

## ARTICLE

## Recording from Snail Motor Nerves to Investigate Central Pattern Generation

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Feeding in pond snails has long been a model system for central pattern generation and its modulation. The pattern is generated by a small set of neurons in the buccal ganglia, which innervate the buccal mass, esophagus, and salivary glands. In this exercise, students observe feeding behavior and then record and quantify rhythmic motor activity and its response to feeding stimulants and neuromodulators. In a standard three-hour class period, students do a dissection, record from several nerves, and perform experimental manipulations such as adding feeding stimulants, serotonin,

or dopamine to the preparation. Depending on the course goals, data can be presented qualitatively or cyclic measurements and spike-rate analysis can be done. This exercise leads to discussion of neural circuitry and intrinsic properties that support pattern generation for rhythmic activities such as feeding, locomotion, and respiration.

*Key words:* feeding, CPG, neuromodulation, buccal, extracellular, serotonin, dopamine, pond snail, *Helisoma*, *Lymnaea*, *Planorbis*

Rhythmic motor patterns responsible for repetitive movements like flying, swimming, walking, and breathing are organized by networks of neurons called central pattern generators, or CPGs (see Marder and Bucher 2001; Marder et al. 2005; Luo, 2021). These networks are capable of organizing all the basic features of a motor pattern, such as periodicity and proper phasing of muscle activity, without detailed timing information from the higher brain networks. Furthermore, the CPG networks do not rely on sensory feedback to produce the basic rhythmic pattern.

The ability of CPGs to modify motor patterns, sometimes very quickly, as when switching from standing to walking to skipping, is an example of network plasticity caused by neuro-active compounds (neuromodulators) such as dopamine and serotonin. Neuromodulators change the strengths of synapses between network neurons and the excitability of individual neurons to determine which neurons are active and how strongly they are active, to produce or modify organized motor patterns (Harris-Warrick, 2011). Booth et al. (2020, Figure 1) summarize select vertebrate and invertebrate preparations in which CPG networks for rhythmic motor output and their sensory and modulatory control have been examined.

Powerful evidence for neural network production of rhythmic motor patterns by CPGs comes from invertebrate model systems such as the motor networks of lobster and crab stomatogastric ganglia (Marder and Bucher (2001), swimming in nudibranch molluscs like *Tritonia* (Katz, 2009), and rhythmic movements of crayfish swimmerets (Mulloney and Smarandache-Wellmann, 2012). Feeding motor programs in freshwater pulmonate gastropods like *Helisoma* and *Lymnaea* are also model systems to study the principles of CPG organization (Kater, 1974; Murphy, 2001; Elliott and Susswein, 2002; Kemenes and Benjamin, 2009; see also Benjamin, 2008), and are more tractable than many other CPG preparations for a student lab exercise to learn about motor pattern production. The CPG for the snail feeding motor programs is a network of neurons in the buccal

ganglia that organize the motor pattern output to muscles of the mouth, pharynx, and radula for feeding movements used in scraping algae from a substrate like the glass walls of an aquarium wall (Figure 1 and Video 1; supplemental videos are linked at the end of this article). Nearly all neurons in the buccal ganglia are motor neurons and interneurons that control the muscles used for rasping and swallowing. The motor program can be initiated and modulated by dopamine (Quinlan et al., 1997). Serotonin and octopamine (a neuromodulator closely related to norepinephrine) also activate and modulate the feeding motor pattern (Vehovszky et al., 1998). Serotonin appears to have a more general arousal effect on feeding behavior, turning on food-searching behaviors in the whole animal as well as activating the feeding CPG (Kemenes et al., 1990).

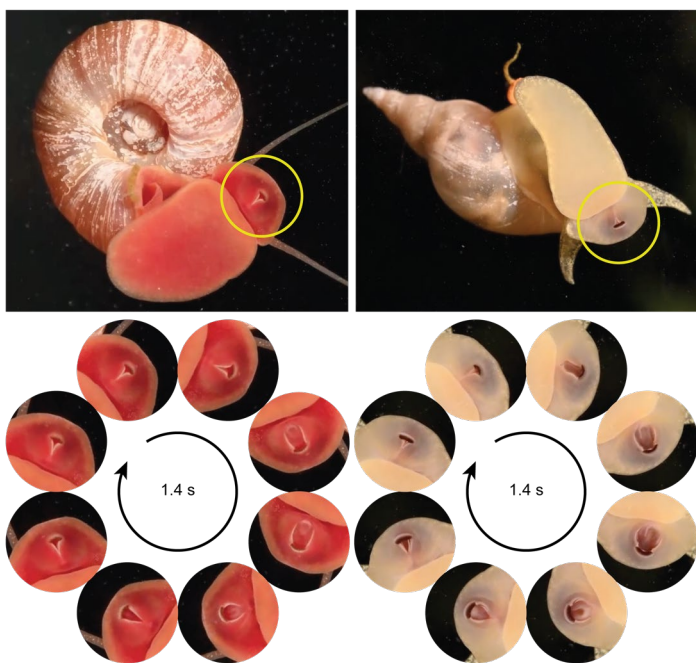
This lab exercise introduces the concept of central pattern generation in the snail model system by first quantifying the behavior (mouth movements); second, measuring neural correlates of the behavior (the feeding motor program) by extracellular recording of motor neuron activity in nerves leaving the buccal ganglia; and third, quantifying changes in the feeding motor pattern due to application of a neuromodulator or feeding stimulus.

**OBJECTIVES**

This lab exercise should develop and improve lab *skills*, result in collection and quantitative analysis of *data*, and introduce or solidify *concepts* surrounding cell and circuit properties responsible for rhythmic neural activity.

**Skills**

Students should gain or improve dissection and recording skills. The dissection is only slightly more difficult than simple crayfish dissections and the difficulty of recording is on a par with other nerve recordings (e.g., crayfish superficial flexor nerve; Wyttenbach et al., 2014). If students already have experience with dissection and nerve recording, this exercise can be done in a three-hour period



**Figure 1.** One feeding cycle in *Helisoma* (left) and *Lymnaea* (right). Yellow circles in the upper images show areas magnified below. The odontophore protrudes back as the mouth opens, the odontophore moves forward such that the radula scrapes the surface, and the mouth closes as the odontophore pushes food back into the esophagus. Each cycle shown here took 1.4 s. These are frames from Video 1, in which snails were feeding on the side of the aquarium.

. We do it after students have recorded from crayfish nerves and performed spike analysis. Students new to dissection and recording may need two lab periods to develop skills and collect data.

### Data

Every lab group should be able to record rhythmic activity in several nerves and measure responses to feeding stimulants and neuromodulators. Data can be shared so that the entire class analyzes a variety of data. Depending on course goals, students can describe these results and changes qualitatively or perform sophisticated measures of cyclic spiking activity in multiple axons per nerve. One strength of this system is the ability to make similar measurements of behavior and neural activity.

### Concepts

Students will see, first-hand, the action of a CPG and its modulation by sensory stimuli and neuromodulators. Ancillary lecture material could cover membrane properties that lead to intrinsic bursting, circuit properties such as reciprocal inhibition that affect oscillation and phasing, and mechanisms by which dopamine and serotonin can modulate the behavior of neurons and circuits.

## MATERIALS

### Snails

Any pond snail can be used. Most published research is on *Lymnaea stagnalis* and *Helisoma trivolvis*, with some work

on *Planorbis* species. In our experience, students find *Lymnaea* easier to dissect, but *Helisoma* more readily generates rhythmic activity in vitro and may also be easier to record from with a suction electrode. *Helisoma* in our videos are albino mutants used in many research labs. Locally collected pond snails can also be used; several web sites can help identify them to family or genus (e.g., Dillon et al., 2019; Nordsieck, ND). *Lymnaea* (great pond snail) is widespread in northern North America and Europe (GBIF, 2019b). *Helisoma* is widespread in North America (GBIF, 2019a). Sellers of pond snails rarely name the species, but snails labeled ramshorn are likely to be *Planorbis* or *Helisoma*. Many aquarium suppliers sell red ramshorn snails, which appear to be albino *Helisoma* (see Aquarium Resources for links to suppliers). Dark ramshorn snails (probably *Helisoma*) are available from biological supply companies (see Aquarium Resources for links to suppliers). These suppliers do not specify sizes but it may be possible to request larger specimens. It would be wise to order far enough in advance that snails can grow to a suitable size.

Keep snails in cool (20–25°C), aerated, and filtered artificial pond water (1.5 g of artificial seawater salts per gallon of deionized water). Cover the aquarium bottom with about 1 cm of crushed oyster shell (sold as a dietary supplement for chickens); rinse off dust first. Change the filter as needed and replace the water if it appears cloudy or smells foul. Snails will thrive on lettuce leaves or carrot pieces. In case of a population crash, change the water and wait for eggs to hatch. It takes 3–5 months from hatching to get snails large enough to work with (10–15 mm diameter for *Helisoma* or 15–20 mm length for *Lymnaea*).

### Dissection

The dissection requires small scissors (curved cuticle scissors work well), student-grade Vannas scissors, and #5 forceps. Dissection and recording take place in a dish at least 20 mm deep and 50 mm diameter (60 or 90 mm crystallizing dishes are ideal) with about 5 mm of Sylgard™ silicone elastomer or similar in the bottom. To hold the preparation, use six #2 insect pins (0.5 mm diameter). A standard teaching lab microscope (6–25× magnification range) with LED or fiber-optic lighting will suffice for dissection and recording.

### Chemicals

Pond snail saline is 1.7 mM KCl (0.13 g/l), 51.3 mM NaCl (3.0 g/l), 4.1 mM CaCl<sub>2</sub>·2H<sub>2</sub>O (0.60 g/l), 1.5 mM MgCl<sub>2</sub>·6H<sub>2</sub>O (0.31 g/l), and 5 mM HEPES buffer (1.2 g/l); bring to pH 7.3 with NaOH (*Helisoma*, Quinlan et al., 1997; *Lymnaea*, Magoski et al., 1994; Vehovszky et al., 2005, use a different saline for *Lymnaea*). Neuromodulators can be mixed in saline. Make concentrated stock solutions (e.g., 10<sup>-2</sup> M) and dilute to the desired concentration by adding small volumes to the prep dish. For example, a 60 mm diameter dish with saline 12 mm deep holds about 35 ml, so adding 350 μl of stock will give a final concentration of 10<sup>-4</sup> M. A drop from a transfer pipette is about 50 μl, so 7 drops give 10<sup>-4</sup> M and 1 drop gives about 10<sup>-5</sup> M. A 90 mm dish holds 100 ml, so adding 1 ml of stock will give a final concentration of 10<sup>-4</sup> M and 2 drops give 10<sup>-5</sup> M. Most chemical stocks can be kept

in solution in the refrigerator, but dopamine oxidizes in water. Make it fresh for each class, keep it frozen when not in use, and discard any stock solution that appears dark.

### Recording

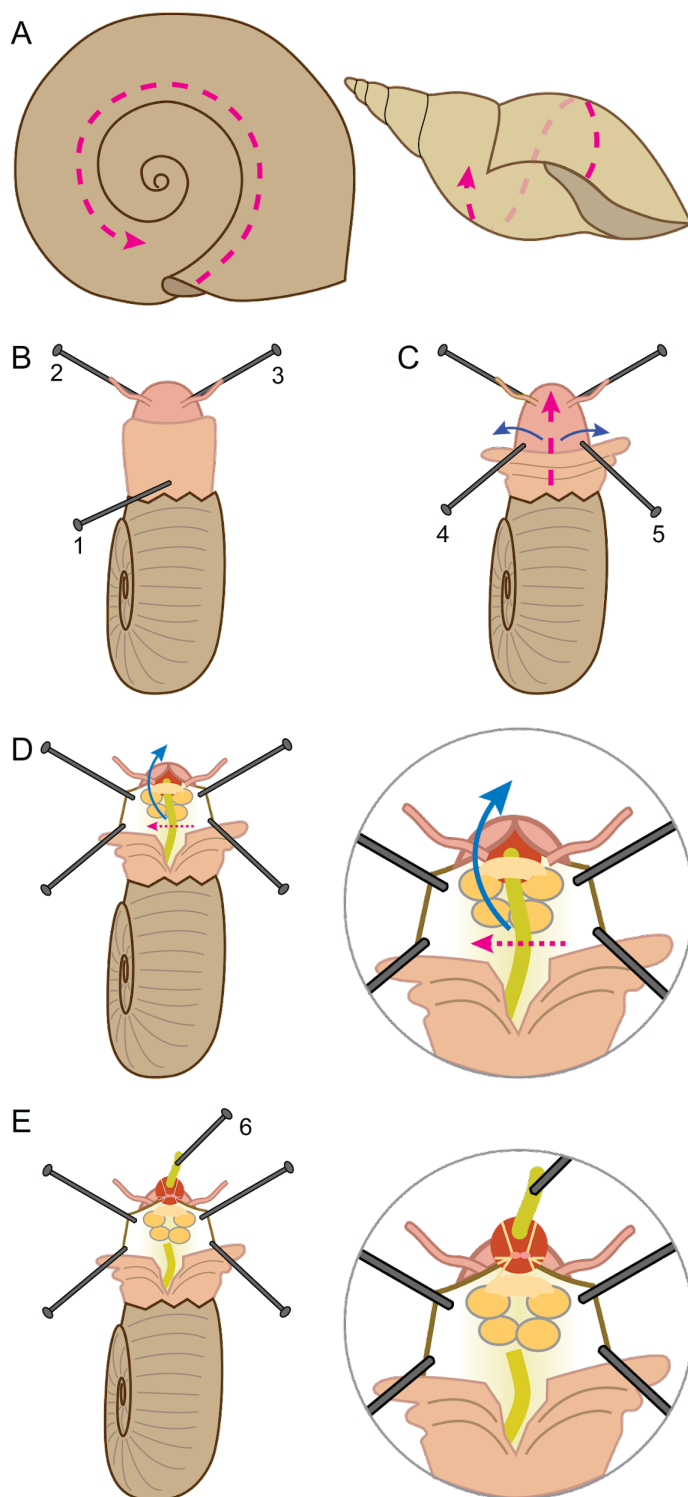
A standard extracellular recording rig (Wytenbach et al., 2018) is sufficient. A Faraday cage is needed for noise reduction but there is little need for vibration isolation. The extracellular (AC) amplifier should have a gain of 1000× to 10000× and a bandpass filter (300-5000 Hz); most teaching lab amplifiers are suitable. A standard suction electrode (Johnson et al., 2007) with 1.5 mm glass works well. Electrode tips can be pulled by hand over a flame or in an electrode puller. Tips can be broken to a variety of sizes and then either fire-polished or smoothed with a ceramic tile (Sutter Instruments part number CTS). Aim for tip diameters of 0.2-0.4 mm. Good tips can be cleaned and reused. Any three-axis micromanipulator will do. An amplified speaker for audio monitoring is not essential but helps one hear rhythmic patterns in the recordings. The analog-to-digital converter should be capable of 10 kHz sampling. See Wytenbach et al. (2018) for equipment suggestions.

## METHODS

### Dissection

Before starting the dissection, anesthetize a snail in cold saline (Ramakrishnan et al., 2014). Alternatively, use a solution of 5% ethanol in pond water but do not leave the snail in ethanol for more than three minutes. The dissection has three parts, as described in Figure 2. Videos 2 and 3 illustrate the process and Figure 3 shows the result.

1. Remove the shell (Figure 2A, Video 2). Insert a blade of the cuticle scissors into the shell opening and cut 1-2 turns around the spiral of the shell, while breaking away pieces of shell. Avoid cutting deeply into the body, but continue even if that happens. *Helisoma* has red body fluid that often leaks. That is generally not a problem. If the entire shell shatters, remove the fragments and continue with the next step, although orienting the snail in the dish may be more challenging.
2. Open the body cavity to expose the buccal mass (Figure 2B-C, Video 3). For this step, it is critical that the body is oriented properly in the dish, with the foot on the floor of the dish. Hold the snail in place with one pin, far from the head, then insert two more pins through the foot, taking care to place them under the tentacles. If pins are placed too medially, the buccal mass and nerves may be damaged. Next, pull the mantle back and secure it with two more pins. Add saline to the dish now or after the next cut. Remove the first pin and use Vannas scissors to open the body cavity.
3. Expose the buccal ganglia and nerves (Figure 2D-E, Video 3). Reset the four pins to hold the body cavity open. Locate the esophagus where it goes through the circumesophageal ganglia and cut it as far behind the ganglia as possible. With forceps, pull the anterior part of the esophagus through the circumesophageal ring to tilt the buccal mass forward, then secure it with the pin.



**Figure 2.** Dissection. A. Remove the shell by cutting around its edge (*Helisoma*, left) or in the middle of the shell opening (*Lymnaea*, right). Continue the cut for at least one full turn of the shell and break off the shell fragments to expose the body. The remaining steps show *Helisoma*; the procedure is the same for *Lymnaea*. B. Hold the snail down with three pins. Add saline now or after the next step. C. Pull the mantle back and pin it, then cut the body cavity open. D. Pin the body walls down, then cut the esophagus and pull it through the circumesophageal ganglia. E. Tilt the buccal mass forward and secure it with a pin through the esophagus.

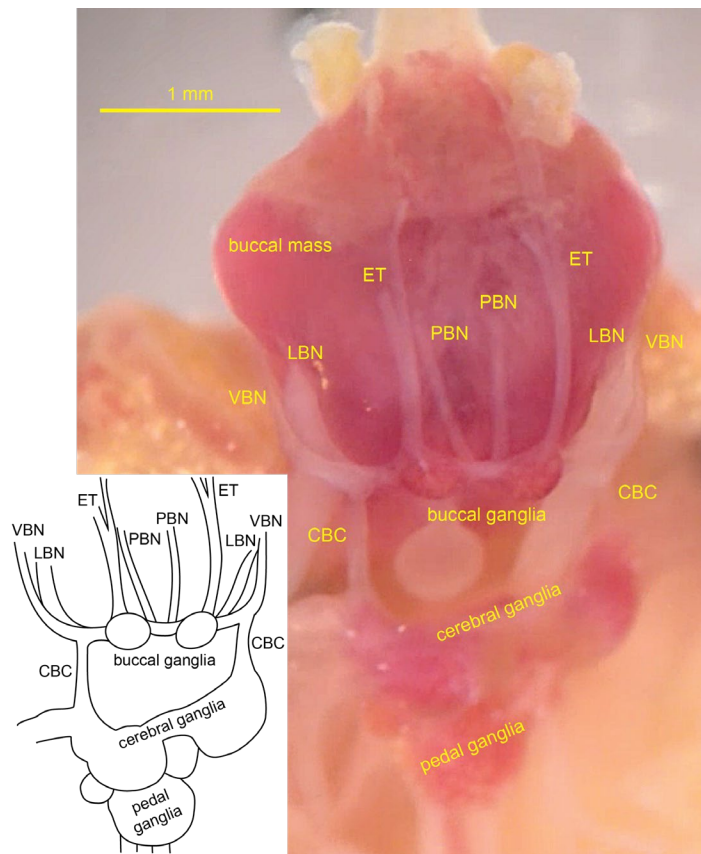


It may be necessary to twist or otherwise move the buccal mass slightly to expose the buccal ganglia and nerves. The result should resemble Figure 3.

Have students pause at times for an instructor to check their progress. (1) After the snail is held down in the dish with a single pin, check that the foot is down and flat enough for the next two pins to be inserted under the tentacles. At this point, students may need help finding the tentacles. (2) After the mantle has been pulled back and the body cavity opened, look for the circumesophageal ganglia and esophagus. If they are not visible, other organs can be pushed aside or removed to reveal them. If the buccal mass has been cut into, check that the circumesophageal ring is intact. If it is broken, the buccal ganglia and nerves may be damaged; check them and start over with a new snail if necessary. (3) Finally, check that the buccal nerves are visible. Rotate the buccal mass as needed to make them accessible for recording, as in Figure 3.

**Recording**

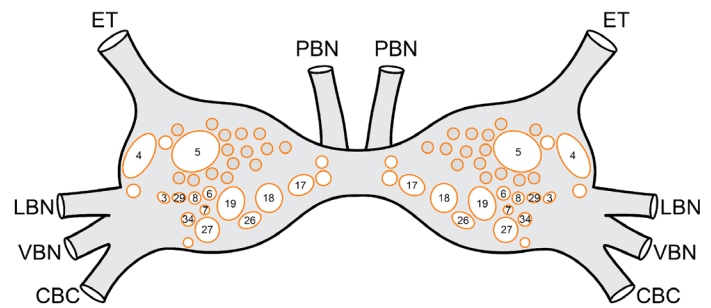
Before recording, empty saline out of the dish and replace it



**Figure 3.** Completed dissection. With the buccal mass tilted forward, the buccal ganglia and nerves should be visible. ET esophageal trunk; PBN posterior buccal nerve; LBN lateral buccal nerve; CBC cerebro-buccal connective; VBN ventral buccal nerve. electrode tip, so only a slight suction may be needed. If the LBN, VBN, and CBC are often joined for some distance before splitting into three distinct branches.

with fresh cold saline. The buccal ganglia will be caudal side up and most of the nerves should be visible (Figures 3-4). Video 4 shows recording from two nerves in *Helisoma*. Select an electrode tip with an opening diameter about 1.5 times the diameter of a nerve (too large is better than too small). Lower the electrode into the saline and pull gently on the syringe until saline touches the wire inside the electrode tip. Place the electrode onto a nerve and apply suction. It is not necessary to suck a loop of nerve into the tip is much larger than the nerve, cover the nerve with it, press it down onto the buccal mass, and apply suction. This should be stable as long as the buccal mass does not move too much.

Unless the signal-to-noise ratio is poor, spontaneous spikes should be obvious in the audio monitor and computer display as soon as the electrode is sucked onto any nerve. Some preparations may also show regular patterns of bursts, but many will not go into a bursting pattern until given a feeding stimulant or neuromodulator. Listen to the audio monitor for burst patterns. Bursts can have very long periods, so slow the display of data acquisition software to show 30-60 s of data per screen.



Neuron and Function	Phase	VBN	LBN	ET	PBN
3 Phase I protractor	I			3	
4	I II			4	
5				5	
6 Phase I protractor	I				6
7 Phase I protractor	I	7			
8 Phase I protractor	I				8
17	II		17		
18	II III	18			
19 Phase III hyper-retractor	III	19	19		
26	II III	26			
27 Phase II retractor	II III		27		
29 Phase II retractor	II			29	
34	I II		34		
110 Phase II retractor	II	110	110		

**Figure 4.** *Helisoma* buccal ganglia and nerves. After dissection, ganglia will be oriented with the caudal side visible and ET nerves pointed away from the body. Many of the neurons have known functions and axonal projections. For example, recording from PBN should show a burst from neuron 6; this would precede dense bursts from 27 and 19, both in VBN (data from Murphy, 2001; see Table 1, Figures 3-6, and associated text).

If the signal-to-noise ratio is poor, work to improve the seal between electrode tip and nerve. Try different tip sizes, increasing suction, and pressing the electrode onto the buccal mass. If buccal movement shifts the electrode on the nerve, try recording from a different location on the nerve. Most of the nerves are long enough to offer many recording locations. As a last resort, the signal may improve if the nerve is cut and the cut end (on the ganglion side) is pulled however the ganglia can flop loosely, making further into the electrode. If that is done with more than 3-4 nerves, recording difficult and making it hard to tell which nerve is being recorded. Note that cutting CBC isolates the buccal ganglia from the circumesophageal ganglia and may reduce sensory input to the buccal CPG. (This could be done deliberately to test the source of sensory input for feeding stimuli.)

## EXPERIMENTS

### Standard Lab Period

To start, students should watch the feeding behavior of intact snails, perhaps capturing video on their phone cameras for later analysis. If snails in the lab are not active, Video 1 would suffice. The feeding rhythm could be measured in several ways. Most simply, one could count the number of cycles and divide by the total time for an estimate of the period. In a video, one could do a more detailed analysis, measuring the duration from mouth opening to mouth closing and the delay until it opens again. These observations and analyses show that the feeding pattern is slow and often irregular.

In the dissected preparation, students can start by just observing spontaneous movements of the buccal mass and trying to record from several nerves. They should see that movement correlates with increased motor nerve activity (Video 4). If the buccal mass does not move, the ganglia are probably still viable; not all preparations are spontaneously active. In a good recording, 3-4 distinct spike classes can be seen; neuromodulators often activate new spike classes. In a three-hour lab period, students should be able to do a dissection, record from several nerves, and perform at least one of the following experimental manipulations:

- Food may start or accelerate the feeding pattern. Crush lettuce in a small amount of saline and add a few drops to the dish. The effect may take a few minutes to develop and wears off over time. Benjamin (2008) reports that sucrose is a feeding stimulus for *Lymnaea*.
- Dopamine ( $10^{-8}$  to  $10^{-4}$  M) may start the *Helisoma* feeding pattern and alter it in a concentration-dependent manner (Trimble and Barker, 1984; Quinlan et al., 1997). At  $10^{-4}$  M, it may start the *Lymnaea* feeding pattern, increase its frequency, and change its phasing (Kyriakides and McCrohan, 1989).
- Serotonin ( $10^{-6}$  M) may start the *Helisoma* feeding pattern (Trimble and Barker, 1984; Quinlan et al., 1997). At  $10^{-4}$  M, it may halt the *Lymnaea* pattern but increase tonic activity (Kyriakides and McCrohan, 1989).
- Octopamine ( $10^{-4}$  M) or acetylcholine ( $10^{-4}$  M) may slow or halt the *Lymnaea* feeding pattern and induce tonic activity (Kyriakides and McCrohan, 1989). We find that

the cholinergic agonist carbachol ( $10^{-4}$  M) slows or halts feeding patterns in both *Helisoma* and *Lymnaea*.

Many of these effects are slow to develop, so students should be patient. If no effect is seen, increase the concentration two- or tenfold by adding more of the stock solution. At all times, students should keep track of which nerve is being recorded.

If several chemicals are applied without changing the saline between, keep track of and report the order and timing of applications. Responses are likely to be history-dependent. A partial washout, however, is possible without ending a recording. Rather than dump and replace all the saline at once, use transfer pipettes to carefully remove and replace half of the saline. If this is done three times, any chemicals that were added will be reduced to  $\frac{1}{8}$  of their original concentration.

### Further Exploration

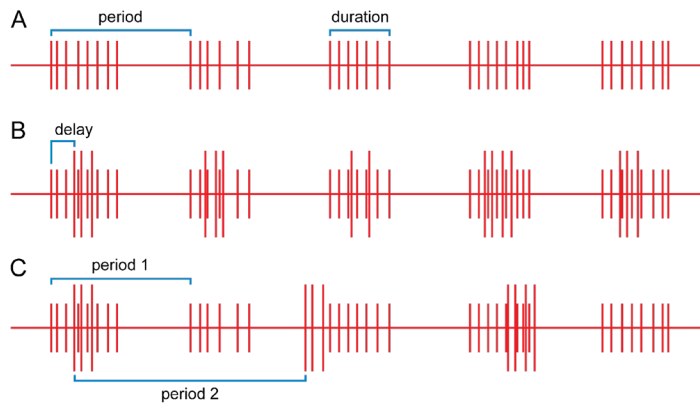
- Are feeding rhythms affected by temperature? In our experience, cold preparations are less active. This could be tested systematically.
- Systematically examine the response to neuromodulators and combinations of them. Which effects are concentration-dependent? What if dopamine is applied before or after serotonin? Does the order matter?
- Record from two or more nerves simultaneously. Are different nerves on the same side (e.g., PBN and LBN) synchronized? If they burst at the same rate, are they in phase? Are the same nerves on opposite sides (e.g., left LBN and right LBN) synchronized and in phase?
- After recording from two nerves simultaneously, switch one electrode from recording to stimulation. Does stimulating one nerve affect activity in another? CBC may be a good candidate for stimulation, since it comes from the circumesophageal ganglia and may modulate buccal circuits. Try single, sustained, and rhythmic stimuli. Do any of the effects outlast the stimuli?
- Stimulate a nerve and observe the effect on buccal mass movements. It may be best to cut the nerve first and stimulate the end that goes into the buccal mass.
- Place a suction electrode directly on a buccal ganglion, over one of the large cells visible on its caudal surface (Figure 4). In a stable preparation, it is often possible to record nearly single-unit activity. See Murphy (2001) or Ramakrishnan et al. (2014) for cell locations in *Helisoma* or Benjamin and Rose (1979) for *Lymnaea*.
- CBC connects the buccal ganglia to the cerebral ganglia and often has rhythmic activity. Cut it and record from each end. Does it carry rhythmic activity from cerebral to buccal ganglia, or from buccal to cerebral ganglia?

## RESULTS

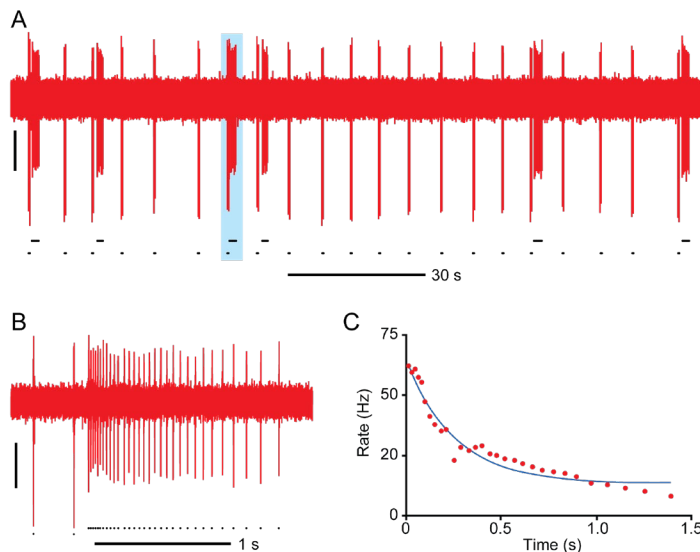
### Data and Analysis

In our teaching labs, students work in groups of 2 or 3 per rig. Each group chooses which nerves to record and what manipulations to do, so the class as a whole should have examples of rhythmic activity and response to experimental manipulations. We share class data, with the proviso that each group does its own analysis for lab reports. At a

minimum, we expect each report to show baseline data and response to manipulation in at least one nerve. Rhythmic patterns should, as far as possible, be described quantitatively (Figure 5). If an existing rhythm changes after a manipulation, before and after values can be given. If manipulation evokes a rhythm where there had not been one, or abolishes an existing rhythm, only the rhythmic activity would be described quantitatively, along with



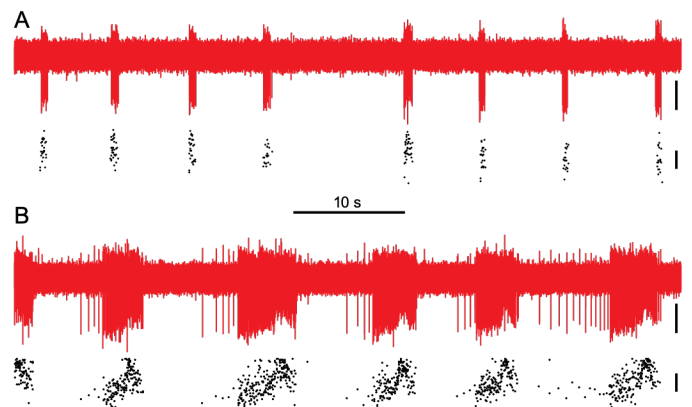
**Figure 5.** Cyclic measurements. *A.* Measure the burst period, burst duration, and number of spikes per burst (if spikes are distinct). Do this for several bursts and report mean and standard deviation. From the means, calculate duty cycle = duration / period (as a percentage). *B.* When more than one spike size has bursts with the same period, measure the duration, spike count, and duty cycle of each. From the delay between the two distinct bursts, calculate phase difference = delay / period (as a percentage). *C.* If there are independent burst patterns, measure the period, duration, duty cycle, and spike count of each.



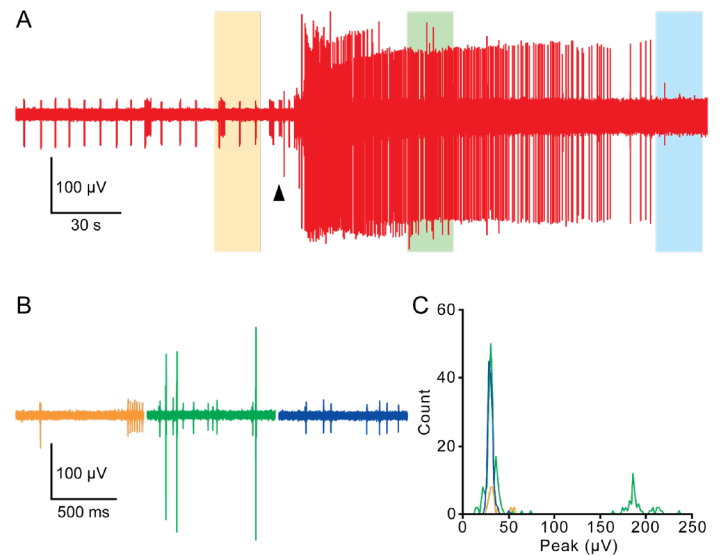
**Figure 7.** Burst phases. *A.* An unidentified nerve in *Lymnaea* had bursts from two neurons. Bursts of large spikes consistently had two spikes and a regular period ( $7.0 \pm 1.1$  s) and duration ( $0.27 \pm 0.01$  s). Bursts of small spikes were less consistent, with  $29 \pm 4.4$  spikes and  $1.3 \pm 0.3$  s durations. These bursts followed the start of a large-spike burst by  $0.90 \pm 0.28$  s, lagging the large-burst period by  $13 \pm 4\%$ . *B.* The area outlined in blue. *C.* Instantaneous spike rate declined exponentially (time constant  $0.26$  s,  $R^2 = 0.95$ ) during the burst in (*B*). Vertical scale bars for traces are  $20 \mu\text{V}$ .

qualitative description of the non-rhythmic activity.

Figures 6-10 show data recorded by students in our classes (analysis, figures, and captions are the work of the authors). Students had previously recorded from crayfish nerves (Wytenbach et al., 2014) but these were their first dissections and recordings from snails. These examples show some of the range of data one might see while also illustrating how analysis might be done and data displayed.



**Figure 6.** Dopamine modifies a rhythm. *A.* This *Helisoma* PBN initially had regular bursts of  $27 \pm 7.5$  spikes, lasting  $0.58 \pm 0.10$  s. Omitting the “missing” burst, the period was  $7.1 \pm 0.7$  s, for an 8% duty cycle. *B.* Five minutes after dopamine was added (final concentration  $10^{-4}$  M), bursts were less frequent but longer and denser. Omitting the sparse spikes leading up to each burst:  $180 \pm 25$  spikes/burst,  $4.6 \pm 1.1$  s burst duration,  $11.3 \pm 1.2$  s period, 41% duty cycle. Vertical scale bars are  $20 \mu\text{V}$ . Spike times came from the analysis tools in Wytenbach et al. (2014).



**Figure 8.** Serotonin disrupts a rhythm. *A.* In the *Lymnaea* nerve shown in Figure 7, serotonin was added at the arrowhead (final concentration  $10^{-4}$  M). Within 10 s, it activated large axons, shut down the larger rhythmically active axon, and increased tonic activity of the small axon. *B.* One-second pieces of the 20-s areas highlighted in (*A*), showing activity before (orange) and after (green and blue) serotonin was added. *C.* Histograms of spike sizes in the 20-s areas ( $2 \mu\text{V}$  bins), showing that increased activity in the small axon persists for several minutes.

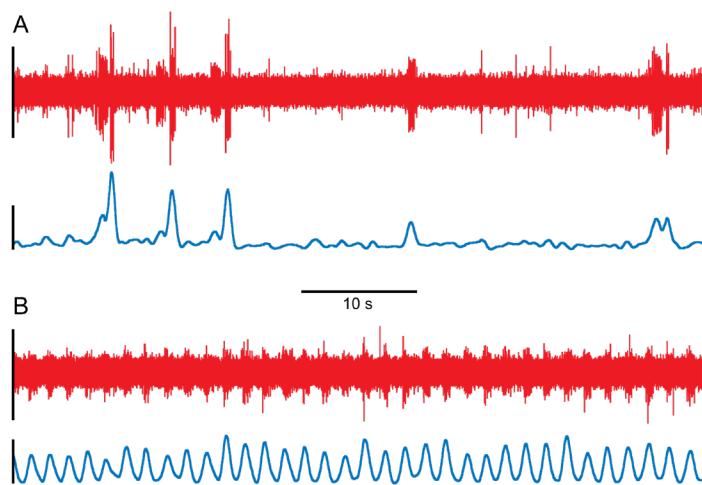
We would not expect all of this in any one lab report.

If spikes are distinct, a window discriminator can give spike times for precise measures of spikes per burst, burst duration, and burst period (Figure 6). If there are distinct spike classes (sizes), the discriminator can give data separately for each spike class. One can then measure these parameters for each spike class and calculate instantaneous spike rates (Figures 9 and 10).

Students may notice variability in extracellular spike sizes (Figure 6B) and ask whether different sizes represent activity in different axons. In this case, they reflect change in the sizes of action potentials during a burst; this often occurs in bursting neurons (see Figures 6, 9, 14 of Benjamin and Rose, 1979; Figure 1 of Quinlan et al., 1997; Figure 3 of Murphy, 2001; Figure 7 of Ramakrishnan et al., 2014).

Unlike some nerves used in teaching labs (e.g., crayfish abdominal nerve 3), the buccal nerves contain many small axons. Thus, extracellular currents are small, so spikes may be hard to separate from background noise. If activity looks like fuzz but there appears to be a cyclic pattern to its amplitude, the recording can be rectified and integrated, then smoothed, to more clearly show the pattern (Figure 9; the response in B would be difficult to separate from noise).

A full quantitative analysis may not always be feasible. In Figure 10, for example, nerve ET was firing irregular bursts. Immediately after lettuce juice was added, new axons became active and activity increased to an extent that would be difficult to quantify on a spike-by-spike basis due to overlapping spikes from different axons. Subsequently, a more regular pattern emerged and then subsided. In this case, students could measure a few bursts before and after application of the feeding stimulant, while qualitatively describing the transitional period.



**Figure 9.** Serotonin modifies a rhythm. **A.** An unidentified nerve in *Helisoma* had occasional bursts in two axons. **B.** Two minutes later, 40 s after serotonin was added (final concentration  $10^{-4}$  M), the larger axon stopped firing and the smaller axon fired regular bursts with a 1.75 s period. During serotonin application, the signal-to-noise ratio decreased slightly, so spikes are smaller. In each case, the recording (red, scale bar 40  $\mu$ V) was rectified and integrated and then smoothed (blue, scale bar 10 nV·s; 1 s

triangular window) to clearly show cyclic behavior. Calculation was done by ADInstruments LabChart 8.1.

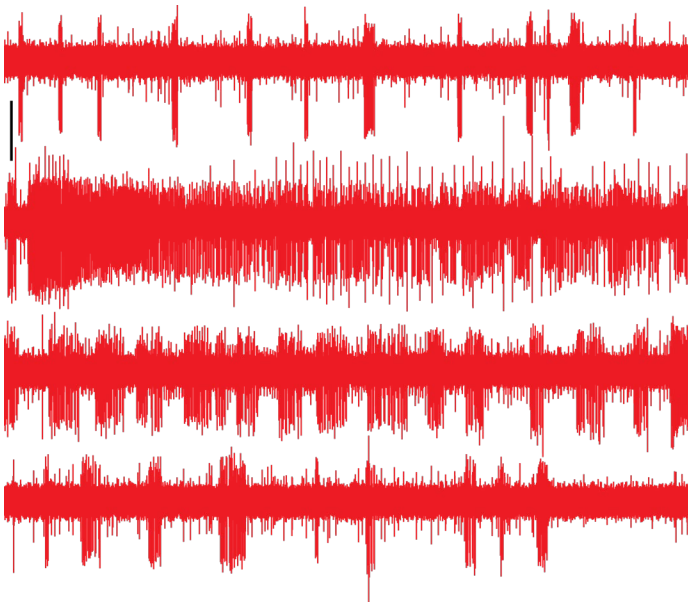
Cyclic measurements (Figure 5) can be done manually by counting spikes and measuring burst onsets and durations from the screen of data acquisition software. Our classes use analysis tools in Crawdad (Wytenbach et al., 2014). Data are copied from LabChart (ADInstruments) and pasted into a window discriminator, which gives a list of spike times and heights. From spike times, it can calculate instantaneous rate and exponential decline parameters (as in Figure 7). Output from Crawdad can be taken into Excel to count spikes per burst (Figure 6) and make histograms of spike height (Figure 8). Before this lab, our students had used these tools to analyze recordings from crayfish sensory and motor nerves. Only the cyclic measurements (Figure 5) are new and are introduced in a lecture. The analysis in Figure 9 uses channel calculations in LabChart; we rarely need to do this and include it only for illustration.

### Questions

In a lab report or assignment, students could address the following questions [*answers italicized*]:

1. If you saw rhythmic bursts, make cyclic measurements (Figure 5). How do they change over time? [*Even in an unmanipulated preparation, rhythms change spontaneously. Students can describe changes qualitatively and make measurements at various points.*]
2. Describe changes that occurred after administration of feeding stimulants or neuromodulators. Compare cyclic measurements before and after. How long do the effects last? [*These effects vary between preparations, between nerves, and even over time in the same snail. The main thing is that students should do quantitative analysis. Effects usually decline after a few minutes, although students often stop recording before then.*]
3. How do your cyclic measurements of nerve activity compare with your cyclic measurements of feeding behavior in the intact snail? If they are significantly different, what might explain the differences? [*If students observe several snails, they will see that, while the period of a feeding cycle varies only a little, there are often long gaps between cycles. Patterns seen in nerve recordings can also be quite variable, with long gaps between bouts of bursting activity. Within those bouts, burst periods are regular. It is our impression that burst periods in semi-intact preparations are longer than during actual feeding. The absence of sensory feedback in many CPG preparations causes longer cycle periods. In addition, sensory feedback can initiate and regulate the timing of activity phase transitions in CPGs (Marder and Bucher, 2001, Frigon, 2012).*]
4. Attempt to infer which neurons were responsible for activity in each nerve. See Murphy (2001) for Figures showing morphology of the neurons and a table of their axonal projections through the nerves. [*Students should come up with several possibilities for each nerve recording, based on neuronal morphology. They can often narrow it down based on known activity patterns of the neurons. Figure 4 gives some possibilities.*]
5. Propose mechanisms by which feeding stimulants or





**Figure 10.** Lettuce juice modifies a rhythm. Nerve ET in *Helisoma* had activity in multiple neurons, with one of them producing bursts. Each row is 30 s, continuing from top to bottom for a total of 120 s. Lettuce juice was added near the end of the top row. Within a few seconds, additional neurons started firing (larger spikes) and the rate and duty cycle of the bursting neuron increased (second row). Over the next 30 s, activity died down and reverted to the original pattern. Vertical scale bar is 20  $\mu$ V.

neuromodulators could initiate or change the feeding motor program (Trimble and Barker, 1984; Quinlan et al., 1997; Marder and Bucher, 2001; Nusbaum and Beehaker, 2002). Try to explain your results in terms of prior work. *[Feeding stimulants activate dopaminergic neurons that start a motor pattern; dopamine itself can have a similar effect, perhaps by stimulating the neurons that get synaptic input from those dopaminergic neurons. Answers could address cellular and synaptic mechanisms behind CPGs; see Discussion.]*

6. Would you expect activity to be synchronous in all buccal motor nerves? Consider different nerves on the same side (e.g., right LBN and right PBN) and the same nerve on opposite sides (e.g., right and left LBN). *[Considering the triphasic motor pattern, we would not expect simultaneous excitation in all nerves; there should at least be phase differences. For the most part, the behavior is symmetric, and many of the neurons described by Ramakrishnan et al. (2014) project bilaterally through the motor nerves. Therefore, we predict similar activity in nerves on the left and right in this case. For locomotion, however, many CPG networks simultaneously activate synergistic muscle groups while alternately activating flexors and extensors or left and right limbs (Frigon, 2012).]*
7. If two different neuromodulators had the same effect (e.g., increasing burst rate), what mechanisms could account for that? *[Some chemicals may mimic the effect of feeding, while others may affect CPG circuitry directly via receptors on pacemaker or other neurons. They may bind to postsynaptic receptors to mimic synaptic input or to non-synaptic receptors that tonically hyper-*

- depolarize neurons or change input resistance. Trimble and Barker (1984) show that dopamine and serotonin activate similar motor patterns through different circuits.]*
8. Effects of feeding stimulants and neuromodulators often diminish over time. Suggest mechanisms that could account for that. *[Sensory neurons may adapt to feeding stimulants (many possible mechanisms). Neuromodulators may be taken up by normal cellular mechanisms that act after synaptic release. They may also break down spontaneously (as in oxidation of dopamine) and be broken down by mechanisms that regulate synaptic transmission. Receptors may desensitize over time.]*

## ASSESSMENT

In our classes, students write reports in standard scientific paper format and are graded on writing, data analysis and presentation, and scientific content of their Introduction and Discussion sections. Questions 1-2 are prompts for Results material, while questions 3-8 would be addressed in the Discussion section. In a class where lab reports are instead done as worksheets, an instructor could write more specific prompts based on these questions.

After several years doing nerve recording as part of a block of intracellular-recording labs, we started using it as a formal exercise in 2018. Dissection and recording videos were produced in Fall 2019. Qualitatively, this lab exercise appears to be a success: students get data, enjoy seeing rhythmic activity, and are excited to see changes due to feeding stimuli and neuromodulation. They often ask about circuit- and molecular-level explanations for these effects.

Concerning our skill, data, and concept objectives, analysis of lab reports at Emory (Spring semesters 2018, 2019, and 2020) is encouraging. These semesters had a total of 23 students in 11 lab groups. All were third and fourth-year students who had already taken an introductory neuroscience course. In a three-hour lab period, each of the 11 groups successfully recorded from 2-4 nerves and did 1-3 manipulations (usually lettuce juice or serotonin, sometimes dopamine or glutamate). This indicates that the skill objective was met. Each lab group co-authored a report that combined nerve recording data with subsequent intracellular recording data. Based on the Results sections of their reports, the data objective was met: 11/11 showed examples of nerve recording, with 7/11 measuring cyclic parameters; 10/11 showed examples of changes due to manipulation, with 7/11 quantifying changes in cyclic parameters. In the Introduction or Discussion sections of their reports, 10/11 covered CPGs and model systems, 8/11 discussed mechanisms that might modulate nerve activity, and 8/11 attempted to determine which identified neurons were responsible for the nerve activity they saw, based on published activity and anatomy of the neurons. This suggests that the concept objective was also met.

## DISCUSSION

Recording from the snail CPG sets the stage for discussion of cellular and synaptic mechanisms of repetitive motor pattern generation. Cellular properties include endogenous bursting to set rhythmic activity, plateau potentials to



maintain firing longer than excitation time, post-inhibitory rebound (PIR) for fast excitation after inhibition, and spike frequency adaptation to slowly reduce firing rate after excitation (Marder and Bucher, 2001). These intrinsic membrane properties interact with synaptic circuitry to force other neurons, through excitation or inhibition, to follow the pacemaker's rhythm, or use reciprocal inhibition between CPG network neurons (half center oscillator) to set rhythmic activity between tonically firing neurons with firing phases regulated by plateau potentials, PIR, and spike frequency adaptation (Marder and Bucher, 2001). Students can delve deeper into CPG network mechanisms with the Networks tutorial in Neurosim (Heitler, 2019; Heitler, 2022).

In our classes, we follow the nerve recording lab with two or more lab periods in which students record intracellularly from the buccal ganglia. These recordings show plateau potentials, post-inhibitory rebound, spike rate adaptation, and other properties related to rhythmic activity. Students can experiment with the effects of neuromodulators on these properties.

Seichter et al. (2014) and Olivo (2019) describe lab exercises based on extracellular recording of the crayfish swimmeret motor pattern. The dissection is slightly more involved than exposing snail buccal nerves but this preparation can show phasing of motor output along homologous segments. The motor pattern is modulated by cholinergic agonists and antagonists. The snail preparation, however offers several advantages: the dissection leaves sensory input intact, one can record different phases of the same behavior by recording from multiple nerves, and more neuromodulators are effective. Furthermore, follow-up work using intracellular recording is easier. In the snail ganglia, one records from large, visible cell bodies; in crayfish, one must record from dendrites. Overall, we see these systems as complementary. Either one could be used by a student whose interest in CPGs was piqued by the other and wanted to follow up with a different system.

Finally, a historical story about the discovery of CPG networks may interest students. Charles Sherrington's seminal and Nobel prize winning research on spinal reflexes convinced him that rhythmic movements like locomotion were not only initiated by sensory stimulation, but also organized by sensory reflex chains. One of his own lab members, Thomas Brown, showed in 1911 that a decerebrate cat with spinal sensory nerves cut could still produce walking patterns on a treadmill, without brain or sensory input. The power of Sherrington's research reputation and personality quashed the spread of Brown's research and ideas (Wickens, 2014; Klarner and Zehr, 2018). It was only in the 1970s that Brown's CPG idea was resurrected by the work of Donald Wilson and others on locust flight (Marder and Bucher, 2001). Today it appears that almost all rhythmic motor patterns are produced by CPG networks that do not require patterned brain or sensory input to produce a basic motor pattern that sensory and brain input can modify.

## SUPPLEMENTAL MATERIAL

[Video 1A, \*Helisoma\* feeding behavior](#)  
[Video 1B, \*Lymnaea\* feeding behavior](#)

[Video 2A, Removing \*Helisoma\* shell](#)  
[Video 2B, Removing \*Lymnaea\* shell](#)  
[Video 3A, Exposing \*Helisoma\* buccal ganglia and nerves](#)  
[Video 3B, Exposing \*Lymnaea\* buccal ganglia and nerves](#)  
[Video 4, Recording from \*Helisoma\* nerves](#)

## AQUARIUM SOURCES

Ramshorn snails (most likely *Helisoma* but possibly *Planorbis*) are available from sellers on eBay and from the following sellers:

### Aquatic Arts

<https://aquaticarts.com/collections/freshwater-snails/products/red-ramshorn-snails>.

### Carolina Biological Supply

[https://www.carolina.com/mollusks/ramhorn-snails/FAM\\_141230.pr](https://www.carolina.com/mollusks/ramhorn-snails/FAM_141230.pr).

### Kazen Aquatic

<https://kazenaquatic.com/10-red-ramshorn-snails-baby-to-juvenile-size/>.

### Niles Biological

<https://www.nilesbio.com/prod345.html>.

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Received March 11, 2022; revised April 25, 2022; accepted April 28, 2022.

While we had recorded from pond snail nerves before, we first learned of the buccal nerve preparation from Anthony Pires of Dickinson College, when he presented it at a Faculty for Undergraduate Neuroscience meeting at Ithaca College in 2014.

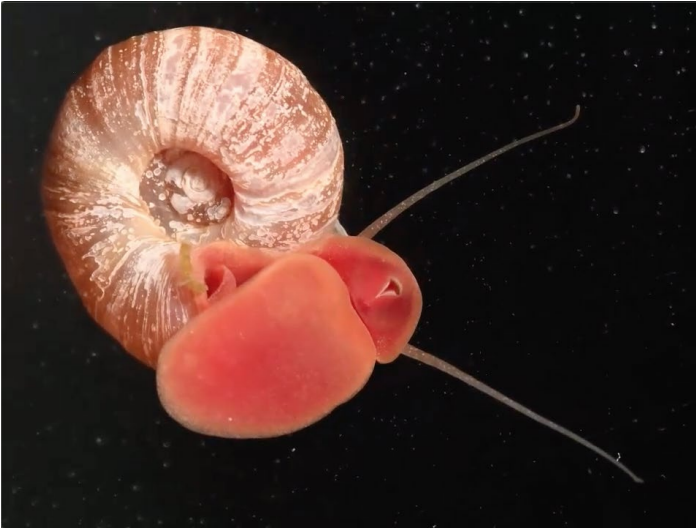
We thank Steven Hauptman of Bowdoin College for sharing his insights into snail breeding and maintenance.

Data shown here come from recordings made by Emory students in NBB 361W from 2015-2019, when these exercises were under development, and 2020, when a first draft of the exercise was tested, and by Cornell students in BioNB 4910 in 2022, with a first draft of this exercise.

This work was funded by an Emory University Winship Award to Robert Wytenbach.

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## APPENDIX: SUPPLEMENTAL VIDEO DESCRIPTIONS



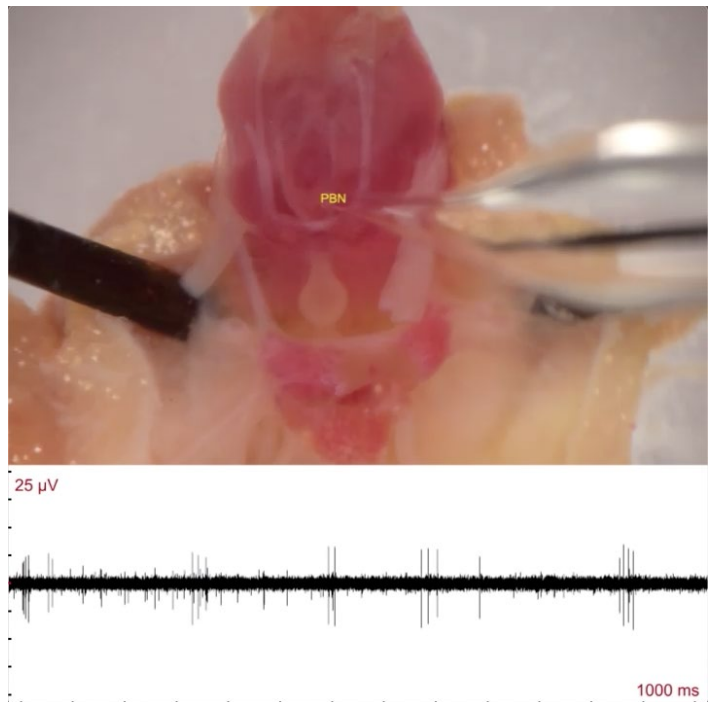
*Video 1. Feeding Behavior.* As a snail moves, it frequently grazes on the substrate. This video shows parts of the triphasic motor pattern. As the mouth opens, the odontophore protrudes backwards. As the odontophore moves forward again, the radula scrapes the surface, loosening food particles. Finally, the mouth closes as the odontophore pushes food back to the esophagus. The rhythm is variable and may include long pauses.



*Video 2. Removing the Shell.* Cut the shell for about one full turn along its spiral. Break the shell away as you proceed. Try not to cut deeply into the snail's body, but do not worry if body fluid leaks. If the shell breaks apart completely, you may need help orienting the body later.



*Video 3. Exposing the Ganglia.* Pin the snail in a dish, fold back the mantle, and cut through the skin to expose the body cavity. Find the circumesophageal ganglia and esophagus (remove other organs or move them aside as needed). Cut the esophagus behind the ring of ganglia and then pull it through the ring. Tilt the buccal mass forward and secure it with a pin through the esophagus.



*Video 4. Nerve Recording.* Pull saline into the suction electrode, move it onto a nerve, and apply suction. This example shows recording from the right LBN and right PBN. Both have bursts of activity that change over time.