chapter 6 Echinoid Fertilization and Development

Fertilization

The events of sea urchin fertilization have been worked out in great detail. You will be studying both normal fertilization and parthenogenesis (development without fertilization). You will be able to ask some very sophisticated biochemical questions once you know some of the details of these events.

We will be injecting potassium chloride (KCl) to induce spawning in both male and female sea urchins. KCl causes a contraction of smooth muscles, and immature gametes will be spawned along with ripe gametes. This can cause problems, and you must be able to recognize a mature egg from an immature one. In the sea urchin, eggs are mature when they are ootids. This means that meiosis is completed and the nucleus is relatively small. An immature egg will be a primary oocyte with a huge nucleus (called a **germinal vesicle**). The germinal vesicle will be easily recognizable under a dissecting microscope, if you adjust the light to obtain good contrast and focus up and down through the egg.

The egg has two extracellular coats: a **vitelline envelope**, which before fertilization fits snugly around the egg surface and cannot be distinguished; and an outer layer of jelly. This jelly contains a chemical sperm attractant (a small polypeptide that is species-specific). It is not present in the jelly of an immature egg. From a mature egg's jelly, this attractant diffuses outward, and sperm swim up the concentration gradient. The jelly also contains a relatively species-specific fucose-containing polysaccharide that activates the acrosome reaction in the sperm. This polysaccharide binds to glycoprotein receptors on the head of the sperm, causing the **acrosomal vesicle** within the head to fuse with the cell membrane and release its enzymes. These enzymes coat the head of the sperm and eat through the jelly, making a path to the egg cell surface for the swimming sperm. In the process, the acrosomal vesicle becomes inverted and greatly elongated by the assembly of actin filaments. This elongate structure, the **acrosomal process**, is what will fuse with the egg cell surface, and it can be seen under the compound microscope under oil immersion if the contrast is maximized.

The sperm first binds to the vitelline envelope using a species-specific cell surface protein called **bindin**, which binds to a bindin-receptor protein on the vitelline envelope. The sperm then fuses with the egg cell membrane, and in so doing causes a brief influx of sodium ions. This influx raises the resting membrane potential of the egg from –70 mV to above 0 mV. Sperm can not fuse with an egg whose membrane potential is above about –10 mV, so this change in membrane potential effectively prevents any additional sperm from fusing. This is called the **fast block to polyspermy**. It takes only one-tenth of a second to occur, but is not permanent, lasting only about a minute. Since the fast block depends on the availability of sodium in the medium, you can circumvent it by keeping the sodium concentrations in the surrounding seawater artificially low.

Binding of the sperm with the egg cell membrane also sets up a second block to polyspermy, the **slow block**. This block takes a minute to occur, and it is permanent. You will see it as a lifting of the vitelline envelope away from the egg cell surface. This membrane then toughens (involving a chemical process much like tanning leather) and is now called the **fertilization envelope**. Sperm cannot penetrate this tough layer. The point of sperm entry can be identified by a cone-shaped elevation called the **fertilization cone**. It represents a tangle of microvilli that have elongated and wrapped themselves around the sperm.

The biochemical events that are causing the lifting of the vitelline envelope are initiated through the **phosphatidylinositol bisphosphate (PIP) cycle**, which is set off by the binding of the sperm to the egg cell membrane. Once the PIP cycle is activated, there is a sudden spike in calcium levels within the egg due to the release of calcium from the smooth endoplasmic reticulum. This spike in calcium levels causes hundreds of vesicles (the **cortical granules**) housed in the cortical cytoplasm of the egg to fuse with the egg cell membrane and to empty their contents into the perivitelline space between the egg cell membrane and the vitelline envelope. The released contents of the cortical granules swell, lifting the vitelline envelope away from the egg cell surface, and tan the envelope, making it tough and impenetrable by other sperm. The PIP cycle also causes a rise in internal pH by activating a sodium–hydrogen ion pump. Sodium ions are pumped into the egg while hydrogen ions are pumped out. The resulting rise in internal pH activates the egg, causing protein synthesis to start and the egg to begin its development.

The details above are sophisticated but not beyond your manipulation with relatively simple reagents. Think hard about what you could do to interfere with one or more aspects of the events of fertilization.

Instructions for normal fertilization

The same instructions can be used for sea urchins or sand dollars, except where noted. You will not be able to sex the animals until they have begun to spawn. Invert five or six animals over dry watch glasses or petri dishes. The five gonadal openings are on the aboral side, and as the animal spawns, gametes will be shed into the dish. On the oral side, you will see the hard white mouthparts ("Aristotle's lantern") surrounded by a tough leathery peristomial membrane. You will be injecting through this membrane into the perivisceral cavity. The length of the needle you choose should be long enough to penetrate into the cavity but not much longer. (When spawning sand dollars, a much shorter needle is used than when spawning sea urchins.) Use a syringe to inject 1–2 ml (0.5–1 ml for sand dollars) of isotonic KCl (0.53 M = 3.9%). This will cause the smooth muscles of the gonads to contract and spawn their gametes. After 2–5 minutes, repeat the injection. This gives a heavier spawning.

As soon as spawning begins (within minutes of injection if the animals are ripe), check the color of the gametes. This will tell you the sex of the animal. Sperm are creamy white; eggs are yellow, pink, or dark red (depending on the species).

For males Immediately pour off the first sperm to get rid of perivisceral fluid, since this will interfere with the sperm's ability to fertilize. Then allow the animal to shed into the watch glass or petri dish without diluting the sperm. This is called **dry sperm**. Dry sperm will be good for 6–10 hours at room temperature and even longer if kept in the refrigerator.

For females Allow the female to shed eggs into seawater by placing her, inverted, on top of a beaker of seawater. The beaker should be small enough that the animal does not fall in. The beaker must be full enough so that seawater touches the aboral side of the animal. As the animal sheds, the eggs will drift down through the seawater and settle in the bottom of the beaker. After shedding is completed, decant off the seawater and add fresh. Do this twice. This washes the eggs of perivisceral fluid which interferes with fertilization. (*N.B.*: Sometimes artificially spawning sea urchins will cause parthenogenetic activation of

the eggs. Examine egg batches for raised fertilization envelopes, which indicates parthenogenetic activation. These eggs could be used in the parthenogenesis section of the chapter.)

To fertilize the eggs, first make a **standard sperm suspension** of 1 drop of dry sperm in 10 ml of seawater. This suspension must be used within 20 minutes and then discarded. Dry sperm are relatively inactive. Diluted, however, they become very active and quickly use up their energy stores. Use sterile procedure and sterile glassware if you are keeping the eggs for culturing. Add two drops of standard sperm suspension to 10 ml of seawater containing eggs. Repeat after two minutes. After 10 minutes, decant off the seawater and add fresh. This culture can be kept for observing normal development. Know the species you are using, and decide on an appropriate temperature (or range of temperatures) for rearing (see Table 6.1). For long-term cultures, refrigerator temperatures are usually adequate. The seawater for culturing should be sterilized by being filtered through a 0.22-μm porosity filter. Streptomycin can be added (2 mg/liter) to retard bacterial growth, but is normally not needed in cultures maintained at cold temperatures.

Watching Fertilization

You can watch fertilization under the microscope by placing eggs on a slide and introducing sperm from one side. One way to do this is to place a large drop of egg suspension on a microscope slide. Put the slide on the microscope stage and have a footed coverslip close at hand. (A **footed coverslip** is made by nicking the corners of the coverslip against some hard paraffin, so that crumbs of paraffin remain attached at each corner. This will give just enough spacer between the slide and the coverslip to avoid crushing the eggs.) Then put a drop of sperm suspension on the slide close to but not touching the drop of eggs. Place the footed coverslip on the slide so that it covers both drops. This will cause the two drops to mix. Immediately focus on eggs that are mature. (Remember, those with large germinal vesicles are immature.) Use a clock with a second hand to time the events that you see. You should be able to determine the exact spot of sperm entry, which will be marked by the fertilization cone (Figure 6.1). Time the raising of the fertilization envelope. Do you see any eggs in which the fertilization envelope starts to rise but doesn't finish? Record any variations you see. Once the fertilization envelope is raised, do sperm continue to be attracted to the egg? Do the sperm bounce off the fertilization envelope or stick to it? Enter your answers in your notebook, along with any other observations you make.

If you can find some immature eggs, place these separately on a slide, and watch as you introduce sperm from the side. Are the sperm attracted to the egg? Compare this with sperm behavior near a mature egg.

Prepare a slide of a fertilized egg, place a footed coverslip over it, and look at it under a 40× objective. Close down the iris diaphragm on your microscope to increase contrast. Focus on sperm that are caught in the jelly surrounding the egg. Can you see any acrosomal processes? Why would you expect to see them? Enter your answer in your notebook, and diagram what you see. Do not go to oil immersion, since this will not work using these wet mounts and will only mess up the microscope by getting seawater on the objective lens.

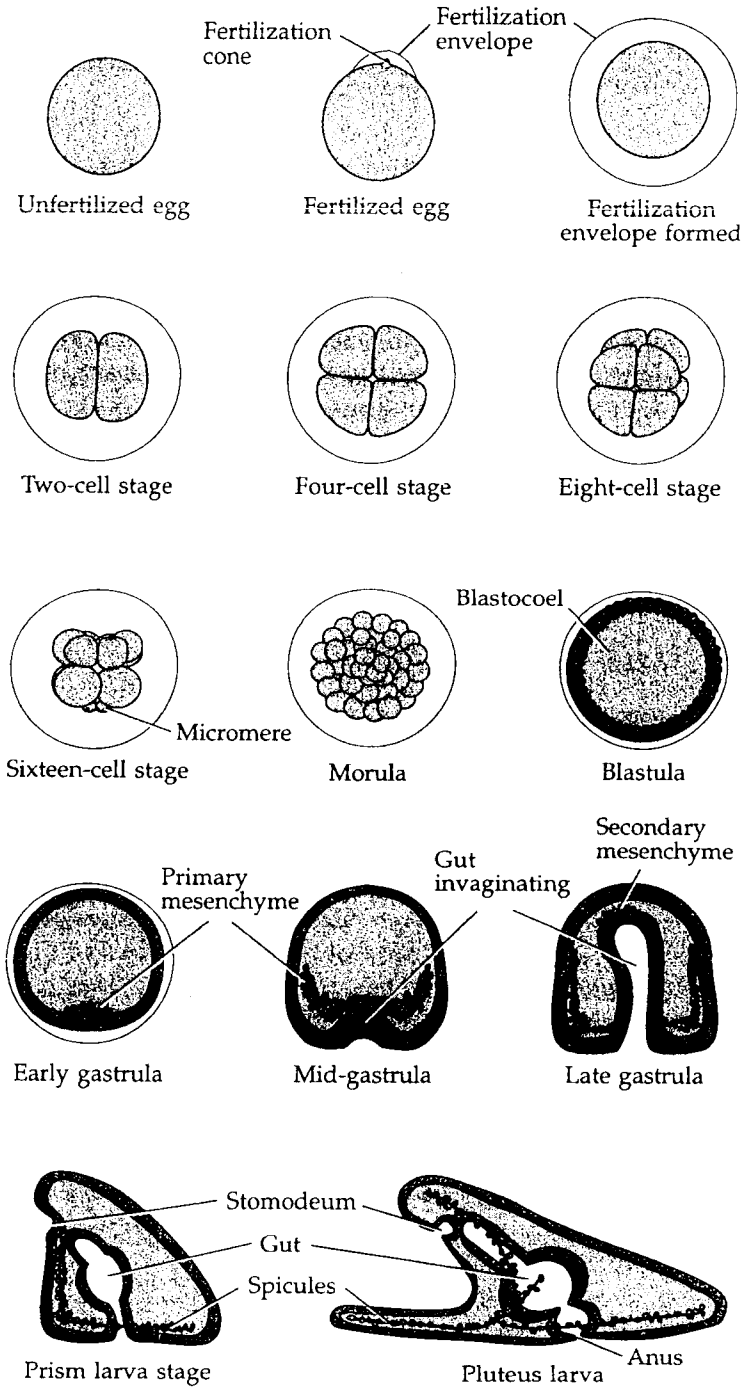

Figure 6.1
Developmental sequence of the sea urchin embryo from fertilization to the pluteus larval stage.

Put a drop of sperm suspension on a slide, and place a coverslip over it. Observe this under a 40× objective, and close down the iris diaphragm to increase contrast. Diagram the sperm. Do you see any acrosomal processes on these? Why? Record your answers.

Cleavage, Gastrulation, and Larval Stages

The first several cleavages can be observed during the laboratory period. But you will have to use your sterile cultures to observe the later stages of development.

The stages and pattern of cleavage can be seen in Figure 6.1. Cleavage is **holoblastic** (the entire egg cleaves) and **radial** (the cleavage planes are parallel or at right angles to the animal-vegetal pole). The first cleavage takes about an hour at room temperature, and subsequent cleavages occur about every half hour. At the 16-cell stage, a small group of **micromeres** are cleaved at the vegetal pole. These are the **primary mesenchyme** cells and will be the first to show gastrulation movements. At 5–6 hours, the embryo is at the **blastula stage**, and by 7–8 hours, the embryo has hatched out of its fertilization envelope and is spinning around the dish using its cilia for locomotion. This is called a **hatched blastula**.

Gastrulation begins at about the hatched blastula stage. Primary mesenchyme cells first migrate into the blastocoel and then form a necklace of cells that will secrete the skeletal supports for the larva, the **spicules**. The spicules are first tripartite rods, and they eventually branch to have several arms. Gastrulation continues by **invagination** of the vegetal plate to form the **archenteron** (meaning "ancient gut"). The forming gut will elongate, capped by a loose collection of cells, the **sec-**

ondary mesenchyme. The secondary mesenchyme aid in elongating the gut with their contractile filopodia, pulling the archenteron toward the far wall of the blastocoel and guiding it to its final destination. They later disperse to form mesodermal organs. As the gut is developing, the embryo goes through a **prism larval stage** (between 18–20 hours), looking like an exquisite rotating jewel, and finally becomes a **pluteus (echinopluteus) larva** (at about 22–24 hours). The pluteus is an ornate organism, projecting long, delicate arms supported by branched spicules.

The timing of development will vary considerably with temperature. You can keep cultures in the refrigerator, at room temperature, or at any other temperatures the laboratory can supply. As soon as you have swimming blastulae, I suggest catching these in a sterile pipette and transferring them to a fresh culture dish with sterile seawater. This will avoid bacterial contamination from the decomposing embryos that didn't make it. Make diagrams of any stages you are able to observe. If you are lucky enough to get larvae, be sure to look at them under the microscope using polarized light. As the larvae rotate, their spicules briefly align with the polarizers with each turn, flashing beautifully for you.

Compare the rates of development at the different temperatures you used by presenting your data in chart and graph form. Notice also (from the cultures that don't make it) that you are establishing viable temperature ranges for the species you are using. You can see from Table 6.2 that complete timetables of sea urchin or sand dollar development are hard to come by. If you develop a complete timetable for one or more temperatures, this is publishable work.

	Strongylocentrotus droebachiensis		*Strongylocentrotus purpuratus*	*Echinarachnius parma*	*Arbacia punctulata*	
Stage	**4°C**	**8°C**	**10°C**	**15°C**	**20°C**	**23°C**
Fertilization	0	0	0	0	0	0
2-Cell stage	5 hrs	3 hrs	3.5 hrs	1.5 hrs	1 hrs	50 min
4-Cell stage	8 hrs	5 hrs	5 hrs	3 hrs	1.8 hrs	78 min
8-Cell stage	10.5 hrs	6.5 hrs	6 hrs	4 hrs	2.4 hrs	1.71 hrs
16-Cell stage	14 hrs	8.5 hrs	8.5 hrs	5.5 hrs		2.25 hrs
32-Cell stage	18 hrs	11 hrs				2.78 hrs
Morula				6.5 hrs		4 hrs
Early blastula				7 hrs	6 hrs	4.5 hrs
Mid-blastula				8 hrs		6 hrs
Hatching blastula	50 hrs	30 hrs	27–28 hrs	12 hrs	10 hrs	7–8 hrs
Early gastrula				25 hrs		12–15 hrs
Mid-gastrula			50 hrs			
Late gastrula			57 hrs	31 hrs		17 hrs
Prism stage	6.5 days	4 days	80 hrs	48 hrs		18 hrs
Early pluteus	11 days	7 days	4.7 days	50 hrs	48 hrs	20 hrs
Late pluteus				72 hrs		24 hrs
Metamorphosis		7–22 wks	63–86 days			5–16 wks

Sources: S. droebachiensis and *S. purpuratus* after Strathmann, 1987; *E. parma* after Karnofsky and Simmel, 1963; *A. punctulata* at 20°C after Costello et al., 1957; *A. punctulata* at 23°C after Harvey, 1956.