INQUIRY & INVESTIGATION

Teaching Principles of Endocrinology Using the Tobacco Hornworm

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ABSTRACT

Insects provide an excellent model for examining concepts in endocrinology in the classroom. They are relatively inexpensive to rear, short-lived, and free from animal welfare regulations. Using the tobacco hornworm (Manduca sexta) *as a model, we have developed a simple laboratory experiment to demonstrate the role of hormones in development. In this experiment, students will use a readily available agonist to disrupt insect development, preventing metamorphosis. This exercise fits well into the AP lab curriculum and the NGSS LSB.1 objectives.*

Key Words: *metamorphosis; juvenile hormone; insect development; growth; (S)-methoprene; hormones.*

○ Introduction

The inclusion of meaningful laboratory exercises into a biology course is a key component in the Advanced Placement curriculum as well as the Next Generation Science Standards. Unfortunately, certain areas of biology are overlooked simply because there are no well-developed model organisms or laboratory exercises. This is particularly evident in endocrinology. Although there are a number of web-based exercises dealing with endocrinology, there are few hands-on laboratory exercises. Presented here is a relatively simple exercise for high school students, who should be made acutely aware of the functions of the endocrine system and the central role it plays in all aspects of life.

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Insects provide an excellent model for examining concepts in endocrinology; they are relatively inexpensive to rear, short-lived, and free from animal welfare regulations. We have developed an insect model that presents a basic aspect of endocrinology—the role of hormones in growth and development. Due to its external, rather than internal, skeleton, growth poses a problem for the insect. As the insect grows, the external skeleton (exoskeleton) expands to compensate for the increased volume; however, its expansion is finite, and as a result, the insect must periodically molt, that is, shed its outgrown exoskeleton for a larger version. The simple laboratory exercise presented here utilizes an easily reared insect, the tobacco hornworm, and a commercially available hormone analog that disrupts development but does not impair growth.

Table 1 provides a comprehensive list of materials and resources, which are discussed in detail below.

○ Endocrinology of Insect Molting

Molting is orchestrated by several hormones, with the major players being 20-hydroxyecdysone (20HE), a steroid, and juvenile hormone (JH), a farnesoid derivative (Figure 1A). An increase in the hemolymph (blood) concentration of 20HE signals epidermal cells, those cells that are involved in exoskeleton synthesis, to initiate the simultaneous breakdown of the old exoskeleton and synthesis of a larger exoskeleton beneath. Once the old exoskeleton is sufficiently loose, the insect sheds the remnants of the old exoskeleton, and the new, highly folded exoskeleton begins to stretch.

The mechanisms that underlie this complex process involve the nervous and endocrine systems. When the exoskeleton can expand no further, signals from the cells that synthesize the exoskeleton are transmitted via the insect's nervous system to the brain, where they are integrated in highly specialized neurosecretory cells. The neurosecretory cells have long axons that exit the brain and terminate in the corpora allata, paired glands that lie posterior to the brain. At the appropriate time, these neurosecretory cells produce a neurohormone termed "prothoracicotropic hormone" (PTTH) that is transported via the long neurons to the

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Table 1. List and sources of equipment and materials required for insect rearing and experimental procedure.

Items	Source
1. Insect Diet and Rearing	
Equipment:	
Refrigerator	
Microwave	
Measuring container (2-plus liters)	Any retail store
Sealable plastic storage containers (for diet)	Any retail store
Blunt knife for preparing individual food cubes	Any retail store
Plastic cups with snap caps (1 oz. and 5 oz.)	Restaurant supply store
Forceps or tweezers to transfer insects	Hardware store
PVC piping and fittings, connectors	Hardware store
Fluorescent shop light (with two fluorescent bulbs: 24″ to 36″)	Hardware store
Plug-in electric timer	Hardware store
Aluminum foil; emergency blanket	Grocery; recreation supply store
Materials:	
Commercial diet	Carolina Biological; Frontier Agricultural Services; MP Biomedicals; Southland Products (see "Rearing in the Classroom" for detailed information)
Water	
2. Experimental	
Equipment:	
Weighing device (± 0.1 g)	Laboratory supply company
Microsyringe or micropipette	Laboratory supply company
Materials:	
Acetone	Laboratory supply company
Precor IGR concentrate	Online supplier (see "Hormone Treatment" for detailed information)
Manduca eggs	Carolina Biological or local source

corpora allata, where the hormone is released into the circulatory system. PTTH then interacts with another gland, the prothoracic gland, which in turn releases a steroid, ecdysone. Ecdysone is converted to the active molting hormone, 20HE, by various tissues. The family of molting hormones that includes ecdysone and 20HE is collectively termed "ecdysteroids."

Whether the insect molts from a larva to another, larger larva or from a larva to a pupa depends on the level of JH. JH is produced by the corpora allata, a paired set of glands that reside just behind the brain. If JH levels are relatively high prior to the molt, the larva will molt to another larva. If, however, JH levels drop, the insect will molt from a larva to a pupa. Conversely, if higher levels of JH are artificially maintained during the last larval stage, the insect will not molt to a pupa but remain a larva (Figure 1B; Akai & Kobayashi, 1971).

Although the hormones are different, there is a remarkable similarity between the corpora allata–prothoracic gland axis in insects and the pituitary-adrenal axis in humans and other vertebrates. In humans, the pituitary is stimulated to synthesize and secrete adrenocorticotropic hormone (ACTH) by upstream factors from the hypothalamus. ACTH is carried via the circulatory system to the adrenal cortex, where it stimulates the production and release of corticosteroids that are responsible for a number of physiological events.

○ The Model

The tobacco hornworm, *Manduca sexta*, progresses through the life stages of egg, larva, pupa, and adult. The larva is relatively large and easy to rear in a classroom. After 3 to 5 days of embryonic

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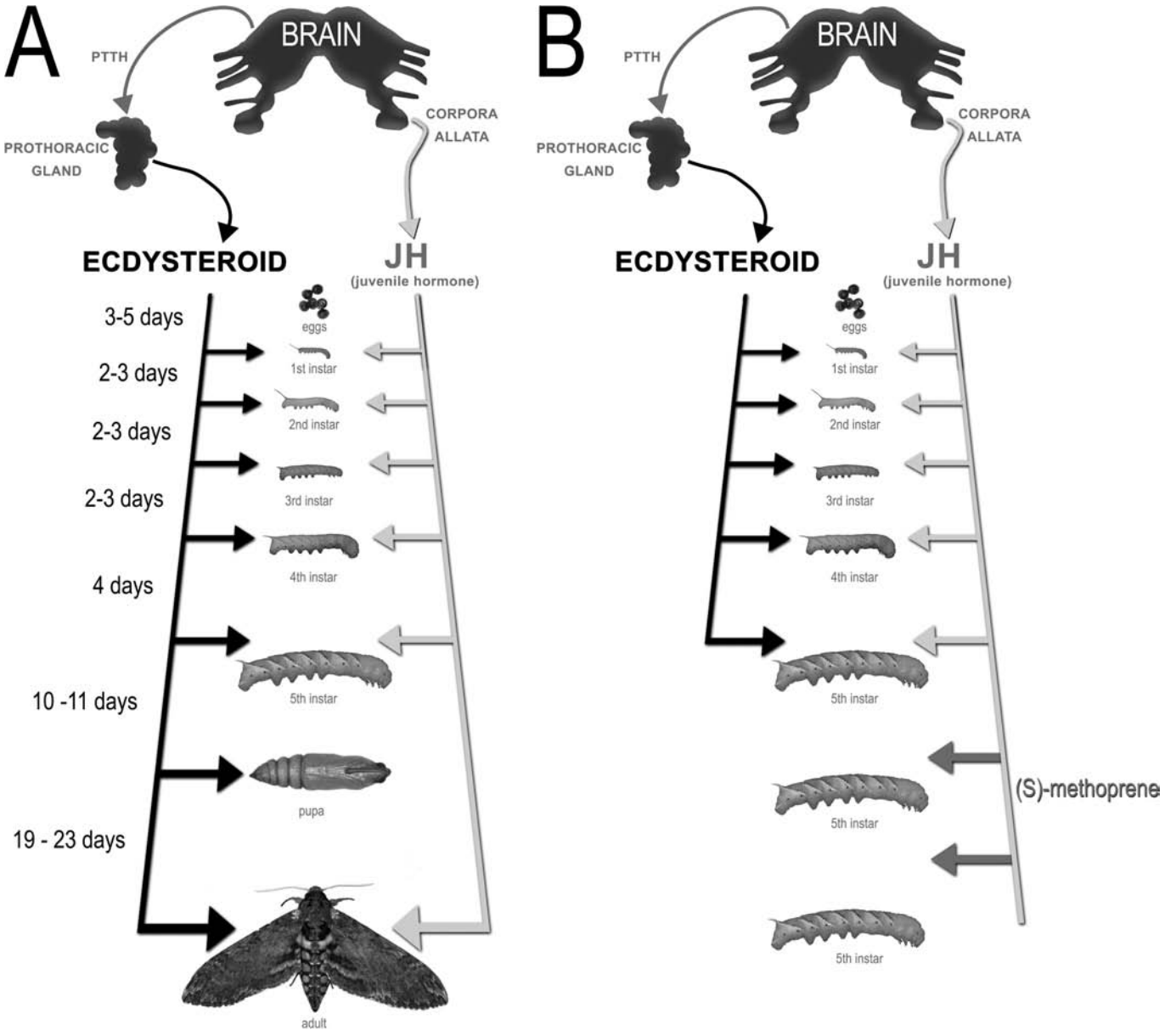

Figure 1. Simplified diagram illustrating the roles of ecdysteroids and juvenile hormone in the development of *Manduca sexta*. Under control from the brain, ecdysteroids are released from the prothoracic gland. Juvenile hormone is released from the corpora allata. **(A)** The combined actions of ecdysteroids and juvenile hormone control development. **(B)** Application of (S)-methoprene to the fifth instar inhibits pupation.

development within the egg, the larva chews its way out of the eggshell and begins to feed. The newly emerged larva is 2 to 3 mm long and has a black "horn" or tail on the posterior end. The pale green larva feeds for several days and then becomes quiescent as it sheds the old exoskeleton. This process is repeated in the development of second, third, and fourth instars (instar = a larva in any one of its periods of postembryonic growth between molts). Development of each instar takes around 3 days, depending upon temperature and nutrition. The fifth instar feeds voraciously for about 5 days, then ceases feeding and burrows underground to pupate. About 1 month later, the adult moth emerges from the soil and mates. Detailed information about the life cycle can be found on Russell Laboratory's *Manduca* website (http://labs.russell.wisc.edu/manduca; Goodman, Carlson, & Nelson, 1985).

○ Rearing in the Classroom

Eggs can be obtained from local university sources where the tobacco hornworm (*Manduca sexta*) is studied, or they can be purchased from Carolina Biological (http://www.carolina.com/). In the classroom, it is best to rear the insect on commercially available artificial diet rather than plants. Artificial diet contains wheat germ, casein, vitamins, minerals, water, and agar only, and can be purchased from: Carolina

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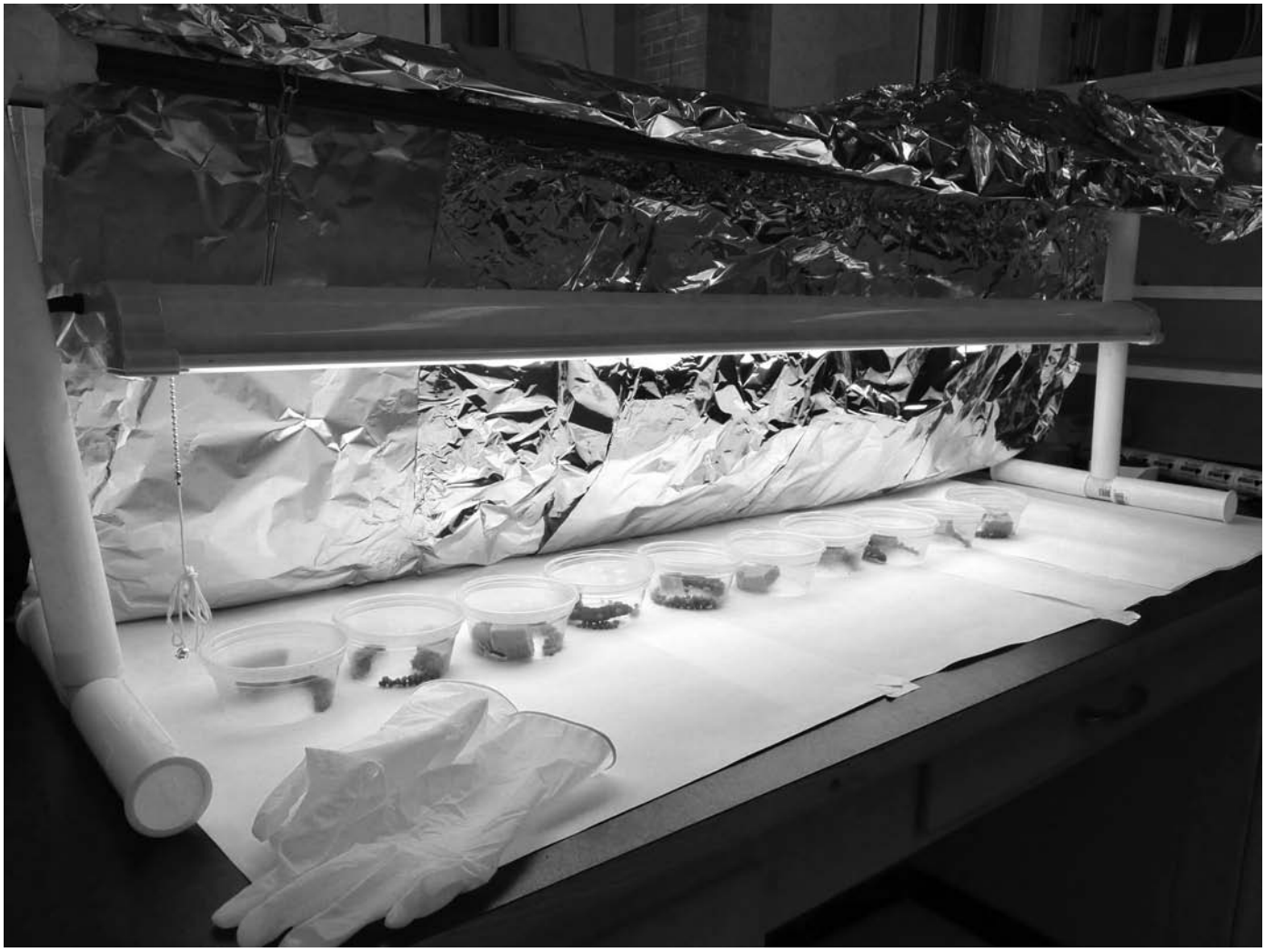

Figure 2. An example of a temporary bench-top "incubator" suitable for the rearing of *Manduca sexta* or other insect species. The incubator is constructed using 2-inch plastic pipe and a double fluorescent shop-light fitting (both available from most hardware stores) together with an emergency blanket (available from most sporting goods stores).

Biological, Hornworm Medium, Dry, product number 143905, http://www.carolina.com/; MP Biomedicals, Gypsy Moth Diet, product number 02960292, http://www.mpbio.com/; Frontier Agricultural Services, Gypsy Moth Diet, product number F9630B, http://www.insectrearing.com/products/indiets.html; Southland Products, Tobacco Hornworm Diet, http://www.tecinfo.com/~southland. A detailed, video-based description of diet preparation is available at http://labs.russell.wisc.edu/manduca.

Manduca sexta growth and development are directly related to light and temperature. Since most classrooms are not equipped with a light- and temperature-controlled incubator, we have developed a simple and inexpensive growth chamber in which to rear the insects. A standard, commercially available fluorescent shop light (24 to 36 inches) with two fluorescent bulbs is suspended from a PVC pipe frame with the bulbs approximately 12 inches from the insect containers (Figure 2). The light is attached to a basic 24-hour plug-in timer set for 16 hours on and 8 hours off. The shop light/PVC pipe frame is draped with aluminum foil or an emergency blanket to create a tent that minimizes temperature fluctuations. Duration of developmental periods varies with temperature; the estimates below assume the temperature is 25°C (77°F).

Maintaining the appropriate humidity is also important. Too little humidity will allow the diet to dry, and larvae derive all their water from the diet. Too much humidity encourages the growth of mold, which can endanger the health of the larvae. Rearing the insects in plastic cups with snap-cap lids, available at any restaurant supply company or online, generally works well under the conditions described below, but cups should be monitored for dry or moldy diet.

We have developed a website for instructors interested in rearing the tobacco hornworm in the classroom (http://labs.russell.wisc.edu/manduca). Briefly, approximately 100 eggs are placed in a plastic 5-oz cup containing a block of diet ~5 × 1 × 1 cm. The eggs must not be in direct contact with diet. Larvae emerge from newly laid eggs in 4 to 5 days; variable temperatures experienced during transport may alter emergence times.

Upon emergence, larvae will make their way to the diet block, where they will feed for approximately 48 hours, become quiescent for approximately 24 hours and then molt. The second instars

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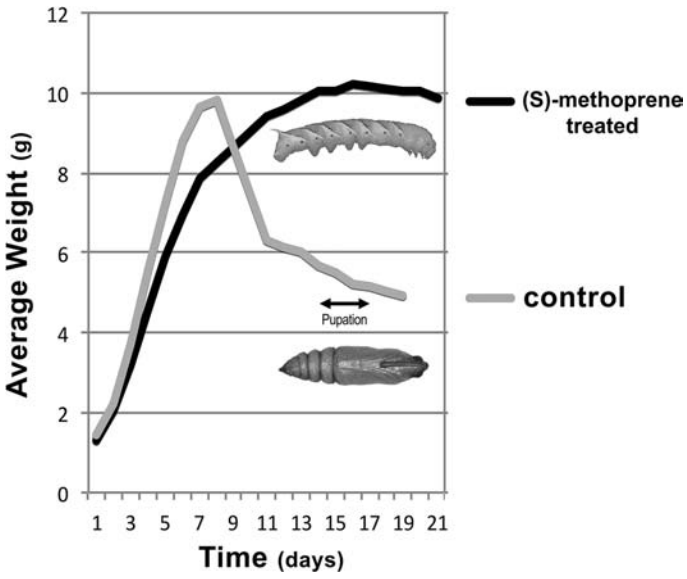

Figure 3. The effects of (S)-methoprene on the weight and development of fifth instar *Manduca sexta*. Control insects (gray line) rapidly develop to achieve maximum weight in approximately 7–8 days. Following this rapid growth period, larvae enter a pre-pupal phase (days 7–10) when they lose weight as they empty the alimentary canal. Control larvae undergo pupation over a 4-day period (days 14–17). All (S)-methoprene treated larvae (black line) fail to empty the alimentary canal and do not undergo pupation.

repeat this process in about the same time. Following this molt, the insects must be separated into individual containers. Transfer is accomplished by gently grasping an insect with forceps, using the elongated tail (horn) as a handle. The larvae are transferred to 1-oz plastic cups containing a diet cube ~1 × 1 × 1 cm. Upon completion of the third larval period (~72 hours), each insect is transferred to a new 1-oz cup containing a fresh diet cube. The fourth larval period usually takes 4 days to complete. Molting is preceded by the forward movement of the old head capsule to form a bubble-like structure in the head region. Once the old head capsule and exoskeleton are shed, transfer the newly molted fifth instar to a 5-oz cup containing a diet cube ~2 × 2 × 2 cm. The waste product, termed "frass," should be removed daily. During the next 4 to 5 days, larvae feed voraciously.

Several markers indicate the larva is preparing for pupation. It ceases feeding; it moves agitatedly around the cup, mashing the diet cube as it attempts to burrow; the dorsal vessel, the major circulatory organ of the larva, becomes noticeably visible as a dark line down the back. During the next several days, the larva will lose about a third to half its weight, and pigmentation will change from the blue-green color to a dull yellow-green color. The larva, previously elongated, shrinks to a football shape and is no longer able to walk due to muscle degeneration. Larvae will pupate approximately 10 to 11 days after the last larval molt.

○ Hormone Treatment

Naturally occurring JH is expensive and highly labile. We have turned to a stable, inexpensive, commercially available analog that displays biological properties similar to JH, (S)-methoprene (Precor IGR concentrate, 1.2% active ingredient; http://www.domyownpest-control.com/precor-igr-concentrate-p-72.html). A note of caution: a number of commercially available products contain (S)-methoprene but also contain insecticides and synergists that enhance pest control effectiveness. Before purchasing an alternative to Precor IGR, check the label.

(S)-Methoprene or acetone (control) is administered to newly molted fifth instars and thereafter every 2 days for up to 2 weeks. The treatments are administered to the first thoracic segment (first segment behind the head). Like the acetone control, the carrier solvent for (S)-methoprene evaporates quickly when placed on the exoskeleton, allowing the (S)-methoprene to be absorbed through the exoskeleton and transported by hemolymph to the target tissues. Application of 1 µl of Precor IGR (~12 µg of (S)-methoprene) from the stock bottle is more than sufficient to inhibit pupation. If a microsyringe or micropipette is unavailable, a fine gauge nichrome wire, such as that used for a microbiological transfer loop, can be formed into a small loop that contains approximately 1 µl. The loop can be carefully dipped into the Precor IGR stock bottle or acetone bottle and the excess removed by touching the loop to the edge of the bottle.

Each insect should be weighed every 2 days until it begins to mush the diet. At this point, the larva is removed from the 5-oz cup, washed under cool water, gently dried and placed into a clean 5-oz cup lined with tissue paper. From this point forward, the insect requires no diet, and hormone treatments cease. It may be useful to obtain 2 egg batches, approximately two weeks apart. Rearing the first batch will familiarize students with the insects. It will also provide an opportunity to define key concepts in endocrinology, such as: What is a hormone? What controls its synthesis and release? How does the hormone induce target tissues to respond? The second batch of insects can be used for the actual experiment.

○ Time Burden

Under the conditions described for rearing (see Figure 1), the instructor would need to set aside:

- ~1 hour to make the temporary incubator once all materials have been purchased,
- ~1 hour to make up sufficient diet to supply all the needs for the next month, and
- ~1 hour to make up the hormone solution.

Student participation during larval growth can be as little as 5 minutes a day and would include changing the diet and making notes.

○ Experimental Outcomes

Figure 3 shows the data from an experiment conducted under the conditions described above. The data indicate that repeated application of (S)-methoprene (1 µl of Precor IGR) will lead to a significant prolongation of the larval stage. This outcome is expected, given the role of JH in preventing larva-to-pupa metamorphosis. By contrast, control insects will cease feeding, turn from the

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characteristic blue-green to a dull yellow color, and lose weight as the alimentary canal is cleared and muscles begin to break down prior to metamorphosis.

○ Building Hypotheses

During the rearing period before the experiment begins, the instructor and students can consider potential variables in the upcoming experiment. These discussion sessions may examine how to generate hypotheses, how to develop an experimental design, and how to employ statistical methods to better understand the results. For example, how many insects should be used in the control and treated groups to derive meaningful data? (See http://www.surveysystem.com/sscalc.htm for a simple method to calculate the appropriate size of the experimental population.) Should the insects to be tested be similar in weight? After the experiment is completed, students can discuss questions appropriate for potential follow-up experiments. For example, students also might be asked to investigate and consider:

- What would happen if an anti-JH were applied?
- The effects of varying the dose of hormone.
- The frequency of administration (e.g., compare every day with every second or third day).
- The timing within the growth cycle to get the effect (e.g., start hormone application at end of the fourth instar).
- The location where the hormone is applied.

Information gleaned from the experiment may stimulate comparisons to human physiology and health. Are there similar hormones in humans? How many hormones do humans have, and what do they regulate? What happens if hormones are not well-regulated? (There are more than 50 well-recognized endocrine-based diseases in humans, with diabetes being the most prevalent.) What would happen if humans were treated with large doses of various hormones? (In considering this question, students can discuss the use of exogenous hormone, such as growth hormone and anabolic steroids, by athletes seeking to increase competitiveness.) Are there gender differences in the hormone response?

The results of this lab underscore the role hormones play in growth and the ramifications of upsetting hormonal balance by introducing exogenous hormone or hormone-like compounds in insects—and, by analogy, humans. These exercises fit well into the AP lab curriculum and the NGSS LSB.1 objectives. For AP Biology, the experiment supports "Big Idea 4: Biological Systems Interact," and these systems and their interactions possess complex properties.

> Additionally, interactions between external stimuli and gene expression result in specialization and divergence of cells, organs and tissues. Interactions and coordination between organs and organ systems determine essential biological activities for the organism as a whole. External and internal environmental factors can trigger responses in individual organs that, in turn, affect the entire organism.

For NGSS, the experiments support HS-LS1 From Molecules to Organisms: Structures and Processes, and, in particular, LS1.B: Growth and Development of Organisms:

> In multicellular organisms, individual cells grow and then divide via a process called mitosis, thereby allowing the organism to grow. The organism begins as a single cell (fertilized egg) that divides successively to produce many cells, with each parent cell passing identical genetic material (two variants of each chromosome pair) to both daughter cells. Cellular division and differentiation produce and maintain a complex organism, composed of systems of tissues and organs that work together to meet the needs of the whole organism. (HS-LS1-4)

Most importantly, projects that incorporate an active, hands-on experience will not only engage the students but promote critical thinking.

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