REVIEW ARTICLE

Molecular Reproduction & Development 78:553–564 (2011)

Laboratory on Sea Urchin Fertilization

VICTOR D. VACQUIER*

Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, California

SUMMARY

Since about 1880, the eggs and sperm of sea urchins have been used for the study of fertilization, the metabolic activation of development and gene regulatory mechanisms governing embryogenesis. Sea urchin gametes are a favorite material for observations of the process of fertilization in advanced high school, community college, and university biology laboratory courses. This article is a laboratory handout, designed for the student to follow in learning about fertilization. In addition to observations of sperm–egg interaction, simple experiments are described that demonstrate some mechanisms involved in the process. The hope is that by making simple observations of fertilization, the student will gain an appreciation for the fact that successive generations of higher organisms are bridged by the fusion of egg and sperm, two very different single cells.

Mol. Reprod. Dev. 78: 553–564, 2011. © *2011 Wiley-Liss, Inc.*

"I present some simple, but fascinating observations and experiments about fertilization that can be carried out in a classroom. . ."

* Corresponding author:
9500 Gilman Drive
La Jolla, CA 92093-0202.
E-mail: vvacquier@ucsd.edu

Published online 8 July 2011 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/mrd.21360

Received 11 May 2011; Accepted 27 June 2011

INTRODUCTION

Sea urchins are an ancient group (at least 450 million years old) of Class Echinoidea in the Phylum Echinodermata, with hundreds of species known in the world's oceans. Sea urchin gametes are a favorite experimental material for the study of fertilization for the following reasons: the sexes are separate, it is easy to obtain the gametes from the adults, the gametes are highly uniform in maturity and purity when released into seawater, fertilization occurs external to the adult body and is therefore easy to manipulate, the financial expense is low, adults are easy to keep in aquaria, and several species are available at marine laboratories and from biological supply companies. There are two reference books on sea urchin embryonic development (Schroeder, 1986; Ettensohn et al., 2004), and much information is available on the Internet by using < sea urchin fertilization > as the query. There are excellent movies on YouTube and Google on sea urchin fertilization. The genome of one sea urchin, *Strongylocentrotus purpuratus*, is known (http://www.spbase.org/SpBase/), and the genomes of other species are being sequenced. The genome size is ~800 Mb (million), compared to the human genome of ~3,200 Mb. The articles describing the sea urchin genome can be found in Science, volume 314: pp 939–962, November 10, 2006. A special issue of Developmental Biology (vol. 300: pp 1–495, 2006) is also devoted to the sea urchin genome.

When injected with 0.5 M KCl (which is isosmotic with seawater) gravid adults of many sea urchin species spawn their gametes (Fig. 1). The K^+ ions depolarize a muscle on the outer surface of the alveolar ovary and testis. The sperm or eggs are forcibly extruded into surrounding seawater through five gonopores on the aboral surface (opposite side from the mouth). Electric shock (6–10 V) can also be used to

Abbreviations: Ap, *Arbacia punctulata*; Sp, *Strongylocentrotus purpuratus*; Sf, *Strongylocentrotus franciscanus*; Lp, *Lytechinus pictus*; Lv, *Lytechinus variegatus*; De, *Dendraster excentricus*; Ep, *Echinarachnius parma*; AR, acrosome reaction; CG, cortical granule; CaFSW, calcium free artificial seawater; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FE, fertilization envelope; FSP, fucose sulfate polymer of egg jelly; HSW, seawater with 10 mM HEPES buffer, pH 8.0; JC, egg jelly coat; PABA, para-aminobenzoic acid; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; VL, vitelline layer of the sea urchin egg.

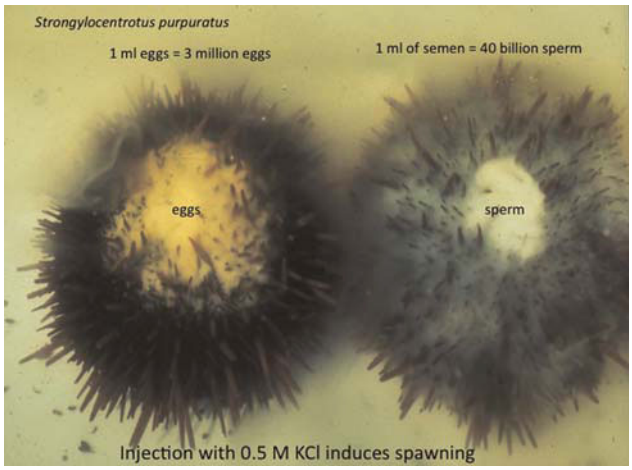

Figure 1. Adult *Strongylocentrotus purpuratus* (left, female; right, male) spawning after injection with 0.5 M KCl. After removal of the bulk of the egg jelly coat by the pH 5.0 method, and gentle sedimentation by hand centrifugation, there are approximately 3 million eggs per ml.

contract the gonad and force the gametes out of the gonopores.

Here I present some simple, but fascinating, observations and experiments about fertilization that can be carried out in a classroom using sea urchin gametes obtained by the extrusion procedure just described. After briefly introducing each type of gamete, I describe several stand-alone lab-exercises to investigate fertilization; these exercises have been tested in many classroom settings, and are reliable and interesting for students. I present the exercises in a format that is designed to function as a lab-handout for use directly by students and instructors to carry out the experiments and observations, and includes information about interpretation of each experiment's significance and expected results.

SEA URCHIN EGGS

Egg diameter is a species-specific characteristic among echinoderms. *Arbacia punctulata* (Ap) eggs, from the North American Atlantic coast, are 80 μm (micrometers) in diameter and dark red/purple due to echinochrome pigment. *Strongylocentrotus purpuratus* (Sp) eggs from the Pacific coast are 80 μm in diameter and yellow-brown in color due to carotenoid pigments. *Lytechinus variegatus* (Lv) from the Atlantic coast, and *L. pictus* (Lp) from the Pacific coast, have light yellow eggs 111 μm in diameter. The eggs of *S. franciscanus* from the Pacific coast are 140 μm in diameter. The Atlantic coast sand dollar, *Echinarachnius parma*, and the Pacific coast sand dollar, *Dendraster excentricus*, can also be used with this handout if sea urchin eggs are not available. Hopefully, the gametes of more than one echinoid species will be available for this laboratory.

Eggs of all species of Class Echinodea (sea urchins, heart urchins, and sand dollars) are unusual in that when they are spawned (released from the ovary), they have finished meiosis and have a complete nuclear envelope containing a haploid amount of DNA; they are actually in the G1-phase of the first mitotic division cycle. In almost all other animals, eggs are spawned and fertilized in a meiotic stage, and finish meiosis shortly after spawning, or after fertilization. Sea urchin eggs are metabolically (biochemically) dormant and undergo an explosive metabolic activation after fusing with a sperm. Protein kinases play key roles in egg activation (Townley et al., 2009).

In this laboratory we will observe some of the phenomena associated with sea urchin sperm–egg interaction (fertilization) and egg activation. A sea urchin egg, with its hydrated jelly coat, is shown in Figure 2. Ap adults may require an electric shock of 6–10 V to induce spawning. As the Ap females spawn, one should collect the dark red/purple eggs with a pipette as they are coming out of the gonopores, and dilute them into cold 100 ml of HEPES-buffered filtered seawater (HSW). This is important because chemicals coming from the surface of the adult females of this species will make the eggs unfertilizable. This is not a problem with the other species, so spawning females can be inverted on top of a 50- or 100-ml beaker filled with HSW so that the HSW is touching the gonopores. The negatively buoyant eggs immediately sink to the bottom.

SEA URCHIN SPERM

The sexes cannot be distinguished until the adult urchin begins to spawn. In all species, the concentrated sperm (semen) is white (Fig. 1). The undiluted semen is collected with a disposable pipette, and kept in 1.5-ml microfuge tubes that are stored packed in ice. If kept undiluted and

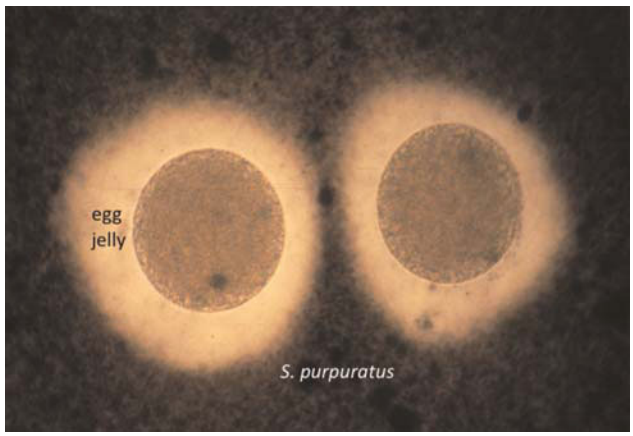

Figure 2. Unfertilized *Strongylocentrotus purpuratus* eggs with hydrated egg jelly coats. The jelly coats are transparent and are made visible by putting India ink in the seawater. The colloidal ink particles do not enter the jelly coat. The eggs are 80 μm in diameter.

packed in ice, the sperm will remain capable of fertilization for 4 days. Sea urchin spermatozoa (sperm) are very small cells that pour out of the male gonopores after injection of 0.5 M KCl. The undiluted semen of Sp species contains 4×10^{10} sperm cells per ml (40 billion). In Sp, this is a fairly constant value among males.

The sperm cell is composed of a tail (flagellum) about 50 μm long and 0.1 μm wide (Fig. 3). Cross sections of sea urchin sperm flagella show they are made of the "9 plus 2" pattern of microtubule doublets characteristic of cilia and flagella, covered by a plasma membrane. Sea urchin sperm have been a valuable model in the study of flagellar motility. The proximal end of the flagellum fits into the "posterior nuclear fossa," a depression in the nucleus, which contains the two centrioles inside the centrosome (which is invisible). Once inside the egg, the centrosome, with its pair of centrioles, replicates, the sister centrosomes separate and form the poles of the first, and all subsequent, mitotic spindles (the cell division spindles). Anterior to the flagellum is the cone-shaped sperm head (~3 μm long by ~1 μm in diameter), consisting of one large mitochondrion, the sperm nucleus, and the acrosomal complex. The most anterior part of the sperm contains the acrosomal complex, consisting of a depression in the nucleus called the "anterior nuclear fossa," which is filled with globular actin. Anterior to this fossa is the acrosomal vesicle (or granule), containing the protein bindin, which will bind the sperm to receptors on the vitelline layer of the egg after it is exposed by the acrosome reaction. You must use a phase contrast microscope with a 100× oil immersion, phase contrast objective, and remove almost all the seawater from the slide, in order to see the acrosomal vesicle.

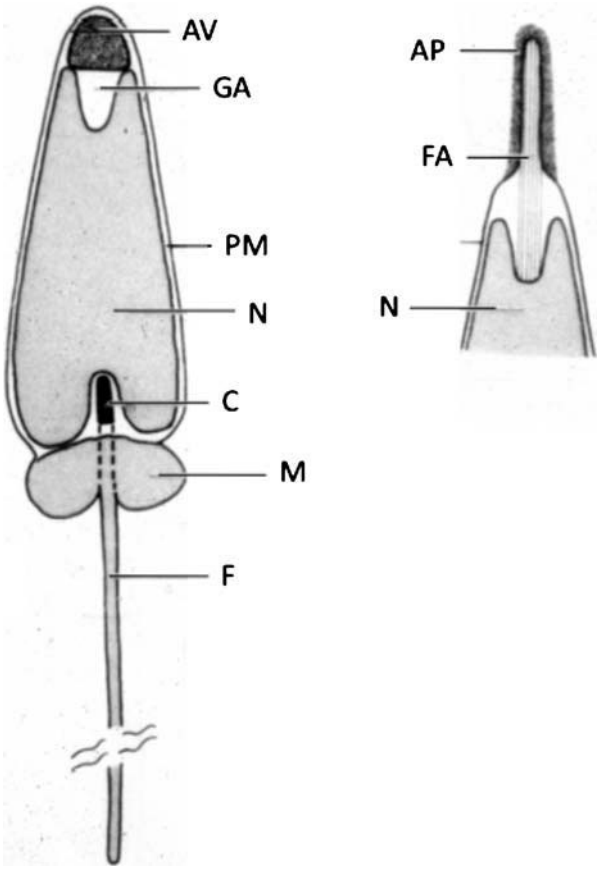

Figure 3. Drawing of *Strongylocentrotus purpuratus* sperm before (left) and after (right) the acrosome reaction. The entire cell is surrounded by a plasma membrane (PM). The acrosome vesicle (AV) contains bindin, the protein that bonds the sperm to the egg. The anterior depression (fossa) in the nucleus (N) contains globular actin (GA). The posterior depression in the nucleus contains a pair of centrioles (C) within the centrosome (not visible). Once inside the egg, the centrosome and centrioles replicate and the two sister centrosomes, each containing a pair of centrioles, separate, go to opposite ends of the egg, and become the poles of the mitotic spindle. The large mitochondrion (M) arises during spermatogenesis by the fusion of several mitochondria. The flagellum (F) is ~50 μm long, the widest part of the sperm head is 1 μm wide and the acrosome vesicle is 0.25 μm wide. The acrosome reaction (right) consists of the exocytosis of the AV and the polymerization of globular actin to form filamentous actin (FA), which forms the cytoskeletal support for the acrosomal process (AP), which is coated with bindin. Fusion between sperm and egg plasma membranes occurs at the tip of the AP. Reprinted with permission, from Endo, 1961; copyright Elsevier.

Almost all animal species have a sperm acrosomal vesicle that must undergo exocytosis before the sperm can fuse with the egg. Exocytosis is the process by which the membrane surrounding the vesicle fuses with the plasma membrane to release the contents of the vesicle extracellularly. All mammalian sperm have an acrosomal vesicle. The intracellular mechanism of induction of the "acrosome reaction" (AR) appears to be similar in all species, in that an increase in both intracellular pH and ionic calcium must occur to trigger the AR. Somehow, membrane fusion of the tiny sperm with the enormous egg metabolically activates the egg, setting it on a biochemical pathway leading to a series of rapid cell divisions known as "the cleavage period of development." How does the tiny sperm do this?

LABORATORY OBSERVATIONS AND EXPERIMENTS

The point of this laboratory is for the student to carefully observe fertilization. It is not important to complete all the experiments and observations described below. Instead they are presented to provide many potential options, from which instructors and students can choose. Use this write-up as a guide, and enjoy watching the phenomena described and conducting the simple experiments.

Caution! It is essential to be very careful to avoid accidentally contaminating the egg-suspension with sperm. To avoid this situation, beakers of eggs should be kept on one side of the room and sperm suspensions, packed in ice, kept on the other side of the room. Follow the instructions below, and do not hesitate to have your neighbor or lab partner add the sperm suspension to eggs while you are observing the cells with low power objectives and no coverslip. Put used disposable glass pipettes, coverslips, and slides in the "sharps" disposal beakers that are on every bench.

Observing Eggs

Put drops of egg suspension on a slide. Be sure to have about 100 μl in the drop with several eggs. Keep the light low to avoid heating the egg suspension. Do not use a coverslip for your first observations. Place the slide on a flat surface, like the microscope stage or bench top. Look at the eggs with the microscope using the low power 10× or 16× objective without a coverslip. You should see that most of the eggs are not touching each other, but are an equal distance from each other. A hydrated, transparent jelly coat surrounds each egg, keeping the egg surfaces from touching each other (Fig. 2). The eggs must be freshly spawned to see the egg jelly coat because it will dissolve with time. If you are having trouble seeing the eggs clearly, check that the microscope is aligned. Focus up and down through the eggs. You should see the egg pronucleus as a circle, lighter in color than the cytoplasm and about 1/20th the diameter of the egg. You might see cells containing a large circle about 1/4th the egg diameter, with a smaller dark circle inside it. These odd-looking cells are oocytes (immature eggs) that have not yet completed meiosis, and should be less than 1% of all eggs present. The nucleus of the oocyte is called the ''germinal vesicle'' and the small dark circular structure within it is the nucleolus. The oocyte has not yet undergone meiosis or released polar bodies. As stated above, mature, fertilizable echinoid eggs are unusual in that they are completely finished with meiosis and have a haploid DNA content, the DNA is decondensed and is surrounded by a complete nuclear envelope.

Observing the Egg Jelly Coat

The thickness of the hydrated, transparent egg jelly coat (JC) is usually about half an egg diameter (Fig. 2). The JC can be seen by mixing one drop of a suspension of 10% India ink (90% HSW) on a slide containing one drop of freshly spawned eggs. The colloidal ink particles do not penetrate the JC, so you see the extent of the JC as a clear zone around each egg (Fig. 2). The sperm must swim through the JC to reach the egg surface. The JC is not fully characterized, but three different molecules in the JC have profound effects on sperm.

1. One is the 10 amino acid peptide, ''speract'' (in the species Sp, Sf, Lv, and Lp), and the 14 amino acid peptide, ''resact,'' for the species Ap. If you do a PubMed search using speract or resact, you will find the recent papers regarding these two egg peptides. They both bind to receptors on the sperm surface, resulting in ion channel opening that changes sperm behavior. Resact, released from the Ap egg JC, is a chemoattractant for Ap sperm. A single sperm cell can sense one molecule of resact bound to its flagellum (Kaupp et al., 2008). Although direct chemoattraction of Sp sperm to speract has not been shown, speract does have profound effects on the swimming behavior of sperm (Darszon et al., 2008).
2. The fucose sulfate polymer (FSP) of the JC is an extremely high molecular mass polysaccharide of >1 mDa that does not contain protein. By weight it can be 80% of the mass of the JC. Depending on the species, the polymer is made of fucose monosaccharides with either a 1–3, or 1–4 glycosidic linkage between the fucose residues. Sulfate groups are attached to the fucose residues at either the −2 or the −4 position of the fucose ring. The patterns of glycosidic linkage and of sulfation are what make the FSP a ''species-specific'' inducer of the sperm's AR (Vilela-Silva et al., 2008). The FSP is always needed for the JC to be an inducer of the sperm AR. The FSP induces the opening of calcium channels in the sperm plasma membrane that are part of the mechanism of AR induction. If you do a PubMed search using ''sea urchin egg jelly,'' you will find the relevant publications. Few animals make unbranched polymers of sulfated fucose, and their biosynthesis has not been studied.
3. The sialoglycoprotein of the JC of Sp species potentiates the FSP-induced AR (Fig. 3). If one adds enough pure FSP to Sp sperm to yield 15% acrosome-reacted sperm, the same sperm batch will yield 80–100% acrosome reacted sperm if a little pure sialoglycoprotein is mixed in with the pure FSP. But, without the FSP, the sialoglycoprotein will not induce the AR. The sialoglycoprotein elevates the sperm's internal pH by about 0.25 pH units, and this increase in pH, plus the increase in calcium ions induced by FSP, triggers the AR. The sperm membrane receptor for the sialoglycoprotein remains unknown (Hirohashi and Vacquier, 2002).

Dissolving the Egg Jelly Coat

One can remove the bulk of the JC (Fig. 2) by treating a suspension of eggs for 2 min with pH 5.0 seawater. This is done by hand stirring an egg suspension and slowly, carefully, dripping in 0.1 N HCl (made up in deionized water) to reach pH 5.0 as you rapidly stir the egg suspension. The pH is then adjusted back to 8.0 by adding a few drops of 1 M Tris pH 8.0 while rapidly stirring. A combination pH electrode is in the egg suspension during the lowering and raising of the pH. Do not use a magnetic stir bar when using the pH 5 method as it will fragment the eggs. The eggs settle, and the supernatant can be clarified by centrifugation for study of the JC molecules. This pH method works well for Sp, Lv, and Lp eggs; with Ap and Sf eggs, the JC can be solubilized, but when the dejellied eggs settle, they irreversibly clump together and cannot be manipulated. With Sp eggs, the pH method removes the bulk of the JC, but JC molecules are still tightly bound to the vitelline layer of the egg; there is still enough FSP on the egg surface to induce the sperm AR. Settle the eggs, decant away or aspirate off the supernatant seawater, and resuspend the dejellied eggs in fresh HSW. If you now look at a concentrated drop of these eggs, you will see that their cytoplasmic surfaces are in contact.

If you carefully drip in 0.1 N propionic acid to solubilize the egg jelly coat at pH 5.0, and then raise the pH to 8.0 with 1 M Tris in 2 min, many of the eggs will parthenogenetically activate and raise fertilization envelopes. Butyric acid (4 carbons) works the best, but it has a terrifically bad

odor, so propionic acid is preferred. The reason why organic acids activate cortical granule exocytosis and biochemical activation of sea urchin eggs remains unknown, but it must involve the release of free calcium ions from the egg endoplasmic reticulum. Although the activated eggs will initiate protein and DNA synthesis, they will not divide because the sperm centrosome is needed to nucleate the microtubule organizing centers (MTOC), which will become the poles of the mitotic spindle. However, if one takes the activated eggs and treat them for 10 min with 2× concentrated SW, and then cultures them in normal HSW at 15°C, some of the eggs will divide into two-cell stage embryos and continue through embryogenesis. Butyric acid treatment of sea urchin eggs is how Jacques Loeb discovered artificial parthenogenesis (Loeb, 1899).

The Egg Vitelline Layer

The vitelline layer (VL) is a lacy meshwork of large glycoproteins under the egg jelly coat that is intimately bonded to the egg plasma membrane. It is very difficult to define and cannot be seen with the light microscope. It elevates away from the egg surface, and is the template for the assembly of proteins coming out of the egg cortical granules (CGs; Fig. 5). The combination of the VL and the CG proteins forms the fertilization envelope (FE) that you will see today. If one slightly digests the eggs with the protease trypsin, or breaks S–S bonds in the VL with dithiothreitol, the VL integrity is destroyed and a FE will not form. In contrast to the zona pellucida of a mammalian egg, the sea urchin egg's VL provides no protection for the unfertilized egg. The sea urchin VL is involved in sperm binding, and it also acts as a template for the CG proteins that assemble on it to form the FE.

Observing Sea Urchin Spermatozoa

Students will need a phase contrast or Nomarski microscope with a 100× oil immersion objective to observe details of sperm cell anatomy. Put a small drop (10–20 μl) of sperm suspension (1:1,000 dilution of semen in HSW) on a slide, and drop on a coverslip. Tip the slide on edge on a tissue paper and drain out the extra seawater. To try a gentle thumb squash, turn the slide over so that the coverslip is face down on a folded tissue and softly press on the slide over the coverslip with your thumb. Do not slip the slide along the coverslip as this will remove almost all the seawater. First observe using the 40× phase contrast or Nomarski objective. You should see the sperm swimming by the propagated bending wave of the flagellum. You should be able to see the cone-shaped head, the bulbous mitochondrion posterior to the head, and finally, the long, thin flagellum (Fig. 3). Now, assuming that one has a phase contrast or Nomarski microscope with a 100× oil immersion objective, put a drop of oil on the coverslip and observe the sperm under high power. You should be able to see the acrosomal complex in the anterior tip of the sperm. The complex consists of the cup of unpolymerized actin in the anterior nuclear fossa, and in front of it, the dark acrosomal vesicle containing bindin. Some of the sperm will have undergone the AR, and you might be able to see the 1 μm long acrosomal process projecting from the anterior (Fig. 3). The acrosomal process can be seen in an assay in which fluorescent phallodin, a mushroom toxin, binds to filamentous actin that is the cytoskeleton of the acrosomal process (Su et al., 2005). The AR can be triggered by nonspecific injury to the sperm plasma membrane, allowing calcium ions to enter the cell. For example, if one puts a drop of a suspension of *Lytechinus* sperm on a slide and then drops a cover slip on the sperm from about 1 in. above the slide, almost all the sperm will acrosome react. Morphologically, the sea urchin sperm AR consists of the fusion of the membrane around the acrosomal vesicle with the sperm plasma membrane (exocytosis), to expose the adhesive protein bindin, and also the polymerization of actin into filaments to form the finger-like acrosomal process coated with bindin (Fig. 3). Usually, about 2–10% of the sperm will be acrosome-reacted without treatment with egg jelly (Vacquier and Hirohashi, 2004). The sea urchin sperm provides three things to the egg: it restores the diploid genome by contributing its haploid nucleus; it biochemically activates the dormant egg setting on an irreversible pathway leading to rapid cell divisions; and it provides the centrosome, which will replicate and form the poles of the mitotic spindle (Epel, 1990).

In mammals the AR consists only of the exocytosis of the acrosomal vesicle and there is no polymerization of actin. Human sperm have an easily seen acrosomal vesicle. If you could specifically block only the human sperm AR, you would have a new type of male contraceptive. Figure 3 is a drawing of the sea urchin sperm before and after the AR. The ionic fluxes across the sea urchin sperm plasma membrane, which are required for AR induction, have been reviewed (Darszon et al., 2001, 2006).

Observing Normal Fertilization

Be careful not to contaminate the beakers of unfertilized eggs with sperm. (That is why the ice buckets with sperm or eggs are kept at opposite ends of the room or lab bench.) The dry sperm have been diluted 1:1,000 in HSW, and the sperm suspension is kept on ice. Put a large drop (50–100 μl) of the egg suspension on a slide, and do not add a coverslip. Have the microscope light turned down so that the stage does not heat to the point of damaging the eggs. Use the 10× or 16× objective. Add 10 μl of dilute sperm to the eggs, mix with the pipette tip, and immediately look at the eggs. If you have a stopwatch, start timing when the gametes are mixed on the slide. If too many sperm are added, the details will be obscured so only 10 μl or less of the dilute sperm suspension should be added. From 0 to 30 sec, one should see sperm bouncing into the eggs, pushing them around and binding to them. At about 30 sec, about the point of fusion between sperm and the egg cell membranes, you will see the VL begin to elevate as a blister from the egg surface and transform into the FE (Figs. 4 and 5). By 60–120 sec (depending on the species) after adding sperm, the FE should be completely elevated

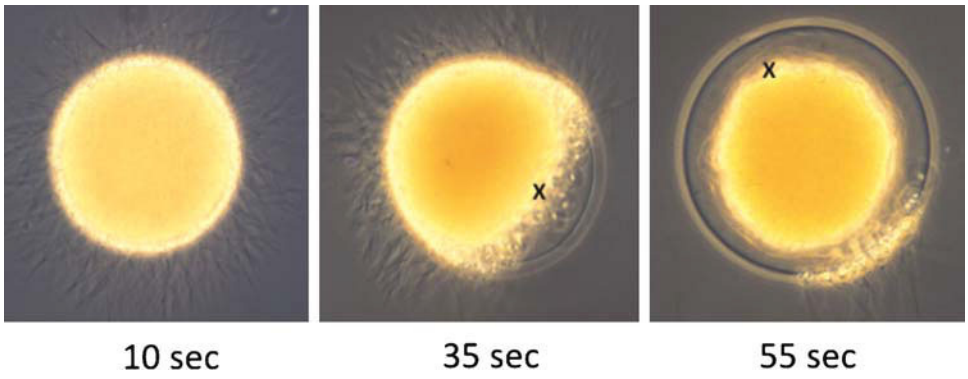

Figure 4. *Strongylocentrotus purpuratus* eggs were fertilized with concentrated sperm, and the sperm bound to the entire egg surface. At 10 sec after insemination, a portion of the egg suspension was fixed in formaldehyde and the unattached sperm washed away. Hundreds of sperm flagella are seen sticking out away from the egg surface. By 35 sec after insemination, a sperm has fused with the egg (x = approximate location of fusion) and cortical granule (CG) exocytosis has begun. The vitelline layer (VL) is elevating and transforming into the fertilization envelope (FE). The CG protease digests the bonds between the plasma membrane and the VL, allowing the VL to elevate and unite with proteins coming out of the CG. The CG protease cleaves the bonds between the VL and supernumerary sperm, which detach from the VL. By 55 sec, the VL has completely elevated from the PM and sperm remain bound to only a small portion of the VL/FE. The x marks the approximate location of sperm–egg fusion. The elevated VL/FE now hardens to become the FE by action of the transamidase and ovoperoxidase enzymes, both of which come from the CG (Wong and Wessel, 2008).

as a ring around the egg, and be in the process of being chemically ''hardened'' (Fig. 4).

Repeat observing normal fertilization several times. As the VL elevates and transforms into the FE, one should see bound sperm detach from the elevating FE. A trypsin-like protease coming out of the egg CGs cleaves the bonds between the VL and the egg plasma membrane, and also cleaves the bonds between the bound sperm and the VL/FE. If you wish to do so, drop on a coverslip and drain out most of the seawater. Observe the eggs using higher power phase contrast objectives. Remember that if seawater gets on an objective, wet a Kimwipe with distilled water and wipe off the objective. Then use lens paper to completely dry the objective lens.

When the blister of the VL first appears, elevating from the egg surface (Fig. 4), that indicates that the fertilizing sperm is in the middle of the blister. Drop on a coverslip and look with the 40× objective. You might have to drain some liquid out of the slide to have the eggs pinned down as the VL is uniting with the CG proteins to create the FE. The fused sperm's tail will be straight and motionless as the egg is drawing it into the egg cortex. It takes about 5 min for the egg to completely take in the fused sperm. Think of the egg cortex as the rind on an orange. With phase contrast microscopy you will see a clear, nipple-like structure around the tail of the fused sperm. This is the fertilization cone and it is made of actin filaments that are drawing the sperm into the egg cytoplasm. The fertilization cone is pulled back into the egg by 10 min after fusing with the sperm, thus it is visible for only a few minutes. If you have an oocyte present, many sperm will fuse with it because it does not have an electrical or physical (the FE) block against polyspermy (more than one sperm fuses with the egg or oocyte). The fertilization cones on oocytes are numerous and long.

How Does the Sea Urchin Egg Prevent Polyspermy?

If more than one sperm fuses with the egg, there will be more than one centrosome in the egg. The first division spindle will be multi-polar, and the egg might attempt to divide into many small cells; development will arrest and the egg will be lost.

Students always ask how the egg guards itself against fusion with more than one sperm. There are two mechanisms that have evolved to protect the sea urchin egg against polyspermy: (1) an incomplete, fast, electrical block, and (2) a complete, slow, structural block.

The egg plasma membrane has an electrical potential across it that is about −70 mV, with the inside being negative. Within 0.1 sec of fusion with the first sperm, the potential reverses and spikes up to +20 mV. This is caused by the opening of sodium and calcium channels in the egg plasma membrane. For an unknown reason, sperm will not fuse with an egg that has a positive membrane potential. The potential has to be lower than −20 mV to permit fusion with the sperm (Jaffe, 1976). The electrical block is called ''incomplete'' several sperm might fuse before the plasma membrane potential reverses if the egg is swamped with thousands of sperm. The membrane potential will remain at +20 mV for several minutes before it returns to −70 mV. During that time, the FE forms and hardens, and that is the ''complete'' structural block because no sperm can penetrate it.

How Does the FE form?

The periphery of the unfertilized Sp egg contains ~16,000 CGs, each about 1 μm in diameter, that fuse with the egg plasma membrane to release their contents extracellularly under the VL (Fig. 5). The fusion of CGs

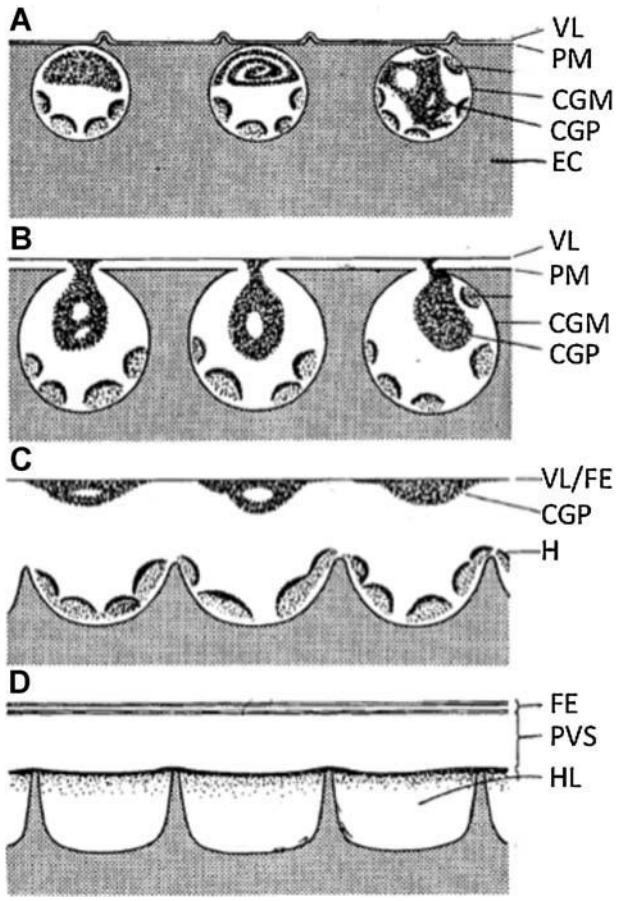

Figure 5. The exocytosis of cortical granules (CG) and the formation of the fertilization envelope (FE). This older drawing, constructed from early electron micrographs, remains the best depiction of the CG reaction in the literature (Endo, 1961). **A**: In the unfertilized egg, the CG are bound to the plasma membrane (PM). The vitelline layer (VL) is a lacy meshwork of fibers that is intimately bound to the PM. **B**: Exocytosis of the CG begins and the VL detaches from the PM. Structural proteins come out of the CG (CGP) and adhere to the VL in a ''paracrystalline'' array. The CG membrane (CGM) fuses with the PM, so the microvilli of the egg surface lengthen. **C**: The VL is now transitioning to become the fertilization envelope (FE) as the CGP unite with it to form the soft FE. Hyalin protein (H) is a calcium-dependent gel that swells and adheres firmly to the PM. **D**: The formation of the FE is complete and appears as ''railroad tracks,'' as seen with the electron microscope. The perivitelline space (PVS) is created in which the embryo develops until hatching. The hyaline layer (HL) has completely gelled and the microvilli from the egg surface are impaled in the HL. At the end of CG exocytosis, the plasma membrane of the zygote is a composite of original PM and CG membrane. Reprinted with permission, from Vacquier, 1986; copyright Elsevier.

with the egg plasma membrane is in response to elevated intracellular calcium ion concentrations, and it proceeds as a circular wave of exocytosis from the point of sperm fusion to a point on the opposite side of the egg. It takes about 30 sec for the wave of CG exocytosis to sweep across the egg (Fig. 4). Many structural and enzymatic proteins are released from the CG, and assemble on the template provided by the VL to transform it into the FE. The FE is then structurally ''hardened'' by two enzymes coming out of the CG, a transglutaminase and a peroxidase, called ovoperoxidase, that crosslinks tyrosine residues in the FE proteins to each other to make the FE into a rigid, tough structure (Wong and Wessel, 2008). The di- and tri-tyrosine crosslinks are extremely stable. The egg actually makes the hydrogen peroxide (H_2O_2) that the ovoperoxidase uses as a substrate to oxidize the tyrosine rings and to link adjacent tyrosines together. The elevation of the FE is visual proof that the egg has been fertilized. The egg has created the FE, which serves as a micro chamber to keep the embryo safe from fusion with other sperm, microbial attack, and physical damage. At the mid blastula stage, when the embryo consists of about 600 cells, the embryo becomes ciliated and secretes a hatching enzyme that dissolves the FE to allow the blastula to swim freely. Almost all animal embryos hatch from some type of envelope at an early stage of development. All mammalian embryos hatch out of the zona pellucida before they can implant in the uterine wall; the FE is analogous to the mammalian egg zona pellucida because it protects the zygote during cleavage, and the embryo must secrete a hatching enzyme to break out of it. The sea urchin FE is so structurally tough that it can be isolated by dissolving the embryos with 5% sodium dodecyl sulfate (SDS).

Species-Specific Sperm–Egg Interaction

Usually, the sea urchin sperm–egg interaction exhibits species-specificity. This means that sperm of species A are quantitatively better at fertilizing eggs of species A compared to eggs of species B. It is rare to have absolute specificity, where sperm of species A fertilize 0% of species B eggs, regardless of the number of species A sperm that are present. However, absolute specificity in cross-species mixtures of gametes is known. Usually, a low sperm concentration can be found that yields 100% fertilization in the homo-specific sperm–egg mixture and 0% fertilization in the hetero-specific sperm–egg mixture. Species-specific (or species-selective) fertilization must be encoded in surface molecules mediating the four sequential events in sea urchin sperm–egg interaction (Vacquier, 1998). These events are: (1) chemoattraction of sperm to the egg, as seen when Ap sperm respond to the egg peptide resact; (2) induction of the sperm AR by egg jelly; (3) attachment of the bindin-coated, finger-like acrosomal process of the sperm to the egg VL; and (4) fusion of sperm and egg plasma membranes. Each of these steps could be encoded by species-specific ''gamete recognition molecules'' that can be thought of as a ''lock and key'' interaction. We know that the structure of FSP makes it a species-specific inducer of the sperm AR. We also know that sperm bindin is species-specific in bonding the sperm to the egg (Zigler, 2008). Bindin binds to EBR1, a large, species-specific glycoprotein receptor that is in the egg VL (Kamei and Glabe, 2003).

Testing the Species-Specificity of Sea Urchin Fertilization

If you have gametes from more than one echinoid species, you can try to make the following observation. Take eggs of one species (call it species A) and inseminate them with a dilute sperm suspension of another species (B). Watch the behavior of the sperm in the proximity of the egg. Do the sperm swim right by the egg, or do they bind to the egg surface? If you add a large amount of sperm, so there are a few hundred sperm per egg, do any of the eggs elevate FEs? Be sure that the stage is cool enough so that the eggs and sperm are still viable. Turn the light down if the sperm are not moving rapidly and allow the stage to cool. After watching the hetero-specific sperm interacting with the eggs, add a drop of a dilute suspension of the homo-specific sperm. If the homo-specific sperm fertilize the eggs, that is the control for the eggs being alive and capable of being fertilized.

Why did species-specific fertilization evolve? One would first think that it evolved to prevent cross-species hybrid zygotes from forming, which might not develop into sterile adults (e.g., mules). But, there is little evidence for this hypothesis. Another hypothesis is that it is a result of ''sexual conflict,'' where eggs try to prevent polyspermic fusion by altering the surface receptors for sperm binding proteins to slow sperm fusion. The eggs that can slow sperm fusion survive to become adults. Variants of the sperm protein binding the ever-changing egg receptor are selected for, and thus the ''cognate pair'' of gamete recognition proteins evolves rapidly and differentiation of the gamete recognition system occurs (for a discussion see Moy et al., 2008).

Sea Urchin Fertilization Depends on Calcium Ions

The only way one knows that an egg has been fertilized is that it elevates a FE. Seawater contains $\sim$10 mM Ca^{++}. Take drops of egg suspension and put them in a tube. Let the eggs settle to the bottom, or give them a few gentle spins with the hand centrifuge. Remove all of the overlying seawater and fill the tube with Ca^{++}-free artificial seawater (CaFSW). You want to wash out the Ca^{++} ions, so the dilution you are getting on each wash is important. Say you get a 1:50 dilution on each wash, and you do two washes into CaFSW. That means that you diluted the original seawater by 2,500 times, so the Ca^{++} will be minimal. Now take 5 μl of concentrated fresh semen (''dry sperm''), and dilute it into 5 ml of CaFSW, making a 1:1,000 dilution of the Ca^{++} ions in the semen.

Mix the sperm and eggs together on a slide, and watch them for 5 min. Keep the light low to avoid heating them. You should not see any FEs form. The reason is because Ca^{++} ions must rush into the sperm from the outside to induce the AR. Without undergoing the AR, the bindin is not exposed and the sperm cannot bind to and fuse with the eggs. The Ca^{++} needed by the egg to induce CG exocytosis comes out of vesicles in the egg cytoplasm. Thus, the Ca^{++} needed for the CG reaction does not need to come from the surrounding seawater.

Testing if the FE has Hardened

The FE will harden in 1–3 min after it elevates from the egg surface (depending on the species). Fertilize some eggs on a slide, and after 5 min add a drop of the powerful ionic detergent 5% SDS in water (sodium dodecyl sulfate). The cell should totally dissolve, but leave the FE intact as a circle. This demonstrates just how hard (chemically stable) the FE really is. To study the amino acid composition of the FE, the FEs are isolated in a buffer containing SDS, and the solubilized proteins washed away with fresh SDS. Then the FEs are washed into distilled water and finally hydrolyzed with 6N HCl at 100°C for 6 hr. Amino acid analysis shows the presence of the di- and tri-tyrosine residues created by the ovoperoxidase.

Blocking the Elevation of the Vitelline Layer to Become the FE

A trypsin-like proteolytic enzyme comes out of the CGs, and into the seawater when the CG membrane fuses with the egg plasma membrane (Fig. 5). If this ''fertilization protease'' is blocked, the VL only partially elevates from the egg surface and the eggs are more susceptible to polyspermy. The protease cleaves the bonds holding the VL to the egg plasma membrane. Sperm also remain bonded to the VL as it is trying to transform into the FE. The protease helps to guard the eggs against polyspermy.

A HSW solution of soybean trypsin inhibitor (SBTI) at 1.5 mg/ml will be available. Put one ml of 1% egg suspension in a tube and allow the eggs to settle, then remove the supernatant HSW with a clean pipette. Add 0.5 ml of SBTI in HSW and swirl the tube to keep the eggs suspended, or keep the eggs on a rocker table. After 5 min, put some of the eggs on a slide and fertilize them with a minimal volume of sperm suspension. Mix the eggs and sperm on the slide and watch if there is a change in the formation of the FE. After 5 min of observing the eggs in STBI, add a drop of 5% SDS in water. Is the FE visible after the egg dissolves? If it is not visible, it was not ''hardened'' when you added the SDS. Be sure to run a control tube of eggs fertilized in HSW without SBTI.

Blocking the Hardening of the FE by Inhibition of the Ovoperoxidase

If the di- and tri-crosslinked tyrosine residues among the FE proteins cannot form, the FE will not harden. Ovoperoxidase, which also comes out of the CG during their exocytosis, can be blocked by para-amino-benzoic acid (PABA), sulfathiazole and para-amino-salicylate. None of these chemicals will kill the eggs. PABA at 100 mM in seawater (pH 8.0) will be provided. One can dilute it with equal volumes of seawater to make 50% dilutions to attain: 50, 25, 12.5, 6.25 mM PABA. Put 1 ml in a clean tube and add one drop of concentrated 5% egg suspension ($\sim$50 μl). Allow 2 min for the PABA to equilibrate with the egg surface. Put one drop on a slide and fertilize the eggs with minimal sperm suspension so as not to dilute the PABA around the eggs. What happens to FE elevation? After 5 min, add one

drop of the 5% SDS and see if the FE dissolves or remains intact. Be sure to run a control tube for this experiment.

One can also make a 1:1 mixture of the stock PABA/HSW solution and the stock SBTI/HSW solution. Put a few eggs in the PABA/SBTI, then after 2 min put the eggs on a slide and add a drop of sperm, mixing well with the pipette tip. Does the VL elevate at all? Do the sperm attached to the egg still remain attached? Add one drop of SDS solution to see if the FE remains or dissolves.

Showing That S–S Bonds Stabilize the Hardened FE

By 3 min after FE elevation, the FE has been crosslinked (''hardened'') by the ovoperoxidase and transglutaminase (Wong and Wessel, 2008). There are few examples in biology where a complex structure made of several proteins is assembled in minutes as a paracrystalline array with an intricate ultrastructure. Put some fertilized eggs with hardened FEs on a slide and mix them with an equal volume of seawater containing 10 mM dithiothreitol (DTT) pH 9.1. You will notice that the FEs expands greatly in diameter but remain circular. This is because the S–S bonds in the FE proteins have been reduced to S–H bonds. Now blow across the slide while you are watching. Blow hard enough to make the eggs move. The FEs will shrink again because the oxygen in your breath is oxidizing the S–H bonds back to S–S bonds. You can perform these cycles of reduction and oxidation of the FE as many times as you wish. You can think of the hardened FE as a series of folded sheets, the folds being held together by S–S bonds in the component proteins. Reducing the bonds allows the folds to stretch out and disappear. Oxidizing them causes the folds to reform. A good analogy is the pleats of an accordion. You can put some of these eggs with DTT-expanded FEs on a slide and see if they will disappear if digested with trypsin. Add1 drop of a HSW solution of trypsin at 0.1 mg/ml to one drop of eggs on a slide and see if the DTT-expanded FEs disappear. The control is a drop of fertilized eggs with hardened FEs that have not been exposed to DTT.

Disrupting the VL Before Fertilization

The object of this exercise is to observe the ''fertilization product'' (the proteins coming out of the egg CG during their exocytosis). Disulfide bonds are somehow involved in holding the molecules of the lacy VL together on the egg surface before fertilization. There are two standard ways to break up the VL before fertilization so that all of the products of the CG are released to the seawater and can be isolated for further analysis. The two methods are, (1) the trypsin procedure and (2) the DTT procedure.

In the trypsin procedure, 0.25 mg of pancreatic trypsin is dissolved in 100 ml of seawater. Take 2 ml of egg suspension and allow the eggs to settle. Remove the supernatant and add 2 ml of the trypsin/HSW solution, and start a stopwatch. Every 5 min, a sample of eggs is placed on a slide and fertilized. One looks for elevation of the FE. By 5, 10, and 15 min of digestion, one should see progressive lack of elevation of the VL/FE. After about 20 min, a test fertilization is done. You should see that there is no VL elevating at all, and by 2 min after sperm addition one sees proteins precipitated around each egg. These are proteins that have been released by CG exocytosis that would have normally united with the VL to form the soft FE. At that moment, add two drops of a 10 mg/ml SW solution of SBTI to completely inhibit the trypsin from continuing digestion. The SBTI can then be washed away by allowing the eggs to settle.

The DTT method is much easier to control. A 10 mM seawater solution of DTT is prepared and the pH adjusted is to 9.1 with 1 N NaOH. This has to be done immediately before use because the DTT oxidizes with time once it is in seawater. Settle the eggs and remove as much seawater as possible with a disposable pipette. Then fill the tube with the DTT solution and agitate the tube for 3 min, then let the eggs settle. Remove the supernatant and wash the eggs twice in HSW to remove the DTT and reduce the pH back to 8.0. Put some of the treated eggs on a slide and fertilize them. In 2 min, you should see large precipitates of CG protein coming out of the eggs. This is one way to separate the soluble from insoluble components of the CG exudate for further study. With a phase contrast microscope you should also see the clear hyaline layer gel around the eggs directly against the cell surface. The hyalin protein comes out of the CG and gels on the egg surface when it binds Ca^{++} ions (Fig. 5). The hyaline layer is important for normal morphogenesis.

Activating Eggs with the Calcium Ionophore A23187

Ionophores are produced by molds and fungi, and are made to fight off bacterial colonization by ionic killing. Ionophores are lipophilic and partition into cell membranes. A23187 is an ionophore that carries both Ca^{++} and H^{+} across membranes from the side of the membrane where the ion is most concentrated to the side of lesser concentration. Ionophores are molecules with ion specificity, but low affinity for the ion, and that is why they can drop off the ion in an area of lower concentration. Seawater contains10 - mM Ca^{++}, while inside the egg (and all other cells) ionic Ca^{++} is $\sim$0.1 μM. That means that there is a difference of 7 logs of Ca^{++} concentration between and inside and the outside of a sea urchin egg. A solution of 2 mg/ml of A23187 in 100% dimethyl sulfoxide (DMSO) has been prepared and stored at room temperature. Do not store it in ice because the DMSO will freeze. Take 10 μl of this solution and add it to 990 μl of HSW at room temperature, then vortex it hard. Immediately add two drops of the egg suspension to this HSW-ionophore solution, swirl the tube for 5 sec to mix, and immediately put some eggs on a slide and look at them at low power without a coverslip. You will see that the elevation of the VL to form the FE occurs all at once all over the egg. How is this different from the activation of eggs by sperm? If you miss this artificial activation, try mixing the eggs with the 1% DMSO-A23187 solution on a slide while you are watching (or get help from your neighbor). After mixing the DMSO-ionophore solution with 99 volumes of HSW-eggs, the

DMSO concentration is 1% and the ionophore concentration is 38 μM.

One variation of this experiment is to wash the eggs into CaFSW and be sure that you have diluted the original seawater at least 1:1,000. Put a drop of these eggs on a slide. In a microfuge tube dilute, 10 μl of the 2 mg/ml DMSO solution of A23187 in 100% DMSO, with 490 μl of CaFSW. The DMSO is now 2%. Add one drop of this solution to one drop of eggs in CaFSW in a microfuge tube. If the drops are of equal volume, the final DMSO concentration is 1% and the ionophore concentration 38 μM. Do the eggs activate in the absence of extracellular Ca^{++}? If they do, that means that the Ca^{++} needed for egg activation comes from a calcium store inside the egg and not from the seawater. We know by other experiments that the Ca^{++} needed for exocytosis of the CG is released from the endoplasmic reticulum of the eggs.

Artificial Activation of Sea Urchin Eggs in Procaine

Sea urchin eggs are metabolically repressed because their internal pH is 6.8. Five minutes after insemination, their internal pH increases to 7.2 (Epel, 1990). The change in pH is what activates microtubule polymerization to form ''asters,'' and also activates protein and DNA synthesis. Local anesthetics, such as procaine-HCl, increase the internal pH of cells very gently. If sea urchin eggs are put in 5 mM procaine in HSW pH 8.0, they turn on protein and DNA synthesis and undergo cycles of chromosome condensation and decondensation. They also cycle the polymerization and depolymerization of microtubules to form quasi-mitotic spindles (Vacquier and Brandriff, 1975). This experiment takes hours to overnight. Put some eggs in HSW containing 5 mM procaine. Have a control dish of eggs in seawater without procaine. Observe the eggs for several hours. What do you see after 6–10 hr in procaine/seawater? Are there changes in the nucleus? Do you see cycles of microtubule formation in that the structure of the cytoplasm appears different from the unfertilized condition?

Twinning of Sand Dollar Embryos in Dithiothreitol

This works very well with sand dollar eggs (*E. parma* or *D. excentricus*) because their cleavage stage cells rely more on cell-to-cell surface interaction to form blastulae than they do to being contained within a thick hyaline layer. Sea urchin eggs rely more on the clear hyaline layer on the egg surface for blastomere interaction to form normal blastulae, and finally gastrulae and plutei. Fertilize some *E. parma* or *D. excentricus* eggs and culture them at room temperature in a medium size petri dish. They should begin to divide in 40–50 min. Watch them closely and when they begin to elongate in early telophase, pour them into a graduated tube and add an equal volume of 100 mM DTT in HSW. They should finish cytokinesis in 5 min. Ten min after adding the DTT, settle the embryos by gentle hand centrifugation, remove the DTT/HSW and fill the tube with fresh HSW. Wash them once by settling or by gentle hand centrifugation, and then resuspend the two-cell stage embryos in pure seawater and culture them. Those zygotes that divided in DTT had the S–S bonds on all their surface proteins reduced to S–H bonds. The reduced surfaces fail to adhere to each other at the two-cell stage and each of the cells goes on to form a twin embryo. It is possible to get 100% twins (Vacquier and Mazia, 1968). Separation of embryo cells at the two- or four-cell stage is the way identical twins (monozygotic twins) form in mammals.

Culturing Normal Embryos

Set up culture dishes of normally fertilized *A. punctulata*, *E. parma*, *L. pictus*, or *L. variegates* embryos in medium size petri dishes (5 cm diameter, 7 ml total volume HSW) for long-term observations of embryogenesis. Do not put more than a few hundred eggs in each dish. If you can see the eggs as less than a monolayer of cells covering the dish bottom, that is plenty. You can keep these cultures at 15–22°C. For culturing Sp embryos you must use temperatures from 12 to 16°C. You should see the cleavage divisions to produce the 2-, 4-, 8-, 16-, and 32-cell stages. After the 32- to 64-cell stage, the embryos begin to form hollow balls one cell thick, which are the blastulae. The blastulae spin inside the FEs before they hatch because every cell has a cilium. The gut forms during gastrulation, and then the larval skeleton begins to form as the prism and pluteus stages develop. The skeletal rods are single crystals of $CaCO_3$ laid down on a complex protein matrix. There is much research on the genes and proteins of the skeleton. Enjoy watching embryonic development.

MATERIALS

Procurement of Sea Urchins

There are biological supply companies that sell gravid echinoid species for classroom observations of fertilization. A telephone call to the supplier will inform you of what species are currently gravid. Some suppliers are: Pacific Bio-Marine Laboratories, Venice, CA; Sea Life Supply, Sand City, CA; Marinus Scientific, Garden Grove, CA; Gulf Specimen Marine Lab, Panacea, FL; Marine Biological Laboratory, Woods Hole, MA; and Santa Barbara Marine Biological, CA. An internet search using ''sea urchin fertilization'' as a query yields many hits with pictures, descriptions, and classroom procedures. The book by Ettensohn et al. (2004) covers procurement and maintenance to adult sea urchins in aquaria. The instructors should demonstrate spawning by 0.5 M KCl injection of sea urchins through the peristomal membrane around the jaws (Aristotle's Lantern). Sand dollars should be injected through the mouth or anus. Between injection of each adult, the hands and the syringe should be dipped for 10 sec in tap water to kill any contaminating sperm.

Gamete Stocks Stored on ICE

Egg suspensions at 1% and 5% v/v in HSW kept in 50 ml screw cap culture tubes on one side of room. Sperm

suspensions at 1:1,000 dilution of undiluted semen in HSW stored on the opposite side of the room. With Sp the sperm concentration will be 4×10^7 cells/ml. The sperm dilutions should be viable for 3 hr. Undiluted semen is stored in capped Microfuge tubes stored in ice. The sperm will be viable for 4 days.

Formulae for Seawaters

If you cannot obtain natural seawater, you can make up HEPES-buffered artificial seawater (HSW) of the following composition: 454 mM NaCl, 9.7 mM KCl. 9.6 mM $CaCl_2$, 26.7 mM $MgCl_2$, 28.9 mM $MgSO_4$, 2,5 mM $NaHCO_3$, 10 mM HEPES at pH 8.0. For calcium-free seawater (CaFSW), use the above formula with two changes: delete the $CaCl_2$ given above and change the NaCl to 469 mM. For 2× concentrated HSW, use the HSW formula, but change the NaCl to 908 mM. If you have access to natural seawater, filter it (paper or Millipore) and then add HEPES to 10 mM and adjust the pH to 8.0 with 1 N HCl and 1 N NaOH (Schroeder, 1986, p. 20).

Equipment

Phase contrast or Nomarski microscopes with 10×, 16×, 40×, and 100× objectives.
Pre-cleaned slides, 1 in. × 3 in. and coverslips 22- or 18-mm square.
KimWipes.
Immersion oil.
Test tube rack and 12 mm × 75 mm disposable glass tubes.
1.5 ml microfuge tubes.
5 cm diameter plastic tissue culture dishes.
Disposable pipettes and pipette blubs.
25, 50, 100 ml beakers, marking pens.
Ice buckets with shaved ice.
Two-liter beakers for glass waste.
pH meter with combination electrode, plastic spatulas.
Pipette man with boxes of tips, for 1,000, 200, 20 μl.
Ionophore A23187, 1 mg dissolved in 500 μl 100% dimethyl sulfoxide, stored at 23°C.

Solutions Made in HSW (Chemicals From Sigma, St. Louis, MO)

India ink or Sumi ink: 10% v/v.
SBTI: 20 ml at 1.5 mg/ml, and 5 ml at 10 mg/ml.
PABA: 20 ml at 100 mg/ml.
Procaine-HCl at 5 mM.
DTT: 100 ml of 10 mM pH 9.1, 20 ml of 100 mM, pH 8.0.
Pancreatic trypsin: 1 mg dissolved in 400 ml, and 20 ml at 0.1 mg/ml.
Propionic acid: 50 ml of 100 mM.

Solutions Made in Deionized Water

0.5 M KCl.
0.1 and 1 N HCl, 100 ml each.
0.1 and 1 N NaOH, 100 ml each.
1.0 M Tris pH 8.0, 25 ml.
5% w/v SDS at room temperature.

REFERENCES

Darszon A, Beltran C, Felix R, Nishigaki T, Trevino CL. 2001. Ion transport in sperm signaling. Dev Biol 240:1–14.

Darszon A, Acevedo JJ, Galindo BE, Hernandez-Gonzalez EO, Nishigaki T, Trevino CL, Wood C, Beltran C. 2006. Sperm channel diversity and functional multiplicity. Reproduction 131:977–988.

Darszon A, Guerrero A, Galindo BE, Nishigaki T, Wood CD. 2008. Sperm activating peptides in the regulation of ion fluxes, signal transduction and motility. Int J Dev Biol 52:595–606.

Endo Y. 1961. Changes in the cortical layer of the sea urchin egg at fertilization as studied with the electron microscope. I. *Clypeaster japanicus*. Exp Cell Res 25:383–397.

Epel D. 1990. The initiation of development at fertilization. Cell Diff Dev 29:1–13.

Ettensohn CA, Wessel GM, Wray GA. 2004. Development of sea urchins, ascidians and other invertebrate deuterostomes: Experimental approaches. Meth Cell Biol 74:1–875.

Hirohashi N, Vacquier VD. 2002. Egg sialoglycans increase intracellular pH and potentiate the acrosome reaction of sea urchin sperm. J Biol Chem 277:8041–8047.

Jaffe LA. 1976. Fast block to polyspermy in sea urchin eggs is electrically mediated. Nature 261:68–71.

Kaupp UB, Kashikar ND, Weyand I. 2008. Mechanisms of sperm chemotaxis. Ann Rev Physiol 70:93–117.

Kamei N, Glabe CG. 2003. The species-specific egg receptor for sea urchin sperm adhesion is EBR1, a novel ADAMTS protein. Genes Dev 17:2502–2507.

Loeb J. 1899. On the nature of the process of fertilization and the artificial production of normal larvae (plutei) from the unfertilized eggs of the sea urchin. Am J Physiol 3:135–138.

Moy GM, Springer SA, Adams SL, Swanson WJ, Vacquier VD. 2008. Extraordinary intraspecific diversity in oyster sperm bindin. Proc Natl Acad Sci USA 105:1993–1998.

Schroeder TE. 1986. Echinoderm gametes and embryos. Meth Cell Biol 27:1–470.

Su Y-H, Chen S-H, Zhou H, Vacquier VD. 2005. Tandem mass spectrometry identifies proteins phosphorylated by cyclic AMP-dependent protein kinase when sea urchin sperm undergo the acrosome reaction. Dev Biol 285:116–125.

Townley IK, Schuyler E, Parker-Gur M, Foltz KR. 2009. Expression of multiple Src family kinases in sea urchin eggs and their function in calcium release at fertilization. Dev Biol 327: 465–477.

Vacquier VD. 1986. Activation of sea urchin spermatozoa during fertilization. Trends in Biochemical Sciences 11:77–81.

Vacquier VD. 1998. Evolution of gamete recognition proteins. Science 281:1995–1998.

Vacquier VD, Brandriff B. 1975. DNA synthesis in unfertilized sea urchin eggs can be turned on and turned off by the addition and removal of procaine hydrochloride. Dev Biol 47:12–32.

Vacquier VD, Hirohashi N. 2004. Sea urchin spermatozoa. Meth Cell Biol 74:524–544.

Vacquier VD, Mazia D. 1968. Twinning of sand dollar embryos by means of dithiothreitol. The structural basis of blastomere interactions. Exp Cell Res 52:209–221.

Vilela-Silva A-CES, Hirohashi N, Mourao PAS. 2008. The structure of sulfated polysaccharides ensures a carbohydrate-based mechanism for species recognition during sea urchin fertilization. Int J Dev Biol 52:551–559.

Wong JL, Wessel GM. 2008. Renovation of the egg extracellular matrix at fertilization. Int J Dev Biol 52:545–550.

Zigler KS. 2008. The evolution of sea urchin sperm bindin. Int J Dev Biol 52:791–796.

MRD