI, USA) and the P-gp inhibitor, PSC833, was a gift from Norvartis (Basel, Switzerland). The primary antibody to P-gp, C219, was purchased from Signet Laboratories (Dedham, MA, USA). The primary antibody to MRP was constructed by Santa Cruz Biotechnology, Inc., using multiple conserved amino acid sequences isolated from MRP genes of the sea urchin, *Strongylocentrotus purpuratus*. Stocks of each chemical were made in either dimethyl sulfoxide or methanol except 1-MA and tributyltin which were stored in filtered seawater (FSW). Paraformaldehyde for oocyte and egg fixation was purchased from Polysciences, Inc. (Warrington, PA, USA) and made into a 4% solution with FSW.

2.2. Animals and oocyte isolation

Adult *Asterina miniata* and *Pisaster ochraceous* were collected during their respective reproductive cycles and maintained at the Bodega Marine Laboratory (BML) in continuous flow-through, sand-filtered Bodega Bay sea water (13 ° C ± 2 °) with weekly feedings of invertebrate food pellets, shrimp or mussels. Immature oocytes were isolated by boring a quarter inch hole in the upper arm near the disc of 2-3 females and removing pieces of the intact ovaries (to avoid confusion, immature oocytes will be called oocytes and mature eggs will be called eggs). Ovarian pieces were transferred to cold calcium-free ASW and shaken to force the release of oocytes from follicular cells and the ovaries. Separated oocytes were separated from the ovarian pieces by filtration through a 250 μm mesh screen and washed three times in 0.7 μm FSW. Oocytes were examined under a phase-contrast microscope for the presence of germinal vesicles and overall appearance and tested for germinal vesicle breakdown (GVBD) after addition of 1-methyadenine (1-MA). GVBD is a standard measurement of oocyte maturation in the sea star. The germinal vesicle is visible in the immature oocyte and disappears within an hour after hormone stimulation. During GVBD, the nuclear envelope separates from the cytoplasmic matrix and becomes ragged or misshapened eventually to disintegrate (Meijer and Guerrier, 1984). Inhibitors of oocyte maturation would decrease the percentage of oocyte maturing and undergoing GVBD.

2.3. Fluorescence imaging of dye efflux

Isolated oocytes were diluted into 15 ml conical tubes filled with 9 ml FSW. 1 ml of 1 mM 1-MA (final concentration 100 μM) was added to tubes labeled for mature eggs for a final volume of 10 ml. Ooctyes were allowed to mature for 1-5 hours (hours post maturation = hpm) before dye was added. At the appropriate maturational time points, 0.5 μM calcein-acetoxymethylester (c-am) was added to 15 ml conical tubes containing oocytes or eggs and incubated for 2 hr at 15 °C on a slow shaker table. After the incubation period and settling, oocytes and eggs were transferred to immunoslides and viewed using a fluorescent microscope. Fluorescent images of 20-30 immature oocytes and mature eggs at 3 and 7 hpm were collected using an Olympus BX50WI fixed stage microscope, equipped with a xenon illumination source, fluorescence objective lenses, and a Roper Scientific CoolSnap HQ cooled CCD camera interfaced to a computer through the imaging program, Metamorph® (Universal Imaging Corp.). Using Metamorph®, filters were designed for an excitation wavelength of 490 nm and an emission wavelength of 535 nm for all dyes used. The mean pixel fluorescence intensity of individual oocytes and eggs was measured and averaged for each time point.

After confirmation of dye efflux activity, a dose response curve for reversin 205 (R205), inhibitor of MDR-mediated dye efflux, was determined for both oocytes and eggs. MDR inhibitors are used to verify functional MDR transport and, if transport activity is present, should result in an increase in fluorescence in relation to the untreated control. R205 (1-20 μM) was added with 0.5 μM c-am to tubes containing oocytes and eggs (1 hpm) and incubated for 2 hr at 15 °C. The samples were analyzed by fluorescence microscopy after the incubation period. A dose of 3 μM R205 was

determined to be the most appropriate final concentration. All further experiments with R205 used this concentration unless noted.

To determine the dye accumulation kinetics during the oocyte maturation transition, oocytes were attached to two polylysine-coated 2-chambered glass slides. Oocytes were incubated in a 1% BSA blocking buffer for 15 min at 15 °C and washed 3 times in FSW. A prepared solution (3 ml) of FSW and 0.5 μM c-am dye was added at t=0 and recordings of fluorescence were started at 10 min intervals for 60 min. At t=60, 30 μl of 10 mM 1-MA was added to the three of the chambers and recordings of fluorescence continued for over 2 hr at 10 min intervals and then 15 min intervals for the final hour. Specific MDR inhibitors were added to two chambers to determine the type of transporter involved in the change in dye efflux during maturation. During experiments with the specific inhibitors, recordings were taken every 15 min for 2.5 hrs (t=150). At t=150, the P-gp-specific inhibitor, PSC833 (10 μM), and the MRP-specific inhibitor, MK571 (10 μM), were individually added to one of the chambers containing matured eggs. Recordings were taken at 10 min intervals for another 90 min (t=240) and analyzed according by MetaMorph®.

For experiments involving cell function inhibitors, oocytes were isolated and prepared for incubation. Maturation was induced by 1-MA and cell function inhibitors were added 20 min later to ensure proper maturation. Transcriptional inhibitors, actinomycin D (AD) and DRB, were used at 1 μM and 100 μM, respectively. Translational inhibitors, cycloheximide (CY) and emetine (EM), were used at 2.5 μM and 100 μM, respectively. The microtubule inhibitor, colchicine (COL), was used at 100 μM. The samples were incubated for 2 hr with the cell function inhibitors. Samples were

washed with FSW because the cell function inhibitors are substrates for MDR transporters (Litman et al., 2001). C-AM (0.25 μM) was added to the tubes and incubated for 2 hr at 15 °C. Samples were prepared for and analyzed by fluorescence microscopy.

To compare the different transporters functioning in each maturational state, samples of oocytes and eggs (1 hpm) were incubated with two dyes, either c-am (0.25 μM) or an MRP-specific dye, CDFDA (10 μM, Zamek-Gliszczynski et al., 2003), for 2 hrs with various transport inhibitors. The specific inhibitors used were PSC833 (5 μM for c-am, 25 μM for CDFDA), MK571 (5 μM for c-am, 25 μM for CDFDA), and BMMA (10 μM for c-am, 25 μM for CDFDA), a putative MRP1 inhibitor (Maguire et al., 2001). Other inhibitors or substrates such as R205 (3 μM for c-am, 25 μM for CDFDA) and rhodamine B (RhoB, 1 μM for c-am, 25 μM for CDFDA) were utilized in the dye accumulation experiments. Rhodamine dye is believed to be P-gp specific (Twentyman et al., 1994) and rhodamine B is not a substrate in systems where MRP is the primary transporter such as the sea urchin egg (Hamdoun et al., 2002; 2004; Toomey and Epel, 1993). A dose response curve for c-am dye accumulation using PSC833, MK571 and BMMA was conducted to determine the appropriate concentration of these inhibitors. Concentrations of rhodamine B were obtained from published data (Hamdoun et al., 2002; Toomey and Epel, 1993).

To determine if reproductive steroids and endocrine disrupting compounds were substrates for the sea star MDR transporter proteins, 17β-estradiol ($E_2$, 1 μM), 17α-ethynylestradiol ($EE_2$, 1 μM), octylphenol (OCT, $2.5 \times 10^{-3}$ μM), tributyltin (TBT, $3 \times 10^{-4}$ μM), and *o,p*-DDD (0.5 μM) were added to the tubes containing either oocytes or eggs (1

hpm) and incubated for an hour. To each sample, 0.25 μM c-am was added and incubated for 2 hr at 15°C. Colchicine (COL, 250 μM) was used a positive control as it is a known substrate for P-gp transporters (Litman et al., 2001) and is a substrate in its GSH-conjugated form for MRP transporters (Loe et al., 1996).

2.4. Immunoblotting

For immunoblots with C219, samples of oocytes, eggs (1, 3, 5 and 7 hpm) and eggs matured in the presence of cell function inhibitors for 5 hpm from both species were collected into conical tubes, hand centrifuged and flash frozen in liquid nitrogen after removal of all FSW. Samples were pulverized using a mortar and pestle chilled with liquid nitrogen and homogenized with 2-3x volume of ice-cold Tris-HEPES-EDTA-sucrose buffer with general protease inhibitor cocktail (Sigma). Protein concentrations of the homogenates were determined using a standard BCA protein assay (Pierce). Solubilized homogenates (10 μg of total protein/sample) were loaded onto 10% SDS-polyacrylamide gels and run at 155V for 90 min at room temperature. Proteins were transferred to nitrocellulose for 1h at 115V in a cooled chamber. Blots were blocked for 2 hr in 4% non-fat milk in Tris-buffered saline (10 mM Tris base, 150 mM NaCl, pH 7.4) and stored overnight in Tris-buffered saline with 1% Tween. P-gp protein was labeled by incubating blots for 2 hr with the mouse monoclonal antibody C219 at 1 μg/ml, followed by the HRP-conjugated goat anti-mouse (1:5000) secondary antibody for 1 h (Sigma). HRP-labeled P-gp protein was visualized by reaction with chemiluminescent reagent (Pierce) according to manufacturers instructions and photographed using a EpiChem

Digital Darkroom. Fish gill homogenate, known to express P-gp, was used as a positive control. Embryos from the sea urchin, *Lytechinus anamesus*, which do not express a C219-reactive P-gp (Hamdoun et al., 2002; Toomey and Epel, 1993), served as a negative control.

For immunoblots with spMRP, samples of oocytes and eggs (1, 3 and 5 hpm) were collected, washed and concentrated to 50 µl volume using a tabletop picofuge. Excess FSW was removed and the samples were transferred to a glass mini-homogenizer. The ice cold homogenization buffer contained 200 µl THE–sucrose buffer with 1 mM PMSF and 50 µl 2x protease inhibitor cocktail (Sigma). Samples were homogenized on ice for several minutes avoiding bubbles. Samples were transferred to 2 ml microfuge tubes and centrifuged for 5 min at 5000 rpm at 4 °C. The supernatant was removed, and prepared for gel electrophoresis by adding an equal volume 2x Laemmli buffer. The pellet was resuspended in the homogenization buffer, mixed and prepared for gel electrophoresis. Protein concentrations of homogenates were determined using a standard BCA protein assay (Pierce). Solubilized homogenates (10 µg of total protein/sample) were loaded onto 10% SDS-polyacrylamide gels and run at 155V for 90 minutes at room temperature. Proteins were transferred to nitrocellulose for 1h at 115V in a cooled chamber. Blots were blocked for 2 hr in 4% non-fat milk in TBS and incubated overnight with primary spMRP antibody (1:1000) in TBS at 4 °C. The blots were incubated in the HRP-conjugated rabbit anti-goat (1:5000) secondary antibody for 2 h at room temperature. HRP-labeled MRP protein was visualized by reaction with chemiluminescent reagent (Pierce) according to manufacturers instructions and

photographed using a EpiChem Digital Darkroom. Homogenized mouse liver was used as a positive control.

2.5. Immunocytochemistry

To determine the movement of the P-gp transporter within the oocyte during maturation, we labeled fixed and permeated ooctyes and eggs (1, 3 and 5 hpm) with C219. The oocytes were extracted from female sea stars and prepared as above. Immature oocytes were immediately fixed in 4% paraformaldehyde in FSW and stored at 4 °C overnight. At the appropriate time points after the addition of 1-MA, the eggs were also fixed and stored. Subsamples of eggs matured in the presence of either AD (1 μM), CY (2.5 μM) or COL (100 μM) for 5 hr were also fixed and stored overnight. The next day, samples were washed three times in Ca-ASW for 10 min followed by a 1 h incubation in 1% NP-40 in phosphate-buffered saline (PBS, Pierce #28374) to permeabilize the cell membrane. The samples were washed for another 3x10 min washing in PBS and transferred to microfuge tubes. Samples were blocked in BSA (4% in PBS) for 1 h and then incubated for 1 h in primary antibody C219 in PBS (1:50) at room temperature. The samples were washed 1x5 min in PBS and incubated for 30 min in a 1:10 dilution of goat serum and washed 3x10 min in PBS. The oocytes and eggs were finally incubated for 1 h in a 1:500 dilution of the secondary antibody, Alexa 488 goat anti-mouse with gentle mixing in the dark. Samples were washed 1x5 min in PBS and resuspended in glycerol mounting media and stored at –20 °C. A secondary control (no primary) of oocytes was also prepared and stored.

Immunocytochemistry using the spMRP antibody was similar to the C219 protocol until the BSA blocking step. After the PBS wash cycle, the samples were blocked in bulk in 15 ml conical tubes in 4% BSA (PBS) overnight at 4 °C (100 μl of 1% sodium azide was added for long term storage). After overnight incubation in BSA, subsamples were transferred to microfuge tubes and washed 1x 10 min in PBS. Primary spMRP antibody (1:100 dilution in PBS) was added to the sample while in fresh PBS for 3-4 hr at room temperature with gentle mixing. The primary antibody was removed followed by another 1x5 min wash in PBS. The sample was blocked in a 1:10 dilution of rabbit serum in PBS for 1 h and washed 3x10 min in 1% BSA in PBS. The secondary antibody, Alexa 488 rabbit anti-goat (1:500) was added to the samples in fresh PBS for 2 h with gentle mixing in the dark. The secondary was removed and, after 1x5 min wash in PBS, glycerol mounting media was added to the samples, mixed and stored at –20 °C. A secondary control was also prepared.

All samples were viewed using an Olympus Fluoview 500 laser scanning confocal microscope (equipped with AOTF for complete channel separation) mounted on an Olympus BX60WI fixed stage upright microscope. Samples were imaged using a 10x fluorescence water immersion objective lens. Images (both fluorescence at 488nm excitation and differential interference contrast) were collected as single scans or as Z series with 5 μm optical sections followed by Z series projection using the Fluoview software (Olympus Corp., NY).

2.6. Data analysis

Fluorescence intensity was measured for individual oocytes and eggs using image analysis macros provided with MetaMorph® (Universal Imaging Corp., Downington, PA) software. All absolute measurements were averaged (± 1 std. dev.). Data were reported either as absolute fluorescence with differences between treatments analyzed using either two sample paired t tests of means or ANOVA ($P<0.05$) or data were reported as a relative fluorescence (treatment/control) and averaged over the three experiments (± 1 std. dev., $P<0.05$). Protein band intensity on immunoblots were analyzed using UVP Labworks® (UVP, Inc; Upland, CA, USA).

**Results**

3.1. MDR expression and dye accumulation during oocyte maturation

To assess the activity of dye transport during oocyte maturation, immature oocytes and mature eggs were exposed to c-am for 2 hours and the amount of dye accumulation was analyzed using fluorescence microscopy (Fig. 1A). The amount of dye accumulation in oocytes was twice the amount recorded in eggs 3 hpm and three times that of eggs 7 hpm. Immature oocytes do have some functional MDR activity as evidenced by an increase in dye accumulation during co-incubation with R205. The decrease in dye accumulation in mature eggs was mostly due to the increase in transporter activity as demonstrated by the equal inhibition of transport activity in treated eggs and untreated oocytes.

Calcein-acetoxymethylester is a non-fluorescent MRP and P-gp substrate (Essodaigui et al., 1998). C-AM is membrane permeable but, once inside the cell, cellular esterases hydrolyzes the acetoxymethylester creating membrane impermeant, fluorescent calcein which accumulates in the cell. Cells with little to no transport activity will accumulate more calcein which is not a substrate for MDR transporters. In cells with a higher level of transport activity, c-am is effluxed before esterases cleave the c-am ester bond and the cells will accumulate less calcein.

Specific inhibitors of P-gp and MRP-mediated transport have been developed for use with chemotherapeutic drugs in the treatment of cancer including PSC833 and MK571. These inhibitors are highly specific and are believed not to interact with other members of the MDR family of ABC transporters. Concentrations in the low micromolar range of both PSC833 and MK571 are known to inhibit the efflux of P-gp and MRP substrates, respectively (Hamdoun et al., 2004; reviewed in Krishna and Mayer, 2000). PSC833 is a P-gp specific transporter and is related to cyclosporin D while MK571 is a MRP specific transporter and is a leukotriene analog (reviewed in Krishna and Mayer, 2000). R205 is a small peptide known to inhibit the efflux capacity of P-gp proteins (Sharom et al., 1999). R205 is considered a general inhibitor of P-gp transporters but the interaction of R205 with MRP proteins is unknown.

Functional MDR activity was not essential for normal oocyte maturation since oocytes incubated with R205 (3 μM), PSC833 (10 μM) or MK571 (10 μM) did not spontaneously mature, and oocytes were not inhibited from maturation via addition of 1-MA (data not shown). However, MDR transport activity may function as a protective mechanism during sea star oocyte maturation as evidenced by the decrease in the

percentage of GVBD (oocyte maturation) when oocytes were incubated with vinblastine, an inhibitor of sea star oocyte maturation (Meijer and Guerrier, 1984), and the specific transport inhibitors. Vinblastine (300 μM), used alone, decreased GVBD by 42 ± 8% but, vinblastine, incubated with PSC833 (10 μM) or MK571 (10 μM), decreased the percentage of GVBD by 69 ± 5% and 73 ± 7%, respectively. Both specific inhibitors significantly increased inhibition of maturation by vinblastine.

The increase in dye efflux (decreasing dye accumulation) after the two hr incubation period correlated with an increase in the intensity of the bands on the immunoblots using the P-gp antibody, C129 (Fig. 1B). The intensity of the 3, 5 and 7 hpm bands were 79%, 103% and 156% greater than the immature oocyte band. The monoclonal antibody C219 specifically binds to a highly conserved amino acid sequence (VQEALD) of the ATP-Binding Cassette domain (Georges et al., 1990). In the sea star, the antibody recognized a protein in the 45 kDa range. When protein samples from oocytes and eggs (1-3 hpm) were not reduced by addition of β-mercaptoethanol to the Laemmli buffer, two diffuse bands at 220 kDa and 45 kDa were apparent (Fig. 1B).

Figure 2 illustrates the kinetics of dye accumulation during the transition from immature oocyte to mature egg and the involvement of P-gp and MRP-like transporters. In the immature oocytes, dye accumulation occurred at a steady rate for 2.5 hr (Fig. 2A). Dye accumulation continued at a slower rate for the next 1.5 hrs. In the maturing egg, dye accumulation was similar to the immature oocyte until 1 hour after 1-MA addition (t=120). The eggs completed maturation at that time and the rate of dye accumulation significantly decreased to ~ 0 units/min for 60 min and slowly increased after t=180. When specific inhibitors were added 1.5 hours after 1-MA (t=150), dye accumulation

increased at a similar rate as early immature oocytes (~23 units/min; Fig. 2B). Increases in dye accumulation with PSC833 or MK571 (10 μM of each) were not significantly different.

To determine what cellular processes were important for the increase in MDR activity at maturation, immature oocytes were incubated in the presence of transcriptional, translational and microtubule inhibitors. Several of the cell function inhibitors (colchicine, emetine) are known to inhibit oocyte maturation (Meijer and Guerrier, 1984); therefore, the inhibitors are added at the end of the hormone-dependent period of oocyte maturation (20 min after 1-MA addition). Data are expressed as relative fluorescence values that are dependent on base level of c-am accumulation. A ratio greater than 1.0 ±1 std. dev. was considered significant. Eggs matured in the presence of inhibitors of translation (cycloheximide and emetine) and microtubule function (colchicine) accumulated c-am at level similar to oocytes but significantly higher than eggs (4 hpm; Fig. 3A). Transcriptional inhibitors (actinomycin D and DRB) did not interfere with the increase in dye efflux in the maturing eggs. The concentration of colchicine used for inhibiting the increase in dye efflux activity was high because colchicine is a substrate for MDR transporters (Litman et al., 2001; Loe et al., 1996). An immunoblot of oocyte and egg protein samples treated with the cell function inhibitors using C219 also demonstrated the importance of translation in the increase in P-gp associated activity (Fig. 3B). Bands from eggs treated with cycloheximide and emetine were 41.1% and 21.1% less intense than the 5 hpm samples, respectively. Eggs treated with colchicine did not have a decrease in band intensity.

A dose response (1-20 μM) with both PSC833 and MK571 in immature oocytes and mature eggs (3 hpm) was conducted to determine the appropriate concentration for inhibition of c-am efflux (Fig. 4A). The specific inhibitors did not affect oocyte maturation at 20 μM. PSC833 was a stronger inhibitor of c-am efflux in both oocytes and eggs than MK571 at all experimental concentrations except at 20 μM. However, PSC833 and MK571 inhibited c-am efflux in mature eggs significantly more than in immature oocytes. At 20 μM, many of the mature eggs burst after the 2 hr incubation period for both specific inhibitors. For the remaining experiments with PSC833 and MK571, the experimental concentration chosen was 5 μM to avoid saturation of the fluorescence signal and adverse effects on the oocytes and eggs.

To determine what type of MDR transporters are involved in both immature oocytes and mature eggs dye efflux and their relative importance, dye accumulation experiments were conducted with three specific inhibitors of mammalian MDR transport. Dye accumulation data were expressed as relative fluorescence values (control fluorescence noted on figures). All inhibitors increased c-am accumulation (compared to control values) significantly ($P<0.05$) in both immature oocytes and mature eggs. The relative c-am accumulation due to PSC833 and MK571 treatment, at 5 μM, was significantly less than dye accumulation with R205 (3 μM, Fig. 4B; $P<0.05$). The relative differences between R205 and PSC833 in inhibiting dye efflux in the oocytes may be a result of differences in affinities between the two P-gp inhibitors. After maturation, the c-am accumulation with all three inhibitors was not significantly different (Fig. 4C).

To broaden the characterization of sea star MDR transport activity, immature oocytes and mature eggs were incubated separately with two dyes (c-am and CDFDA, a reported MRP-specific dye) and an assortment of inhibitors or substrates (R205, MK571, PSC833, BMMA and RhoB). C-AM efflux in the immature oocyte was inhibited 2 to 3-fold by R205 (Fig. 5A). BMMA, a putative MRP1 inhibitor (Maguire et al., 2001), did not inhibit c-am efflux significantly in the immature oocyte, suggesting that little to no MRP1-mediated dye efflux was occurring in the oocyte. The P-gp substrate, RhoB (Toomey and Epel, 1993), did inhibit dye efflux in the immature egg as did a combination of BMMA and RhoB. In the mature egg, c-am efflux is inhibited significantly by all treatments including BMMA alone (Fig. 5B).

In immature oocytes, CDFDA efflux was inhibited by R205 and MK571 (Fig. 6A). R205 did not inhibit CDFDA efflux to the same degree as c-am efflux. BMMA, PSC833 and RhoB did not significantly increase dye accumulation. In mature eggs, R205, MK571 and BMMA increase CDFDA accumulation while RhoB and PSC833 did not (Fig. 6B). The most notable difference between oocytes and eggs in terms of CDFDA accumulation was the potency of BMMA. The decrease in dye accumulation in the mature eggs compared to immature oocytes was suggestive of an increase in the overall population of MRP transporters.

CDFDA is one of the few commercially-available specific dyes for detection of MRP transporters in mammalian cells and is a known substrate for at least three types of MRP transporters (MRP1-3) (Zamek-Gliszczynski et al., 2003). However, the substrate specificity for other MRP transporters (MRP4-9) for CDFDA is unknown. CDFDA is an diacetate ester of CDF (5 (and 6)-carboxy-2',7'-dichlorofluorescein) that is permeable to

plasma membranes and, like c-am, is hydrolyzed to a membrane impermeant form by cellular esterases. CDFDA is a substrate for MDR transporters in the esterified form, but unlike c-am, is also a substrate for MRP transport in its nascent structure at a lower affinity (Zamek-Gliszczynski et al., 2003). Inhibition of MRP-mediated transport should therefore increase the cellular accumulation of CDFDA.

3.2. Immunocytochemistry with P-gp specific antibodies

To determine the location of P-gp-like transporter in the oocyte and its movement during maturation, oocytes and eggs were fixed and incubated with a P-gp or MRP-specific antibodies and viewed using confocal microscopy. In immature oocytes, the P-gp was localized throughout the cytoplasm and the periphery of the cell. The germinal vesicle was the only portion of the oocyte without labeling (Fig. 7A). At 1 hpm, P-gp was primarily located at the periphery of the maturing egg although a number of foci remained localized to the cytoplasm. A Z-series through an immature oocytes demonstrates the cytoplasmic distribution of the protein surrounding the germinal vesicle. Another Z-series through a mature egg (1 hpm) illustrates localization of P-gp at or near the cell surface (Fig. 7B). During the next 4-5 hr, an increase in antibody labeling occurred suggesting that protein translation and translocation to the periphery occurred within the 5 hr time period (Fig. 8). The maturing eggs fixed at 4 hpm exhibited more P-gp protein localized to the cytoplasm than the 3 or 5 hpm eggs (data not shown).

In maturing eggs exposed to cell function inhibitors during the maturation process (until 5 hpm), the increase in cortical/surface P-gp may have been a result of both

translation and trafficking through the vesicle-to-membrane translocation pathway (Fig. 8). Cycloheximide (CY) inhibited the amount of antibody recognition when compared to the 5 hpm (Fig. 8). Colchicine (COL) inhibited the translocation of the protein to the periphery and resulted in increased antibody recognition in the cytoplasm compared to the 5 hpm (Fig. 8). Actinomycin D (AD) did not result in any significant differences compared to the 5 hpm (Fig. 8).

3.3. Immunoblotting and immunocytochemistry with MRP-specific antibodies

Protein analysis using a polyclonal antibody to conserved peptide sequences of sea urchin MRPs reveals three bands in immature oocytes with affinity for the antibody. These bands are approximately at 180 kDa, 80 kDa and 65 kDa (Fig. 9A). In mature eggs (5 hpm), the lower molecular weight band has disappeared leaving only the 180 kDa and the 80 kDa bands. Band intensities are not significantly different.

To determine changes in the location of MRP-like transporter proteins in the immature oocyte and mature egg, oocytes and maturing eggs were fixed and incubated with a sea urchin polyclonal antibody, spMRP. Unlike C219, the transporters localized with spMRP were distinctly localized throughout the cytoplasm in the immature oocyte without evidence of a germinal vesicle (Fig. 9B). At 1 hpm, MRP was translocated to the periphery in a similar manner as P-gp (Fig. 9B). At 5 hpm, the labeled proteins increased in density and again were localized throughout the cytoplasm of the egg (Fig. 9B). Actinomycin D (AD) did not interfere with the increase in density and translocation (Fig. 9B). Cycloheximide (CY) very slightly decreased the amount of MRP labeling after 5 hr,

and colchicine (COL) appeared to decrease the translocation of the protein to the periphery after 5 hr (Fig. 9B).

**Discussion**

This study has shown that MDR transport activity is present in immature oocytes, and that following *in vitro* maturation, dye accumulation decreases 2 to 3-fold within hours of maturation, demonstrating a dramatic increase in MDR activity. Transporter activity increases within one hour of 1-MA addition and the post-maturation upregulation of MDR activity is associated with both P-gp-like and MRP-like transport activities. The increase in post-maturation MDR activity entails both an initial translocation of stored MDR transporters to the periphery of the maturing egg followed by a cycle of translation of mRNA and translocation of new transporter proteins.

In immature oocytes, the MDR inhibitor, R205, appears to saturate the MDR-mediated transport of c-am. Depending upon c-am concentration, oocytes incubated with R205 (3 μM) would often exhibit fluorescence that was at or above signal saturation. The apparent saturation of MDR transport activity by R205 in oocytes suggests a low number of functional transporters. Increases in c-am accumulation in the presence of the P-gp-specific inhibitor, PSC833, occurs in immature oocytes indicating functional presence of a P-gp-like transport activity. P-gp-like activity is also suggested by the inhibition of dye efflux by rhodamine B since rhodamine dyes are thought to be P-gp specific (Twentyman et al., 1994). Analyses using immunoblotting and immunocytochemistry with the P-gp monoclonal antibody, C219 is further confirmation

of a P-gp-like transporter in the immature oocyte. The distinct labeling of the P-gp-like protein at the immature oocyte surface indicates that P-gp may be the primary transporter at this stage.

The sea star P-gp transporter has two characteristics that are different from vertebrate P-gp transporters. First, the C219-reactive protein migrates at ≅ 45 kDa rather than the 150-180 kDa range that vertebrate P-gp transporters migrate to on SDS-PAGE (Litman et al., 2001). Second, the presence of two bands in the unreduced immature oocyte samples suggests a linkage by disulfide bonds in the C219-reactive protein that is similar to the breast cancer resistance protein (BCRP, Doyle and Ross, 2003). BCRP can function as a half transporter and is linked by a disulfide bond. The reduced BCRP migrates to 70 kDa while the unreduced homodimer migrates to 140 kDa. Mitoxantrone, a known substrate for BCRP, did not affect c-am accumulation in either oocytes or eggs indicating that the C219-reactive protein may not be a BCRP-related transporter, rather it may be a novel P-gp-like transporter. Further research is needed to determine if the 45 kDa protein is a fully functional transporter or a non-functional subunit observed with reduced SDS-PAGE and immunoblotting. Complete sequencing of the sea star P-gp and an analysis of the secondary structure for cysteine bridges will confirm or deny this hypothesis.

Increases in c-am and CDFDA accumulation in the presence of the MRP-specific inhibitor, MK571, occurs in immature oocytes. The inhibition of dye efflux by MK571 suggests that a portion of dye efflux activity is MRP-mediated. The MRP1-specific inhibitor, BMMA, did not significantly affect c-am or CDFDA accumulation in the immature oocyte indicating that MRP1 is not functional in the immature oocyte. This is

consistent with the suggestion that P-gp is the primary transporter in the immature oocyte as discussed above. In the oocytes, the low intensity labeling of MRP-like transporters at the cell membrane by the polyclonal spMRP antibody also suggests that MRP-mediated efflux is not a major contributor to dye efflux. The spMRP antibody also binds to at least three specific proteins in the immature oocyte. MRPs usually migrate around 190 kDa on SDS-PAGE (Litman et al., 2001) which is similar with one of the protein bands in the immature oocyte.

PSC833 and RhoB, both thought to be P-gp specific, lacked any efficacy in inhibiting CDFDA efflux, thus confirming MRP-specificity of CDFDA. The MDR inhibitor, R205, does not inhibit CDFDA transport as effectively as c-am transport. R205 is known to interact with P-gp transporters (Sharom et al., 1999) but the interactions with MRP transporters are not defined. The data in this paper suggest that at least in the sea star oocyte, R205 does interact with MRP transporters yet is a superior substrate for P-gp transporters as compared to MRP.

The relative amount of dye efflux activity increased 2-fold three hours after addition of 1-methyladenine. Dye accumulation did not saturate the detection method even in the presence of 10 μM R205 (data not shown). Increases in c-am accumulation in the presence of PSC833 and rhodamine B indicate that P-gp transporters are functioning in the mature eggs. The greater relative inhibition of dye efflux by PSC833 in the mature eggs compared to immature oocytes suggests an increase in the amount of P-gp-like transporters. The increase in P-gp-mediated activity is supported by the increase in band intensity of the C219-labeled protein in the immunoblots and the increase in labeling of

the protein at the cell membrane 3 to 5 hpm as seen in the immunocytochemistry experiments.

In the mature eggs, labeling of MRP by spMRP increased with increasing hours post-maturation, suggesting that MRP may be a greater contributor to dye efflux in the mature egg than in the immature oocyte. Also in mature eggs, the spMRP binds to two proteins; one less than in immature oocytes. Increases in c-am and CDFDA accumulation in the presence of MK571 indicate that MRP-like transporters are functioning, together with P-gp transporters, in the mature eggs. The MRP1-specific inhibitor, BMMA, did significantly increase c-am and CDFDA accumulation in mature eggs unlike in immature oocytes. The change in efficacy of BMMA in eggs versus oocytes indicates an increase in the MRP1-like transporter population. Other MRPs (MRP2-9) could also be involved in the increase in transport activity at maturation since MK571 is not specific for a certain MRP protein.

The incubation of oocytes and eggs with calcein and estrogens or other environmental toxicants also illustrates the changes in transporter populations between maturational stages (Table 1). The differences in substrates specificity suggests an increase in the MRP-mediated transport activity since MRPs are known to transport conjugates of estradiol (reviewed in Krishna and Mayer, 2000) and pesticides and endocrine disrupting compounds (Leslei et al., 2001; Tribull et al., 2003) and may transport *o,p*-DDD and octylphenol.

The period of maturation in the sea star is completed within an hour of 1-methyladenine addition which, according to the time lapse kinetics data (Fig. 3A), is the point when MDR transporters increase the active pumping of the dye. The 1 hpm time

frame also correlates with the translocation of both the P-gp and MRP-like transporters as demonstrated with in the immunocytochemistry experiments. A P-gp-like protein is stored in the immature oocyte cytoplasm possibly in vesicles bound for plasma membrane insertion. The MRP proteins are as conspicuously translocated to the cell periphery as P-gp within 1 hpm but the MRP protein density does not increase until 5 hpm. Colchicine, a microtubule inhibitor and an inhibitor of oocyte maturation (Meijer and Guerrier, 1984), reduces the translocation of both transporters. MDR transporters are likely stored in cytoplasmic vesicles or the endoplasmic reticulum, which is altered during the reorganization of the oocyte during maturation (Jaffe and Terasaki, 1994), and transported during cytoplasmic restructuring by microtubules and microfilaments which is consistent with known translocation of oocyte cytoplasmic constituents during oocyte maturation (Santella et al., 1999). The storage of MDR transporters in the cytoplasm and the subsequent translocation to a functional state in the cell membrane has not been reported in an immature oocyte although there is functional and molecular evidence of storage in the mature sea urchin egg (Hamdoun et al., 2004).

Storage and translocation of transporter proteins in the cytoplasm of various cell types has been previously reported (reviewed in Bryant et al., 2002; Santamarina-Fojo et al., 2001). Translocation is involved in the upregulation of MRP1 in bilirubin removal from neurons (Gennuso et al., 2004) and in microvillar natural defense function (reviewed in Lange and Gartzke, 2001). The proposed translocation of sea star MDR transporter during maturation is similar to the storage and translocation of cortical granules duration maturation (Santella et al., 1999) or the activation of amino acid transporters during fertilization in the sea urchin (Epel, 1972). The storage of MDR

transporter proteins and its induction by a hormone, 1-methyladenine, is also comparable to the insulin-induced translocation of the GLUT4 transporter in human cells (Watson and Pessin, 2001).

Data from this study suggest that translation of new transporter proteins is partially responsible for the increase in dye efflux during oocyte maturation. The role of translation is evidenced by the negative effect of translational inhibitors in both the immunoblotting and immunocytochemistry experiments. Increases in immunoblot band intensity do not occur until 3-5 hpm since we assume that the nascent P-gp transporter is stored in the immature oocyte, thus increasing the basal level of C219-reactive protein. It is suspected, though not determined, that the transporters in the immature oocyte cytoplasm are not functional until they reach the plasma membrane. Transcription of new transporter mRNA does not occur during the maturation process and is supported by the insensitivity of the system to transcriptional inhibitors. It is not surprising that transcription influences MDR activity since little to no transcription occurs in the egg and early cleavage embryo (Davidson, 1999; Tomek et al., 2002).

During the maturation process in the sea star, many important signaling molecules are activated or inhibited including protein kinase C, cAMP, phosphatases and calcium (Meijer and Mordret, 1994; Kishimoto, 1998). Translation of proteins in the maturing sea star oocyte is initiated after 1-methyladenine addition and is associated with an increase protein kinase activity especially protein kinase C (Xu et al., 1993). Protein kinase C is also involved in the activation of MRP2 transporters in shark rectal gland (Miller et al., 2002) and may play a role in the sea star oocyte during maturation.

The purpose of MDR transporters in immature oocyte and its subsequent upregulation is as yet undetermined. Transporter activities appear to not be necessary for oocyte maturation to occur even though MDR transporters appear to be expressed in ooctyes while in the ovary of the female sea star. Furthermore, oocyte maturation is not activated or inhibited by the inhibition of MDR transporters using specific transport inhibitors in the immature oocyte (data not shown). Since the internal body cavity of sea stars is known to equilibrate with the external environment (Prusch and Whoriskey, 1976), potentially allowing for the exposure of oocytes to toxicants, the transporters may function in a secondary protective role in the immature oocyte *in ovario* by exporting endogenous or exogenous compounds. The secondary protective role hypothesis is supported by results showing that there is a increase in the inhibition of GVBD by vinblastine when oocytes are co-incubated with a specific MDR inhibitor. This hypothesis is also supported by the transporter substrate dye experiment using estrogens and environmental toxicants (Table 1). The transporters may protect the oocytes and eggs from these compounds by exporting the steroids and the environmental toxicants before toxicity occurs.

The change in MDR transport activity during sea star oocyte maturation has several major similarities and differences with the change in MDR transport activity during sea urchin fertilization (Hamdoun et al., 2004). First, in both systems, there is a dramatic increase in MDR transport activity in the final state versus the initial (egg > oocyte; 2-cell embryo > spawned egg) coinciding with the completion of cytoplasmic reorganization. Secondly, both systems appear to utilize stored transporter proteins which are eventually translocated to the periphery. Finally, both processes increase

MRP-mediated transport activity during the transition. A major difference is the role of translation in sea star oocyte maturation and the lack thereof in sea urchin fertilization. In sea star oocytes, translation plays a significant role in the increase of MDR activity after maturation while in the sea urchin egg, the upregulation of MDR efflux is mediated post-translationally after fertilization. Unlike sea urchin eggs, the immature sea star oocyte and mature egg have a predominant, functional population of P-gp-like transporters. A comparison of mature sea star eggs with mature sea urchin eggs illustrates the differences in MDR activity (dye accumulation; Figure 10). The significant increase in c-am accumulation with a lower concentration of PSC833 in the sea star egg compared to the sea urchin egg demonstrate that the sea star egg has significant population of P-gp transporters and the sea urchin egg does not.

A discussion concerning the specificity of the dyes, substrates and inhibitors used in this study is warranted. There is an inherent risk when dyes and inhibitors termed 'transporter-specific', based on mammalian cell studies, are used in experiments with invertebrate systems. Our assumption is that molecular sequence homology indicates functional homology and that their inhibitory or substrate characteristics are similar in non-mammalian systems. Therefore, data using these specific molecules should be carefully interpreted when used in invertebrate systems. Within the scope of this study, we do not assume that data using specific dyes and inhibitors determines with absolute certainty the presence and function of specific MDR transporters but a combined approach with inhibitors, dyes, and antibodies collectively suggests their presence and functional importance. Only studies using molecular tools such as real-time Q-PCR and

siRNA designed from isolated transporters from sea stars can confirm the proportional contribution of P-gp and MRP transporters during sea star oocyte maturation.

In summary, MDR transport is fully functional in immature oocytes in the ovaries of female sea stars. A P-gp-like transporter appears to be the dominant MDR transporter in the immature oocyte and may be fully functional *in ovario*. At maturation, both P-gp-like and MRP-like transporters are expressed and transported to the plasma membrane within an hour(s) of maturation. The immediate increase in P-gp transport activity at maturation from translocation may have a protective role during spawning and before fertilization. Mature eggs are usually spawned within hour(s) of maturation and fertilized within 2-3 hours of maturation in the external environment. The increase in MDR activity at 3 to 5 hpm may not be relevant to natural conditions and is possibly an artifact of delayed fertilization. The increase in MRP-mediated transport may be only relevant after fertilization and during early development both by transporting endogenous compounds for controlling cell regulation and as a secondary role of protection in an adverse aqueous environment (see Table 1; Hamdoun et al., 2004). In the sea star, the purpose of MDR transport in the immature oocytes and mature eggs is unknown and can not be determined until further *in vivo* and *in vitro* experiments utilizing molecular tools and techniques are developed.

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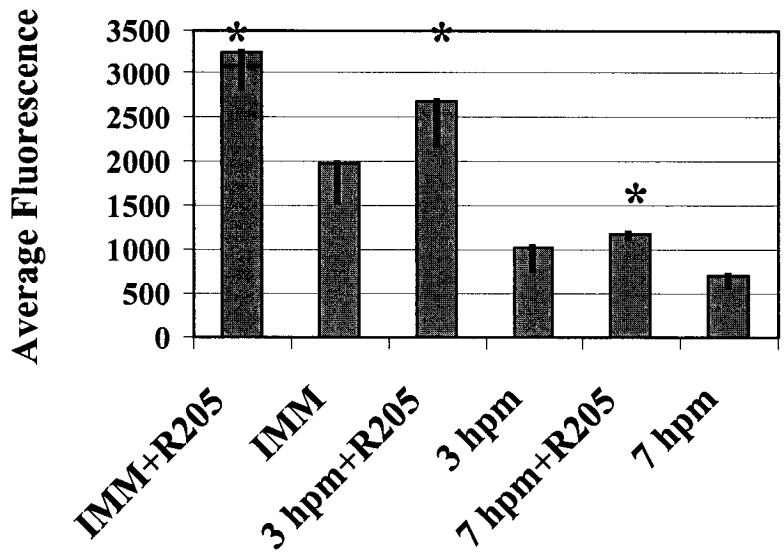
Table 1.

| Substrate | Immature oocyte | Mature egg |
|---|---|---|
| **$E_2$** | 1.23 ±0.10* | 1.72 ±0.46* |
| **$EE_2$** | 1.23 ±0.10* | 1.73 ±0.30* |
| **OCT** | 1.15 ±0.22 | 1.68 ±0.43* |
| **TBT** | 1.23 ±0.14* | 1.86 ±0.14* |
| **DDD** | 1.07 ±0.16 | 1.90 ±0.12* |
| **COL** | 1.37 ±0.21* | 1.84 ±0.21* |

Relative fluorescence (mean ± std) (c-am) of immature oocytes and mature eggs incubated with estradiol, ethynylestradiol and environmental toxicants. Estradiol (**$E_2$**), ethynylestradiol (**$EE_2$**) and tributyltin (**TBT**) are substrates (significantly increase dye accumulation over the control fluorescence) for mdr transporters in the immature oocyte but not octylphenol (**OCT**) and *o,p*-DDD. In the mature egg, all compounds are substrates. Colchicine (**COL** ) used as a positive control. * denotes a significant treatment ($P<0.05$).

Figure 1. Changes in dye accumulation during oocyte maturation and protein analysis using the monoclonal P-gp antibody, C219, from immature oocyte and maturing egg samples. A: Immature oocytes and mature eggs (1 and 5 hpm) were incubated with 0.5 μM c-am with or without 3 μM R205 for 2 hr at 15 °C. The mean pixel fluorescence (± std) intensity of 20-30 individual oocytes and eggs was measured and averaged for each maturational time point. (*) denotes significant differences using paired t-test between inhibitor pairs ($P<0.05$). B: Sea star oocyte and egg protein samples analyzed by SDS-PAGE gel and immunoblot using the monoclonal P-gp antibody, C219. Reduced samples of controls, immature oocytes (IMM) and mature eggs (1-7 hpm) and unreduced samples of immature oocytes and positive control were analyzed on separate blots.



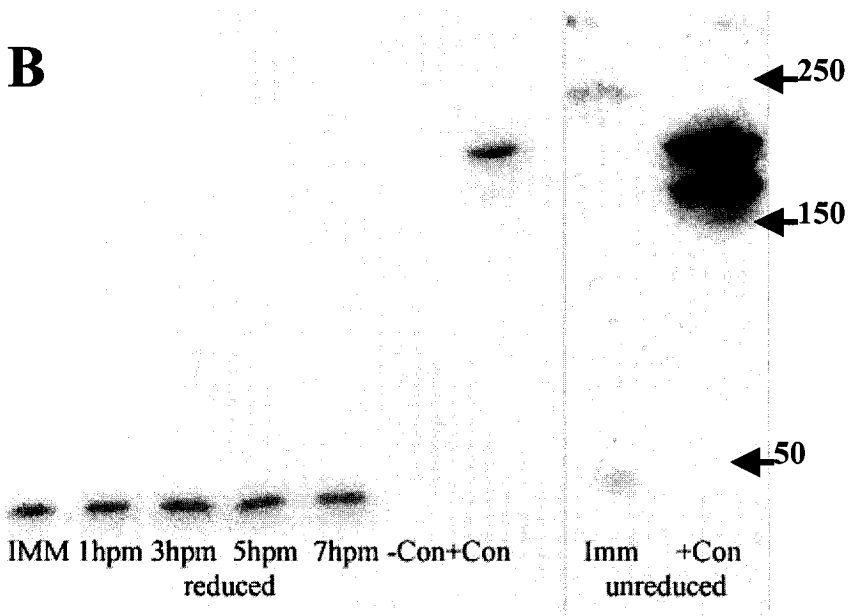
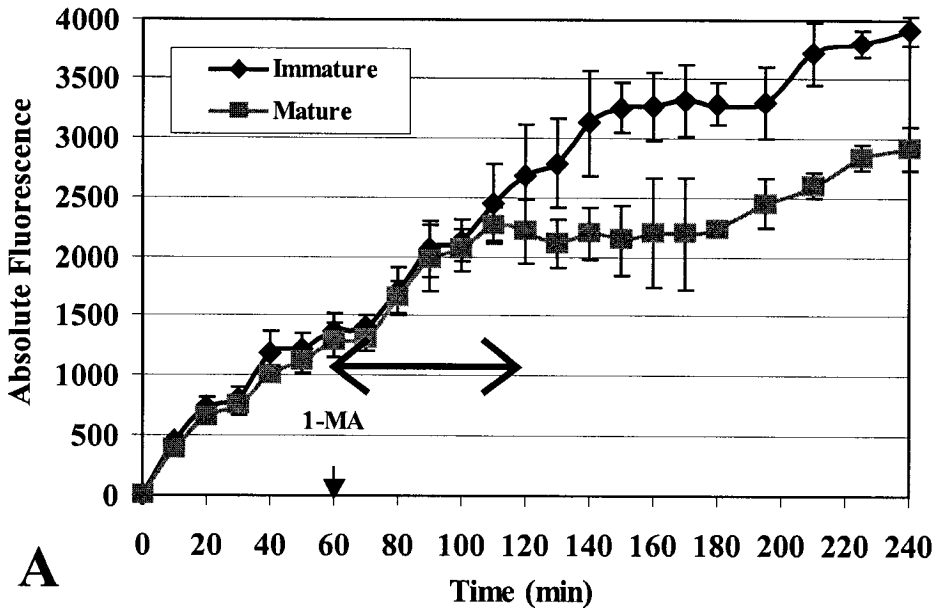



Figure 2. Time-lapse kinetics of c-am accumulation in immature oocytes and mature eggs with and with specific inhibitors (mean ± std). A: Recordings of fluorescence from the same 7-10 oocytes/eggs after addition of 0.5 μM c-am were taken at 10 min intervals for 60 min. At t=60, 1 mM 1-MA (Arrow) was added to the appropriate chambers. Recordings continued for over 2 hr at 10 min intervals and then 15 min intervals for the final hour. B: For experiments with PSC833 and MK571, recordings were taken every 15 min for 2.5 hr (t=150). At t=150 (Arrow), PSC833 (10 μM) and MK571 (10 μM) were individually added to one of the chambers containing matured eggs. Recordings were taken at 10 min intervals for another 90 minutes (t=240) and analyzed according by MetaMorph®. Arrowed line indicates the duration of oocyte maturation determined by germinal vesicle breakdown.



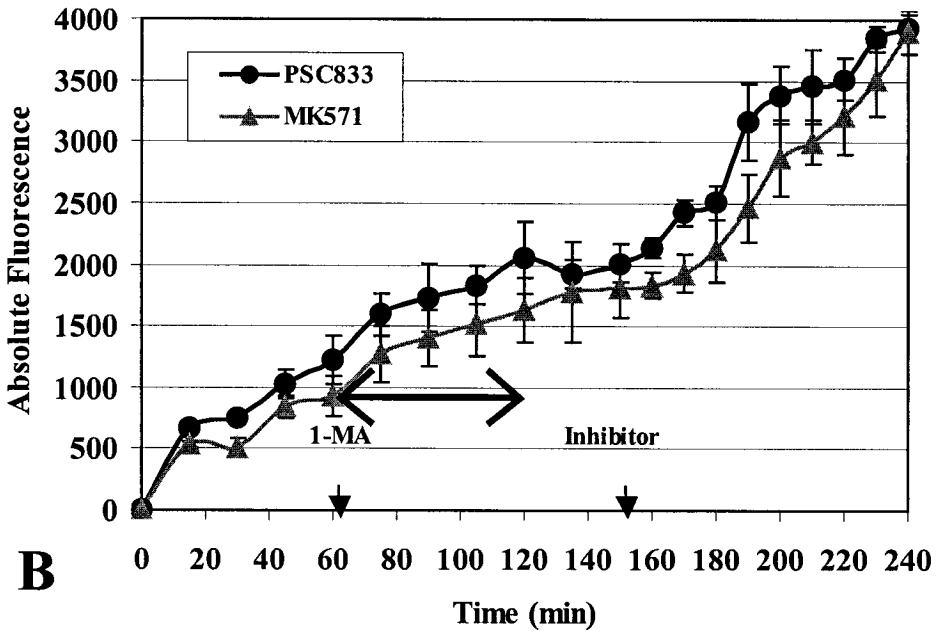
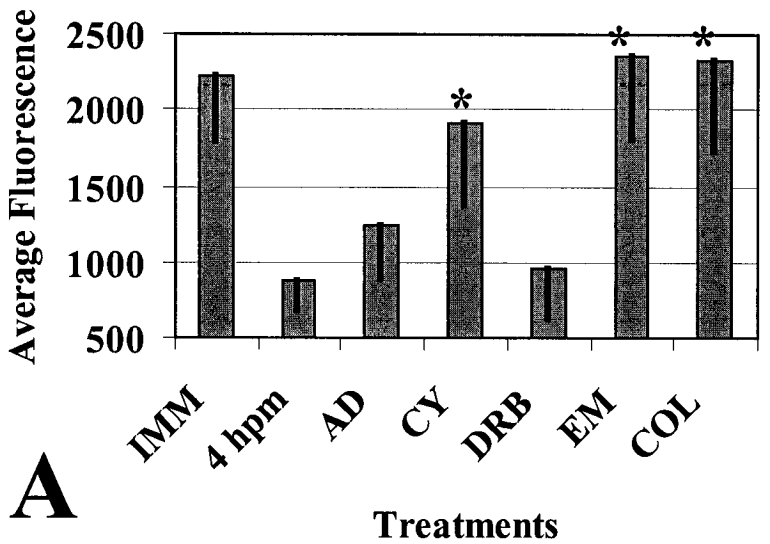



Figure 3. The effects of cell function inhibitors on the decrease in dye accumulation after oocyte maturation and protein analysis using the monoclonal P-gp antibody, C219, from samples of oocytes, eggs and eggs incubated with cell function inhibitors. A: Oocytes were incubated for 2 hr with cell function inhibitors 20 minutes after 1-MA addition. C-AM (0.25 μM) was added to the tubes and incubated for 2 hr at 15 °C. Transcriptional inhibitors, actinomycin D (AD) and DRB, were used at 1 μM and 100 μM, respectively. Translational inhibitors, cycloheximide (CY) and emetine (EM), were used at 2.5 μM and 100 μM, respectively. The microtubule inhibitor, colchicine (COL), was used at 100 μM. The mean (± std) pixel fluorescence intensity of 20-30 individual oocytes and eggs was measured and averaged for each maturational time point. (*) denotes significant differences using paired t-test between the inhibitor and the untreated mature eggs ($P<0.05$). B: Sea star oocyte and egg protein samples analyzed by SDS-PAGE gel and immunoblot using the monoclonal P-gp antibody, C219. Samples of controls, 5 hpm mature egg and mature eggs incubated with cell function inhibitors during maturation.



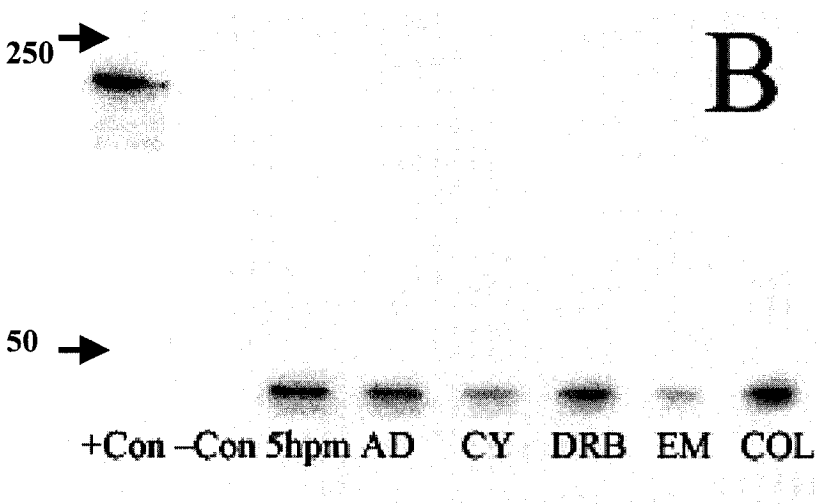
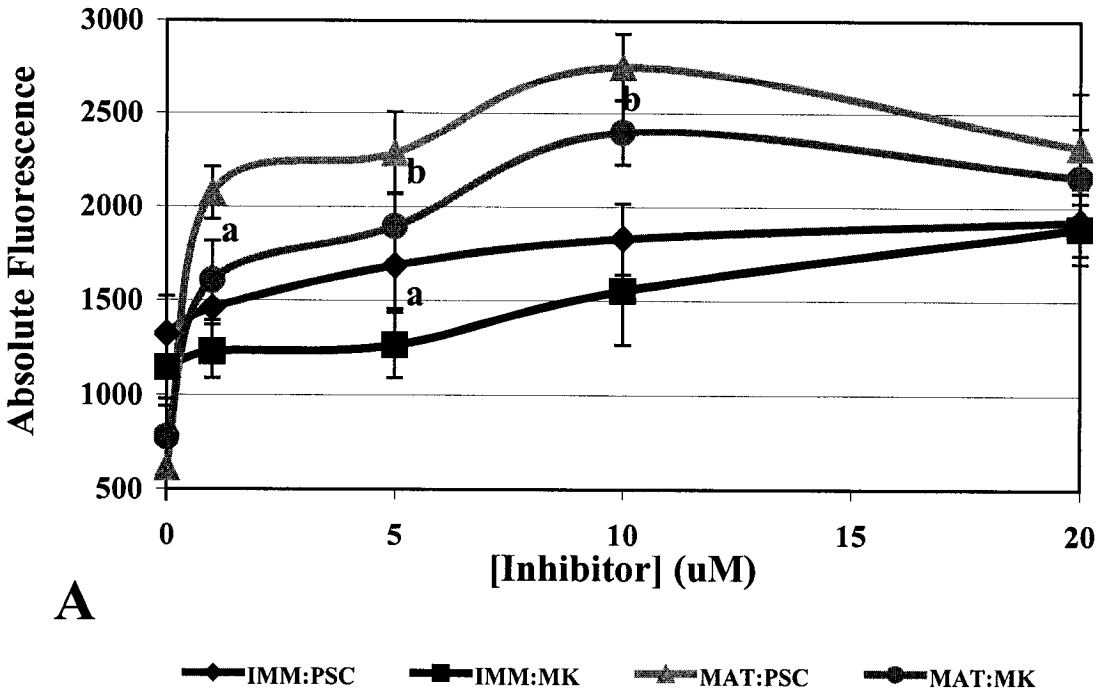
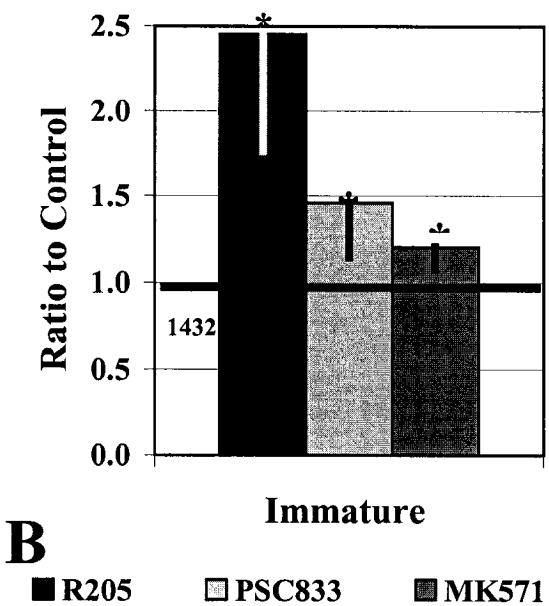



Figure 4. The differences in PSC833 and MK571 dose response between ooctyes and eggs and the effects of R205, PSC833 and MK571 on c-am accumulation during oocyte maturation. A: Immature (IMM) oocytes and mature (MAT) eggs were (1hpm) were incubated with c-am (0.25 μM) for 2 hrs with the mdr specific inhibitors, PSC833 (1-20 μM), a P-gp inhibitor, and MK571 (1-20 μM for c-am), an MRP inhibitor. The mean (± std) pixel fluorescence intensity of 20-30 individual oocytes and eggs was measured and averaged for each maturational time point. (a) denotes significant differences using paired t-test between data points at a similar concentration for either immature oocytes or mature eggs between the two inhibitors ($P<0.05$) and (b) denotes significant differences using paired t-test between data points ($P<0.1$) at a similar concentration for either immature oocytes or mature eggs between the two inhibitors. B & C: Relative fluorescence of immature oocytes and mature eggs incubated with R205 (3 μM), PSC833 (5 μM) and MK571 (5 μM) for 2 hr at 15 °C. Darkened line marks the level of control fluorescence which is given in absolute fluorescence units. (*) denotes significant differences relative to the control absolute fluorescence ($P<0.05$).



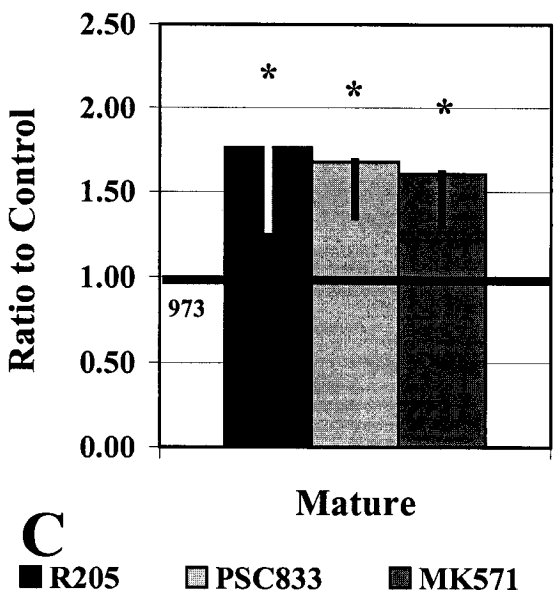
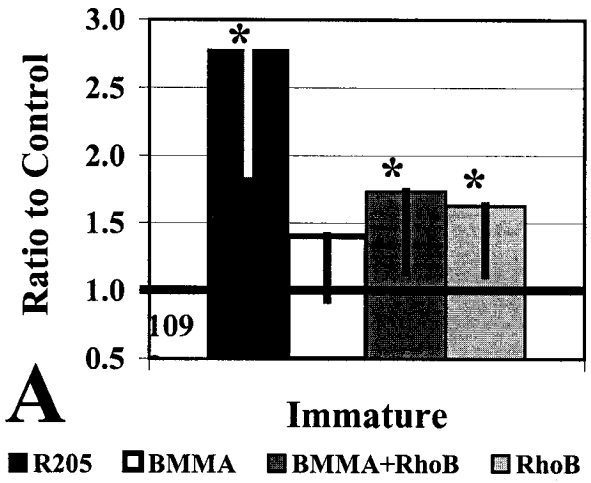



Figure 5. Dye accumulation in immature oocytes and mature eggs using calcein-AM incubated with specific and non-specific inhibitors of mdr transport. A & B: Relative fluorescence of immature oocytes and mature eggs were (1hpm) were incubated with c-am (0.25 μM) for 2 hrs with R205 (3 μM), BMMA (10 μM), an MRP1 inhibitor, RhoB (1μM), a P-gp substrate, or a combination of BMMA and RhoB. The mean (± std) pixel fluorescence intensity of 20-30 individual oocytes and eggs was measured and averaged for each maturational time point. Darkened line marks the level of control fluorescence which is given in absolute fluorescence units. (*) denotes significant differences relative to the control absolute fluorescence ($P<0.05$).



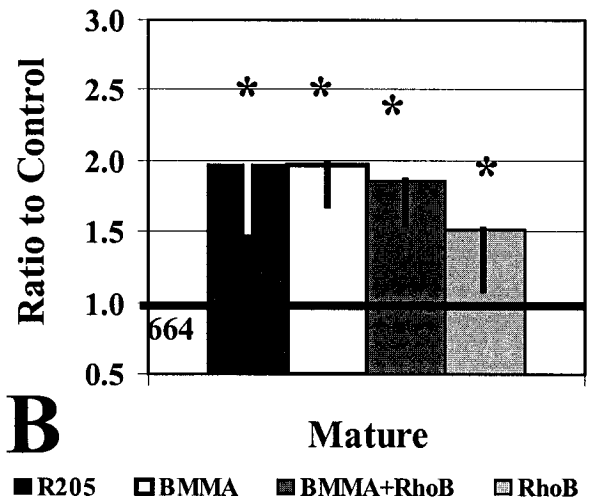
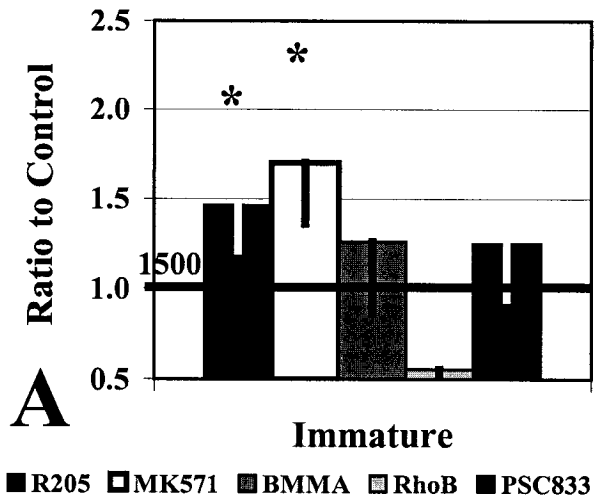
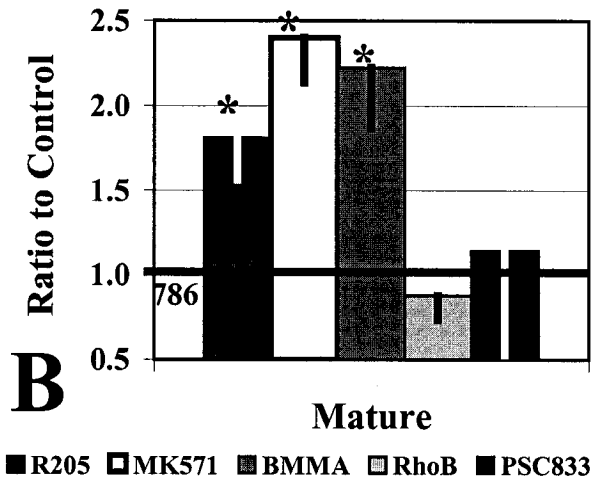



Figure 6. Dye accumulation in immature oocytes and mature eggs using an MRP-specific dye, CDFDA, incubated with inhibitors of mdr transport. A & B: Relative fluorescence of immature oocytes and mature eggs were (1hpm) were incubated with CDFDA (10 μM), an MRP-specific dye, for 2 hr with R205 (25 μM), MK571 (25 μM), BMMA (25 μM), RhoB (25 μM) and PSC833 (25 μM). The mean (± std) pixel fluorescence intensity of 20-30 individual oocytes and eggs was measured and averaged (± std) for each maturational time point. Darkened line marks the level of control fluorescence which is given in absolute fluorescence units. (*) denotes significant differences relative to the control absolute fluorescence ($P<0.05$).



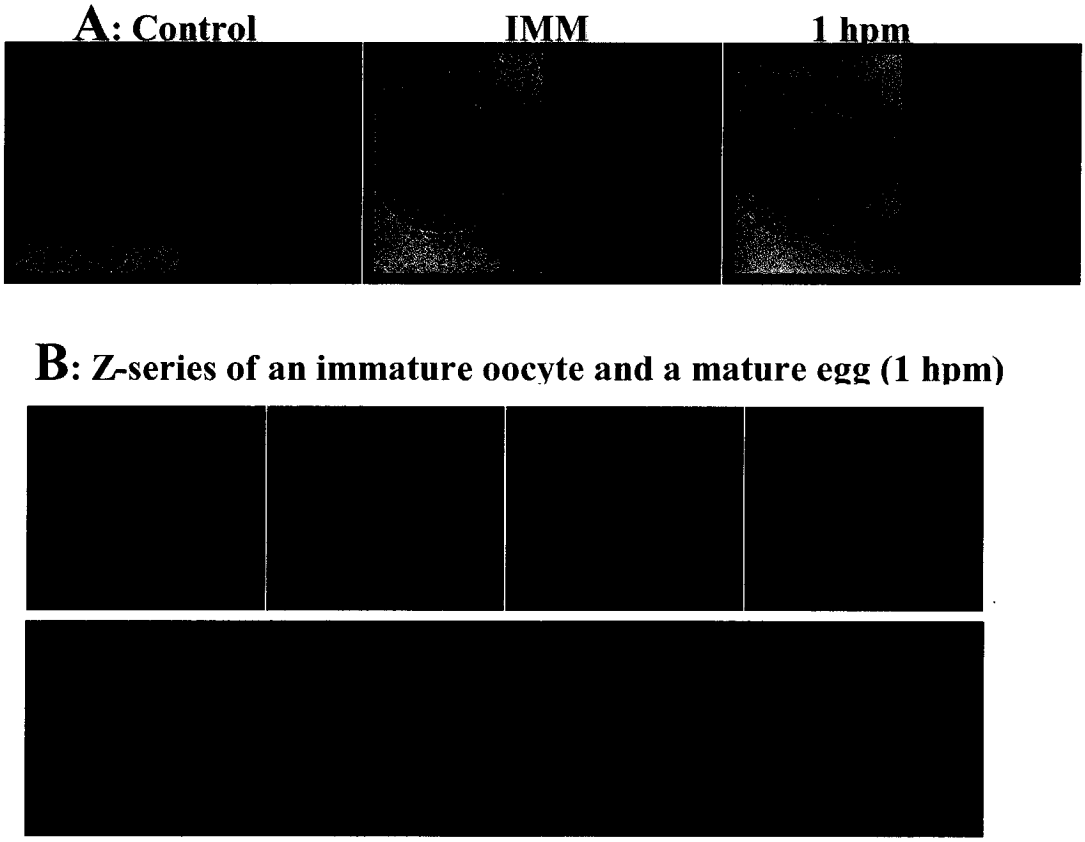



Figure 7. Immunocytochemistry of immature oocytes and mature eggs incubated using the monoclonal antibody, C219. A: Fixed and permeated immature (IMM) ooctyes and mature eggs (1 hpm) were incubated with the P-gp monoclonal antibody C219. A secondary control (no primary) of oocytes was also prepared. B: Z-series through an immature oocyte and mature egg labeled with C219. The samples were viewed using an Olympus laser scanning microscope. Photomicrographs were analyzed using Fluoview® (Olympus Corp.) software.

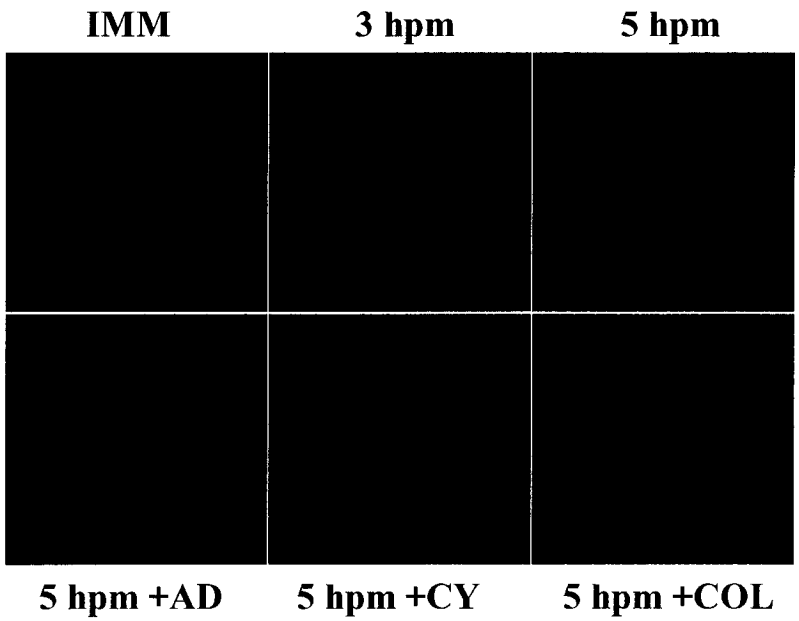



Figure 8. Immunocytochemistry of mature eggs incubated with cell function inhibitors using the monoclonal antibody, C219. Fixed and permeated immature oocyte (IMM), mature eggs (3 and 5 hpm) and 5 hpm mature eggs matured with cell function inhibitors were labeled with the P-gp monoclonal antibody C219. 5 hpm eggs were matured in the presence of either actinomycin D (AD, 1 μM), cycloheximide (CY, 2.5 μM) or colchicine (COL, 100 μM). The samples were viewed using an Olympus laser scanning microscope. Photomicrographs were analyzed using Fluoview® (Olympus Corp.) software.

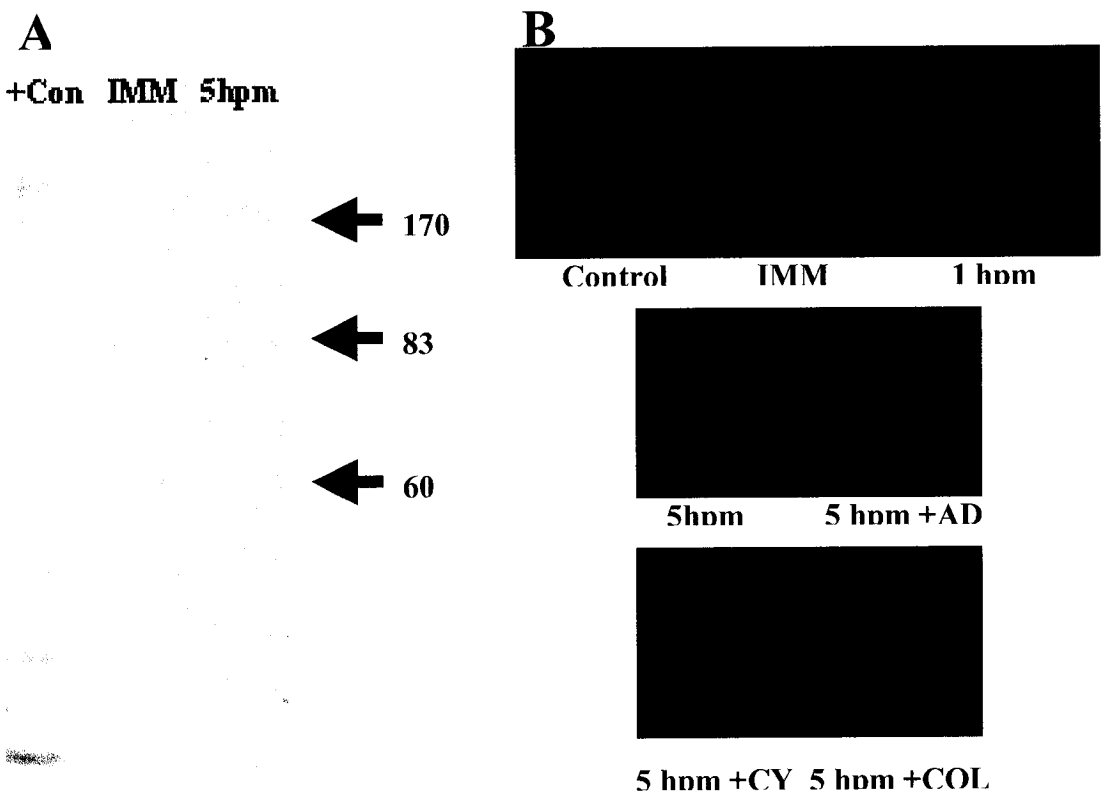



Figure 9. A: Sea star oocyte and egg protein samples analyzed by SDS-PAGE gel and immunoblot using a polylclonal spMRP antibody designed from sea urchin MRP sequences. Reduced samples of positive control (mouse liver), immature oocytes (IMM) and mature eggs (5 hpm). B: Immunocytochemistry of immature oocytes, mature eggs and mature eggs incubated with cell function inhibitors using a polyclonal spMRP antibody designed using sea urchin sequences. Fixed and permeated immature ooctyes, mature eggs (1 and 5 hpm) and 5 hpm mature eggs matured with cell function inhibitors were labeled with the P-gp monoclonal antibody C219. 5 hpm eggs were matured in the presence of either actinomycin D (AD, 1 μM), cycloheximide (CY, 2.5 μM) or colchicine (COL, 100 μM). A secondary control (no primary) of oocytes was also prepared. The samples were viewed using an Olympus laser scanning microscope. Photomicrographs were analyzed using Fluoview® (Olympus Corp.) software.

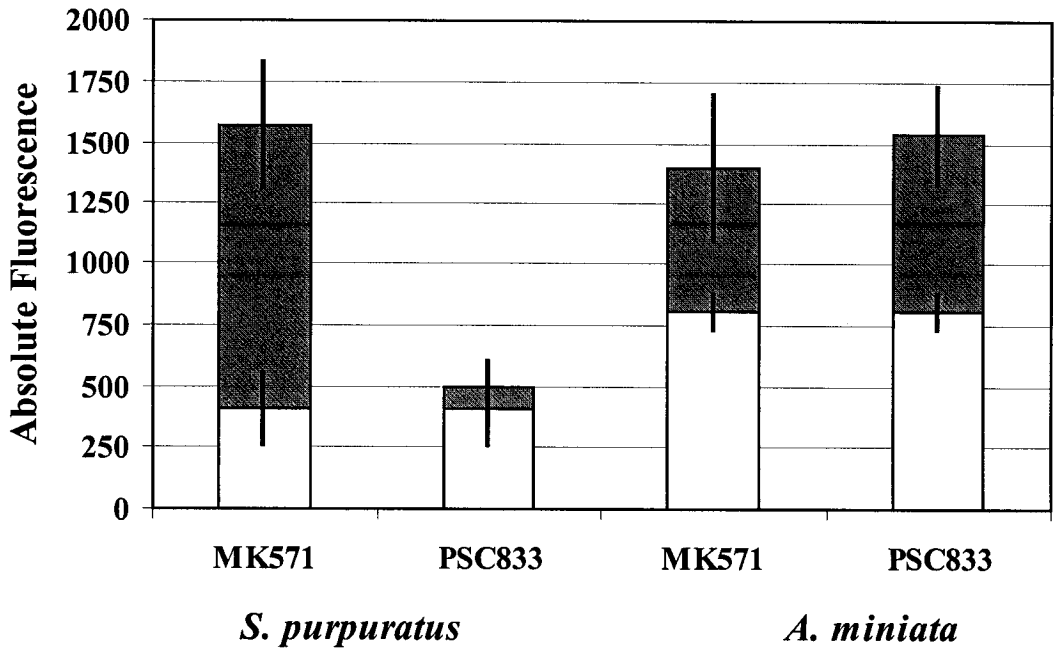



Figure 10. Comparison of dye accumulation with specific mdr inhibitors between mature sea star and sea urchin eggs. The white (control) and gray (treated) bars represent the mean (± std) increase in fluorescence intensity (after 120 min of incubation) of sea star (*A. miniata*) and sea urchin (*S. purpuratus*) eggs when treated with the P-gp specific inhibitor, PSC833 (10 μM for *S. purpuratus*, 5 μM for *A. miniata*) or the MRP specific inhibitor, MK571 (10 μM for *S. purpuratus*, 5 μM for *A. miniata*). Significant increases relative to control occur in sea star eggs treated with either inhibitors and in sea urchin eggs treated with MK571($P<0.05$) but not with PSC833.

# CHAPTER 4

## Expression of multidrug resistance transporter proteins during fertilization and early development in the sea star

**Abstract**

Previous studies in echinoderm oocytes, eggs and early embryos have demonstrated an upregulation of multidrug efflux activity during oocyte maturation and fertilization involving both permeability glycoprotein (P-gp) and multidrug resistance-associated (MRP) transport activity. In this study, functional and molecular evidence is presented for MDR-mediated drug efflux in eggs and embryos of the sea star, *Asterina miniata*. Functional analyses of drug efflux activity in sea star eggs and embryos support the hypothesis that both P-gp and MRP transporters are present during sea star development. Sea star eggs and embryos can extrude a non-specific fluorescent substrate such as calcein-AM (c-am) and an MRP-specific substrate such as CDFDA. C-am efflux is sensitive to both P-gp and MRP inhibitors at all stages of development while CDFDA is only sensitive to MRP-specific inhibitors and a general multidrug resistance transporter (MDR) inhibitor, R205. Unlike in sea urchins, drug efflux activity in eggs as compared to early embryos shows that transport is not activated at fertilization. The inhibition of multidrug transport activity by specific inhibitors significantly reduced first cleavage in fertilized sea star eggs, and the presence of inhibitors potentiated the inhibition of early cleavage by vinblastine. Using a cDNA probe corresponding to the highly conserved nucleotide binding domain of ATP-Binding Cassette (ABC) transporters, we identified several transcripts encoding drug efflux transporters from sea star egg cDNA libraries. Comparison of these transcript sequences to known MRP transporter sequences suggests sea star eggs possess at least three unique MRP-like transcripts with homology to MRP1, 5 and 8. Using cDNA macroarray technology, the presence of MRP 5, 8 and 9 were also confirmed in the sea star. Immunolabeling of fertilized eggs and embryos with an

antibody raised against sea urchin MRP illustrated the changes in MRP protein distribution during the later stages of embryogenesis. This study demonstrates that in the sea star, both MRP and P-gp-like transport occurs during embryogenesis and that these transporters may play important roles in early cleavage events.

keywords: MDR, P-gp, MRP, sea star, embryo

**Introduction**

The ATP-Binding Cassette (ABC) superfamily of transporter proteins function by utilizing the hydrolysis of ATP to pump substrates across the plasma membrane. Of the twelve families of ABC transporters identified in humans, three are known to perform the transport of endogenous waste and xenobiotic compounds (Borst et al., 2000; Dean et al., 2001). Multidrug resistance (MDR) efflux transporters are members of the ABC superfamily and are known to transport moderately hydrophobic compounds, lipids, conjugated organic anions, hormones and xenobiotics (reviewed in Krishna & Mayer, 2000 and Kruh and Belinsky, 2003). Two important MDR transporter families are the permeability glycoproteins (P-gp)/MDR (ABCB) transporters and multidrug resistance associated proteins (MRP)/CFTR (ABCC) transporters (Borst et al., 2000; Dean et al., 2001). These transporters have important endogenous functions including the efflux hydrophobic compounds from exocrine and endocrine glands (Sarkadi et al., 1994), epithelial microvillar transport function (reviewed in Lange and Gartzke, 2001) and, in the case of MRP, the transport of glutathione-conjugated compounds (Leslie et al., 2001).

MDR transporters are expressed in an assortment of developmental cell types. MRP and P-gp transporters are expressed in human trophoblast cells and other placental tissues (Atkinson et al., 2003; Utoguchi et al., 2000). Pig oocytes express the mRNA for both P-gp (MDR1) and MRP (MRP1) transporters (Takebayashi et al., 2001). P-gp proteins are expressed in amphibian embryos that actively extrude P-gp dye substrates (Bonfanti et al., 1998). In invertebrates, embryos from diverse phyla express MDR transporters including nematode, mollusc and echinoderm (Broeks et al., 1996; Hamdoun

et al., 2004; McFadzen et al., 2000; Minier et al., 2002). Two recent studies demonstrated that a dramatic upregulation of MDR activity occurs in sea urchin eggs at fertilization and in sea star oocytes during maturation (Hamdoun et al. 2004; Roepke et al., in preparation, see chapter 3). In both events, the increase in dye efflux activity is post-translationally regulated although there are differences in the transporter populations between the sea urchin and the sea star. Both MRP- and P-gp-like transporters are present in sea urchin and sea star eggs, but the majority of transport activity in the sea urchin egg is associated with MRP while dye efflux in the sea star egg is associated with both transporter types (Hamdoun et al., 2004; Roepke et al., in preparation, see chapter 3).

Little is known about the endogenous function of these transporters during embryonic development. In plants (*Arabidopsis*), the function of similar efflux transporters is the establishment of auxin gradients required for development (Friml et al., 2003; Gaedeke et al., 2001). In slime molds (*Dictyostelium*), an ABC transporter, RhT, transports the endogenously produced differentiation factor (DIF-1) to regulate terminal stalk cell differentiation (Good and Kuspa, 2000). The role of the MDR transporters in the early development of animals is unclear, although the expression of the transporters has been reported in the embryos of such diverse phyla as the soil nematode *Caenorhabditis elegans* (Broeks, 1996), the marine echiuroid worm, *Urechis caupo* (Hamdoun et al., 2002; Toomey and Epel, 1993; Toomey et al., 1996); and mouse embryos (Elbling et al., 1993). In all these systems, a protective role for these transporters has been suggested including defense from heavy metals, microbial toxins and hydrocarbon biodegradation products and other xenotoxins.

The expression of MDR transporters during the early developmental events such as first cleavage, blastula formation/hatching and gastrulation has not been thoroughly examined in marine invertebrates. This study seeks to characterize the dye efflux activity during fertilization, first cleavage, blastula formation and gastrulation in embryos of the sea star, *Asterina miniata* using dye accumulation studies, immunocytochemistry and molecular techniques. In addition, the importance of functional transport activity is examined during first cleavage to compare to recent evidence that the inhibition of MRP activity during early cleavage in the sea urchin retards mitosis and may be necessary for early cell division (Hamdoun et al., 2004).

## Materials and Methods

### 2.1. Reagents and chemicals

1-methyladenine (1-MA), reversin (R-205), rhodamine B, 1-benzoyl-5-methoxy-2-methylindole-3-acetic acid (BMMA), Nonidet P-40 and the horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Calcein-acetoxymethylester (c-am), 5 (and 6)-carboxy-2',7'-dichlorofluorescein diacetate (CDFDA), Alexa Fluor® 488 goat anti-mouse IgM and Alexa Fluor® 488 rabbit anti-goat IgM was purchased from Molecular Probes (Eugene, OR, USA). The MRP inhibitor, MK571, was purchased from Cayman Chemical and the P-gp inhibitor, PSC833, was a gift from Norvartis (Basel, Switzerland). The primary antibody to P-gp, C219, was purchased from Signet Laboratories. The

primary antibody to MRP was constructed by Santa Cruz Biotechnology, Inc., using multiple conserved amino acid sequences isolated from MRP genes of the sea urchin, *Strongylocentrotus purpuratus*. Stocks of each chemical were made in either dimethyl sulfoxide or methanol except 1-MA and tributyltin which were stored in filtered seawater (FSW). Paraformaldehyde for oocyte and egg fixation was purchased from Polysciences, Inc. (Warrington, PA, USA) and made into a 4% solution with FSW.

2.2. Animal care and embryo sampling

Adult *Asterina miniata* sea stars were collected during their respective reproductive cycles and maintained at the Bodega Marine Lab (BML) in continuous flow-through, sand-filtered Bodega Bay sea water (13°C +/- 2°) with weekly feedings of invertebrate food pellets, shrimp or mussels. For experiments with embryos, adult sea stars were induced to spawn by injection of 3-5 ml of 1 mM 1-MA. Gametes were collected in 0.7 μM filters seawater (FSW). Spawned eggs were washed three times with FSW and fertilization tests were performed to ensure maximum amount of development and overall health of eggs. Fertilized eggs were transferred into batch culture pans containing 4 L of FSW and allowed to develop at 17 °C. At each developmental stage, embryos were collected, washed and transferred to 50 ml conical tubes for dye accumulation experiments or concentrated for immunolabeling.

2.3. Fluorescence imaging of dye efflux

To determine the dye accumulation kinetics during fertilization, spawned eggs were attached to two polylysine-coated 2-chambered glass slides. All eggs in the four chambers were incubated in a 1% BSA blocking buffer for 15 min at 15 °C and washed 3 times in FSW. A prepared solution (3 ml) of FSW and 0.5 μM c-am dye was added to the chamber labeled spawned egg. To the other three chambers, the same dye solution was added plus dilute sperm. PSC833 (10 μM) was added to the third chamber and MK571 (10 μM) was added to the fourth chamber. At t=0, after treatments were added, images of fluorescence emission of the same 7-10 eggs were collected at 10 min intervals for 120 min and analyzed according by MetaMorph®.

For the comparison of dye accumulation over time in each developmental stage, dilute samples were added to 15 ml tubes filled with 10 ml FSW. Duplicate tubes were used for each maturational stage containing 0.25 μM c-am. At 30 min intervals over a two hr (4 time points) period, one tube from each treatment was centrifuged and prepared for fluorescent microscopy. Photomicrographs were collected and analyzed according to the protocol above. The unused samples in the tubes were placed back in the incubator at 15 °C and used for a subsequent time point. The tubes were rotated on a slow Nutator throughout the experiment.

To compare the different transporters at each developmental stage, spawned eggs, early embryos (2-4 cell), blastula (unhatched), and late gastrula embryos were diluted into 15 ml conical tubes filled with 9 ml FSW and incubated with either c-am (0.25 μM) or an MRP-specific dye, CDFDA (10 μM, Zamek-Gliszczynski et al., 2003), for 2 hr at 15 °C on a slow Nutator with or without dye efflux inhibitors. After the incubation period and settling, spawned eggs or embryos were transferred to immunoslides, covered

and viewed using a fluorescent microscope. Fluorescent photomicrographs of 20-30 eggs and embryos were taken using an Olympus BX50WI fixed stage microscope equipped with DIC optics and a Roper Scientific CoolSnap HQ cooled CCD camera using the imaging program, Metamorph© (Universal Imaging Corp.). The mean pixel fluorescence intensity of individual eggs and embryos was measured and averaged for each maturational time point.

The specific inhibitors used were PSC833 (5 μM for c-am, 25 μM for CDFDA), MK571 (5 μM for c-am, 25 μM for CDFDA), and BMMA (10 μM for c-am, 25 μM for CDFDA), a putative MRP1 inhibitor (Maguire et al., 2001). Other inhibitors or substrates such as R205 (3 μM for c-am, 25 μM for CDFDA) and rhodamine B (RhoB, 1 μM for c-am, 25 μM for CDFDA) were utilized in the dye accumulation experiments. R205 is a small peptide known to inhibit the efflux capacity of P-gp proteins (Sharom et al., 1999). R205 is considered a general inhibitor of P-gp transporters but the interaction of R205 with MRP proteins is unknown. Rhodamine 123 is believed to be P-gp specific (Twentyman et al., 1994) and rhodamine B is not a substrate in systems where MRP is the primary transporter such as the sea urchin egg (Hamdoun et al., 2002; Toomey and Epel, 1993).

2.4. Cleavage inhibition by vinblastine and MDR inhibitors

To determine the role of MDR transporters in early cleavage, fertilized eggs were incubated with the cleavage inhibitor, vinblastine, with or without specific MDR inhibitors. Shortly after fertilization, fertilized eggs were transferred to 6-well plates

containing FSW. Forty-five min after fertilization, vinblastine was added across a range of concentrations (10-1000 nM) alone or with PSC833 (5 μM or 10 μM) and MK571 (5μM or 10 μM). Embryos were incubated at 17 °C for another 105 min and then fixed 100 μl of 4% paraformaldehyde in FSW. The number of divided embryos was counted in each sample using a phase-contrast microscope. Embryos were also incubated with only the specific inhibitors during the same time period to determine if the inhibitors affected early cleavage.

2.6 cDNA Library Screening and Gene Sequencing

Packed oocytes were flash frozen in liquid nitrogen; homogenized in a liquid nitrogen-cooled ceramic mortar and thawed into 10 volumes of homogenization buffer (Kalinowski et al., 2002) containing proteinase K (200 μg/ml) (Invitrogen, Carlsbad, CA). Homogenates were incubated for 45 min at 37 °C before phenol-chloroform extraction and ethanol precipitation. RNA was further purified by 8 M lithium chloride precipitation (Sambrook and Russell, 2001). cDNA was synthesized with an oligo-dT 12-18 primer and Superscript Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Degenerate MDR and MRP primers (Allikmets and Dean, 1998, Lee et al., 1998) were used to amplify fragments of the corresponding transporter cDNAs by RT-PCR. PCR products were checked for size and yield on 1% agarose gel prior to TA cloning using the dual promoter pCRII-TOPO vector (Invitrogen) and cloning kit. Aliquots of plasmid minipreps (Qiagen) of positive clones were sent to Davis Sequencing for sequencing. Results were analyzed by tBLASTx comparison to existing

sequences at NCBI and a multiple sequence analysis was conducted using ClustalW comparing the sea star sequences to human and invertebrate MDR sequences.

A positive cDNA clone containing a 230bp cDNA of echinoderm MRP, corresponding to a highly conserved region of the MRPs, was used to construct an echinoderm MRP probe. This fragment was random primed and used to screen cDNA macroarray filter libraries of *A. miniata* (Clark, 1999; Kalinowski et al., 2002) using standard filter hybridization techniques (Sambrook and Russell, 2001). Clones that tested positive in the cDNA library screen were obtained from cDNA libraries maintained by Laurinda Jaffe at the University of Connecticut. Clones were grown on selective plates and individual colonies were selected for sequencing. Inserts in plasmid miniprep cDNA were analyzed for size on agarose gels and SP6 and T7 ends were sequenced (Davis Sequencing) to confirm the identity of each clone.

2.5.Immunocytochemistry

To determine the location of MRP-like transporter in eggs, samples of fertilized eggs, early embryos, blastula and gastrula embryos were immediately fixed in 4% paraformaldehyde in FSW and stored at 4 °C overnight. The next day, samples were washed three times in Ca-free ASW for 10 min followed by a 1 h incubation in 1% NP-40 in PBS to permeabilize the cell membrane. The samples were washed for another 3x10 min washing in PBS (Pierce #28374) and were blocked in bulk in 15 ml conical tubes in 4% BSA (PBS) overnight at 4 °C (100 µl of 1% sodium azide was added for long term storage). After overnight incubation in BSA, subsamples were transferred to

microfuge tubes and washed 1x 10 min in PBS. Primary spMRP antibody (1:100 dilution in PBS) was added to the sample while in fresh PBS for 3-4 hr at RT° with gentle mixing. The primary antibody was removed followed by another 1x5 min wash in PBS. The sample was blocked in a 1:10 dilution of rabbit serum in PBS for 1 h and washed 3x10 min in 1% BSA in PBS. The secondary antibody, Alexa 488 rabbit anti-goat (1:500) was added to the samples in fresh PBS for 2 h with gentle mixing in the dark. The secondary antibody was removed and, after 1 x 5 min wash in PBS, a PBS/glycerol mounting medium was added to the samples and stored at –20 °C. A control for non-specific secondary antibody binding was also prepared in which no primary antibody was used but all other steps were included.

All samples were viewed using an Olympus Fluoview 500 laser scanning confocal microscope (equipped with AOTF for complete channel separation) mounted on an Olympus BX60WI fixed stage upright microscope. Samples were imaged using a 10x fluorescence water immersion objective lens. Images (both fluorescence at 488nm excitation and differential interference contrast) were collected as single scans or as Z series with 5 μm optical sections followed by Z series projection using the Fluoview software (Olympus Corp., NY).

2.6.Data analysis

Fluorescence intensity was measured for individual oocytes and eggs using image analysis macros provided with MetaMorph® (Universal Imaging Corp., Downington, PA) software. All absolute measurements were averaged (± 1 std. dev.). Data were

reported either as absolute fluorescence with differences between treatments analyzed using either two sample paired t tests of means or ANOVA ($P < 0.05$) or data were reported as a relative fluorescence (treatment/control) and averaged over the three experiments (± 1 std. dev., $P<0.05$).

**Results**

3.1. Efflux activity during fertilization

After fertilization in the sea urchin, *Strongylocentrotus purpuratus*, a conspicuous upregulation of dye efflux activity occurs within 25 min of fertilization primarily involving MRP transporters (Hamdoun et al., 2004). During fertilization in the sea star, *Asterina miniata*, no upregulation of dye efflux occurred after fertilization (Figure 1). Dye accumulation in spawned eggs and fertilized was statistically similar during the two hours time-lapse experiment. Both P-gp and MRP transporters were present in the fertilized egg of the sea star much like in the mature spawned egg (Roepke et al., in preparation, see chapter 3). The inhibition of dye accumulation by PSC833 and MK571 was not significantly different until 100 minutes after fertilization when PSC833-mediated inhibition of dye efflux was 37% greater than MK571.

3.2. Inhibition of first cleavage

The cytokinesis inhibitor, vinblastine, inhibited first cleavage 50% in sea star embryos at 100 nM and completely inhibited first cleavage at a concentration of 1000 nM (Figure 2A). Vinblastine was also effective at significantly inhibiting first cleavage at lower concentrations (10 to 50 nM). Vinblastine, in the presence of specific MDR inhibitors, PSC833 and MK571, inhibited first cleavage ~30% more (80% inhibition) at 100 nM with greater percentage difference at lower concentrations. There was no difference in the potentiation of vinblastine-mediated cleavage inhibition between specific inhibitors nor was there a difference between the concentrations (5 and 10 μM) of specific inhibitors.

In Figure 2A, the specific MDR inhibitors when used in the absence of dye or vinblastine reduced first cleavage by ~10-20%. In subsequent experiments, both specific inhibitors used alone reduced first cleavage in a dose-dependent manner (Figure 2B). The P-gp-specific inhibitor, PSC833 inhibited first cleavage by 13% at 5 μM and by 19% at 10 μM. The MRP-specific inhibitor, MK571, reduced first cleavage by 6% at 5 μM and by 12% at 10 μM. All concentration of specific inhibitors reduced first cleavage significantly over controls ($P<0.05$). As a group, PSC833 was a stronger inhibitor of first cleavage than MK571 ($P<0.05$). The two concentrations of PSC833 were not significantly different from each other, but the higher concentration of MK571 (10 μM) was significantly more inhibitory at first cleavage than 5 μM of MK571 ($P<0.05$). For those embryos that continued to divide, the specific inhibitors caused the two initial macromeres to divide unequally during second cleavage (data not shown).

3.3. Dye accumulation at developmental stages using specific and non-specific dyes and inhibitors

To determine the differences in c-am dye accumulation during certain developmental stages, recordings of dye accumulation at 30 min time intervals were taken of spawned eggs, early embryos (2-cell), blastula and gastrula embryos. Dye accumulation in spawned eggs was 2- fold higher than dye accumulation in the 2-cell embryo (Figure 3) suggesting an increase in the dye efflux capacity of the 2-cell embryo following fertilization. Dye accumulation in blastula embryos was also ~2-fold greater than in the 2-cell embryo and about 1.5-fold greater in gastrula embryos suggesting further changes in the dye efflux capacity during development of the sea star embryo. The rate of dye accumulation was linear over time in spawned eggs and gastrulae but appeared to have plateaued 30 min following the addition of dye for a total duration of 60 min in both the 2-cell and the blastula embryo.

Two dyes were used to characterize the dye efflux activity during development in the sea star. The first dye, calcein-acetoxymethylester (c-am), is a non-fluorescent substrate for MRP and P-gp transporters and the second dye, CDFDA, is one of the few commercially-available MRP-specific dyes in mammalian cells (Zamek-Gliszczynski et al., 2003). C-am is membrane permeable but becomes impermeant to the cell membrane once intracellular esterases hydrolyzes the acetoxymethylester. The loss of the ester bond creates the fluorescent calcein that accumulates in the cell if transporters are absent or inhibited. CDFDA is a known substrate for at least three types of MRP transporters (MRP1-3) but the substrate specificity for other MRP transporters (MRP4-9) for CDFDA

is unknown (Zamek-Gliszczynski et al., 2003). CDFDA is membrane permeant diacetate ester of CDF (5 (and 6)-carboxy-2',7'-dichlorofluorescein) and, like c-am, is hydrolyzed to a membrane impermeant form by cellular esterases. Inhibition of both P-gp and MRP-mediated transport would increase the accumulation of c-am in the cell while inhibition of only MRP-mediated transport would increase the cellular accumulation of CDFDA.

Specific and non-specific MDR inhibitors are used to verify functional MDR transport and, if transport activity is present, should result in an increase in fluorescence in relation to the control. The general MDR inhibitor, R205, is a small peptide known to inhibit the efflux capacity of P-gp proteins (Sharom et al., 1999) but its interactions with MRP transporters has not been characterized although there is evidence for MRP inhibition in the mature sea star egg (Roepke et al., in preparation, see chapter 3). Specific inhibitors of P-gp and MRP-mediated transport have been developed including PSC833 and MK571. These inhibitors are highly specific and do not interact with other members of the MDR family of ABC transporters in mammalian systems. MK571 is an MRP specific transport inhibitor that is a leukotriene analog while PSC833 is a P-gp specific transport inhibitor that is related to cyclosporin D (reviewed in Krishna and Mayer 2000).

In spawned eggs, a 2-fold increase in dye accumulation occurred with R205 and PSC833 over controls (Figure 4A). The rhodamine B (RhoB), a known substrate for P-gp transporters, did not appear to significantly inhibit c-am efflux. MK571 and the MRP1-specific inhibitor BMMA, both inhibited c-am efflux, although MK571 significantly inhibited dye efflux at a greater level. MK571 was also the strongest inhibitor of c-am efflux in the spawned egg. In the 2-cell embryo, R205 and PSC833

both inhibited dye efflux while RhoB did not (Figure 4B). Inhibition of c-am efflux by PSC833 did not significantly change following fertilization but c-am efflux inhibition by R205 increased by 50% (~3-fold). The sensitivity of the 2-cell embryo to MK571 and BMMA was not different than the spawned egg. In blastula embryos, the inhibitory effectiveness of R205 increased by a 150% over spawned egg and by 33% over 2-cell embryo (Figure 4C). Inhibition of dye efflux by PSC833 in blastula was similar to the 2-cell embryo, however, in blastula embryos, RhoB inhibited dye efflux significantly by 2-fold. The inhibition of dye efflux by MK571 increased in the blastula compared to the early embryo while BMMA did not significantly change. In gastrula embryos, all inhibitors increased dye accumulation although not as strongly as in early embryos or during blastula formation (Figure 4D). The sensitivity of the embryos to R205 and MK571 decreased by 46% and 37%, respectively. The control levels of fluorescence decreased by ~50% following fertilization and then increased during later embryonic stages.

MRP-mediated transport was further characterized by using CDFDA accumulation in eggs and embryos. In spawned eggs and all embryonic stages, the P-gp inhibitors PSC833 and RhoB were not effective at inhibiting CDFDA dye efflux (Figure 5A-D). However, the general MDR inhibitor R205 did increase dye accumulation in all stages, but was more effective at the later embryonic stages (Figure 5A-D). The MRP-specific inhibitors MK571 and BMMA increased dye accumulation in eggs and all developmental stages. MK571 was the most effective inhibitor of CDFDA efflux and caused a 2- to 3-fold increase in dye accumulation. MK571 was most effective in spawned eggs, but in early embryos MK571 was 40% more effective at inhibiting dye

efflux as R205 and BMMA. In blastula and gastrula, the sensitivities to the inhibitors were similar in the 2-cell except that MK571 was not as strong an inhibitor in the gastrula stage. The absolute fluorescence levels in untreated controls did not change after fertilization but increased by 2-fold in later stages of development.

3.4. Sequence analysis of sea star MRP transporters

Using degenerate primers to MRP ABC domains, partial sequences from at least three types of MRPs were isolated (Figure 6 & 7). One translated sequence from sea star was similar to vertebrate and invertebrate MRP1 amino acid sequences (Figure 6A & B). The sea star MRP1-like transporters shared highly conserved peptide sequences with MRP1 transporters in the sea urchin, *S. purpuratus*; the mammalian model rat species; the mosquito, *Anopheles* sp. and the elasmobranch skate, *Raja* sp (Figure 6B). The sea star MRP1-like transporters had an 80% amino acid identity with the Rat Conjugate Export Protein (E value = $6.5 \times 10^{-20}$) and 73% with the Human MRP1 (ABCC1) ($5.4 \times 10^{-23}$) and the *C. elegans* MRP1 ($1.4 \times 10^{-18}$). At least three other sequences aligned with human and nematode MRP5/MOAT-C transporters and one sequence aligned with nematode MRP8. No sequences aligned with any P-gp/MDR transporter from other invertebrates or vertebrates.

3.5. cDNA macroarray data

Using cDNA libraries from *Asterina miniata* eggs, we were able to detect a cDNA corresponding to an MRP with high homology to the mammalian sulfonylurea receptor (MRP 8 and MRP 9 ). These transcripts were characterized by the presence of approximately 14 additional (relative to P-gp) amino acids between the highly conserved Walker A and Walker B motifs of ABC transport proteins (Walker et al., 198: Cole and Deely, 1998). Identification of at least one *Asterina miniata* cDNA clone with homology to mammalian MRP 5, a canalicular organic anion transporter supports the hypothesis that more than one type of MRP transporter is involved in sea star egg and embryo transport activity. A P-gp-like cDNA was not detected in the sea star egg.

3.6. Immunolocalization of MRP using a sea urchin MRP antibody

To localize MRP transporters in fertilized sea star eggs and embryos, fixed and permeabilized samples were incubated with a panMRP antibody (Figure 8). MRP transporters were localized throughout the cytoplasm of fertilized egg although brighter labeling occurred on the cell surfaces. Similar localization occurred in the early 2-cell embryo showing brighter cortical labeling as well as cytoplasmic localization. In blastula stage embryos, the cells surrounding the blastocoel were intensely labeled, particularly at the apical membrane. The inner circle of labeling in the blastocoel were from those cells on the opposite side of the embryo. In the gastrula embryo, the cells at the opening of the archenteron, and the archenteron itself, were not labeled by the antibody, however the cells of the ectoderm did label with the MRP antibody.

**Discussion**

In echinoderms, an echinoid and an asteroid have now been shown to exhibit an upregulation of dye efflux activity that occurs during discreet events in the reproductive/developmental cycle. In the sea star, an upregulation of dye efflux activity occurs at oocyte maturation (Roepke et al., in preparation, see chapter 3), but another increase in efflux activity does not occur following fertilization as it does in sea urchin (Hamdoun et al., 2004). The other major difference between these two species is the type of transporters involved in the increase in dye efflux activity. Sea stars utilize both MRP- and P-gp-mediated efflux transport, while sea urchins predominantly utilize MRP transport. The significance of this difference in MDR transport expression and function may be a result of the difference in life histories and feeding strategies. Both species are scavengers but *A. miniata* is primarily a carnivore while *S. purpuratus* feeds extensively on kelp and algae.

The inhibition of first cleavage by the specific MRP inhibitor MK571 has been reported in sea urchin 2-cell embryos (Hamdoun et al., 2004) and has been confirmed in this study in another echinoderm species. In the sea urchin, MK571 (but not PSC833) inhibited first cleavage, however, unlike the sea urchin, the P-gp specific inhibitor PSC833 inhibited first cleavage in the sea star. MK571 inhibited first cleavage in the sea star but not as significantly as PSC833. Cleavage inhibition by MDR inhibitors may be a result of a non-specific inhibition of an enzyme(s) involved in mitosis or by inhibition of the removal of toxic metabolic compounds or regulatory substances necessary for cell division. Substrates of MRP transporters include glutathione conjugates of cellular waste

products and other toxic byproducts of cell function. These conjugated compounds may be trapped in the cell as a result of the inhibition of MRP transport activity, thereby increasing the chance of cytotoxicity. The substrates for P-gp transporters include peptides from ubiquitin-tagged proteins marked for degradation. The inhibition of peptide transport may itself alter cytoplasmic homeostasis, thus altering normal cytokinesis during early cleavage development.

One possible function of these transporters is to protect the eggs and embryos from natural and anthropogenic toxins in the aquatic environment. The sensitization of early cleavage embryos to vinblastine by inhibitors of both P-gp and MRP transporter activities supports this hypothesis. MDR transporters in vertebrate somatic cells are thought to play a protective function in epithelial tissues as the placenta (Leazer and Lassen, 2003; Pascolo et al., 2003), the intestine (Miller et al., 2002b), the blood-testis barrier (Melaine et al., 2002) and the adult blood-brain barrier (Miller et al., 2002a). This is also consistent with the suggested role of the transporters in marine invertebrate embryos (Hamdoun et al., 2002; Toomey and Epel; 1993, Toomey et al., 1996).

During sea star development there appears to be two or more changes in c-am dye efflux capacity, although CDFDA accumulation does not change. The first change is an increase occurring following fertilization in early cleavage embryos when the amount of dye accumulation during a two-hour incubation with c-am decreased by over 50%. The second change occurs when the dye efflux capacity decreases during blastula formation, but increases again during gastrulation. The second change in dye efflux capacity may be a result of the depletion of maternally-derived transporters during the later stages of early embryogenesis. The second increase in dye efflux capacity occurs near the initiation of

embryonic transcription at the time of hatching (Davidson, 1999) and the potential expression of new transporters during gastrulation. These changes may also be the result of a decease in cellular esterase activity during early embryogenesis since esterases are critical for conversion of c-am to calcein. However, the ratio of dye accumulation in inhibitor-treated embryos compared to control embryos does not significantly change until the gastrula stage of development indicating that no change in esterase activity is occurring at least until the gastrula stage.

Functional evidence of P-gp-like transport activity exist for the three stages of development. R205 is more effective in early embryos and blastulae while PSC833 inhibits dye efflux at a consistent level (2-fold) at each stage. The difference between the two inhibitors suggest that either R025 is interacting with other non P-gp transporters or that expression of P-gp transporters sensitive to PSC833 is consistent during development. P-gp transporters have a broad range of substrates and chemosensitizers or modulators including xenobiotic compounds and endogenous peptides that may be either hydrophilic and hydrophobic (Litman et al., 2001). Peptide substrates relevant for the proper functioning of the developmental program include peptides from the ubiquitinated protein degradation and the proteolytic cleavage apparatus. Also, during early development in the marine environment, sea star embryos are exposed to numerous exogenous compounds with potential toxic effects. These compounds are probable substrates for P-gp transporters.

Inhibition of MRP-mediated efflux occurs during each stage of development demonstrating the functional expression of this family of transporters. Substrates for MRP transporters include organic anions; conjugates of glutathione, glucuronide and

sulfate; nucleotides and their analogues and anionic peptides (Litman et al., 2001). Other substrates of MRP transporters may be involved in paracrine signaling during early development such as leukotrienes derived from the oxidation of arachidonic acid by lipoxygenases (Funk, 2001). Two changes in the MRP-mediated efflux occur during sea star development – an increase in MRP-mediated transport during blastula formation and a decrease in MRP-mediated transport following gastrulation.

The localization of MRP transporters by the panMRP antibody in the fertilized egg and early embryos are another validation of MRP activity during fertilization and early embryo development. The localized transporters in a later developmental stage are found primarily in the ectodermal layer of cells in the gastrula embryo and were not localized in the archenteron. The lack of protein staining suggests either the embryos were not sufficiently permeabilized blocking the access of the antibody to transporter located in the archenteron, or that MRP transporters during this stage of development are not expressed during invagination of the primitive gut and are therefore not important for the developmental program in these cells. The sea urchin panMRP antibody has not been fully characterized, however the partial sequences of at least three types of MRP transporters (MRP1, 5, 8) justify the use of this antibody for immunocytochemistry. Due to vendor issues with the P-gp primary antibody, we were unable to repeat the immunocytochemistry with the P-gp specific antibody, C219, which had previously been used in prepared sea star eggs (Roepke et al., in preparation, see chapter 3).

We propose that the presence of several MRP transporter types observed in the alignments of the partial amino acid sequences to vertebrate and invertebrate MRP transporters implies multiple roles for these transporters in sea star embryos. The partial

characterization of an amino acid sequence similar to MRP1 in sea star eggs corroborates the value of the putative MRP1 inhibitor, BMMA. The sequence aligned with MRP8 transporters, suggesting a potential role in the regulation of $K^+$ conductance during embryogenesis. MRP8/SUR (sulfonylurea receptors) transporters function as heteromultimeric complexes with inwardly rectifying $K^+$ channel (Kir6.1-6.2) subunits coupling membrane excitability to cellular metabolism (Ibrahim et al., 2003). Three other sequences aligned with MRP5 transporters. Plant homologues of MRP5 transporters regulate root development by establishing and maintaining auxin gradients during plant growth (Friml et al., 2003; Gaedeke et al., 2001). These transporters are presumably present and functional during early embryogenesis indicating endogenous functions separate from the role of cellular protection in the marine environment.

A transcript encoding for a P-gp efflux transporter similar to those found in other invertebrates or vertebrates was not identified during screenings of cDNA libraries from the sea star egg. The use of cDNA macroarray technology also did not locate a P-gp-like transcript in the sea star egg. While there is functional evidence for P-gp transport activity (inhibition of dye efflux by PSC833) and protein recognition by a well-characterized P-gp-specific antibody in mature sea star eggs (Roepke et al., in preparation, see chapter 3), the lack of molecular data confirming a P-gp transporter is confounding. A P-gp-like transporter has been sequenced in the sea urchin (Hamdoun et al., 2004) using the same degenerate primers employed in this study. There are several plausible explanations for the lack of P-gp molecular data in the sea star. First, degenerate primers applied to the cDNA libraries did not function appropriately with the sea star egg RNA or the transcript level for P-gp transporters was too low for the routine

RT-PCR techniques utilized. Second, the sea star P-gp-like transporter may be a unique variant of the ABCB family, and this has been suggested in the previous chapter (Roepke et al., in preparation, see chapter 3) based on electrophoretic behavior. Three sequences in Figure 7A do not align closely with any MRP (or P-gp, not shown) and may represent partial sequences from such a unique transporter.

In summary, MDR transport is fully functional in all developmental stages of the sea star, *Asterina miniata*. Both P-gp-like and MRP-like transporters are expressed and functional during the three stages of development examined. The use of specific dyes and inhibitors based on mammalian cell studies gives rise to a caution when used in experiments with invertebrate systems since results are weighted on the assumption that molecular sequence homology correlates with functional homology. Conclusions using data from experiments with these dyes and inhibitors should be carefully inferred in invertebrate systems. This assumption also applies to the apparent endogenous role of these transporters in cleavage. Nevertheless, the roles of these transporters may include regulation of cell division by removal of endogenous compounds and protection from environmental and endogenous toxins. The multiple functions for these transporters can not be determined with certainty until further *in vivo* and *in vitro* experiments utilizing molecular tools and techniques are developed.

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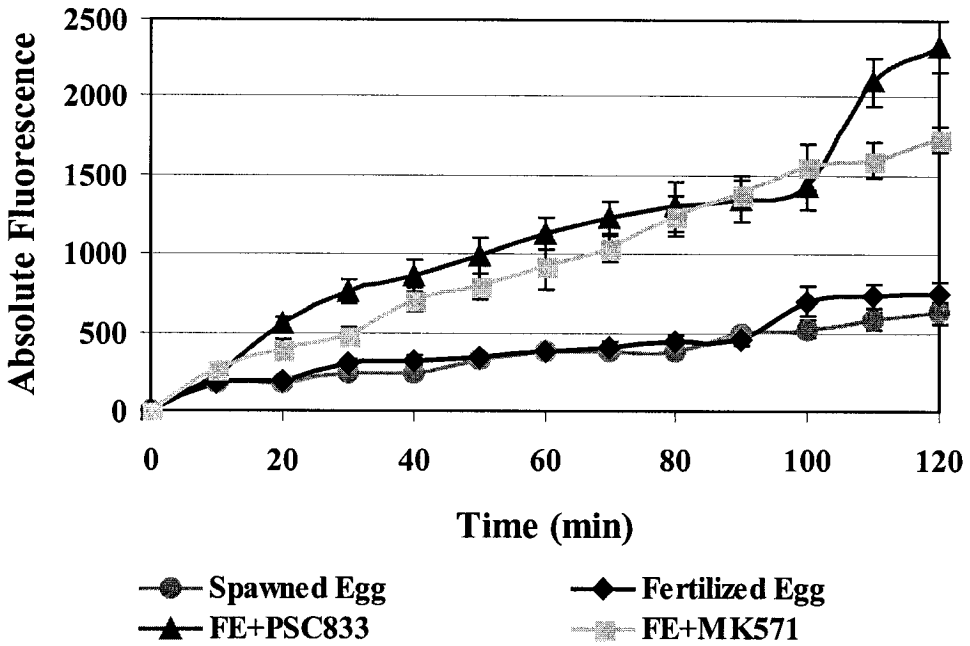
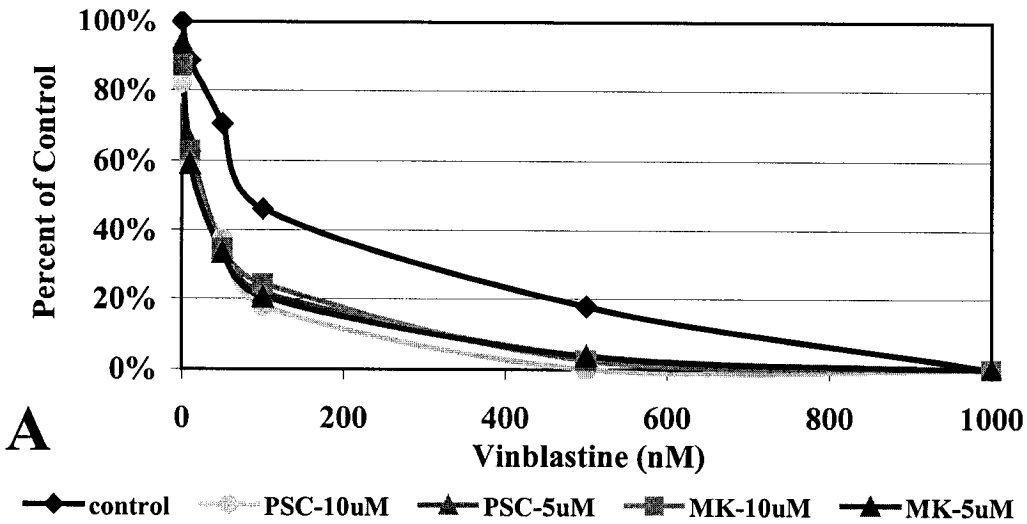



Figure 1. Time lapse kinetics of c-am accumulation in spawned eggs and fertilized eggs with and without specific inhibitors. Recordings of fluorescence from the same 7-10 eggs were taken every ten (10) min for 120 min after addition of 0.5 μM c-am. Spawned eggs were attached to poly-lysine coated chambered slides. At t=0, c-am was added to spawned egg chamber #1; c-am plus dilute sperm was added to chamber #2 (fertilized egg) and c-am plus dilute sperm and specific inhibitor was added to chamber #3 and #4 (PSC833 (10 μM) and MK571 (10 μM)) Recordings were analyzed according by MetaMorph®. The response of the fertilized eggs was not significantly different than the spawned eggs but the responses of the fertilized eggs treated with specific inhibitors were significantly different from untreated fertilized eggs at all time points except for 10 min ($P<0.001$).

Figure 2. A: Potentiation of cleavage inhibition by vinblastine with specific inhibitors. Fertilized eggs were incubated in FSW at 45 min post-fertilization with vinblastine (10-1000 nM) alone or with PSC833 (5 and 10 μM) or MK571 (5 and 10 μM) for 105 min. All samples were fixed with 100 μl 4% paraformaldehyde in FSW and counted using an inverted phase contrast microscope for first cleavage (two-cell embryos). B: Fertilized eggs were incubated with two concentrations of PSC833 or MK571 (5 and 10 μM) 10 min post-fertilization for 140 min in FSW. Samples were fixed and counted for first cleavage (two-cell embryos). (a) denotes significant difference between the two specific inhibitors as a group ($P<0.05$) using ANOVA. (b) denotes significant difference between the two concentrations of MK571 ($P<0.05$) using ANOVA.

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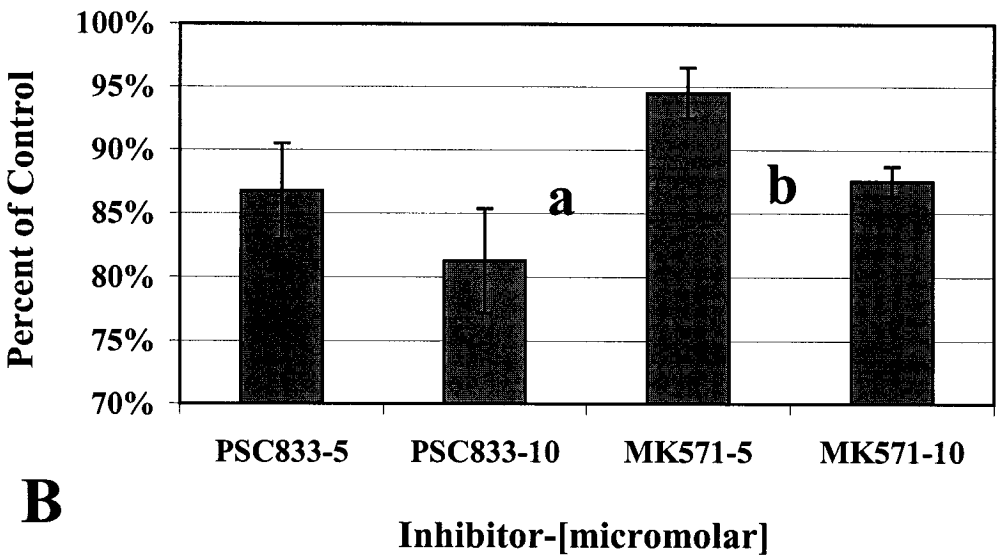


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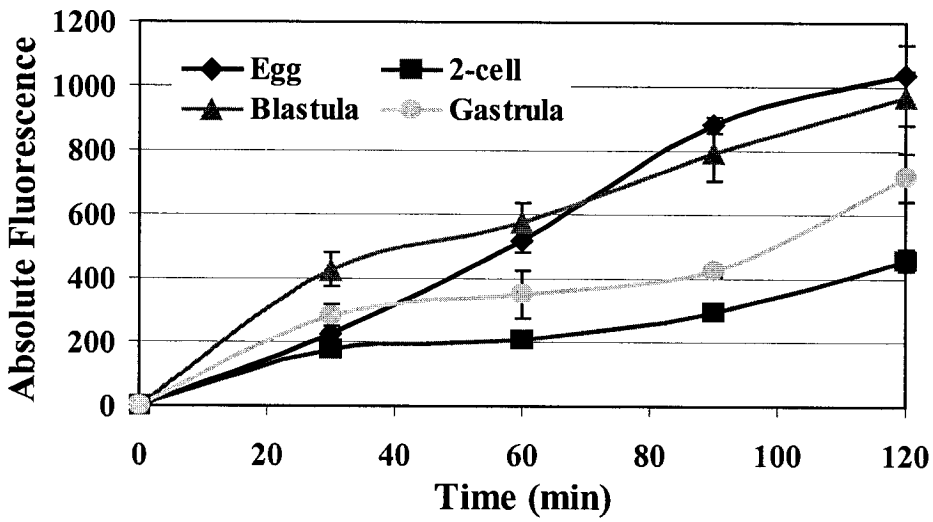



Figure 3. A comparison of the kinetics of c-am accumulation in spawned eggs and 2-cell, blastula and gastrula embryos. Recordings of egg or embryo fluorescence were taken at 30 min intervals for 120 min after addition of 0. 5 μM c-am. Samples were incubated with dye in 15 ml conical tubes at 15 °C for 30 min. Samples were at each time point hand centrifuged and a subsample was examined with fluorescence microscopy. Samples were placed back in incubator for the next time points. Recordings were analyzed according by MetaMorph®.

Figure 4. Dye accumulation in spawned eggs and embryos using calcein-AM incubated with specific and non-specific inhibitors of MDR transport. A-D: Relative fluorescence of spawned eggs, 2-cell embryos, blastula and gastrula incubated with c-am (0.25 μM) for 2 hr with R205 (3 μM), PSC833 (5 μM), RhoB (1μM), MK571 (5 μM) and BMMA (10 μM). The mean pixel fluorescence intensity of 20-30 individual eggs or embryos was measured and averaged for each maturational time point. Darkened line marks the level of control fluorescence which is given in absolute fluorescence units. All ratios were significantly different from 1.0 ($P<0.05$) except for those denoted by (*).

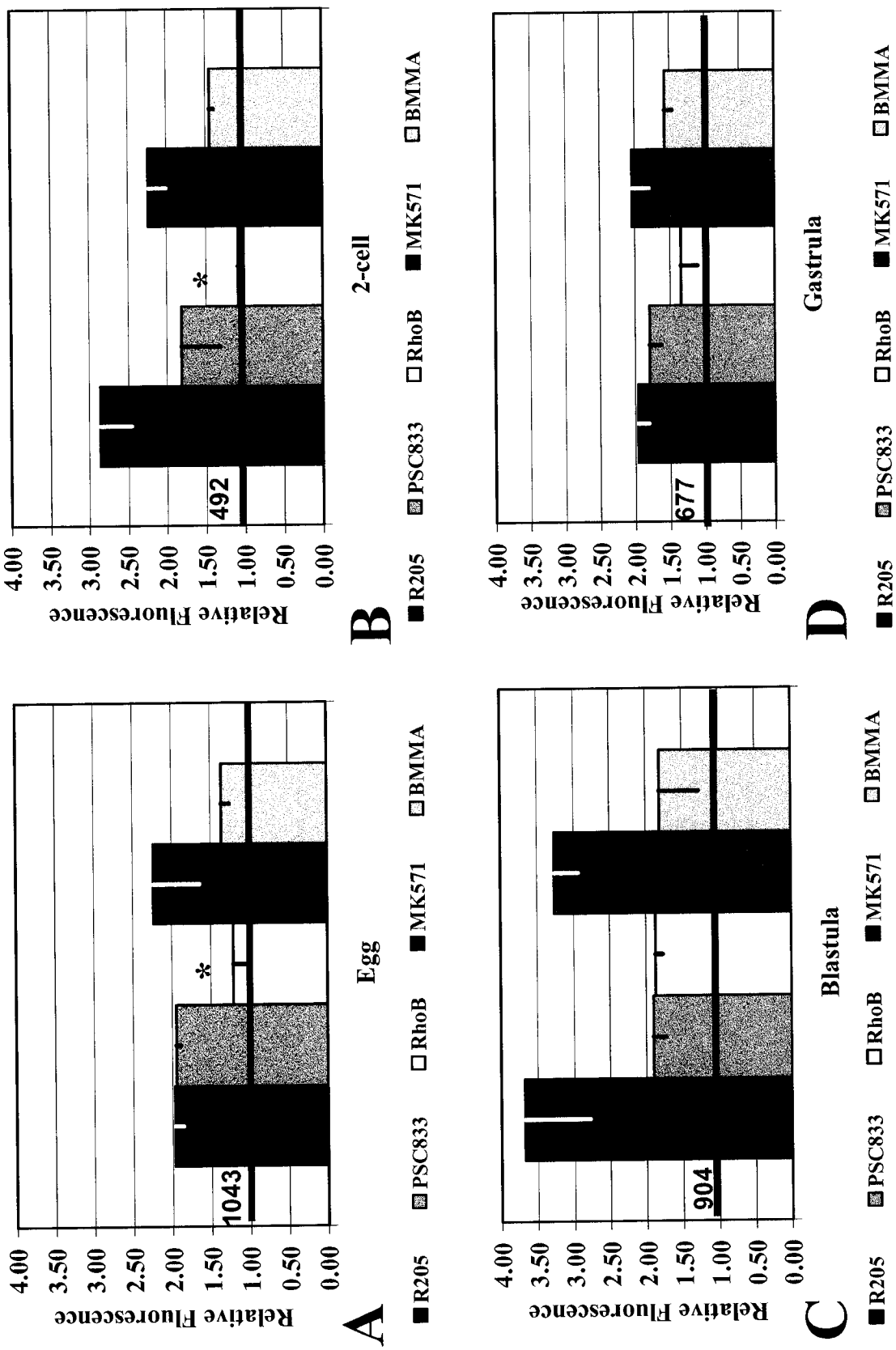
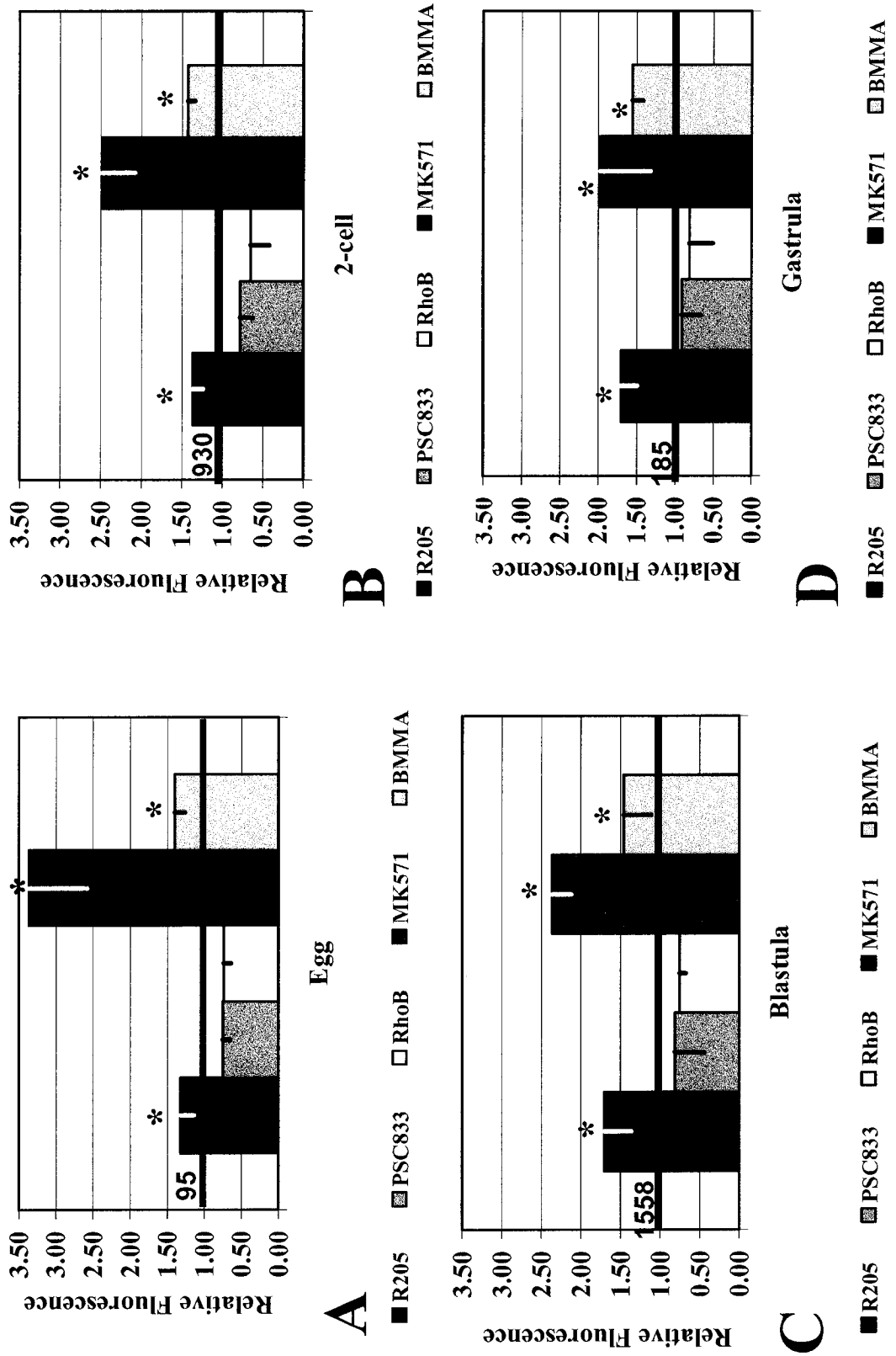


Figure 5. Dye accumulation in spawned eggs and embryos using an MRP-specific dye, CDFDA, incubated with inhibitors of MDR transport. A-D: Relative fluorescence of spawned eggs, 2-cell embryos, blastula and gastrula incubated with c-am (0.25 μM) for 2 hr with R205 (3 μM), PSC833 (5 μM), RhoB (1μM), MK571 (5 μM) and BMMA (10 μM). The mean pixel fluorescence intensity of 20-30 individual eggs or embryos was measured and averaged for each maturational time point. Darkened line marks the level of control fluorescence which is given in absolute fluorescence units. (*) denotes significant differences relative to the control absolute fluorescence ($P<0.05$)



**A.**

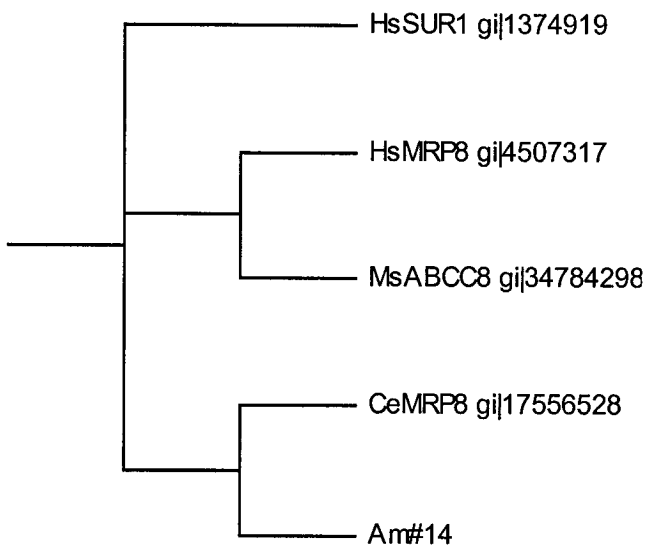
```
TCGGGATCCAGGGAGAATATACTGTTTGGGGCACCATTGCAAGATGCAGAGTACCAGCGAGTGATCGAAGCCTGCGCGCT
S  G  S  R  E  N  I  L  F  G  A  P  L  Q  D  A  E  Y  Q  R  V  I  E  A  C  A  L

GGCCCCTGATCTGGACATGCTCCCAGCTGGAGATCTAACGGAGATTGGAGAAAAGGGAATTAACCTGAGTGGTGGTCAGA
A  P  D  L  D  M  L  P  A  G  D  L  T  E  I  G  E  K  G  I  N  L  S  G  G  Q

AGCAGAGGGTCAGTTTAGCTCGAGCCGTTTACAACAATGCCGACATCTATCTCCTTGACGACGAATTCCG
K  Q  S  V  S  L  A  R  A  V  Y  N  N  A  D  I  Y  L  L  D  D  E  F  X
```

**B.**

| | |
|---|---|
| *Asterina* | RENILFGAPLQDAEYQRVIEACALAPDLDMLPAGDLTEIGEKGINLSGGQKQRVSLARAV |
| *S. purpuratus* | KDNVLFGEKLRDSKYRKVIGACALNPDIDMLPAGDQTEIGEKGINLSGGQKQRVSVARAL |
| Rat | RENILFGRPLQEHCYKAVMEACALLPDLEILPSGDLTEIGEKGVNLSGGQKQRVSLARAV |
| *Anopheles* | KDNILFGKPMDQRRYARVIEACALKPDIEMLPGGDMTEIGEKGINLSGGQKQRVSLARAV |
| *Raja* | QDNILFGMKMEDSRYQEVLEACALPQDLELLPAGDLTEIGERGINLSGGQKQRVSLARAV |
| Consensus | **N LFG Y V ACAL P D LP GD TEIGE G NLSGGQKQRVS ARA** |

Figure 6. A: Partial nucleotide sequence to a sea star MRP1-like transporter and the corresponding amino acid sequence. B: Partial translated sequence of cloned *Asterina miniata* and *Strongylocentrotus purpuratus* MRP transporter proteins compared with other MRP sequences. This sequence corresponds to the highly conserved nucleotide binding domains.

**A.**




**B.**

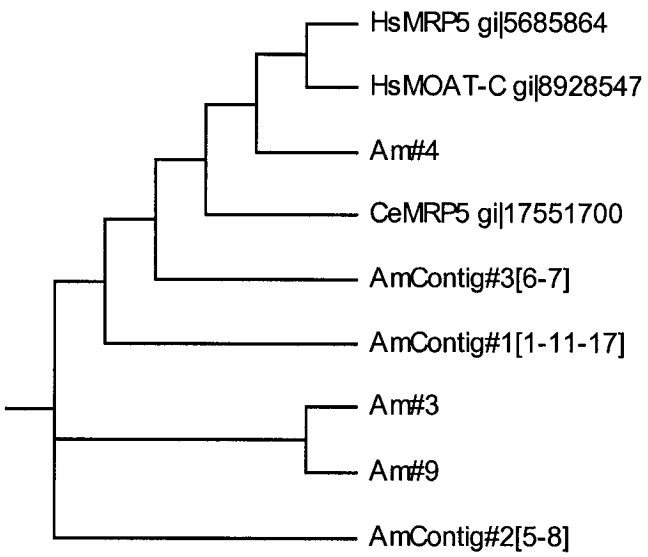



Figure 7. Multiple sequence alignment (ClustalW) demonstrates the alignment of partial amino acid sequences of *Asterina miniata* multidrug transporter proteins with vertebrate and invertebrate MRP sequences. A. One sequence aligned with MRP8. B. Three sequences align with MRP5.

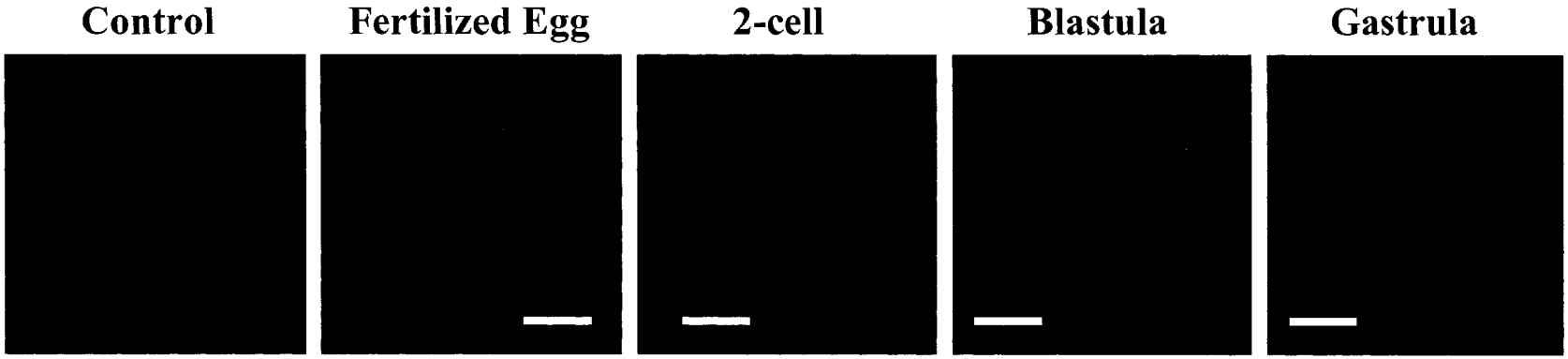



Figure 8. Immunocytochemistry of fertilized eggs and 2-cell, blastula and gastrula embryos using a polyclonal spMRP antibody designed using sea urchin sequences. Fixed and permeated eggs and embryos were incubated with a polyclonal antibody to sea urchin MRP sequences. A secondary control (no primary) of oocytes was also prepared. The samples were viewed using an Olympus laser scanning microscope. Photomicrographs were analyzed using Fluoview® (Olympus Corp.) software. White bars equal 100 μm.

# CONCLUSIONS

Several relevant questions remain to be answered about the role of estradiol in sea urchin embryo development and whether or not a canonical steroid nuclear receptor-mediated pathway is involved. Initially, BLAST searches of the ongoing Sea Urchin Genome Project for the estrogen receptor and other steroid nuclear receptors should continue as well as gene identification using an array of molecular techniques including cDNA macroarray technology and RT-PCR/cloning. Subsequent experiments should involve radiolabeled estradiol ligand-receptor binding studies using embryonic, larval and adult tissues. To address the question presented in Chapter 2 about the role of SpSHR2 in estradiol and estrogenic compound teratogenicity, this particular receptor protein should be expressed, purified and prepared for ligand-receptor binding studies with radiolabeled estradiol, other hormones and relevant estrogenic compounds.

The sea urchin embryo is a developmental model in which many of the important signaling molecules have been identified. As hinted at in Chapter 2 with the regulation of SpSHR2 by maternal exposure to estradiol, experiments measuring the regulation of other genes known to be responsive to estradiol and the ER in vertebrate systems should be conducted in maternally-exposed adult females and males and their subsequent embryos. Potential genes expressed during development include MDR genes, phospholipases, phosphodiesterases, phosphatases, insulin-like growth factors, interleukin receptors, Coenzyme A synthase and glutathione S-transferases (Hagen, et al., 2000; Chen and Yager, 2004: Ee, et al., 2004; O'Lone, et al., 2004; Naciff, et al., 2005). Subsequent experiments should examine the effects of estradiol and estrogenic compounds on the regulation and expression of these signaling molecules.

Also in the sea urchin developmental model, the differentiation of important cell types during embryogenesis has been elucidated. Because cellular exposure to toxicants can induce cell death either by necrosis or apoptosis and one of the obvious effects of estradiol and EDC exposure is abnormal embryonic development, experiments measuring the occurrence of apoptosis during development may illustrate the relevant cell types involved in estrogenic teratogenicity. Spiculogenesis does not appear to be a primary target of the estrogenic compounds possibly precluding primary mesenchyme cells as targets for estrogenic teratogenicity. Conversely, the evidence that estradiol and EDC alter embryonic polarity, the cells types involved in the animal/vegetal polarity are excellent candidates for teratogenic targets during early embryo development. Another particularly interesting question is the relative insensitivity of the gastrula embryos to estradiol and whether or not estradiol has a role in the invagination of the archenteron.

One of the obvious gaps in the MDR portion of my dissertation is the lack of molecular evidence of a P-gp-like transporter in the sea star. Additional sequencing protocols should be used to identify and sequence the transporter that is inhibited by the P-gp specific inhibitor PSC833. Once an antibody raised against the recently cloned sea urchin P-gp transporter is created, the immunoblotting and immunocytochemistry experiments should be repeated. This would not only confirm the presence of a P-gp-like transporter in the sea star but could potentially address the low molecular weight issue presented in Chapter 3. Real-time Q-PCR assays have been developed for sea urchin P-gp and MRP transporters and should also be developed and utilized in the sea star embryo. Both of these tools, antibody and real-time Q-PCR, would also assist in measuring the expression of these transporters through development and into adulthood

and allow experiments addressing the potential inducible changes due to stress and toxicant exposure.

One of the relevant topics for the MDR transporter expression during development is identification of endogenous role(s) the transporters play during development other than a protective function guarding against environmental assault. There are indications in the sea star and the sea urchin that MDR transporters are important for cell division in early development. Subsequent experiments should examine the effects of the transport inhibitors on early cleavage and what signaling mechanism(s) are at play in the reported inhibition. While no particular role for these transporters was suggested by the oocyte maturation data (Chapter 3) other than protection from maturation inhibitors, an interesting question is what are the endogenous roles of the transporters particularly P-gp during oogenesis? The expression of the transporters imply a function of some variety, however any experiments addressing this question would have the difficulty of using an uncharacterized reproductive and cellular system (sea star gametogenesis and reproductive endocrinology).

Developmental bioassays and maternal exposure experiments combining the effects of estradiol and EDCs on development with the expression and activity of MDR transporters would be a unique synthesis of this dissertation. I would suggest repeating the maternal exposure experiments with estradiol, estrogenic compounds and any other relevant environmental toxicant such as pharmaceuticals in tandem with measuring the dye efflux capacity in spawned eggs prior to and following maternal exposure. Samples of the spawned eggs and subsequent developing embryos could be prepared for real-time Q-PCR and immunoblotting to determine the changes in expression of both P-gp and

MRP transporter mRNA and protein due to maternal exposure. Sampling of the adult tissue after exposure could also be informative to determine the expression of transporter mRNA and protein as well as the orphan receptor, SpSHR2. Additional experiments with MDR transport inhibitors added to the developmental bioassays should also be conducted with and without estradiol and the EDCs.

After reading this dissertation, one may asked of the relevance of this data to real world issues involving both the effects of estrogenic compounds on development and the expression of MDR transporters during reproduction and development. The sensitivity of the sea urchin embryo to estrogenic compounds and other EDCs may be useful as a toxicology assessment technique for measuring and detecting pollutants in coastal marine environments. Furthermore, the regulation of steroid nuclear receptor mRNA by environmental toxicants in invertebrates may be developed as biomarker tool to also assess contamination in coastal waters especially those near heavy industrial development and sewage treatment plants. While most MDR transporter research in invertebrates is focused on their potential protective function and their part in the cellular stress response concomitant with heat shock protein expression, the use of echinoderm models may elucidate the other endogenous role(s) these transporters have in vertebrate development and reproduction. Such answers have relevance in gestational pharmacology, cancer therapies and developmental patterning.

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