Use of *Renilla* Bioluminescence to Illustrate Nervous Function

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Abstract: *Renilla mulleri* is a bioluminescent soft coral with a nervous system composed of a simple nerve net. Bioluminescence in these animals occurs in a bright wave across the surface, and is coordinated by nervous transmission. Groups of six students clustered around an animal can easily see the light wave without the use of magnification. The bioluminescent wave can be initiated by mechanical, electrical or chemical stimulation, and can be used to illustrate several concepts related to nervous function, including threshold, refractory period and adaptation. Students complete a homework assignment prior to the lab, in which they research mechanisms and functions of bioluminescence in *Renilla* and in other organisms. In the lab, each group gives a brief summary of the findings from the homework assignment; and examines the bioluminescent response of *Renilla* to touch. Students then make hypotheses about effects of varying the frequency and intensity of electrical stimulation, and about neurotransmitter and drug effects. These hypotheses are then tested by altering settings of the Grass stimulator, and by adding solutions of epinephrine, propranolol, and other chemicals. I used this lab in the context of the nervous systems unit of a sophomore Animal Physiology class, but the lab would also work well with more advanced classes, in which nervous control pathways could be better defined, or with introductory courses, which might involve discussions of bioluminescence or defense from predators. In this workshop, we will examine the morphology of *Renilla* specimens and initiate bioluminescence using mechanical, electrical and chemical stimuli. We will discuss applications of these experiments to various topics, and might also discuss other biological functions of these animals, including water transport, locomotion, and feeding responses.

Student Outline

Objectives:

- To describe the cnidarian nervous system.
- To explore the mechanisms & functions of bioluminescence.
- To investigate effects of electrical and chemical stimulation on nervous function.

Introduction:

The sea pansy (*Renilla spp.*) is a soft coral, a cnidarian of the class Anthozoa. These animals live on the ocean floor in shallow waters, and can often be found on the beaches of the southeast United States at low tide. *Renilla* polyps have various forms, including the tentacled feeding polyps, called autozooids, and the polyps that regulate water circulation, called siphonozooids (Figure 1). These polyps share an umbrella-shaped colonial structure, the rachis, and a foot-like structure, the peduncle (Figure 1). Both the rachis and the peduncle contain a water vascular system, muscular tissue, and nervous tissue, and the coordinated movements of these structures allow *Renilla* to anchor into the sand and move along the sea floor

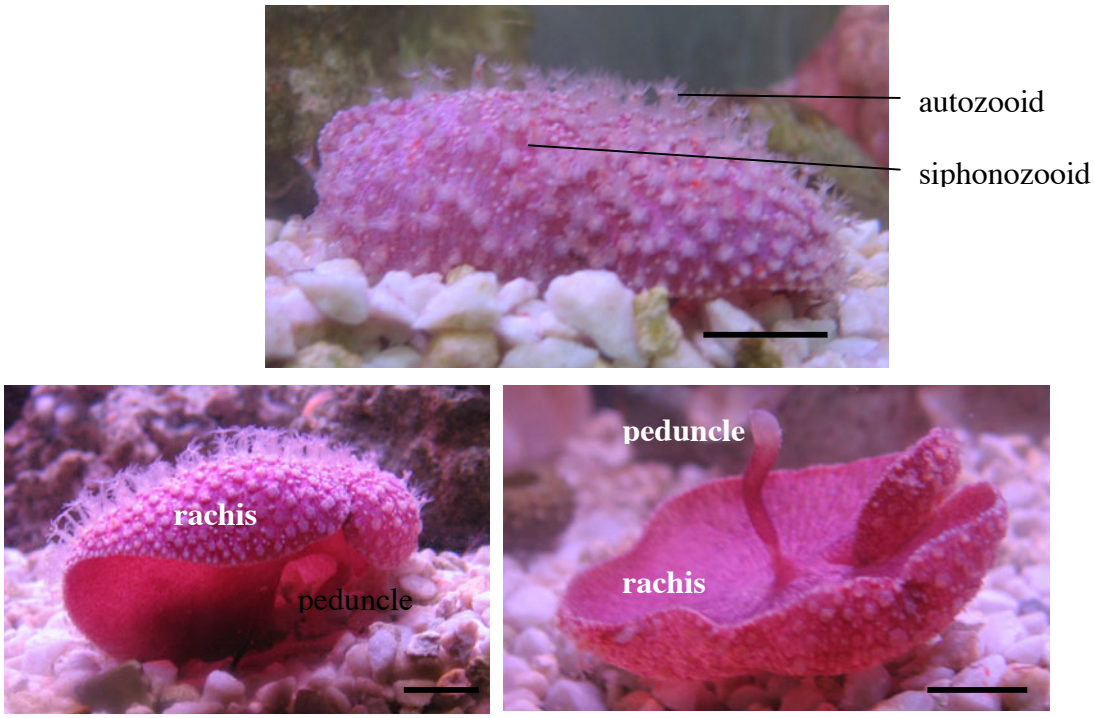

Figure 1. Anatomy of *Renilla muelleri.* Scale bars are approximately 1 cm in length.

Renilla, like other animals of phylum Cnidaria, has a nervous system in the form of a nerve net. The nerve net coordinates colonial responses including contractions of the rachis and peduncle, polyp movements, and bioluminescence. While the exact neurotransmitters used by the cnidarian nerve net are poorly understood, some signaling chemicals are similar to those used by vertebrates.

Bioluminescence is the generation of light via chemical reactions in living organisms. Bioluminescent organisms include fireflies, some jellyfish, dinoflagellates that cause 'sea sparkle,' and many deep-sea animals. The specialized bioluminescent cells of cnidarians, the photocytes, contain membrane-bound vesicles called luminelles. When Ca^{++} ions move into the photocyte, as results from nervous stimulation of these cells, a chemical reaction occurs in which a luciferin substrate reacts with oxygen in the presence the enzyme luciferase to produce light.

Bioluminescence has a number of useful functions for the luminescent organism. The flashes of light can be used to attract prey, hide from predators, distract predators, or attract mates. *Renilla* bioluminescence occurs in response to mechanical disturbance of the colony. When the colony is touched during the night phase, the nerve net sequentially activates the photocytes starting at the point of contact, resulting in a wave of light passing over the colony. This light pattern may function to distract predators – it has been reported that *Renilla* bioluminescence repels crabs, which may be predators of the soft coral, and lowers the heart rate of these predators.

In this laboratory exercise we will test the effects of mechanical and chemical stimuli on nervous function in *Renilla.* We will visualize the path of conduction through the nerve net by observing the wave of luminescence over the colony surface.

Procedure:

Note that the room must be very dark while you are conducting the procedure. Be sure that you are familiar with the equipment and protocol before the lights are turned out!

You will get two *Renilla* specimens in finger bowls. You may use red light to help to find your materials, as this light will not cause the specimens to go into their day cycle (the bioluminescence reaction only occurs at night!), but the light wave can be quite faint, so it is best to have your work area as dark as possible.

Mechanical stimulation

1. To initiate bioluminescence, apply mechanical stimulation by gently poking the edge of the animal with your finger. What do you observe?

   Why does the wave of light only travel in one direction? (Hint: think about refractory periods)

2. Now, use mechanical stimulation to initiate the action potential on opposite sides of the animal. Based on what you know about refractory periods, what do you expect to see?

   What do you observe?

Electrical stimulation

For electrical stimulation, we will use a Grass stimulator and attached electrode (Figure 2). Be sure that you are familiar with the controls before we turn out the lights!

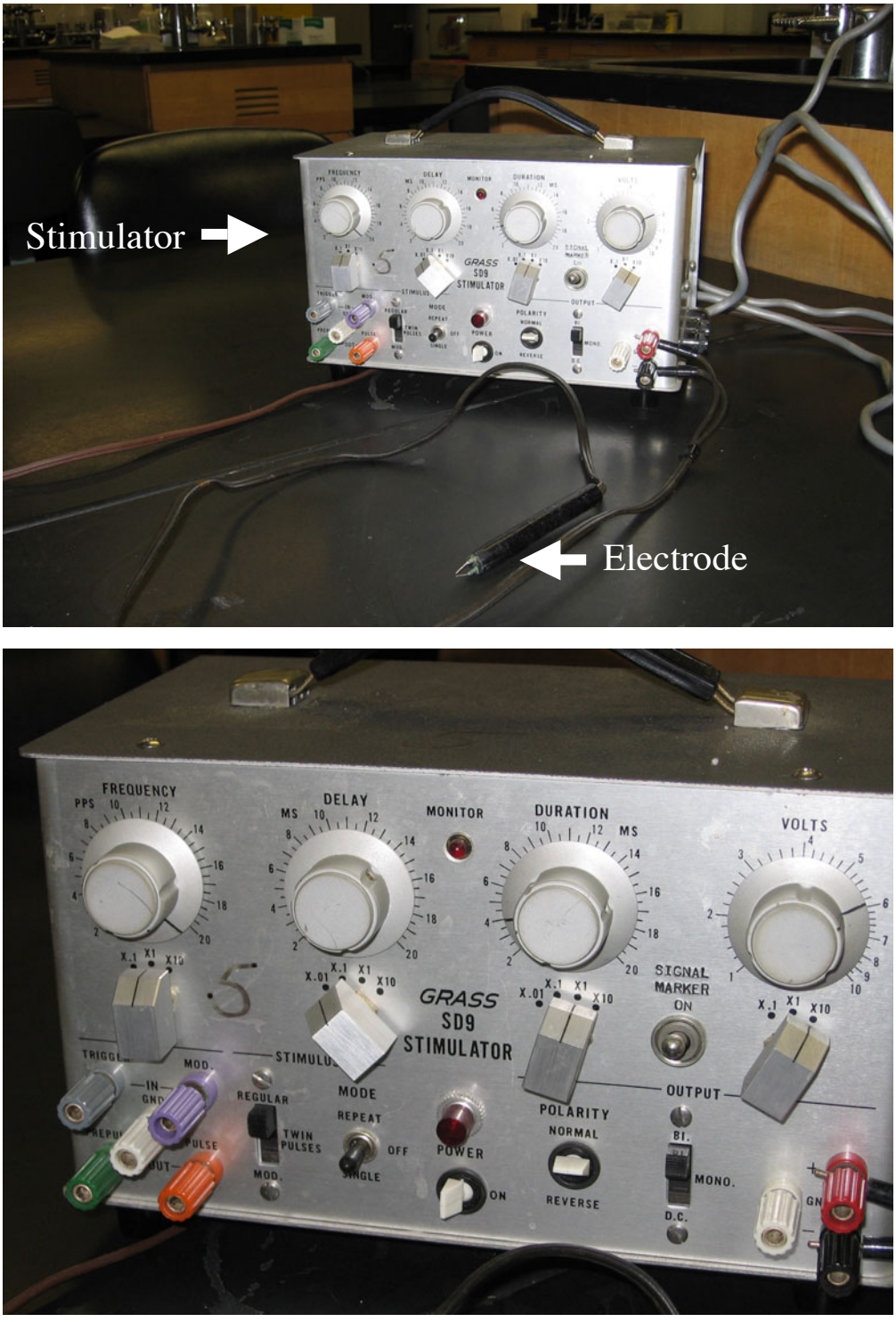

Figure 2. Grass stimulator.

1. Note the location of the power switch and make sure that the controls on the Grass stimulator are set appropriately:
   Frequency: 2 pulses per second (note the multiplier switch below the frequency dial!)
   Delay: minimum (note the multiplier switch below the frequency dial!)
   Duration: 10 ms (note the multiplier switch below the frequency dial!)
   Volts: 5 V (note the multiplier switch below the frequency dial!)
   Stimulus: Regular.

   To initiate the stimulus (don't do this until you are ready to start the simulation of the animal!), you will press the "mode" switch down toward the word "single."

2. When you are ready, gently touch the electrode against the side of the colony, keeping your fingers on the plastic arm of the electrode. DO NOT have your fingers in the water while an electrical stimulus is applied! Apply several stimuli. What do you observe?

   What is 'threshold' in the context of nervous function? Have we provided a threshold stimulus?

3. We will now increase the stimulus intensity. Increase the voltage gradually, not exceeding 50 V. What do you expect will occur?

   What do you observe?

4. Next, increase the frequency with which the stimuli are applied (temporal summation). To apply multiple stimuli, you will press the "mode" lever up toward "repeat." The frequency of pulses is set by the "frequency" dial, which you have already set to 2 pulses per second.

   Set the voltage to a level just below threshold. Start the pulses at 2 pps, then increase the frequency by turning the "frequency" dial clockwise. Predict what will happen as the stimuli are applied with greater frequency.

   What do you observe?

Chemical stimulation

The two major vertebrate neurotransmitters used for communication between nerves and effector tissues are acetylcholine and norepinephrine. Of these two neurotransmitters, only one has an effect on *Renilla* nervous function. You will determine whether the *Renilla* nerve net responds to cholinergic or adrenergic signals.

1. Use a micropipettor to apply 50 µl of a neurotransmitter in the vicinity of the organism by positioning the pipet tip at the bottom or side of the finger bowl and then moving the *Renilla* so that it is just next to the pipet tip. Alternatively, you can hold the *Renilla* in your gloved hand and use one of your fingers to maneuver the pipet tip near the surface of the colony. Release the solution and observe the response.

   Is the *Renilla* bioluminescence response mediated by cholinergic or adrenergic receptors?

Sources:

Anctil M. (1994) Monoamines and elementary behavior in a coelenterate. in *Perspectives in Comparative Endocrinology*. Davey, Peter, Tobe, eds, p. 449-454. National Research Council of Canada, Ottawa.

Awad EW and Anctil M. (1993) Identification of b-like aderenoceptors associated with bioluminescence in the sea pansy *Renilla koellikeri. J. Exp Biol* 177:181-200.

Grober MS. (1990) Luminescent flash avoidance in the nocturnal crab *Portunus xantusii. J. Exp Biol* 148:415-426.

Wilson T and Hastings JW. (1998) Bioluminescence. *Annu Rev Cell Dev Biol* 14:197-230.

Parker GH. (1920). Activities of colonial animals; II. Neuromuscular movements and phosphorescence in *Renilla*. *J. Exp Biol.* 31:475-515.

Thurman CL (2005) The chemical and neural basis for control of bioluminescence. in *Laboratory Manual for Physiology.* Silverthorn, Johnson, Mills, eds, p. 821-829. Benjamin Cummings, San Francisco.

Wilson T and Hastings JW. (1998). Bioluminescence. *Annu. Rev. Cell Dev. Biol.* 14:197-230.

Instructor Notes

Student background:

This lab was incorporated into the nervous system unit of a sophomore level Animal Physiology course. The students had already learned about nervous physiology and types of animal nervous system in the lecture, and had done a simulation of nervous action potentials in the lab. All students in the class had taken Introductory Biology, and most had also taken Zoology. This exercise could also be done as a demonstration in an introductory biology class, or as part of a cnidarian lab exercise for Zoology.

Time required:

I conducted this lab in one three-hour time block. A shorter time block could be used, particularly with a smaller class size or with the ability to darken the entire lab room. Since I had to bring students into the dark room in groups, the students not conducting the experiments looked at nervous system models and completed a case study assignment involving animal neurotoxins.

Renilla specimens:

I ordered the *Renilla muelleri* specimens from Gulf Marine Biological Labs at a cost of $9.20 per specimen plus shipping. Most reports of *Renilla* function use *Renilla kollikeri* specimens from the Pacific coast, but I was unable to find a supplier for this species. In fact the *R. muelleri* specimens proved easier to use and maintain, as they thrive in room-temperature water, as opposed to the colder temperature preferred by *R. kollikeri.*

Renilla bioluminescence only occurs when the specimens are in their night phase. The animals must be dark-adapted for several hours before they will respond well to stimuli; I recommend having them shipped to arrive the day of the lab and kept in the dark until the lab is completed. If you order a few spare specimens, you and the students can examine morphology using dissecting scopes prior to the experiments; otherwise, animals can be examined in the light after the bioluminescence studies are completed. The animals are shipped cold, but do not put the finger bowls on ice to examine bioluminescence – I found that this completely inhibited the response. The animals are not harmed in the experiments described here, though surgical studies could be conducted to better analyze the path of nervous conduction.

The animals can be maintained following the completion of the lab. I have ordered several batches of animals, which have survived as long as 7 months in our aquarium, though 3 months is more typical. The fluorescent lamps on the aquarium should be plugged into a timer to maintain a 12 hr light/12 hr dark cycle. If you are conducting experiments during the day, be sure to keep the aquarium in a dark room where the dark cycle can take place during working hours. I maintain the animals in an established 55-gallon tank containing live rock and a few *Aiptasia* and brittle stars. The seawater is room temperature, filtered and aerated; we replace half of the water with fresh artificial seawater (Instant Ocean) once per month. Salinity is maintained at a specific gravity between 1.020 and 1.022. The animals are fed 3 times per week with Cyclopeze, available in frozen blocks from aquarium stores or online suppliers. We feed by mixing a small piece of the Cyclopeze with seawater and then pipetting drops of this suspension directly onto the animals.

Darkened lab room:

This lab must be conducted in a dark room. I used a prep room adjacent to my lab room, and therefore could only work with six students at a time; the students not actively conducting the experiments looked at nervous system models and completed a case study exercise concerning neurotoxins. It is critical that the students understand the procedure prior to turning out the lights – although red lights may be used at the work stations, this light will not be sufficient to read the lab handout or become familiar with the Grass stimulator. It takes awhile for everyone's eyes to adjust to the dark; during this initial time I had the lab groups discuss the answers to a homework assignment focusing on *Renilla* and bioluminescence.

Lab setup

This might take about an hour. Each workstation will need:

- 1 desk lamp with a red light bulb, or flashlight with red filter paper
- 1 light-safe box (e.g. Styrofoam shipping box) containing:
- at least 2 *Renilla* specimens in a finger bowl with seawater, or two finger bowls with one specimen in each. Use the seawater that the specimens came in, if possible.
- Extra seawater (1 liter or so); can be made from Instant Ocean powder
- Grass stimulator and electrode
- P200 micropipettor and pipet tips
- 100 μM solutions of epinephrine and acetylcholine, dissolved in seawater (add 1 mg/ml ascorbic acid to epinephrine to prevent oxidation), clearly labeled so that the bottles can be distinguished in dim light
- 1 dissecting scope and/or magnifying glass – to look at animals in the light

Protocol notes:

- The *Renilla* may be inflated with water or deflated when you use them, and will likely be 2-5 cm in diameter. An inflated animal might be 2 cm high, while a deflated animal will be almost perfectly flat. An animal that started out inflated will likely deflate during the course of stimulation. Be sure to provide at least two specimens per group, as some are more responsive than others.
- In animals that are particularly sensitive, or are given a very strong stimulus a 'frenzy response' can occur rather than a single wave of light. The frenzy response will either be a sequence of concentric light circles or a spiraling pinwheel pattern of light that will continue uninterrupted for as long as several minutes following the stimulus.
- For mechanical stimulation, I found that a light squeeze with the fingers worked better than a light poke with the glass rod.
- Tape can be adhered over the lights on the Grass stimulator to darken the apparatus.
- For chemical stimulation, Dr. Carl Thurman at the University of Iowa uses a syringe attached to fine rubber tubing to apply chemicals to the top surface of the animal.

Additional lab applications:

- Other examples of bioluminescence can be displayed in addition to that of *Renilla*. Ward's sells a firefly bioluminescence kit, and an excellent protocol for dinoflagellate bioluminescence can be found at www.lifesci.ucsb.edu/~biolum/organism/dinohome.html.
- For more advanced courses, different concentrations, combinations & means of delivery for neurotransmitters and drugs could be used. Other neurotransmitters expressed by *Renilla* include melatonin, serotonin and antho-RF-amide, though in my preliminary study none of these chemicals initiated bioluminescence.
- The neural conduction pathways could be explored by disrupting the nerve net either surgically or chemically using magnesium sulfate. Bioluminescence can then be observed following alteration of conduction paths through the rachis, or connections between the rachis and the peduncle (references below).
- Rachidal peristalsis and feeding polyp retraction are also mediated by the nervous system; these responses, too, could be examined (in the light!).
- *Renilla* feeding polyps withdraw in a wave following exposure to certain chemicals (for example, invertebrate food); this is likely mediated by the nerve net and could be explored further.
- *Renilla* bioluminescence can be quantified using a luminometer (references below). Indeed, in dual luciferase reporter systems for gene promoter activity, *Renilla* luciferase is used as a transfection control.

Student feedback:

Students said that they enjoyed the lab, but aside from asking questions relating to the lab topics on the lecture exam, I did not formally assess how well learning objectives were met. One student found the topic so interesting that she has chosen to examine the contributions of nervous function and circadian rhythm to the *Renilla* feeding response as her senior thesis project.

Lab source:

I first learned about *Renilla* bioluminescence from Dr. Carl Thurman's lab writeup in the Benjamin Cummings *Laboratory Manual for Physiology*. Dr. Thurman was an excellent source of information and tips for conducting the lab.

Useful references:

Anctil, M. 1994. Monoamines and elementary behaviour in a coelenterate. In *Perspectives in Comparative Endocrinology*. Davey KH, Peter RE, Tobe SS, editors. Ottawa: National Research Council of Canada, p. 449-454.

- Summary and images nervous-controlled *Renilla* functions, including bioluminescence.
- Available online at: http://abonnes.collegebdeb.qc.ca/profs/ggermain/redaction/exemple2.html

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Germain, G and M. Anctil. 1996. Evidence for intercellular coupling and connexin-like protein in the luminescent endoderm of *Renilla koellikeri* (Cnidaria, Anthozoa). *Biol Bull* 191:353-366

- Use of luminometers to quantify *Renilla* bioluminescence.

Buck, J. 1973. Bioluminescent behavior in *Renilla.* I. Colonial responses. *Biol Bull* 144: 19-42.
- Provides descriptions and diagrams of the various waveforms for bioluminescence.

Nicol, J.A.C. 1955. Nervous regulation of luminescence in the sea pansy *Renilla koellikeri. Exp Biol* 32:619-635.

Nicol, J.A.C. 1955. Observations on luminescence in *Renilla* (Pennatulacea). *J Exp Biol* 32:299-320.
- Experimental analysis of facilitation, adaptation and summation in nervous control of *Renilla* bioluminescence.

Parker, G.H. 1920. Activities of colonial animals. I. Circulation of water in *Renilla. J Exp Biol.* 31:343-367.

Parker, G.H. 1920. Activities of colonial animals. II. Neuromuscular movements and phosphorescence in *Renilla. J Exp Biol.* 31:475-515.
- Great descriptions of *Renilla* structure and basic biology, as well as functions such as bioluminescence, peristalsis and water circulation.
- Diagrams of surgical patterns and use of magnesium sulfate to block nervous transmission.

Pieribone, V. and D.F. Gruber. 2005. *A Glow in the Dark: The Revolutionary Science of Biofluorescence.* Belknap Press, Cambridge, MA.
- Written for non-scientists.
- Contains interesting historical anecdotes and scientific information about bioluminescence and fluorescence.

Wilson, T. and J.W. Hastings. 1998. Bioluminescence. *Annu Rev Cell Dev Biol.* 14:197-230.
- Excellent review of the chemistry of bioluminescence reactions in *Renilla,* fireflies, dinoflagellates, bacteria, etc.

DVD: The Shape of Life series (NSF), Episode 2: Life on the Move – Cnidarians.
- This whole series is a terrific way of introducing students to current research approaches with the major phyla of the animal kingdom.
- There is a 6-minute segment (at 24-30 minutes) in this episode describing the cnidarian nervous system.

About the Author

Anne Goodwin received a B.A. in Biology from Albion College (Albion, MI) and a PhD in Experimental Pathology from Harvard University, specializing in angiogenesis. She developed this lab with great assistance from Carl Thurman (University of Northern Iowa) while teaching Animal Physiology at Simmons College; this exercise is an adaptation of a protocol written by Dr. Thurman. She will teach a variety of courses as an Assistant Professor of Biology at the Massachusetts College of Liberal Arts (North Adams, MA) starting in July 2007, and will continue to conduct research on cnidarians and tunicates.