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A Holistic Approach to Horseshoe Crab Biology by Studying Easily-reared Larvae

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During this workshop, participants will explore recording the body contractions of horseshoe crab larvae that have been placed in five salinities ranging from 0-50 ppt. This will be conducted in petri dishes under a dissection scope. This experiment will set the tone for studying horseshoe crab biology. Background information will be provided on the horseshoe crab, including medicinal uses and their decline due to overharvesting. Sources of larval horseshoe crabs will be suggested. This workshop is part of a holistic approach to learning about an ancient species in decline. We suggest to the students that the results are important in lieu of increased harvesting pressure and possible ecological changes in salinity due to climate change. For example there may be a decrease in salinity over time due to increased rains that could result from climate change. We also discuss selective pressure and evolution with them. We invite them to explore additional experiments that could be conducted to further fine-tune our knowledge of the horseshoe crab and its biology.

Keywords: horseshoe crab larvae, inquiry-based experiments, invertebrates

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Introduction

Aren't horseshoe crabs amazing? These Darth Vader look-alikes have remained unchanged in looks since their appearance in the fossil record from over 445 million years ago (Rudkin et al., 2008). Once abundant, they are in danger worldwide. Various pressures have contributed to their decline worldwide. In Asia, habitat loss due to the building of sea walls is the culprit (Botton, personal communication). Along the Atlantic seaboard, horseshoe crabs are used for bait for a burgeoning eel fishery and whelk, and, for, of all things, their blood (Tanacredi, 2001). Their hemolymph elicits a general antibody response against pyrogenic bacterial toxins, and so is used to test pharmaceutical preparations for sterility. Leschen and Correia (2010) offer a good background on this medicinal use and also the capture of horseshoe crabs as bait for fisheries such as whelk. Horseshoe crabs are also very important in an estuarine ecosystem. Their eggs serve as food to many migratory bird species, notably the red knot (Niles et al., 2009).

Greene et al. (2011) studied heat shock proteins produced by horseshoe crab larvae that were exposed to different salinity concentrations. They hypothesized that, since horseshoe crabs have to withstand a wide range of salinities because of the tides, these proteins protect the animals. The literature offers many other studies of the immunology, genetics, anatomy, physiology, development and ecology of the horseshoe crab. Students might be assigned to present results from researching the literature along with their lab report.

Another activity that can be used in conjunction with this wet lab is to assign the students to read Niles et al. (2009) from which we make a list of stakeholders who might be interested in the horseshoe crab welfare, which has included

folks from those in the medicinal to the hotel business---the latter would cater to bird-watcher tourists who flock to see flocks of birds that are fattened on horseshoe crab eggs. More details on this activity that adds context to the lab activity can be found in Nolan (2009).

In searching for a "doable" experiment that would awaken an interest in this mysterious creature, we turned to hatching larvae from horseshoe crab eggs that were sitting in my refrigerator. Nolan obtained several thousand of these pinhead sized green eggs (but no ham) from Mark Botton, a horseshoe crab researcher at Fordham College. Botton has studied the developmental stages and ecology of horseshoe crabs and has written extensively on the topic (Botton et al., 2010). Botton said to keep a finger bowl of them in my refrigerator, change the water every week or two (20 parts-per-thousand Instant Ocean) and he said they would hatch into larvae and last at least through November. Well, they actually lasted till around April without feeding! Nolan and her students devised an inquiry-based experiment in which horseshoe crab larvae were exposed to various salinities and body contractions were counted. The trilobite or first instar stage is what we have used for this exercise (Fig. 1.) (See Botton et al. (2010) for a developmental timetable complete with photographs.)

Other ideas the students derived after this pilot experiment (as yet untested) are to repeat the experiments while varying the temperature and/or the amount of light. An idea from toxicology studies would be to see what salinity would cause fifty-percent of the larvae to die (lethal dose of LD_{50}).

Another inquiry-based experiment (based on one found in the literature) is to see what chemical odors either attract or repel larvae. Medina and Tankersley (2010) found that larval and juvenile horseshoe crabs changed orientation and sometimes movements toward or away from visual and chemical odor cues (or a combination thereof). They hypothesized that this aided them in their quest to survive against predation, or to consume prey. Saunders et al. (2010) found that adults used various sensory cues in finding and procuring mates.

Figure 1. Trilobite or first instar stage of horseshoe crab larva (photograph by Lauren Clarke, taken with a Motic camera under a dissection scope).

Student Outline

Materials and methods

- Horseshoe crab larvae in a common tank or finger bowl maintained at 20 ppt Instant Ocean™
- Dissection scopes
- Plastic droppers---tips can be cut off with scissors to make them a little wider for larger larvae
- Small petri dishes
- Sharpie marking pens
- Dropping bottle of various saline concentrations made with Instant Ocean™ ranging from 10 parts per thousand to 50 parts per thousand. (If you are required to make the solutions, use a stir bar to get the salts in solution. Ten parts per thousand is weight/volume; add 10 grams of Instant Ocean™ to a liter of water.)
- Motic or other cameras for recording body movements and contractions (optional)
- Watches, timers, stop watches or cell phones with timers

1. Label five small petri dishes with the five saline concentrations. Label a sixth with 20 ppt, as that is what the larva have been maintained in. Add the appropriate salinity to cover the bottom of the petri dish with a dropping pipette (a few mLs.)
2. Find two different horseshoe crab larvae from a common tank or finger bowl , preferably of different sizes. You will call #1 “small” and #2 “large”. Put these in our sixth small petri dish labeled “20 ppt”
3. At time “0” and working with a partner, place the larger larva in the petri dish with the desired concentration, beginning with 20 ppt, and start counting body contractions. Do not confuse this with leg movements—those can be too quick to count, or could be the data for another experiment. Count for one minute and record.
4. Transfer to the next salinity and repeat.
5. Repeat the experiment for the larger larva.
6. Put your data in Table 1 and also add to an Excel™ spreadsheet that is projected for the class to see. The class data are the replicates.
7. Conduct simple statistics such as mean, median, range, variance, and standard deviation of contractions and record your data in Table 2.
8. Is there a significant difference in the number of contractions in the larva at the various salinities?

Table 1. Sample spreadsheet for collecting the raw data for number of body contractions in horseshoe larvae at different salinity levels.

Salinity Level (parts per thousand)								
Larva #	**Size (lg/sm)**	**0**	**10**	**20**	**30**	**40**	**50**	**#Body Contractions/min**

Table 2. Number of body contractions in horseshoe crab larvae at different salinity concentrations; selected results from student experiments*

	0 ppt	**0 ppt**	**10 ppt**	**10 ppt**	**20 ppt**	**20 ppt**	**30 ppt**	**30 ppt**	**40 ppt**	**40 ppt**	**50 ppt**	**50 ppt**
2012	sm	lg	sm	lg	sm	lg	sm	lg	sm	lg	sm	lg
1/26	56	15	27	17	23	9	32	30	56	44	45	32
2/1	20	52	18	28	22	31	34	29	46	51	59	56
2/3	17	42	21	34	30	44	27	29	35	40	18	34
2/25	22	40	27	34	63	68	42	35	56	54	56	64
3/5	45	34	34	22	78	77	35	50	54	66	64	69
3/8	19	21	24	36	25	54	26	45	18	46	12	34
Mean	**29.83**	**34.00**	**25.17**	**28.50**	**40.17**	**47.17**	**32.67**	**36.33**	**44.17**	**50.17**	**42.33**	**48.17**

*(See statistical analysis in Appendix)

Notes for the Instructor

This lab could be part of a general biology survey course, an invertebrate zoology lab or an ecology lab. It could possibly be used as part of developmental biology lab, or, if one has expertise in physiology, it might be tied into that field.

We were provided with our initial set of eggs by Mark Botton from Fordham College, and with larvae for this workshop from Sixto Portilla and John Tanacredi of Dowling College. The Marine Biological Laboratory of Woods Hole, Massachusetts (**http://www.mbl.edu/**) and the Gulf Specimen Marine Lab will mail eggs and seawater to researchers affiliated with a school at cost. Farley (2012) reported a slower development time of the Massachusetts versus the Florida Gulf specimens. It would be interesting for students to test to see if there was a difference in developmental times based on geography. Botton et al. (2010:550) note that "the rate of larval development is highly plastic and is influenced by temperature, salinity, dissolved oxygen, and the presence of pollutants."

We conceived this idea for the ABLE workshop because the eggs stay viable for a long time in the refrigerator. Out of 1000 eggs, many larvae obtained in August when stored in the refrigerator will still be at the first instar stage after six months. If you are able to obtain a few hundred eggs from a researcher in the summer, or from a supply house, they will keep for the whole semester in uncovered finger bowls in the refrigerator. Change the water using Instant Ocean for a concentration of 20 parts-per-thousand every two weeks.

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Kathleen A. Nolan, Ph.D. is a professor of biology and Chair of the Biology and Health Promotion Department at St. Francis College. She has been a long-time ABLE and has presented numerous major and mini-workshops at ABLE conferences. She is interested in fish population genetics and biology laboratory education.

Mamuna Faizi, Alina Zhyvotovska, Lauren Clark, and James Foo are biology majors at St. Francis College who assisted in conducting the experiments.

Neeti Bathala, assistant professor at the University of the Arts, initially collected horseshoe crab eggs with Bob Ketchum on an ABLE expedition to the Delaware Bay and infected K. Nolan with her enthusiasm for learning about all things marine!

Sixto Portilla, Ph.D. candidate at Dowling College, John Tanacredi, professor of biology, Chair of the Department of Earth and Marine Sciences, Dowling College and Director of the Center for Estuarine, Environmental and Coastal Oceans Monitoring Facility (CEECOM), West Sayville, NY, and Mark Botton, professor of biology at Fordham College are really the horsehoe crab experts and were instrumental in providing eggs, larvae and background knowledge in order that this project could be presented.

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