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# Developing an Intracellular Neurobiology Lab Sequence for Use in an Interdisciplinary Neurobiology Course

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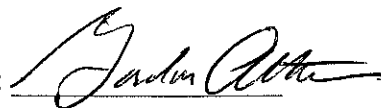Andrews University


Honors Thesis


Developing an Intracellular Neurobiology Lab Sequence for Use In An Interdisciplinary
Neurobiology Course


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Primary Advisor Signature: _____

Department: _____

**Abstract:**

The goal of my research was to evaluate procedures currently used in the Intracellular Leech Lab in order to modify the Neurobiology ZOOL475 Lab to increase the rate of successful intracellular recordings for future Neurobiology students. Two approaches were used: the first was to evaluate the possibility of using a less expensive leech species in order to allow students unlimited attempts at recording from ganglia without budget constraints. The second was to evaluate various raising conditions (water, temperature, and age) to optimize the quality of the leech ganglia. Both approaches provided useful information to improve lab success.

**Introduction:**

The concept for this project stemmed from an attempt to improve Andrews University's Neurobiology Lab. Historically, undergraduate level Neurobiology courses often did not offer laboratory sections to accompany their classes. Due to a grant from the National Science Foundation, Andrews University became one of the first universities to offer a laboratory course at the undergraduate level.

In recent years, the demographics of the students taking Neurobiology at Andrews University has changed - the enrolees were primarily upper division Biology majors, but currently there are both upper and lower division students from the Biology and Psychology Departments. The class size has likewise increased from 2 students in 2008 to 15 enrolled students in 2011. As the demographics have altered, so has the lab. No manuals for an interdisciplinary Neurobiology Lab exist, thus one has been composed by Dr. Atkins. At the end of the Neurobiology Lab Sequence, there are a series of 3 labs entitled "Intracellular recordings from Leech neurons" in which the students attempt to obtain and record and intracellular action potential recordings from a leech ganglion.

The reason that the *Hirudo medicinalis* leech is the species of choice for these labs is in its use in both research and medicine – doctors use leeches post-operatively for skin grafts, replantation of the digits, periorbital hematomas, and in a variety of other surgeries to restore blood flow and reduce inflammation through leeching (Mory *et al.* 2000). Researchers chose leeches based on the rhythmic dorsoventral contraction that is involved in their swimming pattern, which they used in order to create a neuronal model for behavior (Chen, *et al.* 2010). *H. medicinalis* became a popular research animal because of the abundance and bilateral, homologous, and linear positioning of the ganglia within each organism (Ort, *et al.* 1974).

Leeches are likewise popular specimens to use in Neurobiology, due to the large size and great number of ganglia enclosed within each animal.

The nervous system of the leech is laid out in a series of connected ganglia. One ganglion contains a bundle of neurons, from which one can measure action potentials. The leech is also the species of choice for intracellular labs due to the opacity of their ganglia, which makes the individual cells in the ganglion easy to identify under the dissection microscope (unlike the cricket ganglia, which take much a higher resistance electrode to puncture the ganglion due to the lesser opacity of their ganglia, which indicates a thicker ganglionic sheath). (Atkins & Pollack, 1987). This is the only undergraduate lab at Andrews University in which the students have the ability to record *intra*cellular action potentials – all other recordings are *extra*cellular.

The limitations of this particular lab at Andrews University have been the high cost of the equipment and supplies. The cost of one medicinal leech, which previous years have used, is $20.00 plus shipping, while a Common leech costs $4.00 plus shipping. Thus, if the ganglion opacity and sheath thickness is similar for a Common Leech, subsequent Neurobiology classes could use the cheaper species, which would allow more Common Leeches to be bought, in turn giving students more ganglia to practice on for the same cost as having fewer Medicinal Leech ganglia.

Yet another limitation in the Neurobiology Lab is the electrode puller. The settings for the electrode puller (both Heat and Solenoid settings) need to be within a narrow, specified range in order to pull intracellular electrodes under the desired resistance range of 30-50 M$\Omega$ (Atkins Lab Manual). This desired range of resistance indicates an electrode tip width that is sharp enough to puncture the ganglion sheath yet is narrow enough not to break the cells.

Leech care conditions have led to difficulties in obtaining "neuronally healthy" leeches that have opaque ganglia that are not excessively rubbery. Qualitative observations ("hunches") by Dr. Atkins in previous years of leech care seem to indicate differences in ganglion sheath thickness and opacity due to differences in: age, water, and temperature conditions.

Due to the difficulties encountered in recent years in obtaining data, I tested a series of variables within the parameters of the laboratory setup in an attempt to increase the number of successful intracellular leech recordings. My main goal in this lab was to ask: How can we improve the success rate of data collection for the Neurobiology Intracellular Leech Lab? To answer this question, I asked specific sub- questions: What are the optimal Pipette Puller settings to pull electrodes with the necessary resistance range (30-50 MΩ)? Is there a difference in ganglia consistency between Medicinal and Common Leech Species? What are the optimal raising conditions to obtain neuronally "healthy" leech ganglia?

Once I began my experiment, I discovered that the electrode puller displayed great variability between individual electrodes pulled under the same setting. Pulling multiple electrodes in series changed the shape of the coil and the puller oftentimes stopped pulling for some amount of time. Multiple pulls within a short interval changed the production of heat and pull by the same heat and solenoid settings. One electrode coil completely burnt out during the pulling process, and the settings had to be recalibrated to pull within the desired resistance range. There was very little repeatability in the electrode pulls; the standard deviations were high, indicating that there was not an optimal combination of heat and solenoid settings that would consistently pull at the desired resistance range.

Another issue I ran into was the difficulty of obtaining intracellular recordings. The time it took to obtain a single intracellular recording from a ganglion was 4 hours, disregarding

preparation and dissection time. In order to obtain an intracellular recording for 3 ganglia from 36 leeches, totaling 108 ganglia, I would have had to spend 432 hours at the lab. Thus, my methodology was changed from obtaining intracellular recordings to an indirect measure of ganglion sheath thickness through a comparison of opacity. Research shows that thicker, less penetrable ganglion sheaths are associated with less opaque ganglia (Atkins, et al. 1987; Muller, et al. 1981). Thus, by testing opacity (optical density) via a photographic method, one will be able to infer penetrability (neuronal "healthiness") of the leech ganglia.

The rationale for my new procedure involved taking 10 leech ganglia recordings from 1 leech since it is not cost effective to test 1 ganglion from 10 separate leeches under each condition (Approximate total cost: $3600 for *Medicinalis* and $720 for Common Leech = $4320 total). I tested for opacity by keeping F-stop the same and recording shutter speed. As a backup method of analysis I kept the F-stop and shutter speed the same and took photographs to compare opacity.

**Methodology**

Initial Methodology

Electrode Condition:

First, I pulled electrodes under a series of heat settings and solenoid settings. My range of heat settings varied from 18.8 to 19.1 and my solenoid settings ranged from 3.0-4.0, by gradations of 0.5. Once the electrodes were pulled, I connected each electrode to the intracellular recording apparatus. Then, I would conduct an Ohm's test in order to measure the resistance of the electrode to verify whether that particular electrode's resistance was in the desired 30-50 M$\Omega$ range. The electrode settings that were not in the desired range were deemed undesirable settings for Neurobiology lab while the electrode settings that were within the desired range were deemed to be the optimal settings for Neurobiology lab.

Leech Care Condition:

The initial methodology involved caring for leeches under a series of varying raising conditions, for temperature, age, and water. The temperature conditions were: 4°C, 10°C, and 21°C. The age conditions were old (3-4 weeks) and young (1-2 weeks). The water conditions were: lab-prepared solution (Basic stock solution per liter: 2.8 g NaCl, 0.05 g KCl, 0.08 g Ca(NO$_3$)$_2$ • 4 H$_2$O, 0.025 g MgSO$_4$ • 7 H$_2$O, 0.56 g Tris Buffer (pH to 7.4), the store-bought freshwater solution, and the Carolina® Biological Supply Company water in which the leeches were shipped (Muller, et al., 1981). One *H. medicinalis* leech and one Common leech would be raised under each combination of the 3 conditions, resulting in 9 young *H. medicinalis* leeches, 9 old *H. medicinalis* leeches, 9 young Common leeches, and 9 old Common leeches. Next, I dissected each of the leeches and removed 3 ganglia from each leech. Each ganglion was pinned down, the electrophysiology equipment was set up, and an electrode with a proper range of

resistance was used to puncture through the sheath of the ganglion. Then, the electrode placement was manipulated until an intracellular action potential was recorded on the LabChart® Program. Finally, I would conduct statistical analysis to find which leech care conditions and which species of leech obtained the most intracellular recordings, to determine which conditions were optimal to suggest for use in subsequent Neurobiology Labs. Due to time constraints and the difficulty of obtaining intracellular recordings, I was not able to continue this initial methodology past the attempts at obtaining intracellular recordings. Thus, I conducted a different methodology, to evaluate optimal leech species and care condition, using an indirect measure of ganglion sheath permeability.

Alternate Methodology

Leech Dissection Condition:

Following the period of leech care under varying conditions (found in the initial methodology), I dissected each of the 36 leeches, and extracted 10 ganglia from each leech. Then, I placed the ganglia under the microscope lens, with a drop of saline, and viewed each of the 10 ganglia under the light microscope. I took a photograph, using an underwater camera, by keeping the F-stop the same (F-stop: 4.4), with the dependent variable being the shutter speed, which I then recorded. Then, I converted the shutter speed fraction to a decimal, which indirectly indicated the optical density. As a backup method of analysis, I took a second photograph of each ganglion, this time keeping the F-stop and shutter speed constant (F-stop: 4.4, Shutter Speed: 1/125), in order to compare the opacity of the photographs in the Lightroom® program. I analyzed the data from the shutter speed fractions, conducting T-tests for age and species, one-way ANOVA's for average electrode resistances, the variability between 3 Leeches under the same condition, slide

preparation wait time, temperature conditions, and water conditions, and a Chi-square test for

Leech mortality under varying water conditions.

**Results:**

The average resistances for the combined heat and solenoid settings for the electrode puller were (Fig. 1): Heat: 18.8, Solenoid: 3.0; 2.918 MΩ with a standard deviation of: 2.49. Heat: 19.0, Solenoid: 3.5; 26.74 MΩ with a standard deviation of: 38.04. Heat: 19.0, Solenoid: 4.0; 75.55 MΩ with a standard deviation of: 38.06. Heat: 19.1, Solenoid: 4.0; 40.82 MΩ with a standard deviation of: 22.87. Heat: 19.3, Solenoid: 4.0; 181.18 MΩ with a standard deviation of: 109.32. +Even though two of the electrode settings, of Heat 19.0 and Solenoid 4.0 and Heat 19.1 and Solenoid 4.0, had average resistances within the desired range, the standard deviations of the averages were too high, indicating inconsistent electrode pulls.

Next, I tested the control of variability within 1 set of care conditions (Fig. 2): Condition - Species: *H. medicinalis* Leech; Water: Carolina Shipment; Temperature: 10°C. Mean: 0.00668 with a standard deviation of: 0.000406. The standard deviation range was small, indicating little variability within one set of care conditions.

I conducted a comparison of Mean Shutter Speed Comparison Between *H. medicinalis* and Common Leech Species (Fig. 3): the Common Leech mean shutter speed: 0.01761 with a standard deviation of: 0.01021. The *H. medicinalis* mean shutter speed: 0.00807 with a standard deviation of: 0.00186. I conducted a 2-tailed T-test p-value: 0.000488, which is less than $p = 0.05$, indicating a statistically significant difference between leech species.

I analyzed the Mean Shutter Speed Comparison of varying intervals of time between leech dissection and slide preparation of *H. medicinalis* (Fig. 4): The mean shutter speed for 0 Hours: 0.007285 with a standard deviation of: 0.00124. The mean shutter speed for 2-4 Hours: 0.00887 with a standard deviation of: 0.00183. The mean shutter speed for 6-8 Hours: 0.00724 with a standard deviation of: 0.00170. The p-value for the ANOVA between the three time

intervals was: 0.33264, which is greater than p = 0.05. The difference between wait intervals was not statistically significant.

The comparison of Mean Shutter Speeds of Varying Temperature Conditions for Common Leech (Fig. 5): Mean shutter speed for 4°C: 0.01267 with a standard deviation of: 0.00673. The mean shutter speed for 10°C: 0.02009 with a standard deviation of: 0.01092. The mean shutter speed for 21°C: 0.02127 with a standard deviation of: 0.01154. The p-value for the ANOVA of 0.43451 is greater than 0.05, indicating no significant difference in shutter speed of Common Leech ganglia under varying temperature conditions.

The comparison of Mean Shutter Speeds of Varying Temperature Conditions for Medicinal Leech (Fig. 6): Mean shutter speed for 4°C: 0.00773 with a standard deviation of: 0.00249. The mean shutter speed for 10°C: 0.00806 with a standard deviation of: 0.00208. The mean shutter speed for 21°C: 0.00832 with a standard deviation of: 0.00171. The p-value for the ANOVA of 0.84517 is greater than 0.05, indicating no significant difference in shutter speed of Medicinal Leech ganglia under varying temperature conditions.

The comparison of Mean Shutter Speeds of Varying Age Conditions for Common Leech (Fig. 7): Mean shutter speed for old leeches: 0.02128 with a standard deviation of: 0.01317. The mean shutter speed for young leeches: 0.01145 with a standard deviation of: 0.00425. The p-value for the 2-tailed T-test was 0.15839, which is greater than 0.05, indicating no significant difference in shutter speed of Common Leech ganglia under varying age conditions.

The comparison of Mean Shutter Speed Comparison of Varying Age Conditions for Medicinal Leech (Fig. 8): Mean shutter speed for old leeches: 0.00804 with a standard deviation of: 0.00179. The mean shutter speed for young leeches: 0.00794 with a standard deviation of: 0.00201. The p-value for the 2-tailed T-test was 0.91053, which is greater than 0.05, indicating

no significant difference in shutter speed of Medicinal Leech ganglia under varying age conditions.

The Mean Shutter Speed Comparison of Varying Water Conditions for Common Leech (Fig. 9): Mean shutter speed for Store Bought Solution: 0.01988 with a standard deviation of: 0.01696. The mean shutter speed for Lab Prepared Solution: 0.02024 with a standard deviation of: 0.00358. The mean shutter speed for Carolina Supply Company Solution: 0.01610 with a standard deviation of: 0.01141. The p-value for the ANOVA was 0.79064, which is greater than 0.05, indicating no significant difference in shutter speed of Common Leech ganglia under varying water conditions.

The Mean Shutter Speed Comparison of Varying Water Conditions for Medicinal Leech (Fig. 10): Mean shutter speed for Store Bought Solution: 0.00767 with a standard deviation of: 0.00097. The mean shutter speed for Lab Prepared Solution: 0.00797 with a standard deviation of: 0.00177. The mean shutter speed for Carolina Supply Company Solution: 0.00810 with a standard deviation of: 0.00216. The p-value for the ANOVA was 0.96089, which is greater than 0.05, indicating no significant difference in shutter speed of Medicinal Leech ganglia under varying water conditions.

I conducted a comparison of Leech Mortality Rate vs. Water Condition for Common Leech (Fig. 11): Store Bought: 5 dead; Lab Prepared: 1 dead; Carolina Supply Company: 0 dead. Chi Square p-value = 0.03010, which is less than 0.05, indicating that the correlation between Common Leech mortality rate and water condition is statistically significant.

I conducted a comparison of Leech Mortality Rate vs. Water Condition for Medicinal Leech (Fig. 11): Store Bought: 3 dead; Lab Prepared: 0 dead; Carolina Supply Company: 0 dead.

Chi Square p-value = 0.04960, which is less than 0.05, indicating that the correlation between

Medicinal Leech mortality rate and water condition is statistically significant.
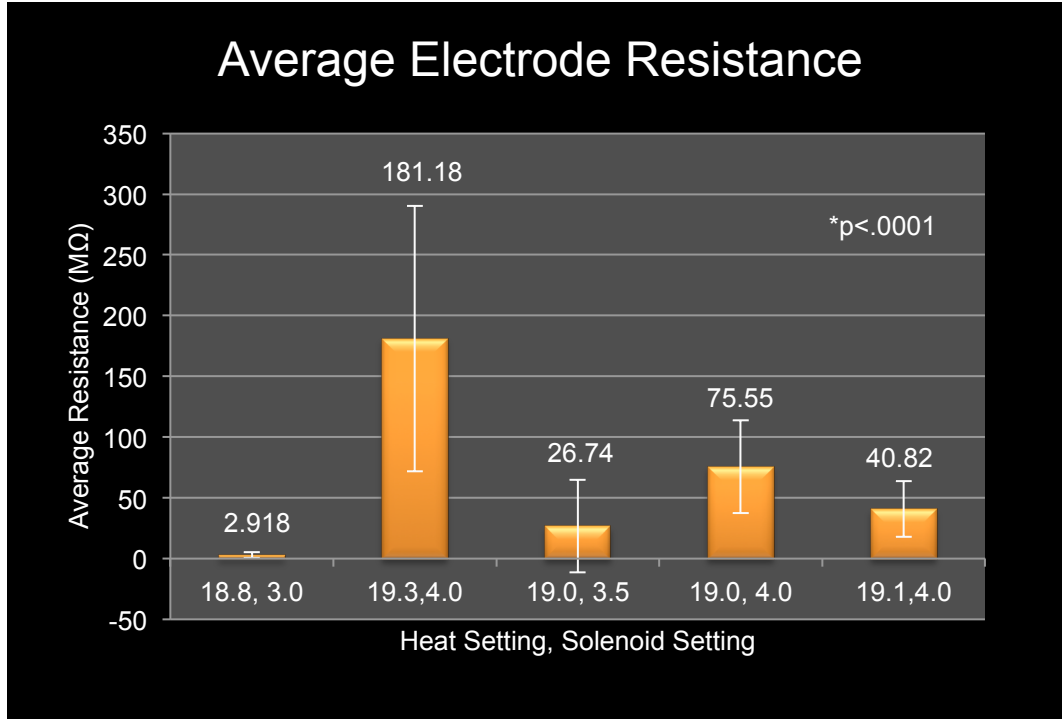


Figure 1: Electrode Pull Settings and Resistance Data
18.8, 3.0, n = 10
19.3, 4.0, n = 4
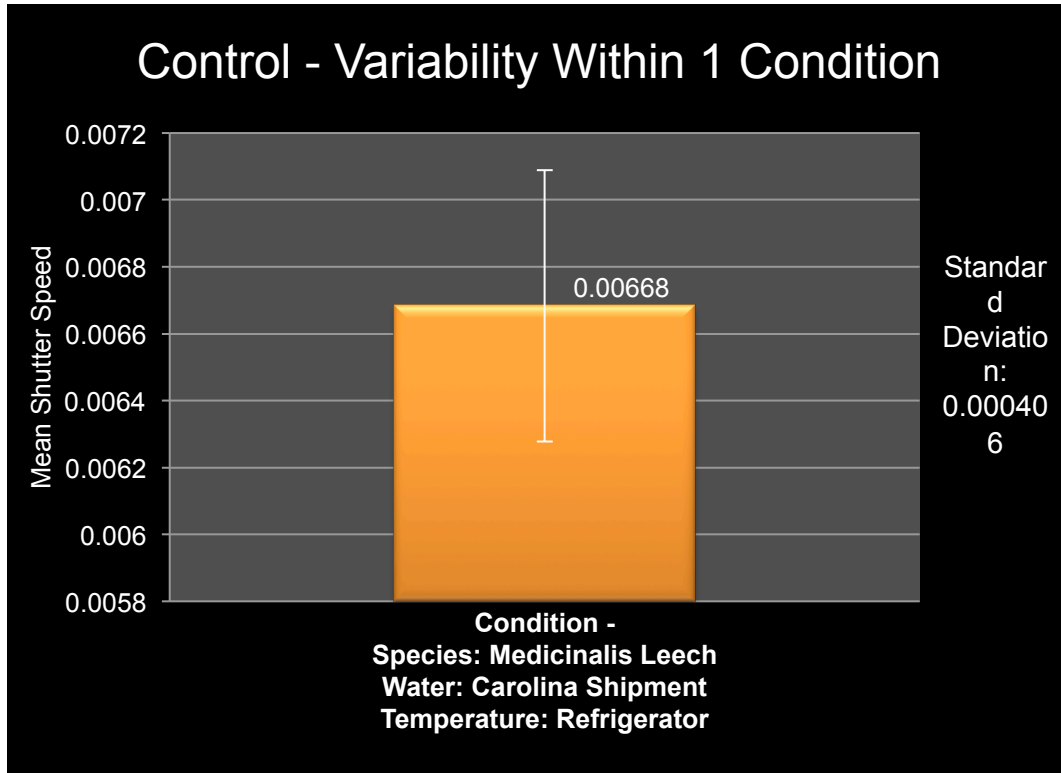19.0, 3.5, n = 3
19.0, 4.0, n = 9
19.1, 4.1, n = 9

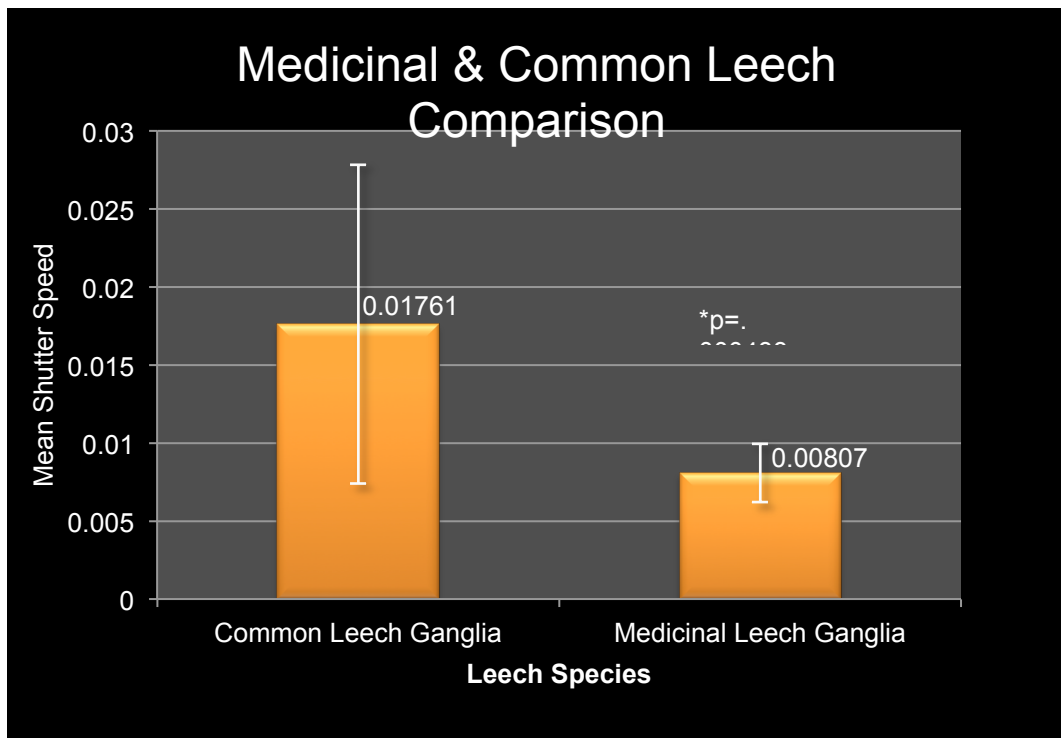Figure 2: Variability between 3 Medicinal Leeches under the Same Condition
n = 30



Figure 3: Mean Difference in Shutter Speed between Medicinal & Common Leech Species
Common Leech Ganglia, n = 16
Medicinal Leech Ganglia, n = 18
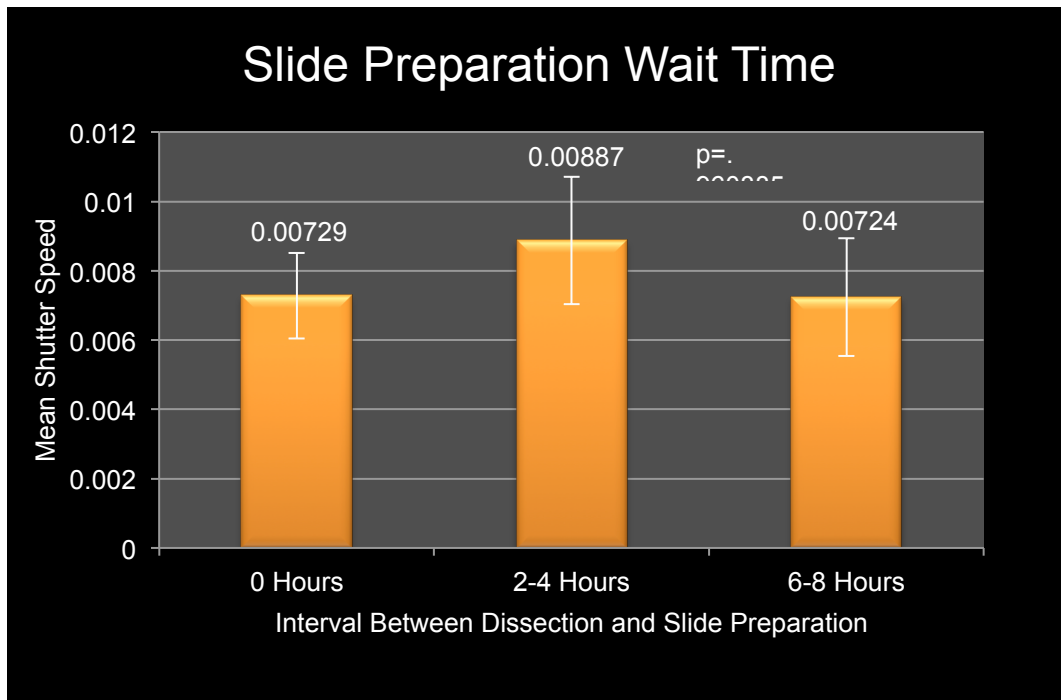
Figure 4: Mean Difference in Shutter Speed in Intervals between Leech Dissections & Slide Preparation
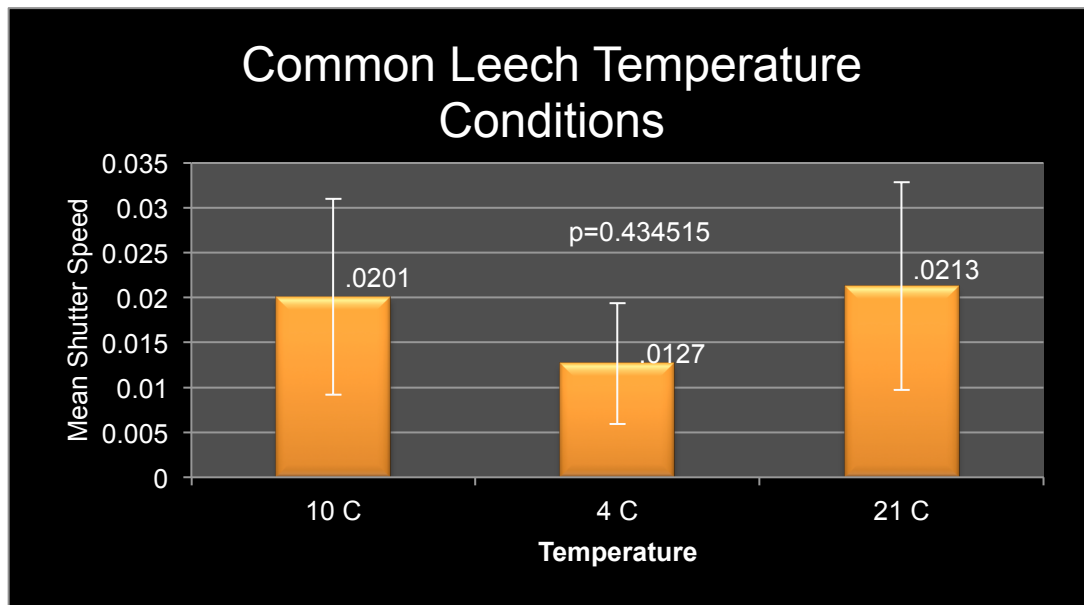0 Hours, n = 6
2-4 Hours, n = 6
6-8 Hours, n = 6



Figure 5: Mean Differences in Shutter Speed for Varying Temperature Conditions for the Common Leech
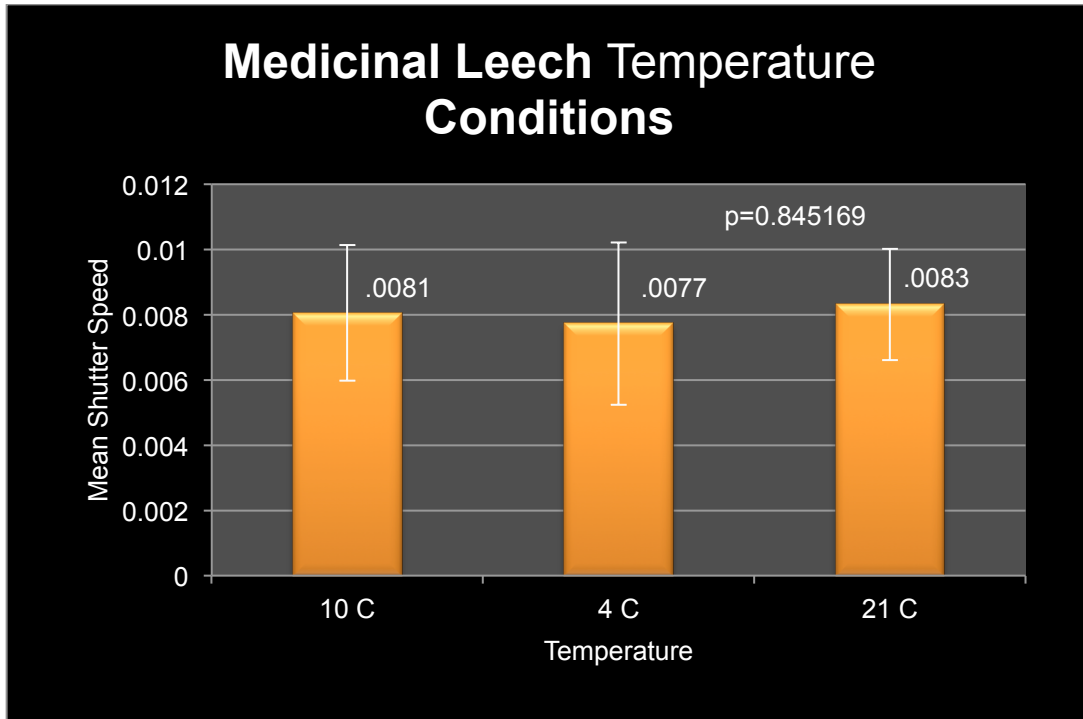10° C, n = 5
4° C, n = 4
21° C, n = 5

Figure 6: Mean Differences in Shutter Speed for Varying Temperature Conditions for the Medicinal Leech
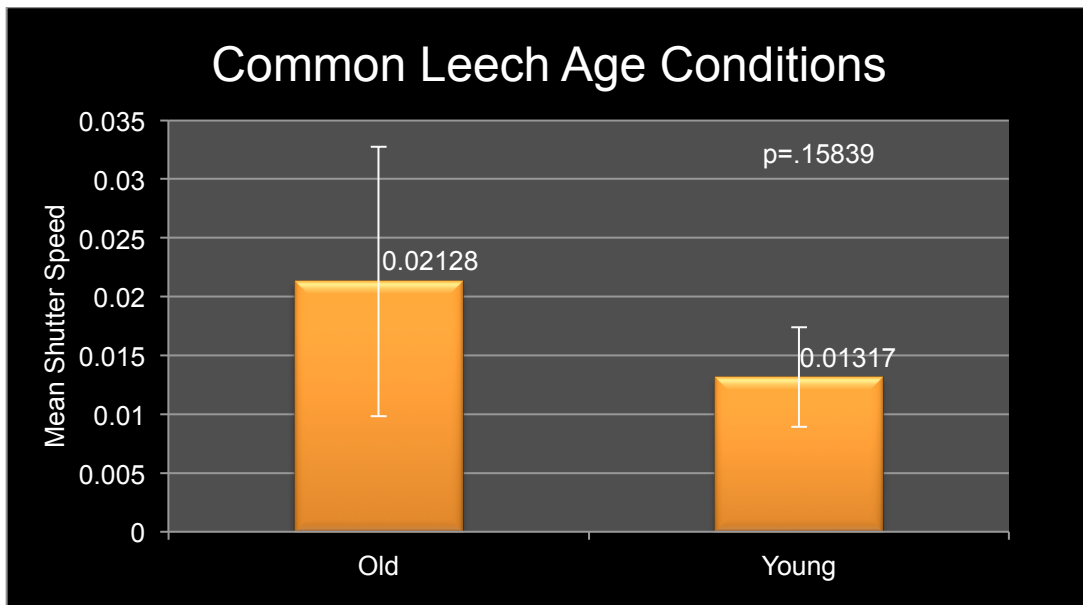10° C, n = 5
4° C, n = 8
21° C, n = 6



Figure 7: Mean Differences in Shutter Speed for Varying Age Conditions for the Common Leech
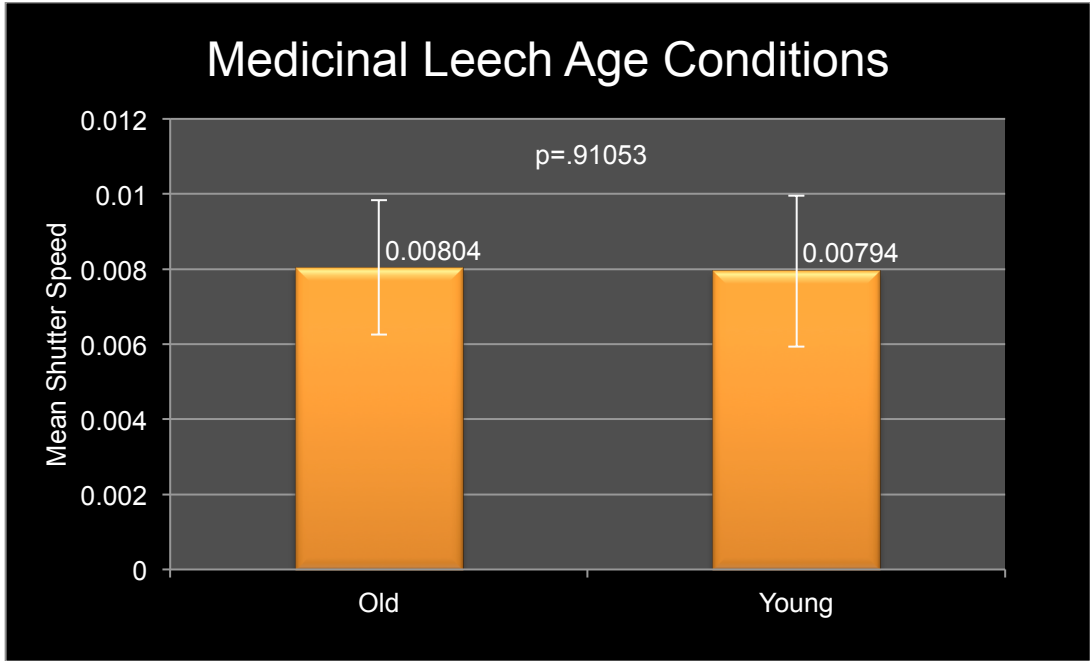Old, n = 9
Young, n = 5

Figure 8: Mean Differences in Shutter Speed for Varying Age Conditions for the Medicinal Leech
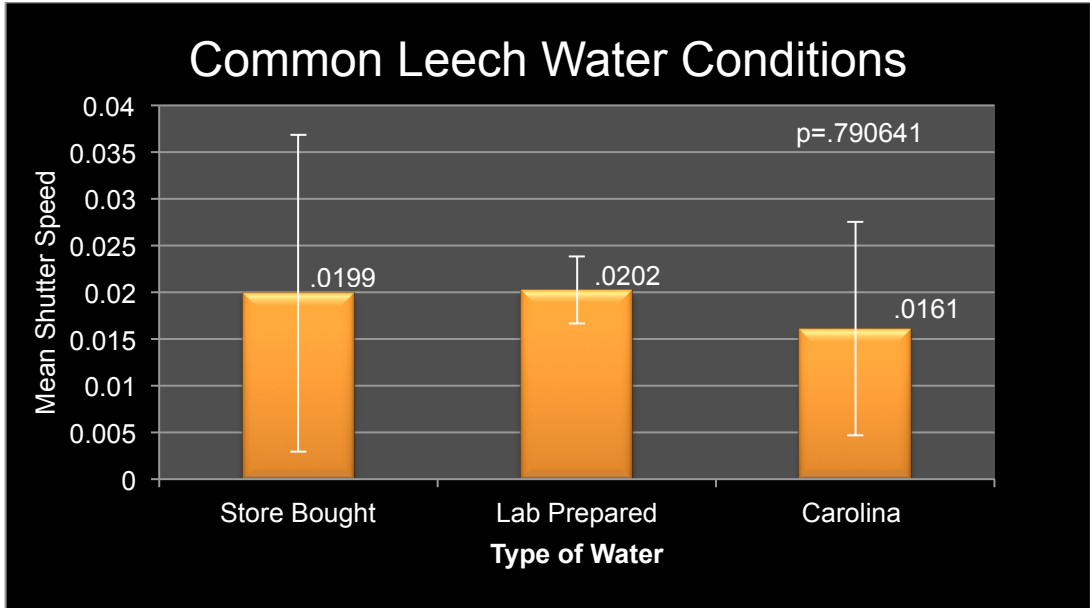Old, n = 11
Young, n = 8



Figure 9: Mean Differences in Shutter Speed for Varying Water Conditions for the Common Leech
Store Bought, n = 3
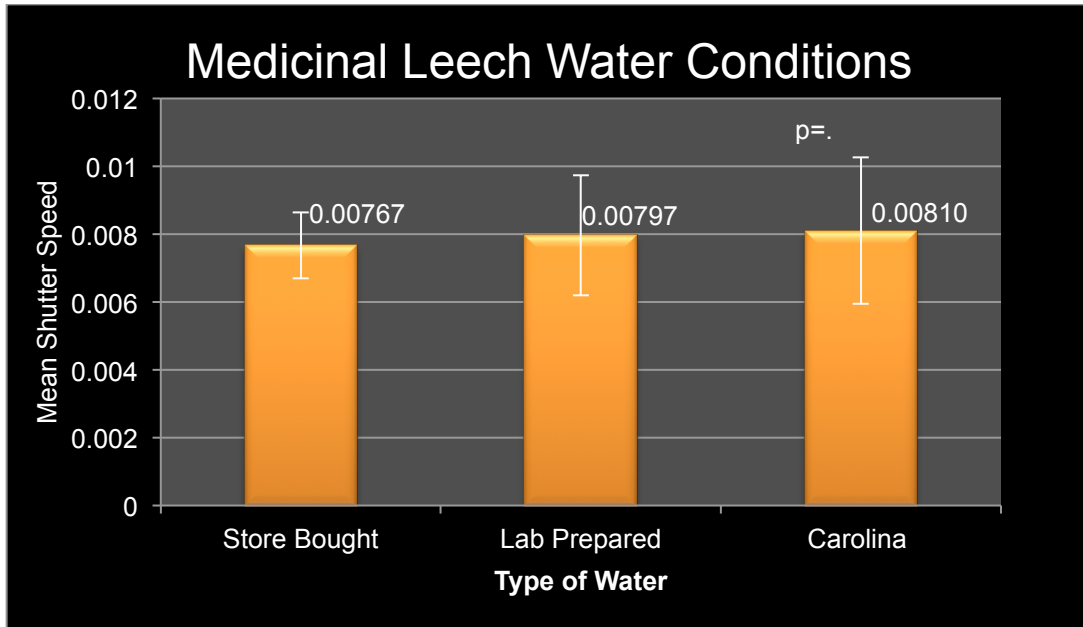Lab Prepared, n = 5
Carolina, n = 6

Figure 10: Mean Differences in Shutter Speed for Varying Water Conditions for the Medicinal Leech
Store Bought, n = 2
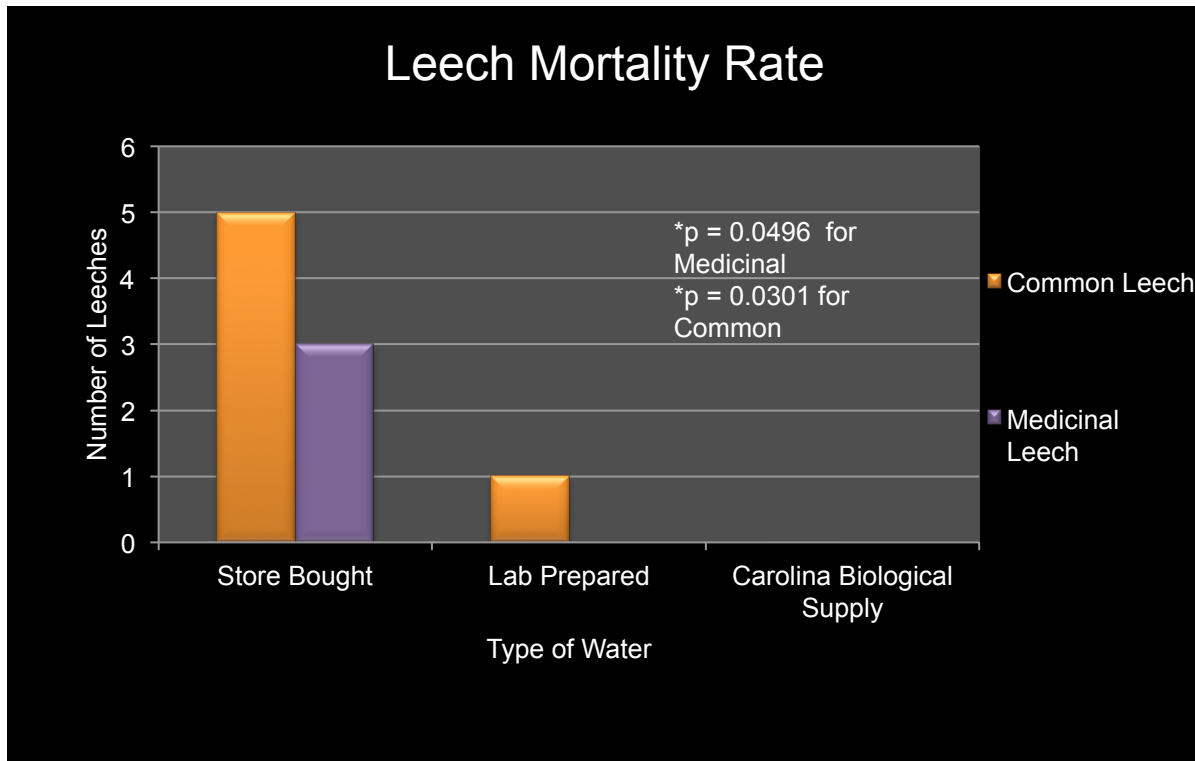Lab Prepared, n = 8
Carolina, n = 9



Figure 11: Leech Mortality Rates of Varying Water Conditions
n = 6 for each of the water conditions for both the Common and Medicinal Leeches

**Discussion:**

From my results, I obtained high standard deviation values for the means of the electrode pull settings, indicating great variability between individual electrodes under the same set of pull conditions. The differences between the varying temperature conditions for both the Common and Medicinal Leeches were not statistically significant. The differences between the varying age conditions for both the Common and Medicinal Leeches were not statistically significant. The differences between the varying water conditions for both the Common and Medicinal Leeches were not statistically significant. From my results, the factors that do not seem to affect the "neuronal" health of leeches are: temperature, age, and water (excluding mortality rates). The interval of time between dissecting the leech and extracting the ganglia and preparing the slides did not seem to make a statistically significant difference in the shutter speeds of the photographs.

Due to these results, my recommendations for future Neurobiology Labs would be to: buy 3 additional electrode pullers – one for each lab station. The cost of one electrode puller is $3000, so it may take a matter of a few years, but it would increase the consistency of the electrode widths to obtain the desired resistance range. Next, I would recommend conducting an Ohm's test each time one pulls an electrode, before attempting to use that electrode to puncture a ganglion, due to the variability in pulls. I would recommend using *H. medicinalis* species as the source of ganglia for the lab. I would advise against the use of store bought solution, to decrease leech mortality.

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