Chapter 6

Inhibition of Gland Development in Insects by a Naturally Occurring Antiallatotropin ("Anti-Hormone")

Dorothy Feir

Biology Department
Saint Louis University
St. Louis, MO 63103

I received my BS from the University of Michigan—Ann Arbor in 1950, my MS from the University of Wyoming—Laramie in 1956 and my doctorate from the University of Wisconsin at Madison in 1960. After finishing my doctoral work I was an Instructor in the Biology Department of the University of Buffalo for one year before returning to my native city in 1961 to be an Assistant Professor in the Department of Biology at Saint Louis University. In 1964 I became an Associate Professor and in 1967 a Professor in that Department. Some of my professional activities in the last few years include Chairman of the Biochemistry and Physiology Section of the XV International Congress of Entomology, Editor of Environmental Entomology, Chairman of the Physiology and Toxicology Section of the Entomological Society of America, President of Saint Louis University Chapter of Sigma Xi, and member of the National Institutes of Health Tropical Medicine and Parasitology Study Section. My current interests include a rather broad spectrum of insect physiology studies. My graduate students and I are investigating the mechanism of action of juvenile hormone in the milkweed bug and the use of maggots in determining time of death for forensic pathologists. My other interests include invertebrate "immunological" reactions and the biology and physiology of the large milkweed bug, *Oncopeltus fasciatus*.

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Introduction

For many years hormone action has been studied by surgically removing the gland that produces the hormone and seeing what physiological or biochemical changes occur. Then the hormone under study was given to the operated animal (replacement therapy) to see whether the associated functions returned. Surgical procedures are always traumatic for the animal and in some cases, such as with small animals or insects, they are quite difficult. The use of an "anti-hormone," a compound that prevents the secretion of the hormone, allows the study of the hormone's actions without surgery. This exercise is designed to demonstrate (1) the value of using anti-hormones or chemical surgery, (2) the functions of insect juvenile hormone, and (3) the possible significance of naturally-occurring compounds in insect-host plant relationships.

The basic exercise as given is suitable for advanced high school students, college freshmen or sophomores. The exercise can be adapted to college junior, senior of first-year graduate levels by adding the procedures listed in Appendix A. Other exercises are also suggested in Appendix A.

This experiment does require that the instructor do some planning to have insects at the proper stage of development. If all stages of the insect are to be treated the insects must be obtained about one month before the day of the experiment. Eggs should be collected each day and placed in a rearing container marked with the date of collection. Procedures for rearing the milkweed bug on milkweed seeds can be obtained in Andre (1934) and Feir (1974). Procedures for rearing the bugs on sunflower seeds can be obtained from Carolina Biological Supply House (Burlington, NC 27215). A sunflower strain of bugs can be purchased from Carolina Biological Supply (also see Gordon 1974). Other milkweed bugs can be collected in the fall of the year, or a few bugs to start your own colony can be obtained from me or other people working with the bug. A student can easily set up the described experiment in 2 hours (Appendix B). However, he must check the bugs and feed and water them at least every other day until he has gathered all necessary data on molting, egg laying, and hatching of eggs. The amount of time that must be spent every other day depends on the number of petri dishes the student has set up. The time per petri dish is only a few minutes. In addition to giving the student data on the experiment, this follow-up work teaches him a great deal about growth and development of insects and the importance of following through on an experiment.

Student Materials

The large milkweed bug, *Oncopeltus fasciatis* (Dallas), belongs to the hemimetabolous order Hemiptera. Hemimetabolous insects have an egg stage, one to five immature or larval stages, and the adult. They do not have a resting or pupal stage. The larvae all look very similar to each other, but they are larger in the later stages, and the wing pads are larger in each succeeding instar. The adults are similar in appearance to the immature stages, but the adults have two pairs of wings and are capable of flight.

The duration of the immature stages has been reported by a number of authors, and it varies with temperature and rearing conditions. Andre (1934) found that at 29.5°C the egg stage lasted 4 days, and the 5 immature stages or stadia lasted 5.8, 5.9, 6.1, 4.5 and 6.8 days, respectively.

The results of precocene 2 treatment on the milkweed bug are clearly given in Bowers et al (1976) and Bowers and Martinez-Pardo (1977). Their results may be summarized as follows: fumigation of milkweed bug eggs with precocene gives normal 1st, 2nd, and 3rd stage larvae, and then a precocious adult is produced. Treatment of one instar usually gives a normal next instar and then a precocious adult. That is, treating the 1st instar gives a normal second instar and then the precocious adult; treating a 2nd instar gives a normal 3rd instar and then the precocious adult; treating a 3rd instar gives a normal 4th and then the precocious adult. Treating the 4th instar frequently results in a precocious adult without the intermediate normal instar. Treating 5th instars does not give precocious adults. Functionally the intercalated normal instar is like the normal fifth instar in which no juvenile hormone is present and adult tissues are being synthesized.

In many cases the morphology and color pattern of precocious adults are the same as in normal adults, but the wings are not fully expanded. The wings have the adultoid color and venation. Normal milkweed bug nymphs have 2-segmented tarsi, and the normal adults have 3-segmented tarsi. Precocious adults from 4th instar nymphs have 3-segmented tarsi, but precocious adults from 2nd and 3rd instar nymphs have 2-segmented tarsi.

Treatment of adult females within 24 hours of eclosion prevents all growth of the ovaries and prevents the corpora allata from developing. If these treated adults are given juvenile hormone III at 120 hours post-eclosion, the ovaries and corpora allata develop normally.

Treatment of adult females with precocene at 120 hours post-eclosion causes the ovaries to stop developing within one day, and then they regress. This treatment also causes the corpora allata to regress.

Precocene has been shown to cause sterility in some adult Lepidoptera, Coleoptera, and Diptera, but these insects do not show precocious metamorphosis.

Instructors' Materials

Procedures

Precocene can be applied topically to the insect or it can be coated on a glass petri dish. Both methods will be described.

Topical application

Dilute the precocene with acetone so that each microliter contains 10μg precocene. Apply 2μl of this dilution to the ventral abdomen of each insect. Use a microliter syringe or a microapplicator. This is a large volume for individual eggs, and it might be easiest to group 10 eggs together and apply 2μl to the whole group. If you find the insects move too quickly for easy handling, you might anesthetize them with CO_2 gas or put them in the refrigerator for 15–30 minutes to slow them down. Control insects are treated with the same volume of acetone.

Petri dish application

For first, second and third instar bugs an amount of precocene equal to 1μg/cm^2 is sprayed on the bottom of a 9 cm petri dish. For the larger bugs, (fourth, fifth and adult stages), an amount of precocene equal to 10μg/cm^2 is sprayed on the bottom of a 9 cm petri dish. A 9 cm petri dish has an area of approximately 64 cm^2, and therefore a total of 640μg precocene is needed per petri dish. The precocene can be diluted in any volume necessary to give this amount of precocene per dish. I have found it convenient to dilute the precocene so that there is 64μg or 640μg/0.1 cc acetone. I distributed this dilution over the bottom of the petri dish with a 0.25 ml or a 1 ml syringe. The control dishes are treated with the same volume of acetone.

After the acetone has evaporated from the dishes (when you can no longer smell it), the bugs can be placed in the dishes. Ten bugs per dish of the 1st, 2nd, 3rd and 4th instars, and 6 bugs per dish of the 5th and adult stages, are used. The bugs or eggs may be left in the dishes for 48 hours and then transferred to clean petri dishes, or they may be left in the treated petri dishes for the entire experiment.

Maintenance of treated bugs

All bugs should be given milkweed seeds and water. Each adult milkweed bug eats an average of 2–3 seeds a day, and it is better to give the bugs a few extra seeds. The old seeds do not have to be removed before adding fresh ones. Distilled water should always be used; an easy way to give the water is to soak 1½ inch cotton dental rolls in the water, squeeze out the excess water and put the wick in with the bugs. Depending on the humidity, the wicks have to be re-moistened at least every other day and sometimes every day. Seeds can be added whenever you think it is necessary. Any moldy seeds or cotton rolls should be discarded and fresh ones used. All dead bugs should be removed from the petri dishes.

When the adult stage is reached check to be sure that there are males and females in your dishes. The bugs from the duplicate dishes for any one condition can be put together. Adult male milkweed bugs usually have 2 complete black bars on the ventral abdomen, and the tip of the abdomen is rounded. Adult females usually have one complete black bar and portions of one or two additional bars on the ventral abdomen, and the tip of the abdomen is pointed and curved slightly downward.

Data collection

Each petri dish should be given a separate number at the time the bugs are treated, and a data sheet (Table 6.1) should be used for each dish. The data sheets can be drawn on a ditto master and a large number of them made. The number of dead, the number and instar of the living bugs, and any other observations should be recorded daily or at least every other day. From these data you can determine the average length of each instar and the effects of precocene on development time, metamorphosis and reproduction.

After adults are produced the dates when eggs are laid and the dates when the eggs hatch should be recorded. Milkweed bugs usually lay the eggs under the seeds, so you have to look carefully for them. The eggs should be removed and placed in a petri dish marked with the same number as the parents' dish and with the date the eggs were laid. I usually make a longitudinal slit in one of the cotton wicks, spread it open and put that in the adults' dish. The adult females will frequently lay their eggs in the cotton, and this makes it easier to remove them each day. Just take out the whole wick.

Adults will usually start laying eggs within a week of being put together. If the adults lay no eggs by 14 days after being paired dissect them to determine the state of their ovaries. Figure 6.1 shows the reproductive system of a female on the day of ecdysis. This is characteristic of an undeveloped female reproductive system. Figure 6.2 shows some development of the ovarioles and Figure 6.3 shows a fully developed reproductive system.

Table 6.1. Data Sheet

Dish No. _____ Date of Treatment _____

Instar Treated _____ Type of Treatment _____

Vol. and Concentration of Precocene 2 _____

Duration of Treatment _____

No. bugs or eggs/dish _____ Date males and females put together

_____

Days Post-Ecdysis	*Date*	*Instar, (No. Dead)*	*Instar, (No. Dead)*	*Living Bugs*			*Date Eggs*	
				Instar (No.)	*Instar (No.)*	*Instar (No.)*	*Laid*	*Hatched*
1								
2								
etc.								
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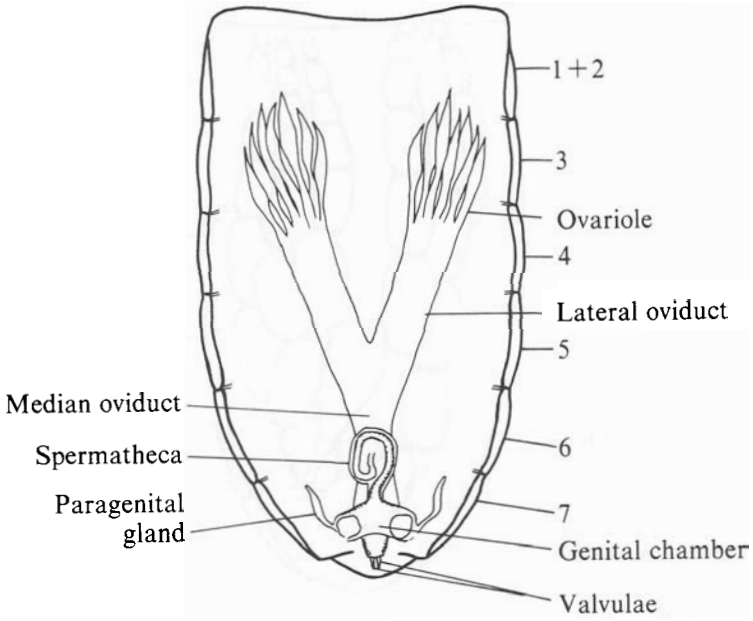

Figure 6.1. Dorsal view of the female reproductive system on the day of ecdysis. Numbers are abdominal segments. (Drawn by George Winkler.)

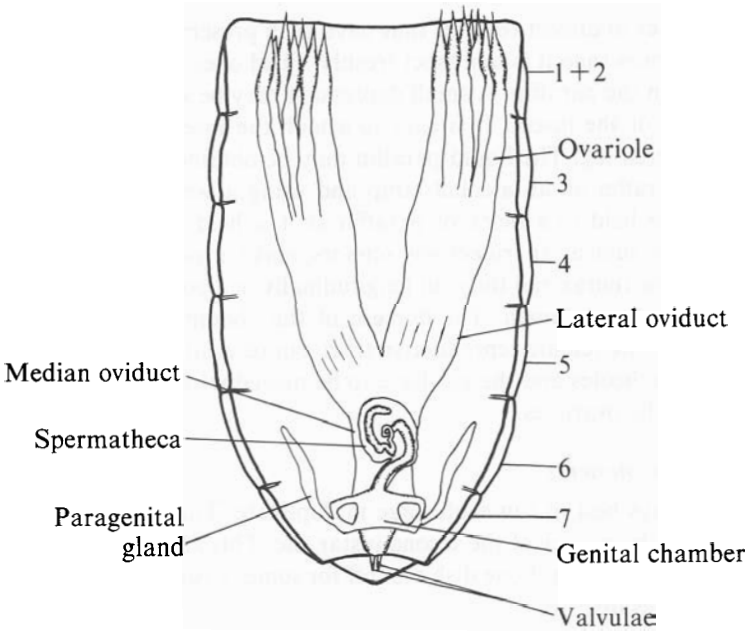

Figure 6.2. Dorsal view of partially-developed female reproductive system. (Drawn by George Winkler.)

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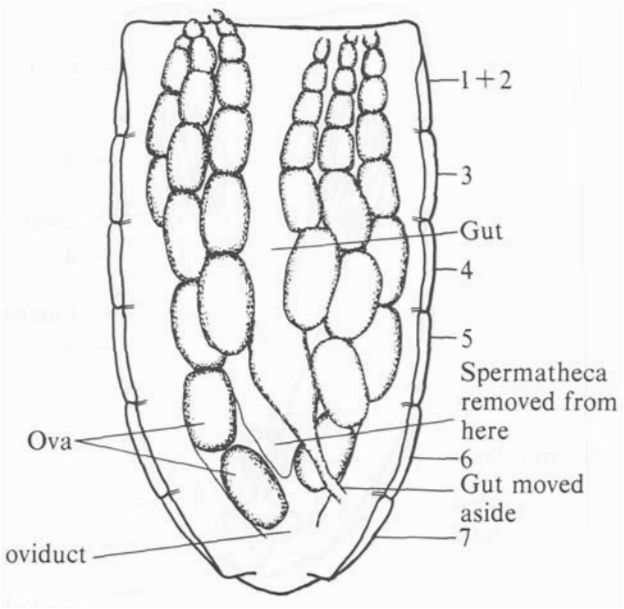

Figure 6.3. Dorsal view of fully-developed female reproductive system. (Drawn by George Winkler.)

Dissection of the female reproductive system

It is easier to dissect females that have been preserved in 70% alcohol for at least 24 hours than it is to dissect freshly-killed ones. Place the female dorsal-side-up on the paraffin. A small depression may be scraped in the paraffin for the body of the insect. It is easy to attach the insect by dropping liquid paraffin on each leg. The liquid paraffin may be obtained by keeping a small beaker of paraffin on an alcohol lamp and using a warm eye dropper, or a match can be held to a block of paraffin as it is held over the legs. With a small scissors, such as an iridectomy scissors, make a transverse cut across the posterior of the thorax and then cut longitudinally in a posterior direction along the edges of the abdomen. The dorsum of the abdomen can then be lifted carefully off. The female reproductive tract can be easily seen. Sometimes fat tissue and tracheoles and the gut have to be moved with dissecting needles in order to see the ovarioles.

General comments

It is always best to run each stage in duplicate. That is, you should have 2 dishes of 10 bugs each of the second instar, etc. This keeps you from missing a whole section of data if one dish dies off for some reason. Both dishes should give similar results.

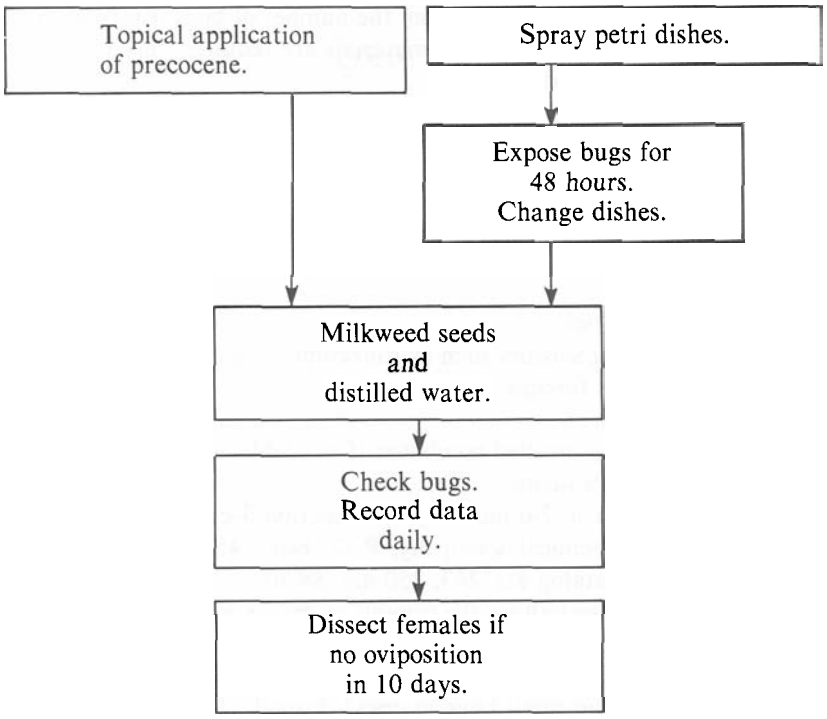

Figure 6.4. Flow diagram of procedures.

The concentrations of precocene that I have suggested may not be the best under your particular laboratory conditions. You should perhaps use lower concentrations if the majority of your bugs die before becoming precocious or normal adults.

Although the bugs can be kept at room temperature, it is better to keep them in an incubator at approximately 27°C. You will get more consistent results under controlled temperatures. In order to lay eggs, milkweed bugs should be kept on 16 hours light and 8 hours dark.

Materials

The amounts of the supplies such as petri dishes will depend on how many stages of the bugs will be treated. If 3 stages of bugs are treated, each stage in duplicate, and 4 petri dishes minimum are allotted for egg laying, a class of 20 students would need approximately 200 petri dishes if each student works independently. The amount of precocene needed must also be calculated from

the number of petri dishes used, or from the number of bugs if topical application is used. Some of the following materials are required only for the advanced exercises in Appendix A.

Milkweed bugs of various stages
Milkweed seeds or sunflower seeds if sunflower strain of bugs used
Glass petri dishes
Microliter syringes
Insect pins
Paraffin
Dissecting dishes
Fine dissecting scissors such as iridectomy scissors
Watchmaker's forceps
Distilled water
Temperature-controlled incubator, if available
Dissecting microscope
Precocene 2 or 6, 7-dimethoxy-2,2-dimethyl-3-chromeme. Sigma Chemical Company, P. O. Box 14508, St. Louis, MO 63178. Catalog #D2263, 250 mg, $8.70.
Grade 80 cheesecloth for the oviposition crocks, etc. The mesh is large enough to allow the female to push her ovipositor through and lay her eggs on top of the cheesecloth, but the mesh is too small to allow the small bugs to crawl through. The eggs can be collected from the top of the cheesecloth each morning with a spatula. Ely & Walker, 823 East Holmes Road, Memphis, TN 38116. Catalog #15-480. Comes in 60 yard bolts. Write for current price.
Cotton dental rolls are available from any dental supply house. American Dental Cooperative, Inc., Milford, DE 19963. 1½" length, No. 2, 2000/box, approximately $20/box; 6" length, No. 2, 500/box, approximately $20/box.
Juvenile hormone, synthetic (also called the Law-Williams mixture) or a juvenile hormone mimic. Calbiochem-Behring Corp., P. O. Box 12087, San Diego, CA 92112. Catalog #430476, 50 mg, $10.00.
Juvenile hormones I, II, and III are also available from Calbiochem-Behring. Write for catalog for current prices.
Milkweed Bug Culture Kit. Carolina Biological Supply Co., Burlington, NC 27215. Catalog #840. This contains eggs of a milkweed bug strain adapted to feeding on sunflower seeds, a rearing container, watering vial, 250 g sunflower seeds and instructions. Eggs, bugs, and seeds are also available separately.

Table 6.2 Student results with precocene sprayed on petri dishes.

Stage Treated (No. Treated)	*Duration of Exposure (Hr)*	*Results*
Eggs (15)	14	**Adults in 28 days; eggs** laid by these adults did not hatch.
2nd (15)	14	Only one precocious adult. Normal (non-precocious) adults did not lay eggs.
3rd (15)	14	Adults produced but did not lay eggs.
5th (10)	48	Eight days to adult; only a few eggs produced which hatched normally.
Adults (12)	48	No eggs; undeveloped ovaries.

Typical results

Results vary tremendously because some students have difficulty handling small insects. The ideal results are those given in Student Materials which are taken from the literature. (Bowers et al 1976; Bowers and Martinez-Pardo 1977.) An actual set of results is given in Table 6.2.

Please note that although these data may be "typical" they do not represent the best possible set of data. Records were not kept well enough to give consistent interpretation of the data for the different stages treated and the different exposure times. If you want good data, you must keep complete records.

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APPENDIX A

Possible Additions to this Experiment

In order to adapt this experiment to junior, senior, or graduate-level students, one or more of the following can be added.

1. Use a series of concentrations of precocene to see which gives optimum results, what the effects of excessive doses are, and whether there is a dose-response relationship or simply a threshold of all-or-no response.
2. Vary the time of exposure to precocene to determine minimum exposure time for a particular concentration. Try to relate concentration and exposure time. (See Masner et al. 1979.)
3. Adults that have been treated with precocene at or near the time of eclosion can be treated with Juvenile Hormone III at 120 hours to overcome the effects of the precocene.
4. Treat the immature intercalary instar with Juvenile Hormone III. (The intercalary instar is the normal instar between the precocene-treated nymph and precocious adult.) The dosage of juvenile hormone can be varied to find the most effective one. A dose to start with is 10μg/μl acetone. One μl can be applied to the ventral abdomen of the adult, and I suggest ¼ to ½ μl for the immature stage.
5. Dissect out the corpora allata and compare sizes for the different treatments. (See Bowers and Martinez-Pardo 1977.) This is a fairly difficult dissection.

APPENDIX B

Selecting Bugs from Stock Colonies

In any physiological or biochemical experiment it is important to know the age of the organism being used. It is easiest to age insects by noting the day post-ecdysis. When an insect undergoes ecdysis (sheds its exoskeleton) it has no melanin, and it takes one to two hours for the melanin or black coloration to appear. When the milkweed bug sheds its exoskeleton it is all orange and can easily be picked out from the rest of the bugs in the stock colony. An hour later you can go back to that stock tray of bugs and pick out those that have undergone ecdysis within the hour. For any single experiment I make hourly collections for a period of no more than 4 hours. In that way I know all the bugs are within 4 hours of age in that particular stage.