Mussels are common marine and freshwater bivalves. *Mytilus edulis* is a common saltwater mussel known as a “blue mussel”. It is used as a model system for many research programs looking at the effects of environmental toxins on aquatic systems. Mussel muscles have some unusual properties, namely the presence of “catch” muscles that can sustain a contraction for a long time period. Neurotransmitters control the contraction and relaxation of the catch muscle. Release of acetylcholine from excitatory motor neurons causes the depolarization of the muscle fibers, an increase in the intracellular concentration of calcium, and muscle contractions. On the other hand, the release of serotonin from inhibitory neurons causes an increase in the intracellular concentration of cyclic AMP and muscle relaxation.

In this experiment, phasic and tonic responses of the anterior byssal retractor muscle (ABRM) to individual and repeated stimulus pulses will be recorded, and the effects of the direct application of acetylcholine and serotonin to the muscles will be measured. The ABRM which has a dual origin at the anterior point of the valves and an insertion at the base of the foot is the focal muscle. The ABRM is an example of a catch muscle, which is able to maintain a steady contraction with very little energy expenditure. This ability is important in anaerobic environments. The ABRM tends to contract tonically by slowly generating force and maintaining the contraction for some time after the excitatory input has ceased. The ABRM is used in this lab, because it is easily isolated, stimulated, and monitored by using a force transducer.

**Goals:**
1. Gain confidence and technique in using transducers and stimulators to examine basic physiological systems.

2. Learn about mollusc biology– from identifying key parts of their anatomy to understanding the connection between muscle physiological properties and their function.

3. Connect the principles of muscle contractions learned in lecture to the actual process of stimulating and recording muscle contractions in lab.

*Much of the text and content of this lab was provided by materials from iWorx.*
What should you bring to lab?
1. Illustrations of mussel internal anatomy, particularly the muscles, to use during your dissection. This can be from your textbook or other scientific/teaching sources. Without good illustrations, it will be very difficult to perform this experiment.
2. Hypotheses for the expected results from the three experiments.
3. Experimental designs for this experiment, preferably in the form of a table with a list of experimental parameters for stimulating the muscle (sample tables are provided below).

With what should you leave lab?
1. A lab notebook with careful illustrations of mussel anatomy from the dissection.
2. A spreadsheet containing the analyzed data from the laboratory.

Is there anything due after lab?
At the end of the lab period (noon), turn in a one page statement of how the results were consistent with, inconsistent with or not interpretable in relation to the hypotheses with which you came to lab. This can be part of the lab notebook pages or it can be turned in as a print-out.

Timeline:
9:05-9:30 am: Overview of the techniques to be used in the lab.
9:30-9:45 am: Practice running an experiment without using an animal.
9:45 -10:00 am: Prepare for your experiment by setting up an experimental design with a table of parameters and tests to be run. After receiving approval from the teaching staff, you may receive a mussel.
10:00-10:15 am: Dissect mussel.
10:15-11:15 am: Run experiments.
11:15 am-12:00 pm: Analyze, save data and write brief statement about findings in relation to original hypotheses.
The General Principles for Running the Mussel Lab

The general principle of this lab is to stimulate the muscle using electrodes and measure the force of muscle contraction using the force transducer setup used in the “physics of physiology” lab. The muscle will be stimulated with a range of different electrical pulses. During the experiments, the amplitude, frequency and duration of these electrical pulses will be varied. In addition, the effects of acetylcholine and serotonin on the muscle contraction will be measured. Analysis of the muscle contraction will include measurements such as contraction amplitude and contraction duration. The relevant stimulus parameters that can be varied are listed in Table 1 and the muscle contraction parameters are listed and defined in Table 2.

Table 1. Electrical stimuli to the muscle can be varied using a range of stimulus parameters which can be entered into Labscribe.

<table>
<thead>
<tr>
<th>Stimulus parameter</th>
<th>Units</th>
<th>Name of box in Labscribe</th>
</tr>
</thead>
<tbody>
<tr>
<td>pulse amplitude</td>
<td>V</td>
<td>Amp</td>
</tr>
<tr>
<td>pulse duration</td>
<td>s</td>
<td>W(s)</td>
</tr>
<tr>
<td>number of pulses</td>
<td>pulses</td>
<td>#pulses</td>
</tr>
<tr>
<td>pulse rate</td>
<td>Hz (pulses/sec)</td>
<td>F</td>
</tr>
</tbody>
</table>

Table 2. Muscle contractions can be measured using these parameters.

<table>
<thead>
<tr>
<th>Contraction parameter</th>
<th>Units</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>amplitude</td>
<td>V</td>
<td>voltage difference between baseline tension and tension at the peak of the twitch</td>
</tr>
<tr>
<td>contraction time</td>
<td>s</td>
<td>duration from the onset of contraction and the time when twitch tension reaches its peak</td>
</tr>
<tr>
<td>relaxation time</td>
<td>s</td>
<td>duration from the time at peak twitch tension to the time when the tension returns to the baseline value</td>
</tr>
<tr>
<td>latency</td>
<td>s</td>
<td>duration from the onset of the stimulus to the onset of contraction</td>
</tr>
</tbody>
</table>
The first experiment: characterizing the muscle’s response to varying voltage stimuli

Guiding questions:
- What is the relationship between the strength of the stimulus and the response of the muscle?
- Why doesn’t the muscle respond to low stimulus voltages?
- Why does the amplitude of the muscle response increase with increasing stimulus voltages? At high stimulus voltages, the muscle response reaches a maximum amplitude. Why doesn’t the muscle response continue to increase with increasing stimulus voltages?

1. Open Labscribe, load IPLMv4Complete.iwxgrp. Then, go to the Settings menu, then Animal Muscle, then select Byssal-Muscle-LS2.

2. In this lab, pressing record will not only record the output of the force transducer, it will also deliver an electrical stimulation to the muscle. Thus, the first step is to check the stimulation settings at the top of the screen. Press the stimulator preferences icon (a picture of two square wave pulses). The options for the stimulator preferences will appear above the recording trace and include pulse amplitude (“Amp”), pulse duration (“W(s)”)) and pulse rate (“Hz”).

3. Construct a table in Excel or your lab notebook that details the stimulus parameters and test number (e.g., Table 3).

4. For a first test, without stimulating the muscle, enter “0” into the Amp box, 1 into the # pulses box and 0.01 into the W(s) box. These settings will deliver 0 Volts, so the muscle will not contract. Press the Apply button (otherwise the settings will not change).

5. Press Record and Mark the treatment as 0 V. Press Stop.

6. Change the Amp value to 0.250 V and enter a label in the Mark box stating 0.25 V. Press Apply.

7. Press Record and then Stop when the muscle has stopped contracting. Note that it can take a long time for the muscle to relax – keep recording until the muscle force is back to the baseline force (the force being registered just before you applied the stimulus).

8. Continue increasing the voltage values at 0.250 V increments, until the amplitude of the muscle contraction no longer increases or you reach 5 V Amp value.

9. Save the file.

10. At the end of lab, you can measure the correlation between stimulus amplitude and muscle contraction amplitude and duration (Table 4 provides an example table for these measurements).
Table 3. Stimulus settings for testing the correlation between stimulus amplitude and muscle contraction amplitude and duration.

<table>
<thead>
<tr>
<th>Test number</th>
<th>Stimulus amplitude (V)</th>
<th>Number of pulses</th>
<th>Stimulus duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.50</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.75</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>6</td>
<td>1.25</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>7</td>
<td>1.50</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>8</td>
<td>We stopped the test because the muscle contraction amplitude was no longer increasing.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Measurements of muscle response to varying stimuli for testing the correlation between stimulus amplitude and muscle contraction amplitude and duration.

<table>
<thead>
<tr>
<th>Test number</th>
<th>Contraction amplitude</th>
<th>Contraction time</th>
<th>Relaxation time</th>
<th>Latency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
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<td>7</td>
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<td>We stopped the test because the muscle contraction amplitude was no longer increasing.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Now, let’s get fancier…

Experiment 2: The effect of number of pulses and pulse rate on the muscle, specifically testing for the presence of twitch summation and tetanus

Guiding questions:
- How does the amplitude of the contraction vary with the type of stimulus used, for example, a long duration pulse versus repeated pulses?
- If contraction amplitude is dependent upon the increases in concentration and persistence of intracellular calcium, why are the contraction amplitudes of single twitches the same?
- Tetanus occurs at a much lower stimulus frequency in this muscle than it does in vertebrate skeletal muscle. How might this be advantageous to a mussel, given the function of the anterior byssal retractor muscle?
- Why is the rate of muscle relaxation much slower after tetanus than after a single twitch?
- After a 5-second current pulse, how long does it take for the muscle to return to its baseline tension? How does this compare to the time it takes for the muscle to return to baseline levels after a series of short pulses?

a. The starting stimulus parameters should be set at:
   - 25 pulses (#pulses)
   - pulse amplitude (Amp) at the minimal value that yielded maximal muscle contraction in the first experiment
   - 0.5 Hz frequency (F)

b. Vary the stimulus frequency in steps up to a maximum pulse frequency of 20 Hz.

c. You can also vary the number of pulses and pulse duration to examine these effects.
Experiment 3: The effects of $10^{-4}$ M acetylcholine and $10^{-5}$ M serotonin on contraction and relaxation of the muscle

Guiding questions:

- How do neurotransmitters affect contraction and relaxation of the muscle?
- Was the maximum contraction with the addition of acetylcholine similar to the maximum contraction produced by electrical stimulation? Why might they differ?
- Compared to previous contractions, did the rate of relaxation change immediately after the addition of the neurotransmitter?
- Did the rate of relaxation and the amplitude of the contractions return to normal after the neurotransmitter was removed? Describe the time course of recovery and explain why it may be so slow.

a. Set the starting stimulus parameters as follows:
   - **pulse amplitude** (Amp) at the minimal value that yielded maximal muscle contraction in the first experiment
   - 0.005 s **pulse duration** W(s)
   - 1 pulse (#pulses)

b. Run a baseline test and then run the test again after adding a drop of $10^{-4}$ M acetylcholine using a dropper.

c. Quickly after running the acetylcholine test, remove the acetylcholine solution using a dropper and then add a drop of $10^{-5}$ M serotonin and run the test again using the same settings.

d. Wait for the muscle to completely relax and then apply the stimulus again.

e. Wait for the muscle to completely relax and then rinse the prep with buffer.

f. Run the same test again on the rinsed muscle and continue doing this every five minutes (keeping the muscle moist with drops of buffer).
The Dissection

The dissection should be started only after you have settled on an experimental design, run a pretend experiment and practiced analyzing the data from the pretend experiment. Solutions should be prepared BEFORE beginning the dissection.

Each group will only receive one animal.

1. Pry open the mussel and cut any remaining muscles that are keeping the valves from opening. Cut only along the posterior side, so that you don’t accidentally cut the anterior byssal retractor muscle. Anterior is on the side of the hinge closest to the origin of the growth rings (umbos); sometimes this looks like the “blunt” end of the shells. The big round, white muscles are the adductor muscles – it’s fine to cut those. Just next to them, however, are the small, white byssal retractor muscles. We will record from the anterior muscles. Once you have found the ABRM, you can cut one branch to the shell you wish to remove and leave the other branch intact.

2. Put the mussel in the dissecting dish and observe its anatomy. Several soft structures surround the tougher muscles. The mantle spreads over the surface of both valves and secretes the material that builds the shell. Much of the rest of the soft structures are the ctenidia, the filamentous structures that move seawater though the mussel and filter out its food.

3. Remove the mantle, the ctenidia, and all other structures except for the adductor muscles, the muscular brown foot and the byssal muscles.

4. Clean out and dry the dissecting dish and dry off the mussel shell. Place a lump of clay in the bottom of the dish and firmly push the mussel shell onto the clay so that it is not easily dislodged. Alternatively, you can bend a paper clip and brace the mussel into the tray using the wire. Pour a small amount of buffer into the shell to keep the muscles moist (do not fill up the dissection dish).

5. The anterior byssal retractor muscle is a white to yellow muscle that extends from its origin at the anterior tip of the shell to its insertion at the base of the foot.

Note: Isolate as much of the ABRM as possible, since it will be used to attach the muscle to the transducer.
Experimental Preparation

1. Place the force transducer on a ring stand.
2. Tie a piece of thread under the head of the hook-shaped pin.
3. Place the hook around the center of the anterior byssal retractor muscle (do NOT puncture the muscle).
4. Attach the thread to the hole on the end of the blade of the force transducer.
5. Adjust the height of the transducer so that the byssal muscle is lifted slightly.
6. Lay the stimulating needle electrodes on top of the byssal muscle on each side of the insect pin hook (do NOT puncture the muscle and make sure that the electrodes do not touch each other or the hook). Secure the needles in position by using a wad of clay to hold the wire along the edge of the dissection dish.
7. The muscle preparation used in this experiment is functional for a limited period of time. If the muscle is bathed periodically in the buffer solution, it will work for about two hours. To conserve time, complete all the exercises in the experiment before analyzing the data.