Tested Studies for Laboratory Teaching
Proceedings of the Association for Biology Laboratory Education
Vol. 33, 95–112, 2012

Effect of Environment and Modulators on Hindgut and Heart Function in Invertebrates: Crustaceans and *Drosophila*

Rachel C. Holsinger[1] and Robin L. Cooper[2]

[1]Sayre School, 194 North Limestone Street, Lexington KY 40507 USA
[2]University of Kentucky, Department of Biology, 675 Rose Street Lexington KY 40506-0225 USA
(**rholsinger@sayreschool.org; RLCOOP1@email.uky.edu**)

Using preserved animals during teacher-led demonstrations and student experimentation may help students appreciate the anatomy of animals but does not allow them to design experiments to develop an understanding of the physiology. The crayfish hindgut allows for easy physiologic recordings through visually monitoring peristaltic activity, which can be used as a bioassay for various peptides, biogenic amines, neurotransmitters and environmental substances. The neurogenic crustacean and the myogenic *Drosophila* larva hearts clearly show the affect of environmental stimuli (temperature, CO_2) and modulators (serotonin, nicotine) that enter the hemolymph. These robust preparations are well suited for training students in physiology and pharmacology.

Keywords: invertebrates, crustaceans, *Drosophila*, neuromodulators, physiology, peristalsis, heart rate

Introduction

The goal of our report is to increase the awareness to the potential of this preparation in student-run investigative laboratories that teach fundamental concepts in physiology and pharmacology. These preparations can be used to investigate a number of experimental questions that will lead to a better understanding of the physiological functions. These labs were used for Fall 2010, Summer 2011, and Fall 2012 semesters of Animal Physiology at the University of Kentucky. The labs have been beta tested and videos showing the procedures are available online at:
http://web.as.uky.edu/Biology/faculty/cooper/ABLE/LAB-GI.htm

These three experiments can be used with any level of student, ranging from an Anatomy and Physiology high school class to an upper level undergraduate course. Additional levels of these experiments can be performed depending on available equipment such as a force transducer to measure force and rate of GI contractions or a dissecting microscope to examine a heart in larval *Drosophila* when inducing a modulation of pacemaker activity. Also multiple influences in the form of environmental and modulator cocktails are able to provide student individuality in experimentation. These labs are designed to be completed in less than 3 hours with the assumption that students have read the protocol and watched the online videos before coming to class.

Drosophila serve as a good model for physiological function. Although they have an *open* circulatory system, the myogenic nature of the larval heart is comparable to the mammalian heart with pacemakers that drive the heart to pump fluid in a direction to be effective in bathing organs. This dorsal vessel is a continuous tube extending from the last abdominal segment to the dorso-anterior region of the cerebral hemisphere. It is divided into anterior aorta and posterior heart. The heart rate (HR) varies throughout larval stages depending on whole animal activity (feeding and crawling) and the HR tends to slow down during pupation. The larval heart is very susceptible to biogenic amines and peptides depending on the food source. The mechanism of action for neurotransmitters and cardiac modulators on the larval heart has not been described at the cellular level to date.

The ghost shrimp (*Palaemonetes* sp.) is an ideal experimental model for monitoring heart rate, due to the organism's heterothermic nature and transparent exoskeleton. In addition, these organisms are well suited for use in the classroom because they are easily acquired and require little maintenance.

It is very easy to dissect and record contractions from the crayfish hindgut. In the dissected preparation, the gut is easily exposed to exogenous substances. The alteration

© 2012 by Rachel C. Holsinger and Robin L. Cooper

in peristaltic activity can be monitored visually or with a force transducer. This preparation is useful to demonstrate pharmacological concepts to physiology students in laboratory settings. It has been in use for over 100 years, yet it still offers much as a model for investigating the generation and regulation of peristaltic rhythms and for describing the mechanisms underlying their modulation.

The details provided in the associated movie and text describe the key steps needed to record the activity in the hindgut of the crayfish *in situ* as well as *in vitro*. The mechanisms underlying regulation of peristaltic waves and their reversal are still not known. The mechanisms for higher control of the entire GI tract are also not fully understood. The question of how higher centers integrate their activity with the autonomic output that directly controls the GI system remains an open area of investigation (Shuranova *et al*., 2006). In addition, the osmoregulatory capabilities of the crustacean hindgut and the functions of osmoregulation during molting and environmental stress have not been fully elucidated. There are still many questions awaiting answers in this preparation.

Student Outline

1. Heart Rate Response to Induced Environmental and Modulators in Freshwater Shrimp

Introduction

The purpose of this exercise is to investigate the effect of various environmental cues on the heart rate of the transparent ghost shrimp (*Palaemonetes*). Heart rate in crustaceans can be altered under many conditions: neurotransmitters, temperature, and chemicals, such as stimulants, can all have an effect. Neurotransmitters act on the organism's heart rate through the nervous system in a parasympathetic-like or sympathetic-like manner. This can either cause an increase or decrease in overall heart rate based on the properties of the neurotransmitter in question. The effects of temperature on heart rate are also variable. Lower temperatures tend to decrease the heart rate. Conversely, high temperatures tend to cause an increase in heart rate due to the increase in metabolic activity and higher rate of chemical reactions within the body. Chemical stimulants also increase the heart rate and blood flow. In this experiment, students will become familiar with these effects by subjecting a species of freshwater shrimp to varying environmental conditions - dopamine, serotonin, and cold water.

Preparation

The ghost shrimp is an ideal experimental model for monitoring heart rate, due to the organism's transparent exoskeleton and low maintenance. Before starting this experiment, become familiar with the shrimp anatomy (Fig. 1).

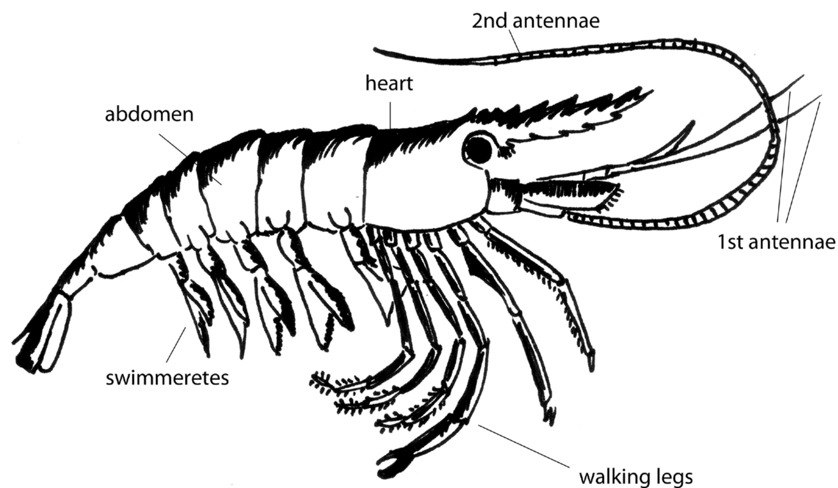

Figure 1. Anatomy of Shrimp

Protocol

1. Dampen a paper towel with distilled water.
2. Place the shrimp in the damp paper towel so that the anterior region is wrapped and the animal's back and tail are free.
3. With a second, dry paper towel gently dab the back of the animal in order to dry it for the glue.
4. Place a small drop of the glue onto the wooden rod and then adhere it to the animal. Ensure that the stick is glued slightly below the back on the tail so that the apparatus does not hide the heart (Fig. 2).
5. While holding the rod in place, place a dab of the quick dry compound over the glue to complete the bonding.
6. Hold the stick in place for approximately five more seconds or until the rod is sufficiently affixed to the shrimp.
7. Rinse the shrimp and rod of any excess chemicals by dipping it once or twice into the beaker of water.
8. Place the shrimp in the grooved Petri dish; ensure that the rod fits securely into both grooves on either side of the dish. This will limit the animal's movement and will allow it to be monitored through the microscope. Fill the dish with aerated water and be sure that the level is over the back of the shrimp so that it survives the experiment.

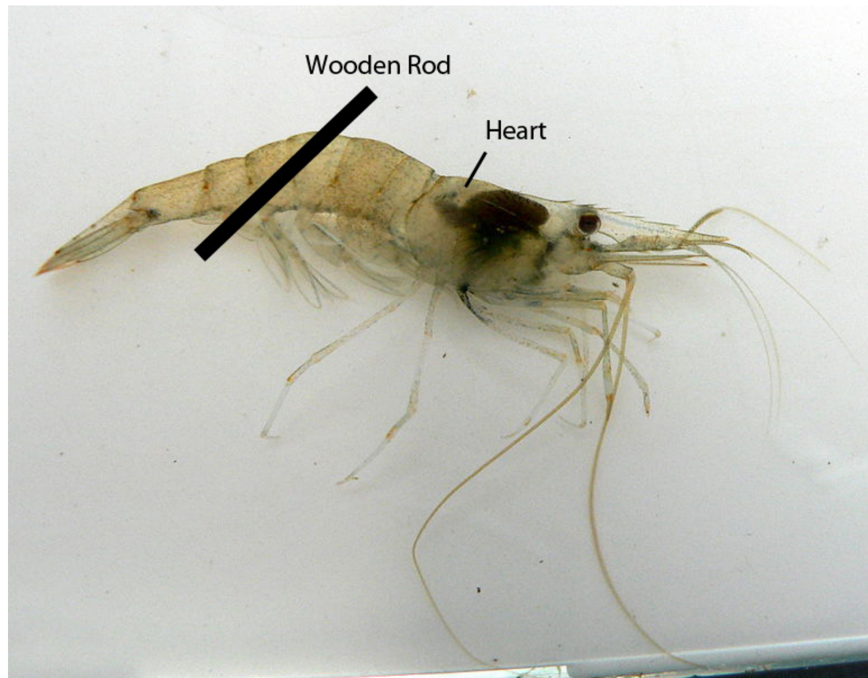

Figure 2. Diagram of wooden rod placement on dorsal side of shrimp.

Methods

Exercise 1

Monitor the heart rate of the shrimp to gain a baseline. To do this, place the shrimp setup under the microscope so that the heart is visible on the back of the animal. Count the number of beats in ten seconds and multiply this number by six to get the beats per minute. To obtain an experimental baseline, do this three times and take the average of the three values and record values in Table 1. **Note:** It may be beneficial to allow the shrimp to acclimate for approximately five to ten minutes before taking a baseline reading to account for the agitation of the animal.

Exercise 2

Note: Your teacher will instruct your group to perform either Part A or Part B of Exercise 2, **NOT** both. You will then exchange recorded data with a group that used a different chemical stimulus.

A. Carefully transport the dish containing the shrimp to the sink and gently pour the contents of the bath out. Make sure that the shrimp is secure and does not fall into the sink. While monitoring the shrimp heart rate through the microscope, fill the bath with the prepared dopamine solution. Take note of the immediate response. After approximately thirty seconds, take note of the number of heart beats in a ten second period. Again, multiply this number by six in order to calculate beats per minute. As in Exercise 1, repeat this process for a total of three times; generate an average beats per minute reading and record it in Table 1.

B. Carefully transport the dish containing the shrimp to the sink and gently pour the contents of the bath out. Make sure that the shrimp is secure and does not fall into the sink. While monitoring the shrimp heart rate through the microscope, fill the bath with the prepared serotonin solution. Take note of the immediate response. After approximately thirty seconds, take note of the number of heart beats in a ten second period. Again, multiply this number by six in order to calculate beats per minute. As in Exercise 1, repeat this process for a total of three times; generate an average beats per minute reading and record it in Table 1.

Exercise 3

Carefully transport the dish containing the shrimp to the collection beaker in the classroom. Gently pour out the solution containing either dopamine or serotonin. Make sure that the shrimp is secure. Rinse the beaker and the shrimp with aerated water to ensure that all of the chemicals have been removed. While monitoring the shrimp heart rate through the microscope, fill the bath with chilled aerated water from the beaker containing the ice. Observe the immediate change. After approximately

thirty seconds, take note of the number of heart beats in a ten second period. Again, multiply this number by six in order to calculate beats per minute. As in the previous exercises, repeat this process three times to generate an average beats per minute reading and record in Table 1. Record the water temperature, _______ °C.

Exercise 4

Gently pour out the cold water. Make sure that the shrimp is secure. While monitoring the shrimp heart rate through the microscope, fill the bath with warm aerated water from the beaker. Observe the immediate change. After approximately thirty seconds, take note of the number of heart beats in a ten second period. Again, multiply this number by six in order to calculate beats per minute. As in the previous exercises, repeat this process for a total of three times; generate an average beats per minute reading and record in Table 1. Record the water temperature, _________°C.

Results

Table 1. Results of various conditions on heart beat in the ghost shrimp.

	Control (beats/min)	**Dopamine (beats/min)**	**Serotonin (beats/min)**	**Cold Water (beats/min)**	**Warm Water (beats/min)**
Trial 1					
Trial 2					
Trial 3					
Average					

In order to compare the effects upon heart rate of the various conditions observed above, plot the average beats/minute of each stimulus in Figure 3.

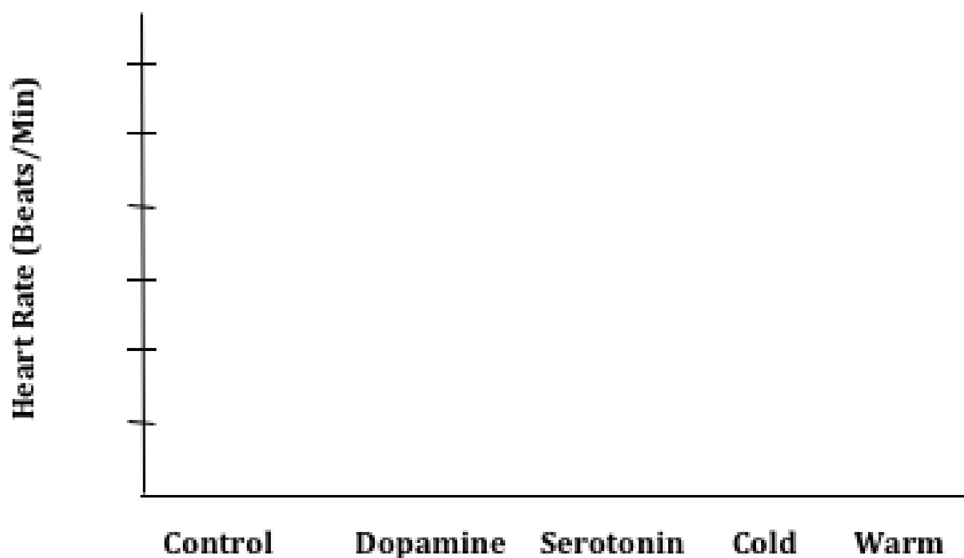

Figure 3. Results of various conditions on the bpm of the ghost shrimp.

To account for the effects of non-target variable conditions such as experimenter noise or changes in the amount of light over the shrimp with passing shadows, it is important to calculate the percent difference from baseline for each of the stimuli observed before drawing any conclusions about generalized reaction trends:

$$\% \text{ Difference} = \frac{\text{absolute difference (initial - experimerimental)}}{\text{initial}} \times 100\%$$

Record the percent difference in Table 2.

Table 2. Percent difference in bpm of ghost shrimp under various conditions.

	Dopamine	**Serotonin**	**Cold Temperature**	**Warm Temperature**
Percent Difference				

2. Heart Rate in *Drosophila*

Introduction

The *Drosophila* heart is known to be myogenic (cells initiate contraction, not nerve impulses) in the larval stage and can be studied after being removed or *in situ*. The myogenic nature of the larval heart is comparable to the mammalian heart as pacemakers drive the rest of the heart to pump fluid in a direction to be effective in bathing organs. The cellular mechanism of action of the neurotransmitters and cardiac modulators in larvae have not been described to date as there has not been sufficient understanding of the ionic currents and channel types present in the larval heart that contribute and regulate pacemaker activity (Cooper, *et al*., 2009). As far as we are aware, there are no reports documenting extracellular or intracellular recordings of myocytes to assess ionic currents to address the mechanistic effects of modulators in larval *Drosophila*. The larval heart is very susceptible to biogenic amines and peptides, which vary in the hemolymph depending on food source or intrinsic state of the animal (Dasari and Cooper, 2006; Johnson *et al*. 1997, 2000; Nichols *et al*. 1999; Zornik *et al*. 1999). Addressing how endogenous or exogenous compounds influence the heart mechanistically is of interest. Possibly novel insecticides with fewer effects on other organisms can be developed if we gain a better understanding of insect physiology and pharmacology.

Preparation

The *Drosophila* larva is an ideal experimental model for monitoring heart rate because of the ease of measuring heart rate by counting the movement of the trachea. There are attachments from the heart on the trachea. Before starting this experiment, become familiar with the *Drosophila* anatomy in Figure 4.

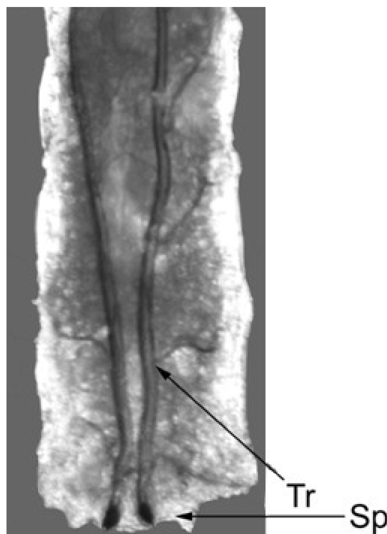

Figure 4. Dorsal view of an intact 3rd instar larva showing trachea (Tr) and spiracles (Sp)

Protocol

1. Take a clean slide and place a cover slip at one end of it.
2. Put a small dab of superglue at one corner of the cover slip.
3. Locate your *Drosophila* larva and remove it from the test tube.
4. Place the larva in a Petri dish and rinse it with a small amount of water to remove any excess food.
5. Soak up remaining food with the corner of a small tissue or paper towel.
6. Gently pick up larva with tweezers and place it on your slide on the opposite end from your cover slip.
7. Place the slide under the microscope and adjust your lens on the larva. The larva should be on its stomach with its back facing upwards. You can distinguish between the two sides of the larva because their backs feature two "racing stripes" which are the trachea. The stomach has faint horizontal grooves running along it with very fine black hairs.
8. If the larva is facing the incorrect way, simply turn the right way by gently flipping it over with your tweezers (Fig. 5).

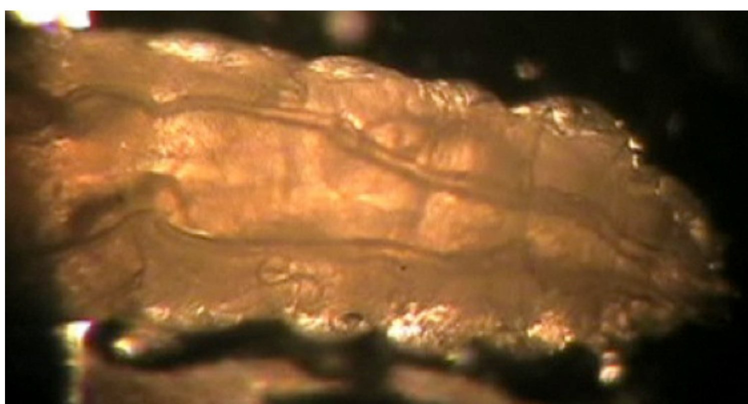

Figure 5. Dorsal view of *Drosophila* larva.

Intact Larvae Protocol

9. Restrain the larva to one location using double stick tape on a glass slide and placing the ventral side of the larva to the tape.
10. Make sure the black mouth hooks are located near or at the edge of the cover slip and neither they nor the brown spiracles come in contact with the tape.
11. Carefully press down on the larva to flatten it out.
12. This approach does not work well if the tape gets wet when feeding the larvae. To avoid the tape getting wet use Vaseline (injected out of a small needle around the base of the larvae and around the tape edge). To free the larvae, moisten the tape to loosen the adhesiveness to the animal.

Permanent Restraining Protocol

13. With a new set of tweezers used specifically for glue take a small dab from the drop at the end of your cover slip and place it at the corner of the opposite end. You should use a fractionally amount of glue, just enough to cover the head of the tweezers. Also, make sure you wipe off the ends of your tweezers so that they do not become glued shut.
14. Under the microscope, double check to make sure the larva is still in the correct position. If it has turned over, see step eight.
15. Now, with the tweezers used to handle larva, pick up the larva and place it gently on the fresh patch of glue. Make sure the black mouth hooks are located near or at the edge of the cover slip and neither they nor the brown spiracles come in contact with the glue.
16. Carefully press down on the larva to flatten it out (Fig. 6).

Figure 6. Attached *Drosophila* larva on cover slip.

Methods

Exercise 1

Monitor the heart rate of the *Drosophila* to gain a baseline. Count the number of trachea movements in ten seconds and multiply this number by six to get the beats per minute. To obtain an experimental baseline, do this three times and take the average of the three values and record values in Table 1.

Exercise 2

Note: Your teacher will instruct your group to perform either Part A or Part B of Exercise 2, **NOT** both. You will then exchange recorded data with a group that used a different chemical stimulus.

A. While monitoring the *Drosophila* through the microscope, use a syringe to add dopamine-laced food to the head of the fly. After approximately thirty seconds, count the number of spiracle movements in a ten second period. Multiply this number by six to calculate beats per minute. Repeat this process three times; generate an average beats per minute and record it in Table 1.

B. While monitoring the *Drosophila* through the microscope, use a syringe to add serotonin-laced food to the head of the fly. After approximately thirty seconds, count the number of spiracle movements in a ten second period. Multiply this number by six to calculate beats per minute. Repeat this process three times; generate an average beats per minute and record it in Table 1.

Exercise 3

Carefully rinse the food off the slide making sure the *Drosophila* is secure. While monitoring the *Drosophila* through the microscope, add chilled water from the beaker containing the ice to the slide. After approximately thirty seconds, count the number of spiracle movements in a ten second period. Multiply this number by six to calculate beats per minute. Repeat this process three times; generate an average beats per minute and record it in Table 1. Record the water temperature, _________°C.

Exercise 4

Carefully rinse the food off the slide making sure the *Drosophila* is secure. While monitoring the *Drosophila* through the microscope, add warm water from the beaker to the slide. After approximately thirty seconds, count the number of spiracle movements in a ten second period. Multiply this number by six to calculate beats per minute. Repeat this process three times; generate an average beats per minute and record it in Table 1. Record the water temperature, _________°C.

Results

Table 1. Results of various conditions on heart beat in *Drosophila* larvae.

	Control (beats/min)	Dopamine (beats/min)	Serotonin (beats/min)	Cold Water (beats/min)	Warm Water (beats/min)
Trial 1					
Trial 2					
Trial 3					
Average					

In order to compare the effects upon heart rate of the various conditions observed above, plot the average beats/minute of each stimulus below:

To account for the effects of non-target variable conditions such as experimenter noise or changes in the amount of light over the shrimp with passing shadows, it is important to calculate the percent difference from baseline for each of the stimuli observed before drawing any conclusions about generalized reaction trends.

$$\% \text{ Difference} = \frac{\text{absolute difference (initial - experimerimental)}}{\text{initial}} \times 100\%$$

Record the percent difference in Table 2.

Table 2. Percent difference in bpm of ghost shrimp under various conditions.

	Dopamine	Serotonin	Cold Temperature	Warm Temperature
Percent Difference				

3. Physiological Experimentation with the Crayfish Hindgut

Introduction

The purpose of the exercise is to monitor peristaltic activity in the *Procambarus clarkii* (crayfish) hindgut with and without the addition of various compounds. Alexandrowicz (1909) identified two nerve plexuses innervating the crustacean hindgut, an inner plexus and an outer plexus, which later proved to be rich sources for identifying and characterizing neurotransmitters. In the 1950s, Dr. Ernst Florey began a series of pharmacological studies on the crayfish hindgut and demonstrated that contractions are modulated by acetylcholine and its related compounds and also by epinephrine and norepinephrine (Florey, 1954). Florey, the discoverer of an inhibitory substance known as "Factor-I," studied the effects of this substance on various preparations, including the GI system in crayfish (Florey, 1961). Factor-I was later shown to be GABA. Thus, the crayfish hindgut played an important role in the early studies of synaptic inhibition.

Although the crustacean hindgut contracts spontaneously following denervation, such contractions are typically weak and disorganized (Wales, 1982; Winlow & Laverack, 1972a). Peristaltic movement requires coordinated motor output from the central nervous system, apparently originating in the last abdominal ganglion (Winlow & Laverack, 1972a, b, c). In crayfish, the motor output is carried to the hindgut through the 7th abdominal root, which contains 75 axons whose cell bodies are localized in the last abdominal ganglion (Kondoh & Hisada, 1986). At least some of the peptides appear to be supplied to the hindgut plexus from neurons originating in the last abdominal ganglion (Dircksen *et al*. 2000; Mercier *et al*. 1991b; Siwicki & Bishop, 1986), but dopamine is supplied by neurons in more anterior ganglia (Mercier *et al*. 1991a). Since motor output through the 7th abdominal root is necessary for large, coordinated contractions, it is likely that some or all of the putative transmitters listed above contribute in some way to peristalsis. Their relative contributions, however, are not known. Some insight may be gained by studying their effects on circular and longitudinal muscles separately (Mercier & Lee, 2002).

It is interesting to note that the anal portion of the hindgut acts not only to expel feces but also to take up the water from the environment for osmoregulation. This region of the gut can undergo forward or reverse peristalsis depending on the animal's needs.

Preparation

1. Crayfish (*Procambarus clarkii)* measuring 6-10 cm in body length should be placed on ice for 5 to 10 minutes to anesthetize the animal before dissection begins.
2. Hold the anesthetized crayfish from behind the claws with one hand. Quickly, cut from the eye socket to the middle of the head on both sides, and then behead the crayfish (Note: the blood from the preparation will be sticky when it dries, so wash the tools when completed).
3. Cut off the chelipeds and walking legs.
4. Cut off the left and right tail fins, only leaving the middle tail fin (uropod).
5. On the dorsal side of the crayfish cut ventrally on both the left and right side of the dorsal cuticle.
6. Cut transversely on the dorsal side of the cuticle, making sure that the cut is shallow to prevent damage to the hindgut. Then remove the lower section of the dorsal abdominal cuticle (Fig. 7).
7. Place the dissected crayfish into a Sylgard-lined dish.
8. Pin the crayfish to the dish at the tip of the tail fin. One may use more pins on either side of the hindgut as necessary to hold the body down. The dissected crayfish should appear as shown in Fig. 8.
9. Fill the dish with crayfish saline covering the hindgut Make sure to continually douse the hindgut with saline using a pipet.

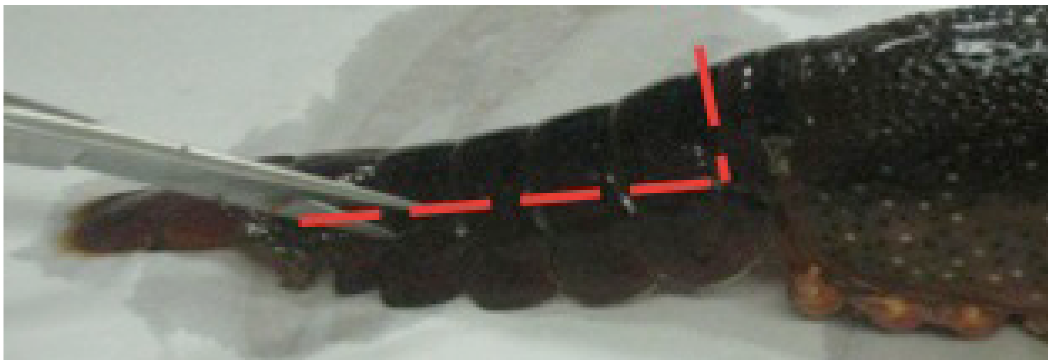

Figure 7. Removal of dorsal cuticle
.

Figure 8. Dissected crayfish with hindgut intact.

Methods

Contractions in the animal

1. Have solutions of the compound to be tested ready and at the same temperature as the bathing saline.
2. Allow the dissected crayfish to sit in the crayfish solution for about ten minutes to allow it to adjust to the shock of dissection and saline exposure. The slow peristalsis contractions from the hindgut should start to occur.
3. Once the contractions begin, record the number of contractions that occur in thirty seconds (note the type of contractions that occur: i.e. peristalsis type, or spastic and the direction of any peristaltic waves) in Table 1.
4. Using a pipet, remove the saline within the cavity of the crayfish's exposed abdomen, and apply saline containing the substance to be examined directly onto the hindgut.
5. Immediately after adding the solution, record the number of contractions that occur after thirty seconds and note the type of contractions that occur (i.e. peristaltic, or spastic) in Table 2.
6. Immediately after recording the response to the test substance, rinse the hindgut several times with normal crayfish saline. Let the preparation stand for 5 minutes, while rinsing about every 30 seconds with crayfish saline.
7. Using a pipet, place some saline containing the next compound to be examined or a varied concentration of the last substance tested directly onto the hindgut. It is best to start with a lower concentration and work one's way to higher concentrations.
8. Immediately after adding each new substance or each new concentration, record the number of contractions that occur after thirty seconds and note the type of contractions in Table 2.

Recording forces of contraction in excised preparations

1. Attach the transducer to the bridge pod.
2. Attach the bridge pod to the PowerLab 26T.
3. Attach the PowerLab 26T to the USB port on the computer.

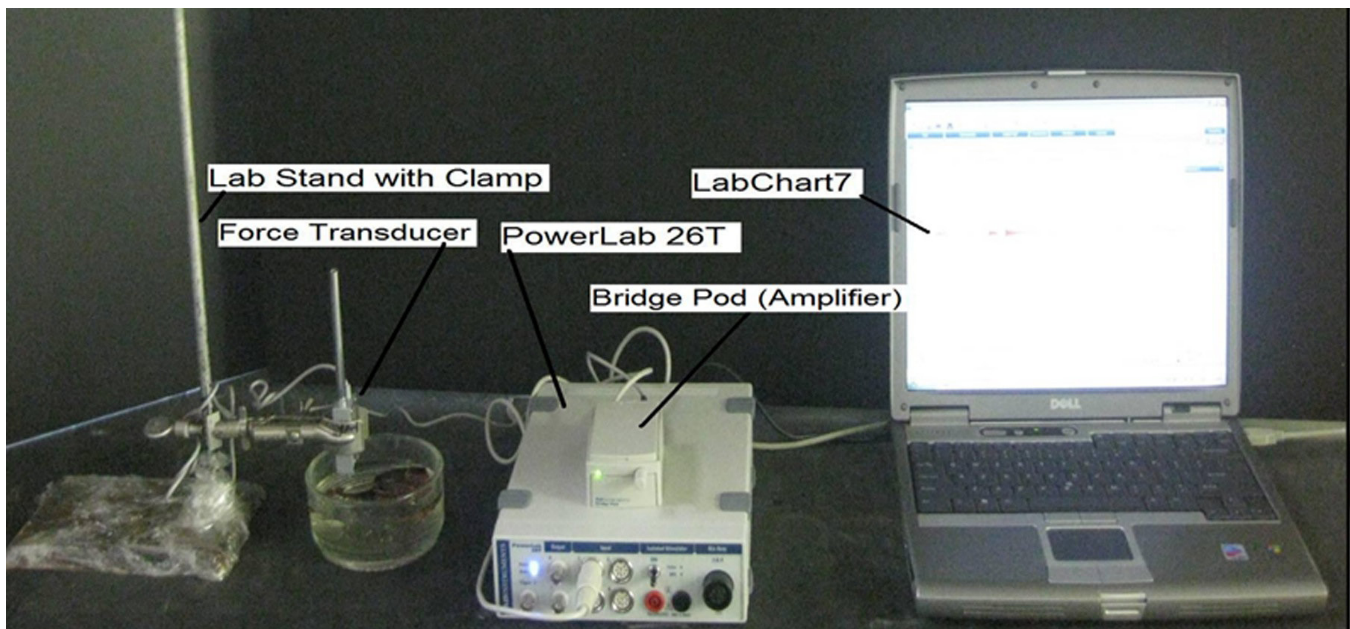

Figure 9. Setup with Powerlab.

4. Open LabChart7, by clicking on the LabChart7 icon on the desktop.
    - The LabChart Welcome Center box will pop open. Close it.
    - Click on Setup.
    - Click on channel settings. Change the number of channels to 1 (bottom left of box); push OK.
    - At the top left of the chart set the cycles per second to about 2k. Set the volts (y-axis) to about 500 or 200 mV.
    - Click on Channel 1 on the right of the chart. Click on Input Amplifier. Ensure that the settings (single-ended, ac coupled, and invert (inverts the signal if needed), and anti-alias) are checked.
    - To begin recording press start.
5. Pick up the preparation and cut the hindgut at the juncture between the thorax and the abdomen. Then carefully cut around the telson part of the tail. Remove the hindgut from the dissected crayfish and place it into the Sylgard dish. There is a blood vessel that runs along the dorsal aspect of the hindgut. Carefully pull this away from the hindgut. Pour fresh crayfish saline onto the preparation.
6. Place the force transducer in the clamp near the dissected crayfish.
7. Hook the force transducer in the crayfish hindgut as shown in Fig. 10.
8. Wait until the hindgut starts to contract, and push start on the LabChart7.
9. Let the program run for about 20 seconds. Push "stop," and add a comment labeled,

   "Saline only." Add the test compounds of interest, and immediately push "start" on the LabChart7.
10. Let the program run for about 20 seconds. Push "stop" and add a comment directly on the chart file as to what substance was added and its concentration.
11. Record compound, concentration and amplitude of contractions in Table 3.

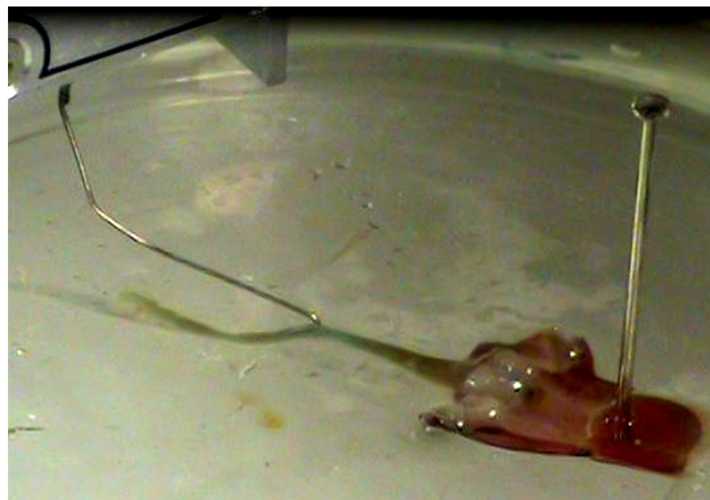

Figure 10. Isolated hindgut of the crayfish in saline attached to the hook of the force transducer.

Results

Table 1. Contractions in Saline of intact Crayfish Hindgut

Number of Contractions	Type of Contractions

Table 2. Contractions with exposure to compounds of intact Crayfish Hindgut

Compound tested	Concentration	Number of Contractions	Type of Contractions

Table 3. Contractions of fully dissected Crayfish Hindgut

Compound tested	Concentration	Number of Contractions	Amplitude
Saline			

Data Analysis

Part A: Graph the number of contractions from each solution (saline, serotonin, glutamate) vs. time. Explain any trends.

Part B: Graph the number of contractions from each solution (saline, serotonin, glutamate) vs. the amplitude of the contractions in mV. Explain any trends.

Measuring the rate and force of contractions

To index the rates of contractions one can measure the time from the beginning on one deflection to the beginning of the next or count the total deflections over a minute or two. The values can then be put in terms of contractions per min or per second depending on how rapid they are occurring.

To index the force of contractions a relative measure could be used to compare different conditions. On the saved file one can use the marker “M” and move to the baseline. Then use the cursor and move to the peak of the deflection and take note of the value listed on the top right of the screen as a delta value (the difference from M to the peak). Note the cursor needs to be kept stationary for the measure of the change. Then move the M to the next waveform of interest and repeat the measure.

Materials

Supplies are listed for groups of three to four students.

1. Heart Rate Response to Induced Environmental and Modulators in Freshwater Shrimp

Palaemonetes (Ghost Shrimp)
Small wooden rod or toothpick
Grooved Petri dish
Compound Light Microscope with external fiber optic microscope lights
2 Pins with Curled Ends
Paper towels
2 Beakers of distilled, aerated water
Beaker of ice water
Beaker of warm water
1 bath 10 µM 5-HT solution
1 bath 10 µM dopamine solution
Drop of SuperGlue (Maxi-Cure)
Drop of Quick Dry (Insta-Set)

2. Heart Rate in *Drosophila*

Drosophila melanogaster (3rd instar larva)
Slide
Cover slip
Tweezers or cotton swabs
Compound Light Microscope with external fiber optic microscope lights
Paper Towels
Beaker of distilled, aerated water
Beaker of ice water
Beaker of warm water
Food laced with 10 µM 5-HT Solution
Food laced with 10 µM Dopamine Solution
Drop of SuperGlue (Maxi-Cure) or double stick tape
Vaseline
Needle syringe

3. Physiological Experimentation with the Crayfish Hindgut

Procambarus clarkii (Crayfish 6-10 cm in length)
Bucket of ice
Crayfish saline
Dissection instruments (coarse and fine scissors, coarse and fine forceps)
Sylgard-bottomed Petri dish
Steel dissecting pins
Beakers (to hold chemical solutions)
10 µM 5-HT saline solution
10 µM dopamine saline solution
Pipets
Parafilm
Ring stand with clamp
Bridge pod (amplifier)
Force transducer
PowerLab
Computer with LabChart 7

Notes to the Instructor

Instructors will be able to view the detailed videos for each of the three labs on the University of Kentucky's Biology web page:
http://web.as.uky.edu/Biology/faculty/cooper/ABLE/LAB-GI.htm

Also some movies are available through JoVE, an online publication of experimental procedures (Cooper *et al*., 2009, 2011).

1. Heart Rate Response to Induced Environmental and Modulators in Freshwater Shrimp

If students are allergic to shrimp they should wear gloves before handling the ghost shrimp. There are two different techniques for making the grooved petri dish for viewing the shrimp heart. Fill the bottom of a small petri dish with Sylgard (see Notes to Instructor 3 for more information) and cut a groove for the wooden rod perpendicular to a groove to rest the shrimp and enough water to cover (See Fig. 10). The other option is to cut or burn two small notches in the wall of a small petri dish to rest the wooden rod and the shrimp will be in the water inside the dish. To find the correct location for gluing the rod hold the shrimp by the tail and it will bend creating a bump on the dorsal side of the organism. Glue should be applied on this bump to make sure that the rod does not cover the heart.

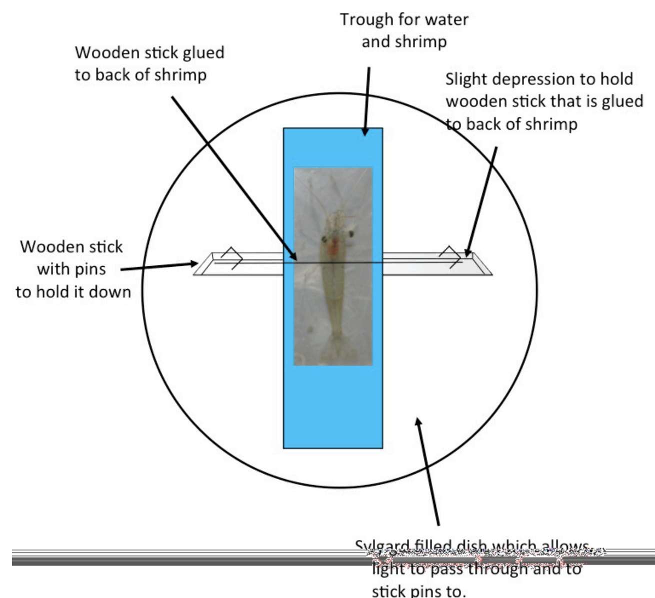

Figure 10. Grooved Petri Dish for viewing Ghost Shrimp.

This lab can use many different chemicals including GABA, glutamate, or nicotine. Students could try serial dilutions to see dose response curves. Be sure to have students use hot water last when performing this lab because if it is too hot the shrimp will die.

In our lab we use Maxi-cure glue by Bob Smith Industries purchased from Hobby Lobby. While we were in New Mexico at the ABLE conference we had difficulty finding glue that would dry quickly enough to hold the organisms to the rod. Be sure to try different types of glue on these animals before trying the experiment with your students.

2. Heart Rate in *Drosophila*

A good means of visualizing an intact beating larval heart is directly with a microscope (adjustable zoom 0.67 to 4.5; World Precision Instrument; Model 501379); however, the larva must remain still enough to count of the contractions. A 2X base objective and tube objective 0.5X is used to gain enough spatial resolution and magnification to cover a 1 cm by 0.5 cm rectangle. The ambient temperature is maintained at 20°C. You can use either the *Intact Larvae Protocol* or the Permanent Restraining Protocol.

Benefits of Intact Larvae Protocol

These approaches could be used to follow an individual larva over extended periods of time if care is used to avoid dehydration. This technique also allows video imaging within a single plane. White light is projected from the underside of the microscope stage with a mirror so that it can be moved accordingly for the best contrast of the heart or the two trachea which move while the heart contracts. These methods can also be used to assess pharmacological agents introduced in the diet or to examine various times in development in mutational lines or with induction of heat shock genes.

Benefits of Permanent Restraining Protocol

If one is not interested in freeing the larvae for experimentation one could use the permanent method of restraining the larva by gluing the animal to a glass slide. With use of super glue method, the animal can eat and even be covered in a moist solution while remaining adhered to the glass cover slip. Make sure students are careful with the glue and they do not to cover spiracles with glue or attach larva to forceps. Try different types of glue before conducting this experiment with your students because some glue will not dry fast enough to adhere the organism to the cover slip before it crawls away.

3. Physiological Experimentation with the Crayfish Hindgut

If students are allergic to shellfish they should wear gloves before handling the crayfish. Different solutions could be used such as serotonin (100 nM, 1 μM), glutamate (1 μM) and dopamine (1 μM) made in crayfish saline as starting substances to be tested. Saline is a modified Van Harreveld's solution (1936), which is made with

205 mM NaCl
5.3 mM KCl
13.5 mM $CaCl_2 \cdot 2H_2O$
2.45 mM $MgCl_2 \cdot 6H_2O$
5 mM HEPES

Adjust to pH 7.4.

To make the solution start with 900 mL of RO water and add
12.00 g of NaCl
0.400 g of KCl
1.980 g of $CaCl_2 \cdot 2H_2O$
0.500 g MgCl2 $\cdot 6H_2O$
50 mL of HEPES

Stir. Add enough RO water to make 1 L and buffer to pH 7.4.

Crayfish tissue is very sensitive; be sure to wash off the pH electrode before placing it in the saline. The saline should be kept cool, but not ice cold, during the experiment. To make the Sylgard dishes, use Dow Corning Sylfard 182 Silicone Encapsulant Kit. Be sure to follow the directions carefully, using one part curing agent to ten parts Sylgard and making the dishes in a hood. They will need to cure in an oven for at least four hours.

Using a force transducer is not mandatory and students can watch the contractions with the gut attached to a hook, pinned down, or it will even crawl along a wooden rod. In our lab we use AD Instruments PowerLab, Force Transducers, software which can be purchased at **http://www.adinstruments.com/products/**.

Acknowledgements

Other contributors to these laboratory exercises were undergraduates from the University of Kentucky: Ann S. Cooper, Bonnie Leksrisawat, Jonathan M. Martin, and Martha M. Robinson. For further readings see Cooper *et al*. (2009, 2011). This laboratory exercise was developed in part due to funding through The Kentucky Non-Public School Commission, Inc. Grant and Department of Biology, University of Kentucky.

Literature Cited

Alexandrowicz, J. S. 1909. Zur Kenntnis des sympathischen Nervensystems der Crustaceae. *Jenaischen Zeitschrift für Naturwissenschaft.* 45: 395-444.

Cooper, A.S. and R.L. Cooper. 2004 Growth of troglobitic (*Orconectes australis packardi)* and epigean (*Oroconectes juvenilis*) species of crayfish maintained in

laboratory conditions. *Journal of Kentucky Academy of Sciences* 65(2):108-115.

Cooper, A.S., B. Leksrisawat, A.B. Gilberts, A.J Mercier, and R.L Cooper, 2011. Physiological experimentations with the crayfish hindgut. J*ournal of Visualized Experiments (JoVE)*. Jove 47: **http://www.jove.com/details.php?id=2324** doi: 10.3791/2324.

Cooper, A.S., K.E.Rymond, M.A. Ward, E.L Bocook, and R.L. Cooper, 2009 Monitoring heart function in larval *Drosophila melanogaster* for physiological studies. *Journal of Visualized Experiments (JoVE)*. 32: **http://www.jove.com/index/details.stp?id=1596**.

Dasari, S. & Cooper, R.L. 2006. Direct influence of serotonin on the larval heart of *Drosophila melanogaster*. *Journal of Comparative Physiology B* 176: 349–357

Dircksen, H., Burdzik, S., Sauter, A. & Keller, R. 2000. Two orcokinins and the novel octapeptide orcomyotropin in the hindgut of the crayfish *Orconectes limosus*: identified myostimulatory neuropeptides originating together in neurons of the terminal abdominal ganglion. *Journal of Experimental Biology* 203:2807-2818.

Florey, E. 1954. Uber die wirkung von acetylcholin, adrenalin, nor-adrenalin, faktor I und anderen substanzen auf den isolierten enddarm des flusskrebses *Cambarus clarkii* Girard. *Zeitschrift für vergleichende Physiologie* 36:1-8.

Florey, E. 1961. A new test preparation for bio-assay of Factor I and gamma-aminobutyric acid. *Journal of Physiology* 156: 1-7.

Johnson E., J. Ringo, and H. Dowse. 1997. Modulation of *Drosophila* heartbeat by neurotransmitters. *Journal Comparative Physiology B* 167: 89-97.

Johnson E., J. Ringo, and H. Dowse. 2000. Native and heterologous neuropeptides are cardioactive in *Drosophila melanogaster*. *Journal Insect Physiology* 1:1229-1236.

Kondoh, Y. and M. Hisada. 1986. Neuroanatomy of the terminal (sixth abdominal) ganglion of the crayfish, P*rocambarus clarkii* (Girard). C*ell and Tissue Research* 243:273-288.

McRae, T. 1999. Chemical removal of nitrite and chlorinating agents from municipal water supplies used for crayfish and aquarium fin fish culture. *Freshwater Crayfish* 12:727-732.

Mercier, A.J. and J. Lee. 2002. Differential effects of neuropeptides on circular and longitudinal muscles of the crayfish hindgut. *Peptides* 23:1751-1757.

Mercier, A.J., Orchard, I. and Schmoeckel, A. 1991a. Catecholaminergic neurons supplying the hindgut of the crayfish, *Procambarus clarkii. Canadian Journal of Zoology* 69:2778-2785.

Nichols R., Kaminski S., Walling E., and Zornik E. 1999. Regulating the activity of a cardioacceleratory peptide. *Peptides* 20:1153-1158.

Wales, W. 1982. Control of mouthparts and gut. In: *The Biology of Crustacea*, Vol. 4; D.C. Sandeman and H.L. Atwood, eds.; Academic Press Inc., London; pp. 165-191.

Winlow, W. and Laverack, M.S. 1972a. The control of hindgut motility in the lobster *Homarus gammarus* (L.). Analysis of hindgut movements and receptor activity. *Marine Behavior and Physiology* 1: 1-28.

Winlow,W. and Laverack, M.S. 1972b. The control of hindgut motility in the lobster *Homarus gammarus* (L.). Motor output. *Marine Behavior and Physiology* 1:29-47

Winlow, W. & Laverack, M.S. 1972c. The control of hindgut motility in the lobster *Homarus gammarus* (L.). 3. Structure of the sixth abdominal ganglion (6 A.G.) and associated ablation and microelectrode studies. *Marine Behavior and Physiology* 1:93-121.

Zornik E, Paisley K., & Nichols R. 1999. Neural transmitters and a peptide modulate *Drosophila* heart rate. *Peptides* 20:45-51

About the Authors

Rachel Holsinger is a high school Biology teacher at an independent school, Sayre School, in downtown Lexington, Kentucky where she teaches 9th grade Biology and 12th grade AP Biology. She received her B.S. in Biology from Dickinson College and is currently perusing a Masters in Biology from the University of Kentucky. Robin Cooper is an instructor of animal physiology at the University of Kentucky. He received a double major with a B.S. from Texas Tech in 1983 and a PhD in Physiology from Texas Tech Medical School in 1989. He has been at the University of Kentucky since 1996.

Appendices

Ordering

Carolina Biological Supply
#142504, Crayfish, Living, Large, Pack of 50. $140 USD

ADI materials and software
http://www.adinstruments.com/

- MLTF500/ST, Teaching Force Transducer that requires no Bridge Pod (0-500 g). $595 USD
- PTB154/S or PTB262 and PTB266, Software LabChart 7 - This software is included with the PowerLab systems

The PowerLab 15T is an integrated data recording unit featuring built-in amplifiers. With appropriate transducers and accessories the PowerLab 15T covers the broad experimental requirements in life science education. The prices are depending on the number ordered and institution. Must contact company for a quote.

Sigma-Aldrich

- #S1679, NaCl 1 kg. $68 USD
- #P1597, KCl 500 g $61 USD
- #C8106, $CaCl_2$ 500 g. $74 USD
- #M9272, Magnesium chloride hexahydrate 100 g. $31 USD
- #H3375, HEPES 100 g. $68 USD
- #H9523, Serotonin hydrochloride 25 mg. $29 USD
- #H8502, Dopamine hydrochloride 10 g. $48 USD

Dow Corning

182 SIL ELAST KIT, Dow Corning Sylgard182, 3.9 kg, $352 USD

Preparation of Solutions

Crayfish saline (modified Van Harreveld's solution)

(in mM: 205 NaCl; 5.3 KCl; 13.5 $CaCl_2 \cdot 2H_2O$; 2.45 $MgCl_2 \cdot 6H_2O$; 5 HEPES adjusted to pH 7.4). For 1 liter

12 g NaCl
0.4 g KCl
1.98 g $CaCl_2 \cdot 2H_2O$
0.5 g $MgCl_2 \cdot 6H_2O$
Add 50 ml of HEPES

Bring to 1 L with double distilled water, Check pH and adjust accordingly to pH 7.4

HEPES
Make up 100 mL of 0.1 M
((238.3 grams)/mole)((0.1 mole)/(1 liter))
(0.1liter)=2.383 grams
then pH to 7.4 with NaOH (1 M) or HCl (1M)

Serotonin
For 10 µM:
((212.68 grams)/mole)((0.00001 mole)/(1 liter))
(0.5liter)=1.0 milligrams

Dopamine
For 10 µM:
((189.64 grams)/mole)((0.00001 mole)/(1 liter))
(0.5 liter)=0.9 milligrams

Maintenance of Animals

Food for flies

We suggest using the Standard medium in use at Bloomington *Drosophila* stock center.
http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm

Their recipe makes about 42.5 liters of food, enough to fill about 4,250 vials with 10 ml of food each. Even when hot the food is thick - be sure your pump can handle it before you make a large batch.

39 L water
675 g yeast
390 g soy flour
2,850 g yellow cornmeal
1,800 g light malt extract (dehydrated) OPTIONAL
225 g agar*
3 L light corn syrup
188 mL propionic acid

*adjust agar concentration according to the gel strength of your agar

Cooking instructions

Bad food is often simply dry food. Seemingly small changes in the cooking process can make a significant difference in the quality of the food if those changes result in increased water loss. If you cook small batches in a large kettle, don't have a covered kettle, need to cook the food longer, or are in a particularly dry environment you might need to increase the proportion of water in this recipe.

1. Measure malt if using it, and corn syrup (separately). Set aside.
2. Hydrate yeast and soy flour with 3 L of water, more if necessary (use hand mixer, or press out larger lumps with fingers or the back of a large spoon). Set aside.
3. Measure cornmeal and agar into a large container, add 5 L of the water, mix until lump-free, using more water if necessary to produce a smooth slurry.
4. Set aside about 4 L of the water.
5. Bring the remaining water to a full boil.
6. Stir cornmeal and agar mixture again (add some of the set-aside water if needed) and pour the smooth slurry into the rapidly boiling water (both points are important to avoid lumps that will clog the pump). Stir the pot at the same time if possible (we have an electric stirrer). If the cornmeal-agar mixture begins to form lumps as it is poured, add more of the set-aside water and mix well before pouring the rest of it into the boiling mixture. Use any remaining set-aside water to rinse the cornmeal-agar dregs into the pot.
7. Stir in malt if using it. Lumps will form at first, but unlike cornmeal lumps, these will cook out.
8. Stir in yeast-soy mixture and then the corn syrup.
9. When mixture has just begun to bubble at the edges start timing and cook 10 minutes, stirring frequently.
10. Turn off heat source.
11. Stir in propionic acid. If you have time, cool to 70°C before adding the acid.
12. Dispense the medium.
13. Cool before plugging if time allows.

Crayfish and Ghost Shrimp

Transfer crayfish from shipping box to individual aquaria 33 x 28 x 23 cm with a water depth of 10-15 cm (we use plastic shoe boxes). Be sure the lid is snapped on tightly because the crayfish will crawl out. The ghost shrimp, obtained from your local pet store, can be held in one aquarium. Both crayfish and shrimp can be feed commercial fish food pellets (Aquadine), which is marketed as “shrimp and plankton sticks: sinking mini sticks.” Fragments of cleaned chicken eggshell also should be placed in the containers as a source of calcium. The chloramines of local water must be removed by carbon-based filters for the aquaria water. Bacteria and algae will help to detoxify any ammonium ions, and the water should be aerated for several days before using (Cooper and Cooper 2004; McRae 1999).

Mission, Review Process & Disclaimer

The Association for Biology Laboratory Education (ABLE) was founded in 1979 to promote information exchange among university and college educators actively concerned with biology learning and teaching in a laboratory setting. The focus of ABLE is to improve the undergraduate biology laboratory experience by promoting the development and dissemination of interesting, innovative, and reliable laboratory exercises. For more information about ABLE, please visit **http://www.ableweb.org/**

Papers published in *Tested Studies for Laboratory Teaching: Proceedings of the Conference of the Association for Biology Laboratory Education* are evaluated and selected by a committee prior to presentation at the conference, peer-reviewed by participants at the conference, and edited by members of the ABLE Editorial Board.

Citing This Article

Holsinger, R.C., and Cooper, R.L. 2012. Effect of Environment and Modulators on Hindgut and Heart Function in Invertebrates: Crustaceans and *Drosophila*. *Tested Studies for Laboratory Teaching,* Volume 33 (K. McMahon, Editor). Proceedings of the 33rd Conference of the Association for Biology Laboratory Education (ABLE), 390 pages.
http://www.ableweb.org/volumes/vol-33/?art=7

Compilation © 2012 by the Association for Biology Laboratory Education, ISBN 1-890444-15-4. All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording, or otherwise, without the prior written permission of the copyright owner.

ABLE strongly encourages individuals to use the exercises in this proceedings volume in their teaching program. If this exercise is used solely at one’s own institution with no intent for profit, it is excluded from the preceding copyright restriction, unless otherwise noted on the copyright notice of the individual chapter in this volume. Proper credit to this publication must be included in your laboratory outline for each use; a sample citation is given above.