

# Blackworms, Blood Vessel Pulsations & Drug Effects

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As biology teachers we want our students to understand and, if possible, witness firsthand the interactions and internal functions of organ systems in animals. As part of this experience, we also want them to appreciate the impact that biologically active substances, such as pharmacological agents (drugs) and toxins, have on these functions. By doing this, we hope they will gain insight and respect for the potent biological effects that such substances have in all animals, including humans.

Classroom activities involving circulatory functions are especially useful because student interest is readily captured by the rhythmic and vivid dynamics of blood pulsations in animals. Feasibility for such laboratory studies may be limited, however, by factors such as:

1. Need to forcefully restrain and/or surgically invade animals in order to access the circulatory system
2. Time, special procedures, and/or apparatus required to measure pulsation rates under normal and treatment conditions
3. Variability in results due to physiological stress related to handling and treatment of animals.

Our intent in this article is to introduce the freshwater oligochaete worm, *Lumbriculus variegatus* (common name: blackworms), as an organism that is well suited for classroom study of its closed circulatory system. We describe a set of simple, fast, noninvasive and inexpensive methods for observing

pulsations of the worm's dorsal blood vessel under baseline conditions and following treatment with two well-known pharmacological agents, nicotine and caffeine. These methods should be widely adaptable for general biology and physiology courses at both high school and college levels.

Earlier versions of this exercise were field-tested during the summer of 1996 by high school biology teachers working at Iowa Lakeside Laboratory (West Lake Okoboji, IA) and at the Woodrow Wilson National Leadership Program for Teachers (Princeton, NJ). Much of the inspiration and initiative for this article arises from these teachers' success, enthusiasm and support in working with this organism.

## Background

Oligochaete worms have a closed circulatory system with bright red blood that resembles vertebrate blood. However, in these worms, the hemoglobin-like respiratory pigment, called erythrocrucorin, is dissolved in plasma rather than in red blood cells (Jamieson 1981). Two major blood vessels are

present along the body of most oligochaetes. These are the dorsal and ventral blood vessels. In addition to these, there are lateral commissural vessels that connect the dorsal and ventral vessels in anterior segments.

In oligochaetes, blood is propelled forward by waves of muscle constriction that begin near the posterior end of the dorsal vessel. Usually, numerous waves are seen at different points of progress along the vessel. In the earthworm, *Lumbricus terrestris*, blood flow is augmented by pulsations in muscular lateral commissural vessels (so-called "hearts") found in segments 7-11. In the freshwater worm, *Lumbriculus variegatus*, there is one pair of lateral commissural vessels in each anterior segment as far back as segment 18 (Stephenson 1930). In addition, the dorsal blood vessel in most body segments gives rise to a pair of branched lateral vessels (Figure 1). These vessels are unusual in two respects: they end blindly rather than connect with the ventral vessel and they are contractile. Contractions in these special vessels are synchronized with those of the dorsal blood vessel,

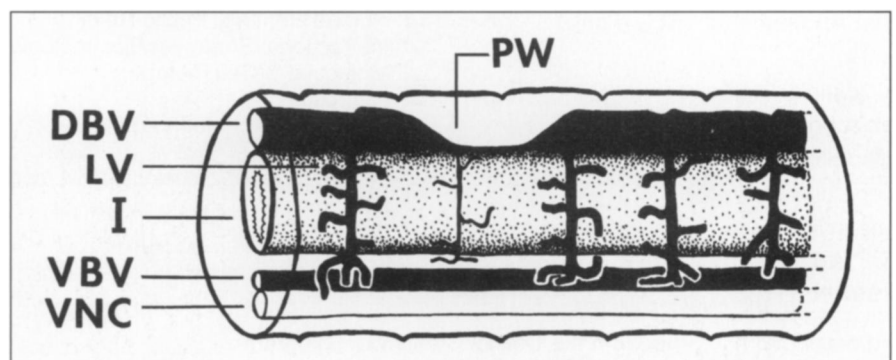


Figure 1. Side view of several body segments showing major blood vessels and internal anatomy of *Lumbriculus*. A pulsation wave (PW) of the dorsal blood vessel (DBV) is shown. Note that contraction of the dorsal vessel is synchronized with contraction of the blind lateral vessels (LV). VNC, ventral nerve cord; VBV, ventral blood vessel; I, intestine.

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presumably leading to more efficient pumping of blood throughout the body.

In earthworms, pulsation waves in the dorsal blood vessel pass the entire length of the worm but, occasionally, a wave fails to progress beyond the middle part of the body (Carlson 1907). This means that pulsation rates may be somewhat higher in segments near the tail end than in segments near the head. The reported basal pulsation rate in the posterior segments of *Lumbricus terrestris* is  $11 \pm 2.1$  SD beats per minute (Fournier & Pax 1971).

Regional differences in pulsation rates are more exaggerated in the blackworm, *Lumbriculus*. The basal rate toward the tail is much greater than in middle or head regions, indicating that many pulsation waves starting at the tail never succeed in traveling the whole length of the worm's dorsal vessel. Our data from laboratory cultured worms indicate that the basal pulsation rate near the tail end ranges from 24 to 32 beats/minute while the basal rate in the middle of the worm ranges from 8 to 12 beats/minute (room temperature: 23° C).

As in many animals, pulsation rates in oligochaete worms are specifically affected by naturally occurring neurotransmitters (e.g. acetylcholine) and related pharmacological agents. In the earthworm, *Lumbricus terrestris*, acetylcholine significantly accelerates pulsation rate in the dorsal vessel. Nicotine, a drug that mimics acetylcholine in many situations, similarly accelerates pulsation rates of the dorsal vessel; at high concentrations, it may arrest pulsations altogether (Laverack 1963). Such effects are striking and reliable, but not readily observed without using restraint and surgical invasion. This makes it difficult to use earthworms as physiological models for student laboratories.

Given the actions of nicotine in earthworms, we asked whether similar effects may occur in smaller, freshwater species, such as blackworms, by merely immersing worms in nicotine solutions. Since the body wall is transparent, blood pulsations may be viewed directly and without surgical invasion, before and after short-term drug treatment.

In this report we introduce laboratory methods for rapid and reliable quantitation of basal pulsation rates in blackworms, *Lumbriculus variegatus*. We also describe simple treatment protocols for studying short-term effects of nicotine and caffeine on pulsation rates.

## Materials Required

- Living *Lumbriculus variegatus* (common name: blackworms). Commercial sources include:  
[www.holidayjunction.com/arol/](http://www.holidayjunction.com/arol/)  
[www.novalek.com/korgde1.htm](http://www.novalek.com/korgde1.htm)  
[www.carolina.com](http://www.carolina.com) (Drewes 1996).
- Dissecting microscope and light source (for use with paraffin block viewing chamber)
- Compound microscope (for use with Parafilm slide viewing chamber)
- Stopwatch timer (or a watch/clock with a second hand)
- Small glass or plastic containers for immersion of individual worms; about 20 ml volume per container; separate container for each worm (e.g. disposable plastic sauce cups from fast-food restaurants)
- Numerous 500-ml beakers for stock solutions and serial dilutions
- Disposable plastic pipets (or eyedroppers)
- Spring water (non-carbonated; most commercial brands will work)
- Nicotine (Sigma Chemical Co., St. Louis, MO)
- Caffeine (Sigma Chemical Co., St. Louis, MO or Carolina Biological, Burlington, NC)
- Parafilm
- Glass slides (25 mm × 75 mm × 1 mm)
- Hot plate (with low heat setting)
- Single-edge razor blade
- Jumbo paper clip (4.6-cm length; 1-mm diameter wire)
- Paraffin blocks (12.5 cm × 6.5 cm × 1.5 cm; five cakes per 16 oz box)
- Metal ruler
- Fine forceps
- Wooden applicator stick
- Straight human hair (2-cm long)
- Paper labelling tape
- A large finger bowl (for temporary storage of worms isolated from the colony)
- Cotton thread (preferably black)

## Viewing Chambers

We designed two different, easy-to-make chambers for viewing dorsal blood vessel pulsations in *Lumbriculus*. Key features of chambers are: (1) a shallow and narrow trough with dimensions slightly wider and longer than a single worm, so that the worm fits snugly into the chamber; and (2) a chamber with hydrophobic walls and a hydrophilic floor.

The hydrophobic walls insure that the small volume of water, used to bathe the worm, coalesces within the trough as an elongate column bounded

by its own surface tension. Thus, the worm tends to stay entrapped by the water surface tension. Worms also tend to stay in contact with the solid wall and floor surfaces of the chamber (thigmotaxis). This tends to minimize crawling activity and optimize viewing and counting of pulsations. We highly recommend taking time to make all viewing chambers prior to scheduled usage in class. The chambers can be rinsed and reused many times.

## Parafilm Chamber

Cut off six rectangular pieces of Parafilm (6.5 cm × 1.5 cm) and remove the backing from each, making sure that the sheets are not wrinkled or stretched. Neatly stack the Parafilm sheets on top of one another and place this stack on a glass slide. Then, using a low-heat setting on the hot plate, heat the underside of the slide until the Parafilm just begins to soften and becomes slightly clear. [Caution: Do not overheat!]

Remove the slide from heat and quickly place a sheet of Parafilm backing paper on the Parafilm stack. Using your fingertips, apply gentle pressure to the backing and work any air bubbles that are under the Parafilm toward the edges of the slide. This step ensures bonding of the Parafilm sheets with each other and with the slide. If necessary, reheat briefly and repeat steps to ensure bonding.

Next, using a new, single-edge razor blade and a metal ruler, neatly trim excess Parafilm from around the edges of the slide. Remove trimmed edges with a fine forceps. Then, using a sharp corner of the blade, cut a rectangular outline (1 mm × 40 mm) in the center of the Parafilm. Make sure that the razor blade slices through all six layers of Parafilm and that the blade is advanced a couple millimeters beyond each corner of the rectangle. Then, use the forceps to remove the center piece of Parafilm, thus forming the trough.

A chamber with dimensions shown in Figure 2 holds one worm and about 40 µl of water. Trough size can vary slightly, but make sure it is not too wide or long. When worms have too much space to explore, the observer has less frequent opportunity for viewing and counting pulsations.

## Paraffin Block Chamber

This is an easy-to-make and economical alternative to the Parafilm slide chamber. Each chamber is made from a block of household paraffin wax, normally used for canning or candle making. Open up a jumbo paper clip

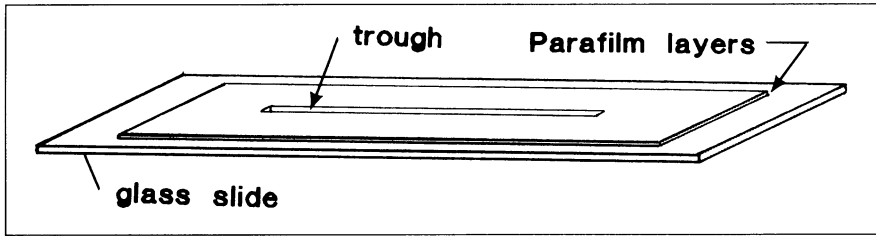


Figure 2. Parafilm slide viewing chamber. Six layers of Parafilm are bonded by heat to a glass slide and a trough is made by cutting the Parafilm with a razor blade. Trough dimensions are: 1-mm width, 1-mm depth, and 4-cm length.

and position one end of it perpendicular to the paraffin block at the starting point shown in Figure 3. Then, press the tip of the wire gently into the wax and begin pulling it smoothly and repeatedly along the edge of a ruler for a length of 4 cm. The idea is to make a straight, shallow trough in the paraffin with final dimensions about 1-mm deep, 1-mm wide, and 4-cm long. Try to keep the depth of the trough uniform from one end to the other and carefully clean away excess wax scrapings.

Next, cut a length of cotton thread (preferably black) slightly shorter than the trough length. Moisten the thread with spring water and place it in the trough. Then, hold a glass slide perpendicular to the block and insert the short edge of the slide into the trough. Press the slide against the floor of the trough so that the thread is imbedded partially but not completely into the paraffin floor. The moist thread will create a hydrophilic surface for water in the trough and a rough surface for worms to crawl upon. Each trough holds one worm and about 50  $\mu$ l of water. Two or three parallel troughs can be made in the same paraffin block, but troughs should be at least 1-cm apart.

### Stock Solutions

1. Prepare a 10 mM stock solution of nicotine (mol wt: 162.2; density 1.0097)

using 0.8 ml nicotine in 500 ml of spring water.

2. Use appropriate amounts of the 10 mM nicotine stock solution to make 500 ml quantities of each of the following dilutions: 1, 0.5, 0.25 and 0.01 mM. Label containers.

3. Prepare a 10 mM stock solution of caffeine (mol wt: 194.2) using 0.39 g caffeine in 200 ml of spring water.

4. Use appropriate amounts of the 10 mM caffeine stock solution to make the 500 ml quantities of each of the following dilutions: 5, 3, 1, 0.1 and 0.05 mM. Label containers.

5. We recommend using solutions within 24 hours after preparation.

### Experimental Design & Lab Groups

Divide the class into groups of three students. Designate one person as pulsation observer, another as timer/calculator, and a third as data recorder. For groups of two students, one person is the observer and the other does both timing and data recording.

For each 45 to 60 minute block of class time, each group should have time to gather complete data sets (i.e. pulsation rates before and after treatment) from about three worms. For convenience, all three worms may be from the same treatment group. A recommended time line for one

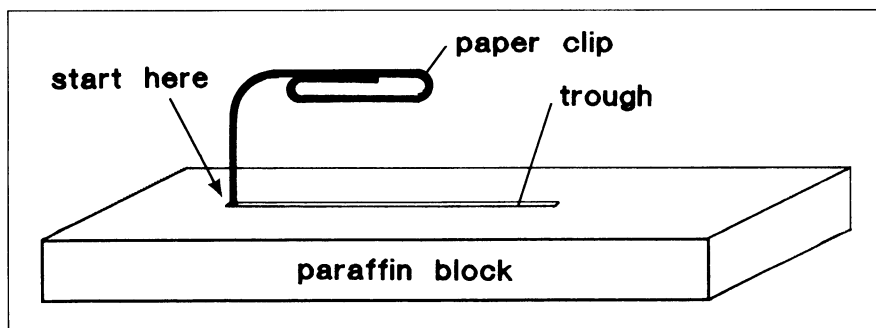


Figure 3. Paraffin block viewing chamber. A trough is made using the end of a paper clip to chisel a groove 1-mm wide, 1-mm deep, and 4-cm long in the wax.

group's activities over a 45-minute interval is shown in Figure 4.

With a class of 21 to 24 students, seven or eight groups may be formed, with each being assigned to one nicotine or caffeine concentration, or to zero concentration (control). Then, class data are pooled to study overall trends.

For nicotine, we suggest using at least these three concentrations: 0.05, 0.25 and 1.0 mM. Similarly, for caffeine, we suggest at least three concentrations: 1, 3, 10 mM. In addition, one or two student groups should be assigned as controls (zero drug concentration), in which all protocols are identical to the drug-treated worms, except that the "treatment solution" is pure spring water.

### Procedures

Because *Lumbriculus* is a small, soft-bodied invertebrate with a relatively thin cuticle and epidermis, many pharmacological agents penetrate the skin and rapidly cause observable effects. To minimize variability and enhance clarity of results, we emphasize the following points related to chemical treatment and measurement of effects:

1. *Selection and isolation.* We prefer to use worms that are uniform with respect to overall body size and segmental dimensions. We avoid using worms with recently regenerated (immature and unpigmented) tail segments. We also recommend isolating worms overnight in a separate container (finger bowl) of clear spring water; this helps to clear the digestive tract.

2. *Transfer and handling.* Using a large-bore pipet, remove a worm from the finger bowl along with a little water. Deposit the worm in an observation chamber (either the Parafilm slide or paraffin block chamber). Use a pipet to carefully remove excess water; the water level in the trough should be about even with the top surface of the Parafilm or paraffin block. Carefully remove any misplaced water with the corner of a paper towel or tissue. A cover slip is not needed or recommended. Let the worm crawl around for about 1 to 2 minutes to adjust to confinement in the trough.

3. *Viewing.* Place the Parafilm slide chamber under the lowest power of a light microscope. Observations should be possible with a minimum amount of transmitted light. For the paraffin block chamber, use the lowest power of a dissecting microscope.

4. *Worm position.* You may need to coax the worm to crawl into a position that allows easier viewing of the dorsal blood vessel. Crawling movements (forward or backward) are readily initiated by very lightly stroking the worm's tail or head with a hand-held flexible probe that will not damage worms. We highly recommend using the tip of a straight human hair to touch the worm and evoke crawling movements. The hair is attached by tape to an applicator stick, with about 1 cm of the hair extending beyond the tip of the stick.

5. *Pulsation rates before treatment.* Obtain a basal pulsation rate from one specific body region, such as a mid-body location. Although you will see contractions of the dorsal vessel at multiple locations along the worm, concentrate your viewing on a single selected segment and count the number of pulsations that move through that particular segment over a 30- or 60-second interval. A stopwatch, or a watch with a second hand, can be used for measuring the elapsed time. A count of 6 pulsations in a 30-second time interval, for example, is a pulsation rate of 12 beats/minute. Obtain an average pulsation rate for each worm based on the mean of two to four separate rate counts.

6. *Transfer to treatment solution.* After obtaining a basal rate, use the pipet to add extra spring water to the trough, then remove the worm with the pipet and transfer it to a small container (20 ml) of treatment solution. The treatment time may be varied, but we recommend 15 minutes to observe full effects.

7. *Pulsation rates after treatment.* After treatment, rinse the worm briefly in spring water; this is important to prevent contamination of the viewing chamber. Place the worm back in the viewing chamber and measure pulsation rates as before. Make sure that measurements are made from the same body location used for pretreatment measures.

8. *Control groups.* Drug effects in treated worms must be compared to "effects" in a control group. Pulsation rates in the control group are determined initially (as in treatment groups) and then again after a 15-minute "treatment" during which worms are immersed in pure spring water.

9. *Comparisons.* Compute and compile mean pulsation rates, before and after treatment, for each worm, as shown in Table 1. Compute overall means, before and after treatment, for each group. Then, calculate the *change in pulsation rate* (rate after treatment

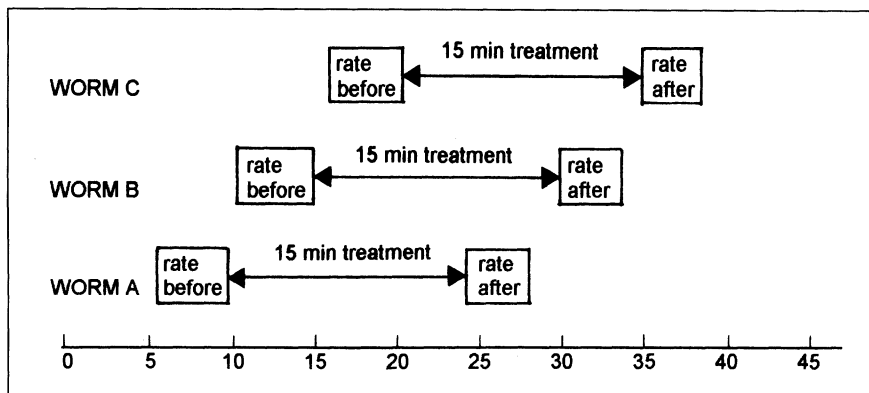


Figure 4. Suggested time line of activities for one student group over a 45-minute interval.

minus rate before treatment) for each group. Graph these changes as a function of concentration to see general trends.

If desired, a variety of statistical applications may be applied to the pooled data; however, the "n" value

(number of replicates) for each group should be at least five worms. Statistical significance between "before" and "after" values within a given group may be determined using a "paired difference" *t*-test.

Table 1. Format of data sheet and sample data for one worm.

Student's name: \_\_\_\_\_ Date: \_\_\_\_\_  
 Compound used: \_\_\_\_\_ Concentration: \_\_\_\_\_ mM  
 Data sheet

	Rate Before Treatment (beats/min)	Rate After Treatment (beats/min)	Change in pulse rate (beats/min)
Worm A rate 1 rate 2 rate 3			
mean A			
Worm B rate 1 rate 2 rate 3			
mean B			
Worm C rate 1 rate 2 rate 3			
mean C			
MEAN			

Sample Data for nicotine treatment (0.05 mM)

	Rate Before Treatment (beats/min)	Rate After Treatment (beats/min)	Change in pulse rate (beats/min)
Worm A rate 1 rate 2 rate 3	12 10 12	19 22 20	
mean A	11.3	20.3	9.0

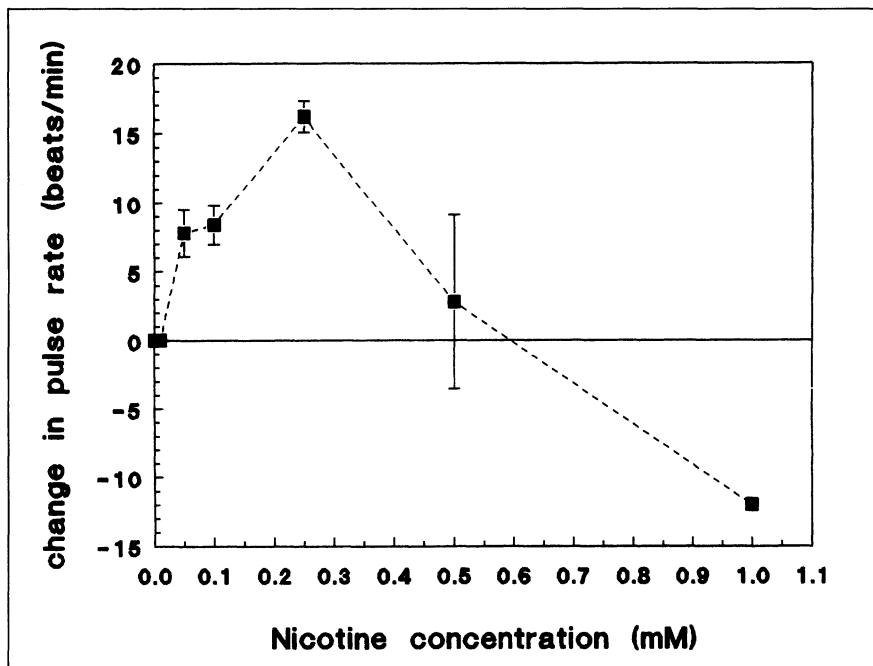


Figure 5. Dose-response graph showing effects of different nicotine concentrations on pulsation rates in *Lumbriculus*. Each point represents the mean change in rate for 10 worms (four measures of rate per worm). Vertical bars indicate  $\pm 1$  S.E.M. The horizontal line at 0 indicates the average basal pulsation rate ( $\approx 12$  beats/min). The maximal increases occurred at 0.25 mM nicotine. At 1.0 mM, pulsations were completely abolished (i.e. pulsation rate decreased from 12 to 0 beats/min).

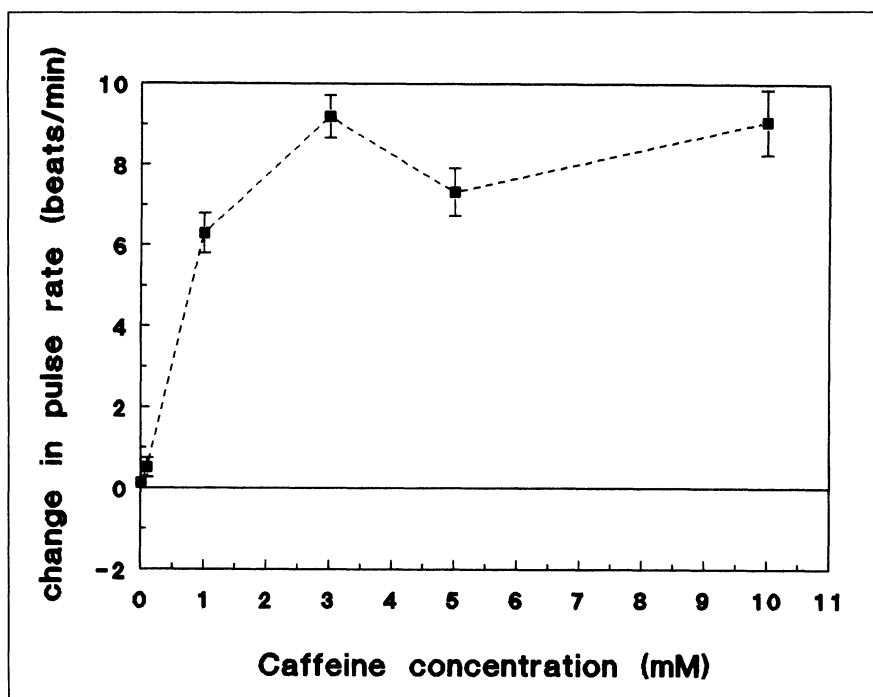


Figure 6. Dose-response graph showing effects of different caffeine concentrations on pulsation rate in *Lumbriculus*. Each point represents the mean change in rate for 10 worms (four measures of rate per worm). Vertical bars indicate  $\pm 1$  S.E.M. The horizontal line at 0 indicates the average basal pulsation rate ( $\approx 12$  beats/min).

### Sample Results: Nicotine

Figure 5 shows typical changes in pulsation rate of the dorsal blood ves-

sel after a 15-minute treatment with nicotine. All data were obtained from the middle part of the body. In the control group and in the lowest nico-

tine concentration (0.01 mM), we found no significant changes in the pulsation rate. Basal pulsation rates in these two groups were approximately 12 beats/minute. However, at the next highest concentration, 0.05 mM, the pulsation rate increased sharply (about 70% increase above basal rates). This indicates that the "threshold concentration" was between 0.01 and 0.05 mM. Maximum effects may be expected at 0.25 mM, at which the rate increased to almost 150% above basal level. However, the pattern of nicotine effects changed dramatically at concentrations  $\geq 0.5$  mM. At this concentration, pulsations were completely blocked (rate = 0 beats/min) in 6 of 10 worms. In the other four worms, pulsation rates were very high. Thus, the net rate change for this group was near zero, but variability of effects was very large. Finally, at the highest concentration (1 mM), pulsations were absent in all 10 worms (rate = 0 beats/min).

Besides effects on pulsation rates, other effects of nicotine occurred. At all concentrations above 0.05 mM, worms were paralyzed and incapable of crawling in response to touch stimulation. Interestingly, all effects of nicotine are sublethal and reversible, provided that worms are allowed to recover in spring water after treatment.

In some biological systems (such as vertebrate skeletal muscle), nicotine is known to mimic the effect of the natural excitatory neurotransmitter, acetylcholine, by acting on acetylcholine receptors (nicotinic type). Studies in earthworms have shown that both acetylcholine and nicotine accelerate the pulsation rate of dorsal blood vessels and, at high concentration, completely arrest pulsations (Laverack 1963). Similar nicotine effects are shown here using the dorsal blood vessel in *Lumbriculus variegatus*. These results support the idea that there is a cholinergic excitatory control of the dorsal blood vessel pulsations in oligochaetes. The paralysis of worms that occurs during nicotine treatment is also expected, since excitatory synaptic transmission at the oligochaete neuromuscular junction is probably cholinergic (Jamieson 1981).

### Sample Results: Caffeine

Figure 6 shows typical changes in mid-body pulsation rates in worms treated for 15 minutes with various concentrations of caffeine. The threshold concentration is between 0.05 and 1.0 mM, while a 3-mM concentration causes a maximum increase in pulsa-

tion rate, about 70% greater than the basal rate. Unlike the effects of nicotine, there is no blocking of pulsations at higher caffeine concentrations. It is not clear whether the excitatory action of caffeine is the result of a direct or indirect effect.

### Alternative Experiment

As an alternative to treatment with pure nicotine, water extracts from a tobacco product, such as cigarette or chewing tobacco, may be used. These extracts also have potent excitatory effects on pulsation rates.

As one possibility, we suggest placing the tobacco contents of one cigarette into 50 ml of warm spring water (about 60° C). Stir this continuously for 15 minutes. Then, filter the solution using standard filter paper and allow it to cool. Dilute this solution to 50%, giving a total of 100 ml of stock solution. The minimum amount of stock solution needed for treatment of each worm is about 3 ml. Be sure that the treatment container is sufficiently small so that worms are totally immersed in the solution.

### Discussion

Few animals are suitable for studying effects of pharmacological agents on circulatory functions in a general biology laboratory. Typical problems one encounters are time limitations for data collection, need for special apparatus, and variability due to animal stress and trauma.

Among vertebrates, frogs and turtles are often used for studying pharmacological effects on cardiac function *in vivo*. Usually, this requires anesthesia (or pithing) and surgical invasion. However, many concerns exist regarding economic, ecological and ethical aspects of using these animals and procedures in general biology laboratories.

Among invertebrates, *Daphnia* has been commonly used for observing heart beats *in vivo* and studying effects of temperature and drug exposure. However, *Daphnia* is not only difficult to immobilize, but the rapidity and variability in its heart rate make it difficult for students to obtain reliable and meaningful quantitative data. Further, *Daphnia* is not easily reared in the laboratory.

Blackworms, *Lumbriculus variegatus*, provide numerous advantages over *Daphnia*. First, they are easily raised

and handled in the laboratory. Second, it is easy to obtain pulsation rate measures, both before and after drug exposure. Third, basal pulsation rates are relatively slow, and exposure effects are robust and consistent from one worm to another.

However, as with any quantitative studies of biological rate functions, instructors will need to have a well planned strategy for students' data acquisition and analysis of results. Decisions about group size and the total number of worms monitored by each group are important considerations. Also, trade-offs may be necessary regarding the number of compounds used, the number of concentrations per compound, and the number of worms in experimental and control groups.

Besides convenience in studying circulatory physiology, blackworms offer unique possibilities for investigating other areas of organismal biology including regeneration of amputated head and tail segments, biomechanics and behavior of crawling and swimming, and neurophysiological recording from giant nerve fibers. We are currently developing and documenting each of these as routine exercises for general biology laboratories. The authors would be pleased to share ideas, information and experience regarding any of these.

### Acknowledgment

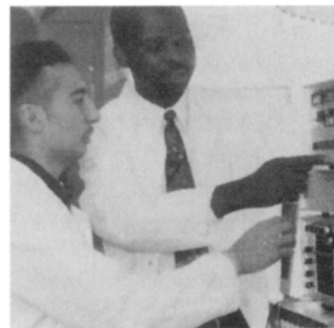
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