In Vitro Spermatogenesis of Gypsy Moth Larvae

Judy Brown Marcia J. Loeb

This laboratory activity is designed to offer students an opportunity to establish simple cell developmental cultures in order to observe the process of spermatogenesis, mitosis and meiosis in living cells. Insect cultures grow at room temperature and do not require a special incubator or complex growth media. The cells are easily observed under a microscope, and the process of spermatogenesis is complete within approximately five days. After mastering the basic culturing techniques, students can design and carry out experiments that explore environmentally safe methods of interrupting spermatogenesis.

Background

Gypsy moths *Lymantria dispar* are formidable environmental pests. They annually defoliate 4 million acres of woodland in the Northeastern United States. Their range extends from New England to as far south as West Virginia and as far west as Ohio and Michigan. In May 1991, eggs from the Asian strain of Gypsy moths apparently hitchhiked aboard Russian freighters leaving from Asian ports in Russia and arriving in Vancouver, British Columbia. Since this strain devours more than 500 species of plants including Douglas fir, larch, spruce, birth, alder, willow and poplar, the entire Northwest timber industry is at risk. The Asian version of this pest poses a special threat because unlike the female moth of the North American strain, the Asian female moths can fly. William Wallner, U.S. Forest Service research entomologist, explains that the Asian female moth "zips right along with a payload of 600 to 700 eggs and will fly in to lay its eggs just like a stealth bomber" (Gibbons 1992). Another concern is that the Asian strain will eventually mate with the moths already here to produce a hybrid that could spread even faster.

Judy Brown is a biotechnology teacher in the Edison Career Center, 12501 Dalewood Dr., Wheaton, MD 20906. **Marcia J. Loeb**, Ph.D., is a research physiologist in the Insect Neurobiology and Hormone Laboratory, U.S. Department of Agriculture, Beltsville, MD 20705.

In vitro insect cell culture is one method that can be used to study the development and physiological processes of these destructive insects in order to develop environmentally safe methods for eliminating them. Simple cultures of developing sperm in late larval instar (the period before the molt to the pupa stage) of male caterpillars are easy to make and develop quickly so that they can be easily observed in five days of laboratory time. Testes provide a ready source of rapidly developing cells that can be observed and used for experiments.

Tests develop slightly more at each larval molt. During the last larval instar, prior to development into the pupa, sperm colonies undergo meiosis and then elongate into mature sperm. In this lab exercise, Gypsy moth testes will be isolated and the spermatocytes cultured *in vitro* allowing the student to observe spermatogenesis. Advanced students can be challenged to develop a procedure that interrupts or halts this process.

The testes of male Gypsy moth larvae can be located at the level of the second false foot on the back of the caterpillar (Figure 1). Testes consist of an outer layer of somatic cells and an interior lumen with an inner sheath that secretes a growth hormone (Figure 2). Sperm stem cells reside in the apical end of each of four follicles within the testes. Stem cells divide and continually release daughter cells coated with a few somatic cells into each follicle. Inside the coating of envelope cells, the daughter cells undergo repeated simultaneous mitosis until the mass reaches a total of 64 diploid cells (Figure 3). These developing germ cells are arranged like an echinoderm blastula and have no vigital or animal pole. Since the apical cells bud off new germ cells continually during the life of the caterpillar, germ cells in all stages of development may be seen when the last instar caterpillar is dissected. However, further development occurs in all categories of spermatocysts and can be observed continually by the students.

After receiving a signal (nature not known), the cells simultaneously enter meiosis resulting in 256 haploid cells per cyst. After the second meiotic division, the cyst elongates and forms sperm. At maturation, the sperm can be clearly seen inside each cyst lined up with all of the heads at the wide end of the cyst. The sperm are held together as a packet until the adult stage, just before mating (Figure 4). This exercise allows students to observe simultaneous cell divisions in living cells as well as maturation of sperm, providing a double lesson in biology.

Materials for 7 lab groups of 3–5 students each

- 7 Sterile 6 well plates with tape to seal lids
- 21 250-ml beakers for each pair of students
- 21 Sterile petri dishes (one for each larva to be dissected)
- 21 Sterile 10-ml pipettes
- 7 Pipette pumps
- 14 Fine tipped forceps
- 36 60-mm petri dishes with fresh dissecting wax
- 72 Gypsy moth larvae (can be collected by students ahead of time).

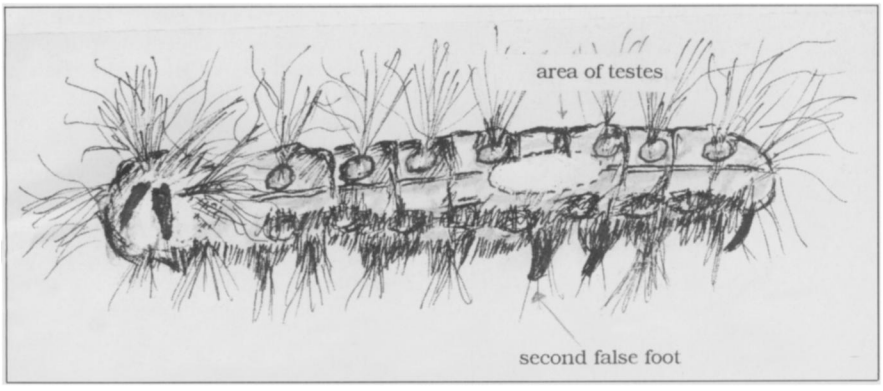

Figure 1. Gypsy moth larvae showing area of testes.

Chemicals

95% ethanol—500 ml
200 ml Ringer's solution
0.5% Penicillin/streptomycin
Grace's Insect medium TNM-FH (Sigma T 3285)
Fetal bovine serum (Sigma F 3018)
Alconox or other strong non-sudsing detergent
Clorox® (hypochlorite)
Sterile distilled water

Equipment

2—Flame sources
7—Dissecting microscopes
1—Inverted microscope (cultures can also be prepared as hanging drops on sterile depression slides and cover slips and can be observed using an ordinary light microscope.)

Directions for Preparing Lab

Plan to start lab on a Monday in order to have maximum observation time. *Culture media* of equal parts of sterile Grace's Insect medium TNM-FH (Sigma T 3285) and sterile Ringer's supplemented with 0.5% penicillin/streptomycin and 7% fetal bovine serum (Sigma F 3018). Prepare 3 ml for each 6 well plate used.

Ringer's Solution

This is needed to maintain tissue osmolarity during dissection.

1.6 g sodium chloride
.004 g calcium chloride
0.04 g potassium chloride
0.04 g sodium bicarbonate
distilled water to a final volume of 200 ml. Autoclave 20 minutes at 220 psi.

Cleaning Solutions

Three cleaning solutions are required. Prepare 1 liter of each.

1. 10% solution Alconox or any non-sudsing detergent
2. 10% hypochlorite (Clorox®)
3. Sterile distilled water

Sterile Dissecting Dishes

These can be made by pouring dissecting wax or paraffin enhanced with a small amount of beeswax into 60 mm petri dishes. Prepare one dish for each larva to be dissected. These are not reused. Store in sealed plastic bags and sterilize with UV light for 1 hour just before use. A goggles sanitizer cabinet can be used by wiping it with ethanol and turning it on for ½ hour *before* putting the opened petri dishes inside the cabinet.

Sterilization of Dissecting Instruments

Lay 2 forceps and 4 insect pins on five layers of tissue (Kleenex®, Kimwipes®) soaked with 70% ethanol and covered with three layers of soaked tissue. Prepare 30 minutes before dissection. Prepare one set of instruments for each pair of students.

Collection of Gypsy Moth Larvae

Gypsy moth larvae can be collected by students from their yards during late April and well into May. Each pair of students should collect at least 12 large larvae since about ⅔ will be females and ⅓ males. Large, mature larvae must be used in order for this experiment to work well. Alternatively, Gypsy moth larva can be ordered as *Lymantria dispar* larvae from the ARS Southern Field Crops Insect Management Laboratory, Stoneville, MS 38776. If Gypsy moths are not available, an alternative species, *Manduca sexta*, can be purchased from Carolina Biological Supply Company (2700 York Rd., Burlington, NC 27215) and will work well.

Procedure for Isolating Testes

Cleaning Larvae

Gypsy moth larvae are messy, hairy creatures with lots of places for

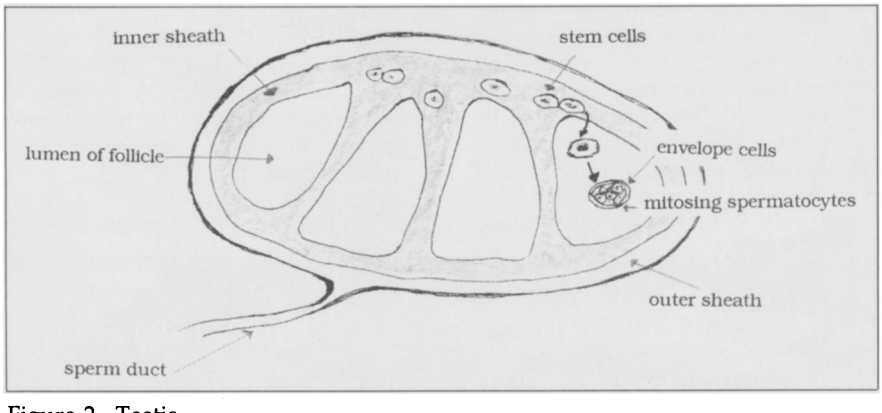

Figure 2. Testis.

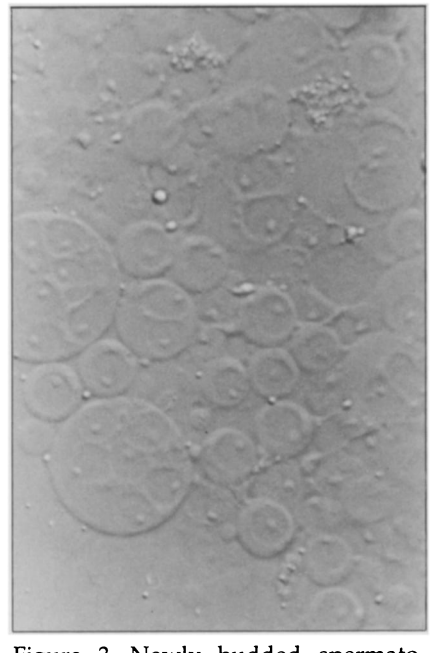

Figure 3. Newly budded spermatocysts. Note 1 cell, 2 cell and 8 cell cysts.

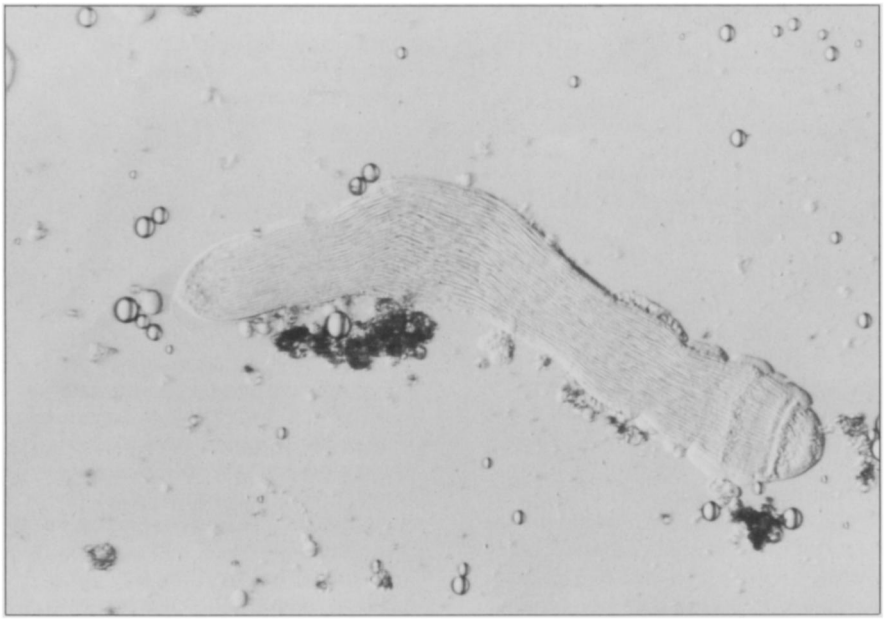

Figure 4. Mature adult spermatocyst. Contains 256 sperm. Tails visible. Elongate heads with nuclei at right. The cyst remains in this state until the adult stage of the moth. Sperm become free and motile after they are deposited in the female.

bacteria to reside. Care must be taken to clean the surfaces of the insect in order to avoid contamination of the cell culture. This cleaning process must be done just prior to dissection in order keep the larvae alive and to have viable cells to culture. Several larvae can be cleaned together. It is best to gently move the larvae using forceps rather than bare fingers since some people have a reaction to the surface proteins on the larval hairs.

1. Soak larvae in a beaker of 10% Alconox solution or other detergent solution for one minute.
2. Soak in a beaker of 10% Clorox® solution for two minutes.
3. Place larvae in a plain sterile petri dish containing sterile distilled water.
4. Proceed immediately to the next steps in the procedure.

Preparation of Well Plates & Dissection

All work must now be performed in a hood using aseptic techniques. Have a bottle of isopropanol and a flame source, sterile 1- or 10-ml pipettes, sterile wax dissecting dishes, and sterile instruments available in or near the hood. *Safety consideration: Be sure students understand flaming techniques and the need to keep the bottle of isopropanol well away from the flame source.*

1. Fill all wells of the well plate with 0.5 ml (500 μl) culture media and cover plate.
2. Remove larvae from sterile water and put into small petri dish containing sterile wax. Cover larvae with 10 ml sterile Ringer's solution.
3. Using sterile insect pins, secure larvae onto the wax of the dissecting dish with the top side of the larvae up.
4. Position the larvae under the dissecting microscope. Flame two fine sterile forceps, cool, and gently pull open the skin on the dorsal side (animal's back) just over the second false foot. The testes will be small, pale yellow egg-shaped structures and can be picked up with the forceps.
5. Reflame and cool forceps and place one testis in each well. Pull sheath of the testis open using the forceps and gently shake the testis to spill the cysts out into the well. Leave the testis sheath in the well since it is a source of necessary growth factors for spermatogenesis.
6. After all the wells contain a testis, cover the plate and use tape to seal the lid in order to prevent evaporation. All wells should contain media in order to maintain equal vapor pressure inside the plate.
7. Plates can be kept on tables at room temperature (20–28° C) or placed in a draft free incubator. They should be observed frequently under the microscope for the next five days as the cells change rapidly. It is wise to initiate the cultures on Monday for optimal observation since the entire process of spermatogenesis generally occurs within a five-day period.

Possible Extended Activities

Once students have mastered the basic culturing techniques, they can be challenged to develop experiments that inhibit or change the process of spermatogenesis. Possible strategies might include the use of: known insecticides including metabolic inhibitors such as sodium azide; colchicine, which prevents mitotic spindle formation; caffeine, which is a Ca^{++} regulator; or leupeptin, a protease inhibitor.

References

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They're hairy, they're hungry, they're here. (1992, May). *Time, 139*, 24.