Developing a Staining Protocol for the Common Loon Syrinx

A Major Qualifying Project

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WORCESTER POLYTECHNIC INSTITUTE

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degree of Bachelor of Science

by

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Report Submitted to:

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Abstract: The Common Loon (*Gavia immer*) has four unique vocalizations attributed to its syrinx. This project aimed to develop a staining and storage protocol for Common Loon syringes. Cannell et al. published a protocol to stain muscle, bone, cartilage, and nerves in avian syringes in 1988. However, this protocol must be modified and adapted to each species, as the morphological changes affect stain uptake and tissue integrity. It was found in this study that the times, temperatures, and materials need to be adjusted in order to achieve optimal stain uptake and minimize tissue damage. Having developed a proper protocol, further studies can be done to determine the makeup of Common Loon syringes and how that contributes to this species' unique bird calls.

I. Introduction

In mammals, the organ responsible for vocal communication through tissue vibrations is the larynx (Bowling et al., 2020). Avian species, however, have a specialized organ for vocalizing known as the syrinx. Individual species have distinct differences in their syringes that affect their ability to produce certain sounds. Common loons, scientifically known as *Gavia immer*, are an avian species with a specialized set of vocalizations, but the anatomical and morphological makeup of their syrinx has never been officially documented. This is of interest to a number of conservationists, as having this information will contribute immensely to behavior, communication, and conservation studies for the species (Bowling et al., 2020; King, 1989). Therefore, this project aimed to study, document, and investigate the anatomy and morphology of the *Gavia immer* syrinx across gender and life development stages. If the anatomy of *Gavia immer* syringes from each gender and various life stages are documented then the structural morphology of gender and development can be quantified through measurements and staining

techniques to account for functional differences across development and gender lines. In order to ensure the continuity of syrinx research on *Gavia immer* going forward, this project aimed to get measurements of prominent structures and perform muscle, cartilage, and nerve stains to serve as a foundational baseline for comparative anatomy with other avian species. However, due to time constraints these measurements were unfortunately not taken, but the protocols developed and the stained samples will be left for future researchers to build upon.

1.1 Classifications of Syringes

The syrinx lies at the junction in the trachea where the bronchial tubes meet. The syrinx is composed of a cartilaginous framework made up of rings and the tympanum supporting a stretched membrane, producing vibrations using fine muscles controlling the tissue's tension, across the tracheal bifurcation (King, 1989; King & McLelland, 1984; Warner, 1972; Erdoğan et al., 2015). The tympanum is the structural cartilaginous framework of the syrinx, composed of multiple tracheal cartilages which fuse the frame to the base of the trachea (King & McLelland, 1984; Erdoğan et al., 2015; Gaban-Lima & Höfling, 2006). There are tracheosyringeal and bronchosyringeal cartilages, two pairs of vibrating tympaniform membranes, located both laterally and medially to the tympanum, and a central pessulus at the tracheal bifurcation (see Figure 1) (King & McLelland, 1984; Warner, 1972; Erdoğan, et al., 2015; Gaban-Lima & Höfling, 2006; Mohamed, 2017).

The pessulus is housed within the tympanum and acts to direct the vertical airflow resulting from a bird's inhalation into each bronchial tube while fine extrinsic syringeal muscles become taut or loosen for each type of vocalization (King & McLelland, 1984; Warner, 1972; Erdoğan, et al., 2015; Gaban-Lima & Höfling, 2006; Mohamed, 2017). The specific morphology

and placement of each component varies depending on age, size, sex, and life stage (King & McLelland, 1984; Ibrahim et al., 2020).

1.2 Types of Syringes: Tracheobronchial, Bronchial, Tracheal

There are three main types of syringes classified by the location of the syrinx relative to the trachea and bronchial tubes: tracheal, bronchial, and tracheobronchial. Tracheobronchial syringes are the most common type. They are found in the majority of birds and exhibit characteristics particular to non-oscine birds (Mohamed, 2017; Warner, 1972). For example, while a songbird may have five or more extrinsic syringeal muscle insertions, non-oscine birds typically have as few as one syringeal muscle, the tracheolateralis (King & McLelland, 1984; Warner, 1972). The density of musculature varies by species and complexity of birdcall. For example, oscine birds have increased syringeal muscles assisting with ornate bird calls whereas non-oscine birds, such as chickens, have fewer syringeal muscles but may have more tracheal muscles (Tsukahara et al, 2008). The typical tracheobronchial syrinx is located ventral to the esophagus and dorsal to the heart. It is made up of the three main regions; the tympanum, the intermediate syringeal section, and a bronchosyringeal section at the caudal region of the syrinx (See Figure 1) (Ibrahim et al., 2020). For an oscine bird, vocalizations are generated when the air sacs attached to the lungs compress, propelling air through the lungs and syrinx where syringeal muscles constrict the inner syringeal aperture causing the tympaniform membranes to vibrate (Tsukahara et al, 2008).

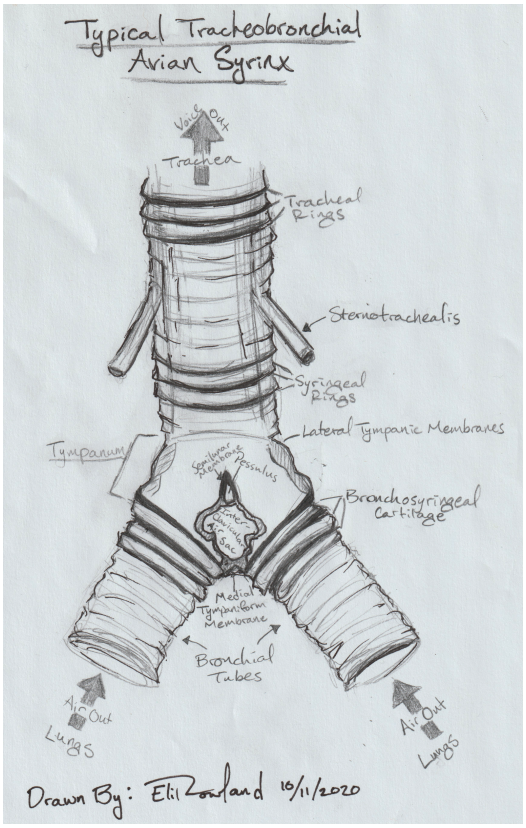

Figure 1. Diagram of a Typical Avian Tracheobronchial Syrinx During Exhalation. (Image generated by Elisheba Rowland, 2020).

The tracheobronchial syrinx pictured in Figure 1 differs from the other types of syringes in a few key features. The presence of a centralized tympanum at the tracheal bifurcation is the hallmark of a tracheobronchial syrinx as well as having tympanic membranes symmetrically placed on the medial and lateral edges of the tympanum and bifurcation (King, 1989). Additionally, tracheobronchial syringes and tracheal syringes typically have an interbronchial ligament which serves no purpose with respect to vocalizations, but is rather structural in function (See shaded tissue stretched between the bronchial tubes in Figure 3) (King, 1989).

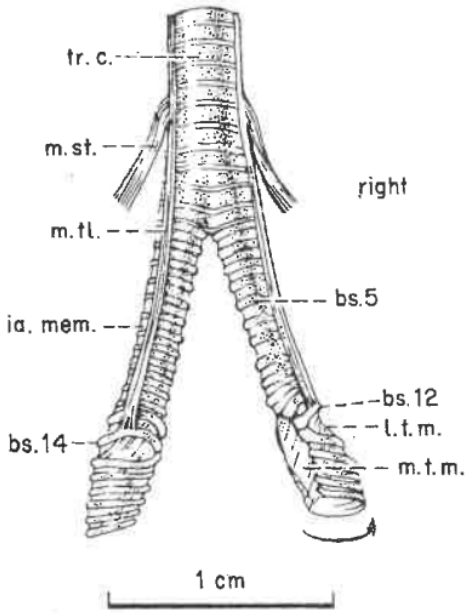

Figure 2. Diagram of a Typical Bronchial Syrinx (King, 1989).

The dorsal view of a typical Bronchial syrinx is shown in Figure 2 above. This particular syrinx is a more extreme case in which the structural differences to either type of syrinx are readily seen (King, 1989). Most notably, there is a total absence of tympanum and no specific tracheosyringeal structures or cartilages, because of this the syrinx is considered to begin at the bifurcation (King, 1989). Additionally, while the other types of syringes typically have one, the bronchial syrinx does not feature an interbronchial ligament. Structures labeled m.t.m. and l.t.m denote the medial and lateral tympaniform membranes, respectively. These are very different from the other syringes as these membranes are not only vastly inferior to the tracheal bifurcation, but also exhibit some bilateral asymmetry as the left membranes are located inferior to the right membranes (King, 1989). The left tympaniform membranes are located at bronchosyringeal cartilage 14th, rather than the 12th, bronchosyringeal cartilage on the right (King, 1989). The medial tracheolateralis (m.tl.) has an insertion point on the interannular

membrane (ia.mem.) and shares another insertion on the tracheal cartilage (tr.c.) with the medial sternotrachealis (m.st.) (King, 1989).

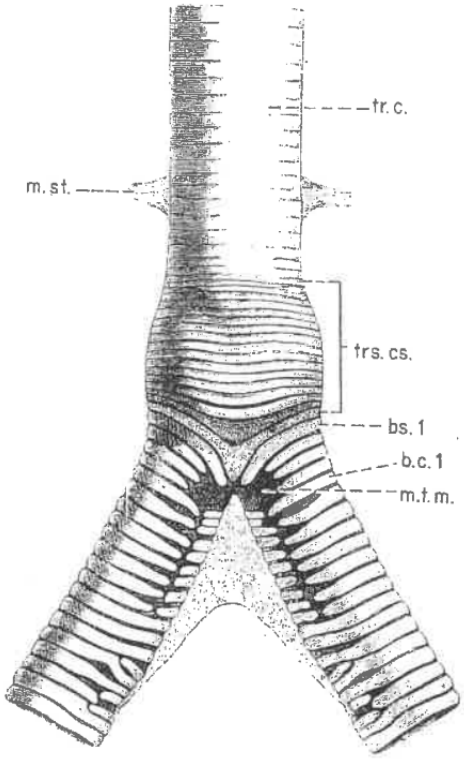

Figure 3. Diagram of a Typical Tracheal Syrinx (King, 1989).

Figure 3 above pictures a standard example of a tracheal syrinx which features no medial tympaniform membrane. Additionally, there are fewer bronchosyringeal cartilages that are present but not fully closed rings allowing for the specialized placement of vestigial medial tympaniform membranes denoted above by m.t.m. The closed rings following these cartilages are not bronchialsyringeal but rather true bronchial tubes (King, 1989). The m. tracheolateralis is sometimes absent in this type of syrinx but if present, as shown in Figure 3, would insert at the point of attachment for the m. sternotrachealis (King, 1989). Due to the tracheal nature of this type of syrinx, the number of tracheosyringeal cartilages are often reduced and sometimes not

present in lieu of a tracheosyringeal membrane, not pictured above, but located on the dorsal side at the segment labeled trs.cs. (King, 1989).

1.3 Communication Differences Based on Age and Sex

We do not know how the syrinx of the *Gavia immer* compares to that from the previous examples because the anatomical and morphological makeup of their syrinx has never been documented. Despite not knowing syrinx structure, the types of sounds that they can produce have been extensively studied. *Gavia immer* can produce four kinds of vocalizations, which are hoots, wails, tremolos, and yodels (McIntyre, 1988).

Hoots typically serve as a means of communication between individual members of a group, but they are also used to communicate with the larger group during migration (McIntyre, 1988).

Wails are used to get the attention of another loon in the population and get them to come over. For example, these calls are widely used to attract mates or communicate with family members, including partners and offspring (McIntyre, 1988). There are three types of wails; these are one-note, two-note, and three-note wails. As is implied by the names, a one-note wail only has one note, a two-note wail contains two, and a three-note wail contains three. The number of notes indicates the level of importance and urgency of the call, with the one-note conveying the lowest level of urgency and the three-note indicating the highest (McIntyre, 1988). Two and three-note wails are particularly interesting because they utilize each side of the syrinx individually to produce two unique sounds that are vocalized at the same time. This is known as the two-voice-phenomenon (McIntyre, 1988). The structure of the syrinx enables this

phenomenon, and mechanisms of such can be investigated by staining the syrinx and studying its composition.

Tremolos are used by *Gavia immer* to warn others about a threat or danger. Similarly to how *Gavia immer* can produce three variations of a wail, they can also produce three types of tremolo calls (McIntyre, 1988). However, this time instead of the amount of notes denoting urgency, that is indicated by the frequency of the call. Type one tremolos are the lowest frequency, which means that they are the least urgent form. Type three tremolos have the highest frequency of the three, so they indicate the highest sense of urgency. In between those two calls is the type two tremolo that has an intermediate frequency; it is more urgent than a type one, but less so than a type three (McIntyre, 1988).

The fourth, and final, type of call that *Gavia immer* can produce is called a yodel, which is typically observed during territorial disputes (McIntyre, 1988). Male murres, another non-oscine bird, have been found to have larger syringes than females (Miller, 2007). This finding would make sense in *Gavia immer* as well given that only males are capable of yodeling. Additionally, this call is very distinct compared to the other three because individuals can be identified by their yodel since no two birds produce one that is exactly the same (McIntyre, 1988). In addition to this sound only being produced by the males of the species, only those over three to four months of age are capable of yodeling (McIntyre, 1988). Until they hit this age milestone, young *Gavia immer* are only able to peep, yelp, and wail which are mostly just utilized to communicate with parents and make distress calls when necessary (McIntyre, 1988). This indicates that the syrinx of a *Gavia immer* undergoes anatomical and morphological changes as it matures. This project aimed to develop a proper staining protocol for common loon

syringes, the development of which has tremendous implications for being able to study these changes in the future.

II. Materials and Methods

Figure 4 below shows the overall protocol for staining the muscle, cartilage, nerves, and bone of common loon syringes. It is based on a 1988 protocol for staining avian syringes (Cannell, 1988) and includes the modifications that were made to adapt this protocol specifically to common loon syringes.

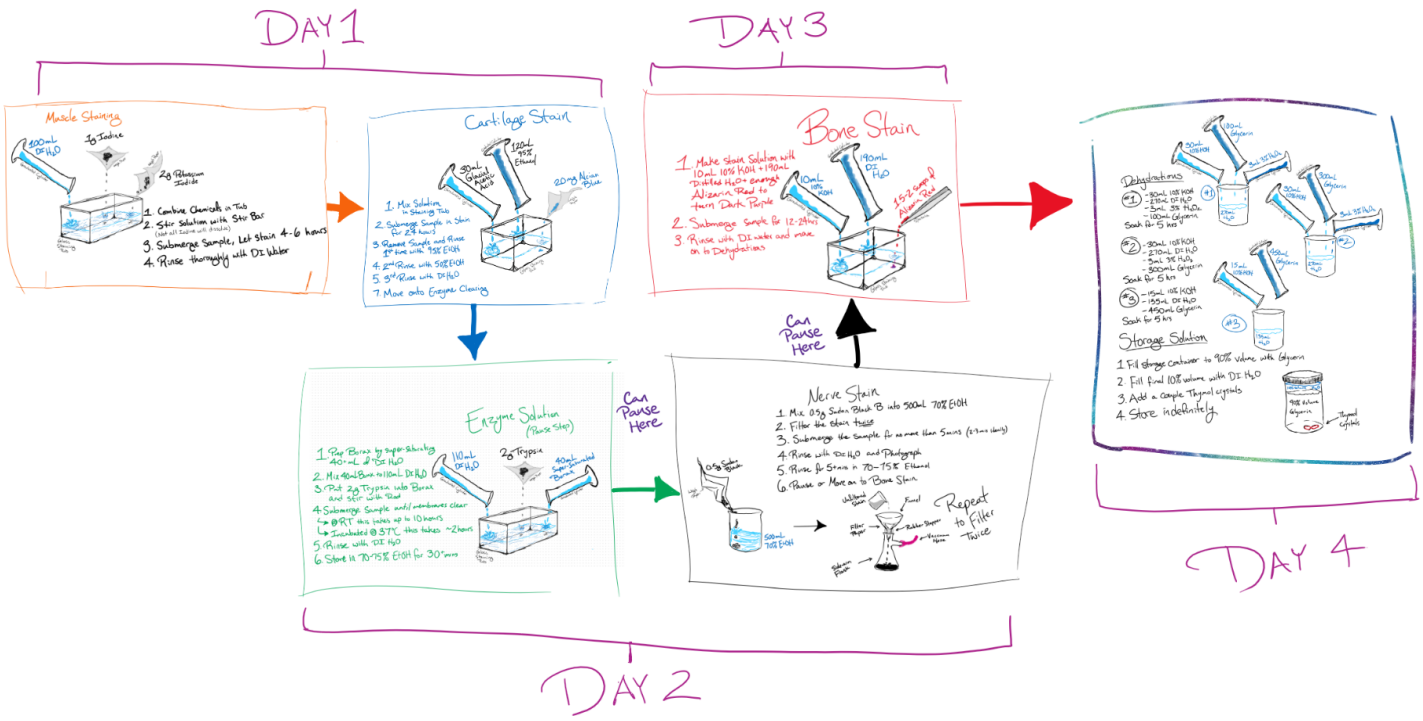

Figure 4. Flowchart diagram depicting the timeline and protocols for staining, based on the Cannell 1988 protocol for staining avian syringes.

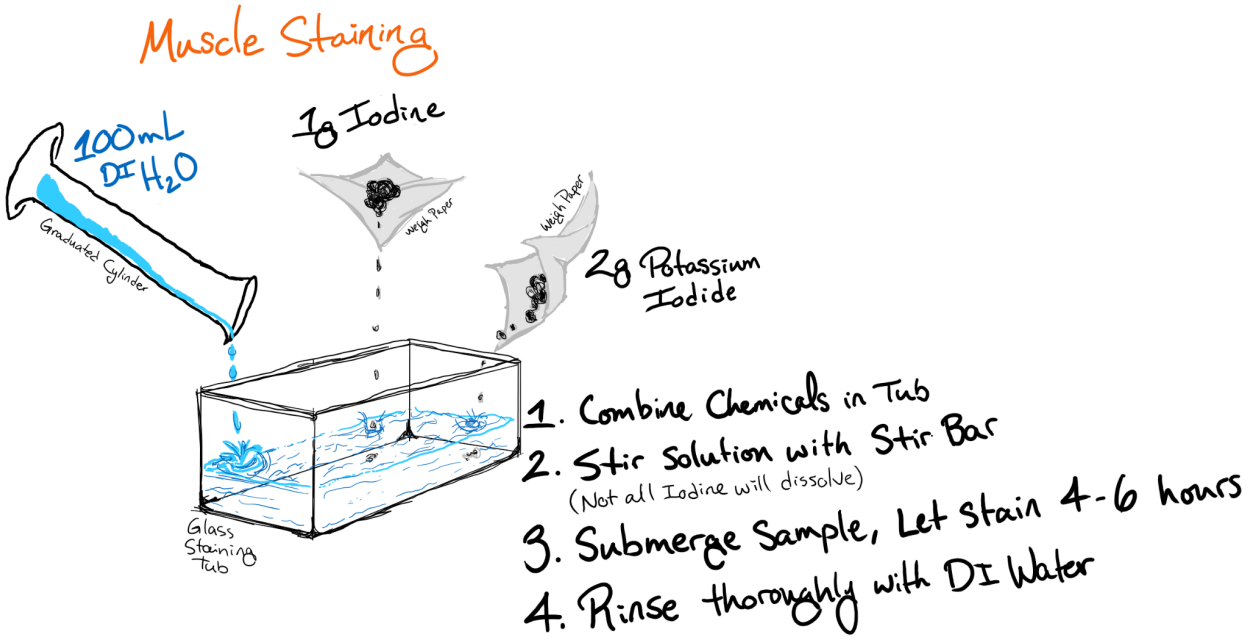

Figure 5. Muscle staining protocol with experimental modification to duration of stain.

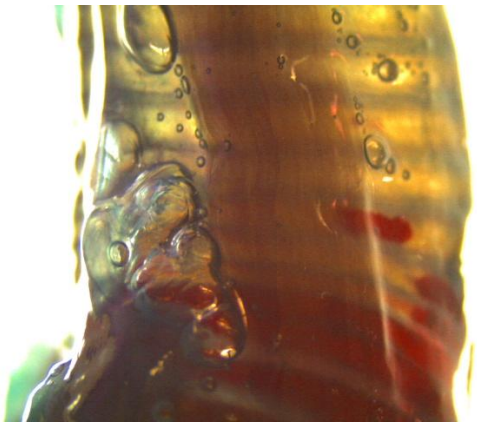

Figure 6. Staining of the sternotrachealis muscle, which is evident by the long brown strip spanning the trachea of the syrinx.

The muscle stain was successful, although the length of time for the sample to remain submerged in the stain was adjusted for optimal stain uptake. Cannell et al. called for the syringes to be submerged in the staining solution for "several minutes" in a dark location. However, it was determined experimentally that 4 to 6 hours was ideal for significant uptake in common loon syringes as can be seen in Figure 6.

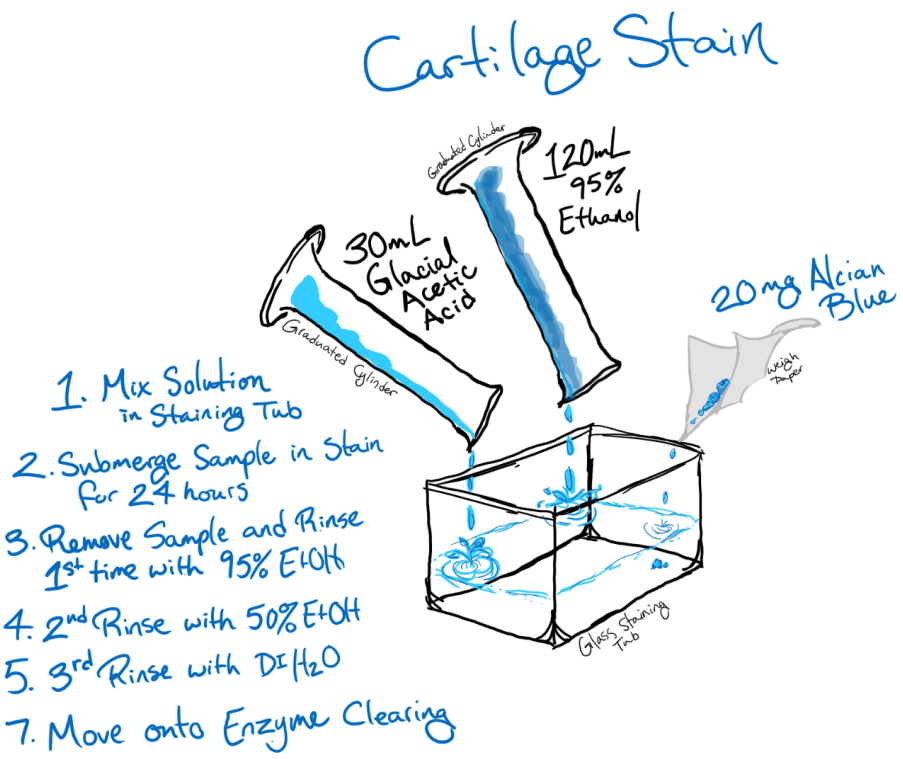

Figure 7. Cartilage staining protocol according to Cannell, 1988.

Figure 8. Blotchy staining that resulted from undissolved alcian blue powder that sunk to the bottom of the staining container.

The cartilage stain shown in Figure 7 also worked as expected, though the staining could be blotchy as seen in Figure 8 due to insoluble alcian blue particulates collected at the base of the staining container. This can be resolved by either mixing the solution with a stir bar on a stir plate for an extended period of time until all of the powder has dissolved or filtering the solution to remove any remaining particulates. After cartilage staining, the entire syrinx will appear blue-green, including target membranes, which requires an enzyme clearing solution (see Appendix II). The ideal conditions for trypsinizing the syringes to clear the blue stain from target membranes was determined experimentally to be approximately 90 minutes to 2 hours at 37°C or 9 hours at room temperature.

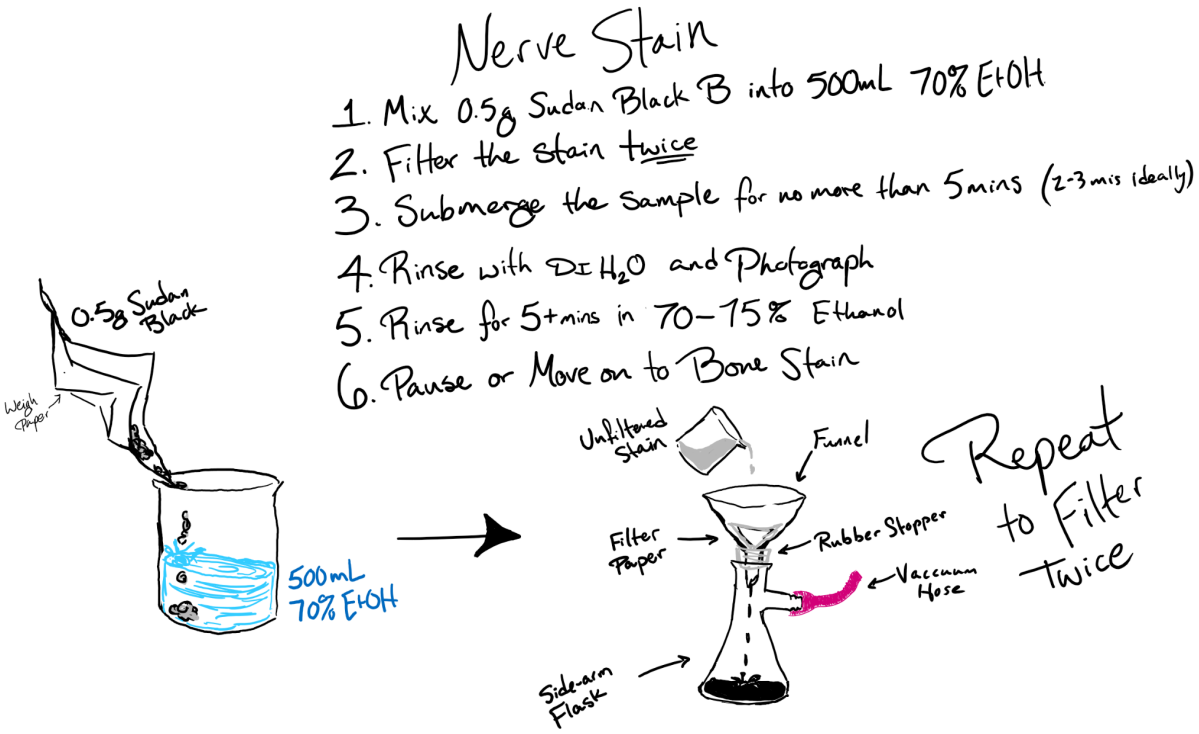

Figure 9. Nerve staining protocol with experimental modifications made to length of time and solution preparation

The nerve stain protocol shown in Figure 9 was successful, but it needed to be double filtered prior to submerging the syrinx in the solution, as the stain was very saturated with Sudan Black B powder when initially mixed. When left unfiltered, the solution was too concentrated and resulted in a very dark blue color that could obstruct previous stains. Additionally, the Cannell, et al. protocol called for the syrinx to be submerged in this staining solution for 30+ minutes, but it was determined experimentally that 2-3 minutes was sufficient to see significant stain uptake in common loon syringes. Lastly, the bone stain in Figure 10 worked exactly as expected and did not require any modifications. Additionally, the dehydration and storage solutions shown in Appendix II keep the stained syringes well hydrated and predominant colors preserved.

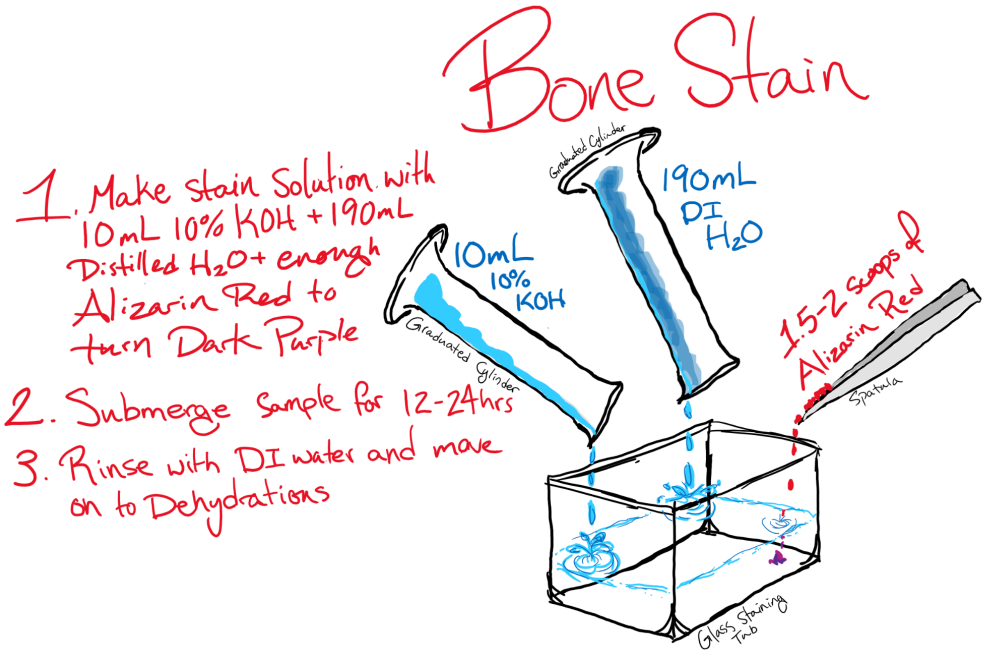

Figure 10. Bone staining protocol according to Cannell, 1988

For further detail regarding the protocols and suggestions for troubleshooting any issues that may arise, please see the functional staining guide (Appendix I).

III. Results

Overall, the stains showed optimal visualization of the muscle, cartilage, nerve, and bone once the appropriate modifications were made. This is known because the stain uptake in the syringes that were used in this experiment was very similar to the expected staining patterns as can be seen in Figure 11 below. This diagram was generated based on the literature detailing the makeup of the syringes of other avian species (King, 1989; Ibrahim et al., 2020; Miller, 2007), and we expected the main anatomical and morphological features to be fairly similar, which was the case here.

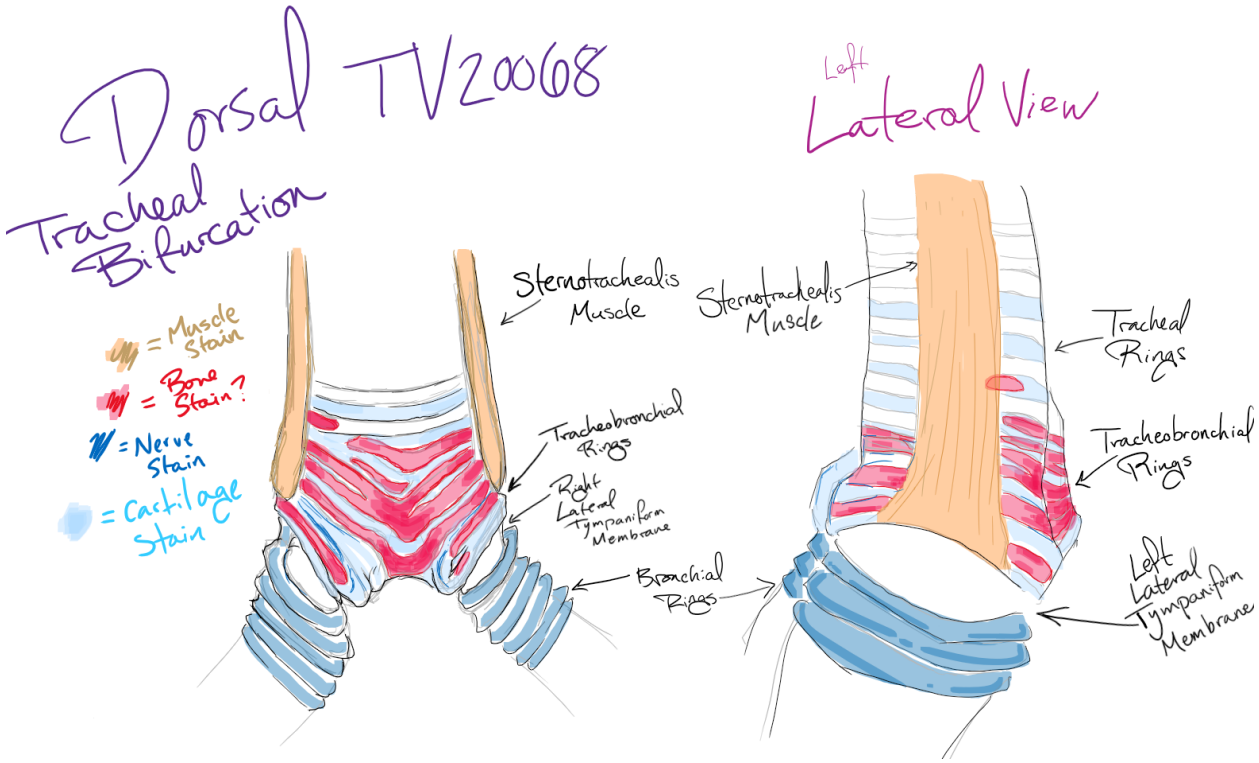

Figure 11. Diagram of stained syrinx TV20068 showing bone stain in red, muscle stain in brown, cartilage stain in light blue, and the outline of nerves in dark blue lines along the bifurcation.

The modifications made to the Cannell protocol were noted in the "Materials and Methods" section above, as well as in the functional staining guide (Appendix I). However, an issue that could not be resolved by modifying the length of time in solutions, temperatures, or concentrations was that the syringes were very damaged after the muscle and cartilage stains. They were very rigid and dehydrated, such that the membranes tore in multiple places (see Figures 12 and 13).

Figure 12. Sample TV20070 showing dry and torn membranes.

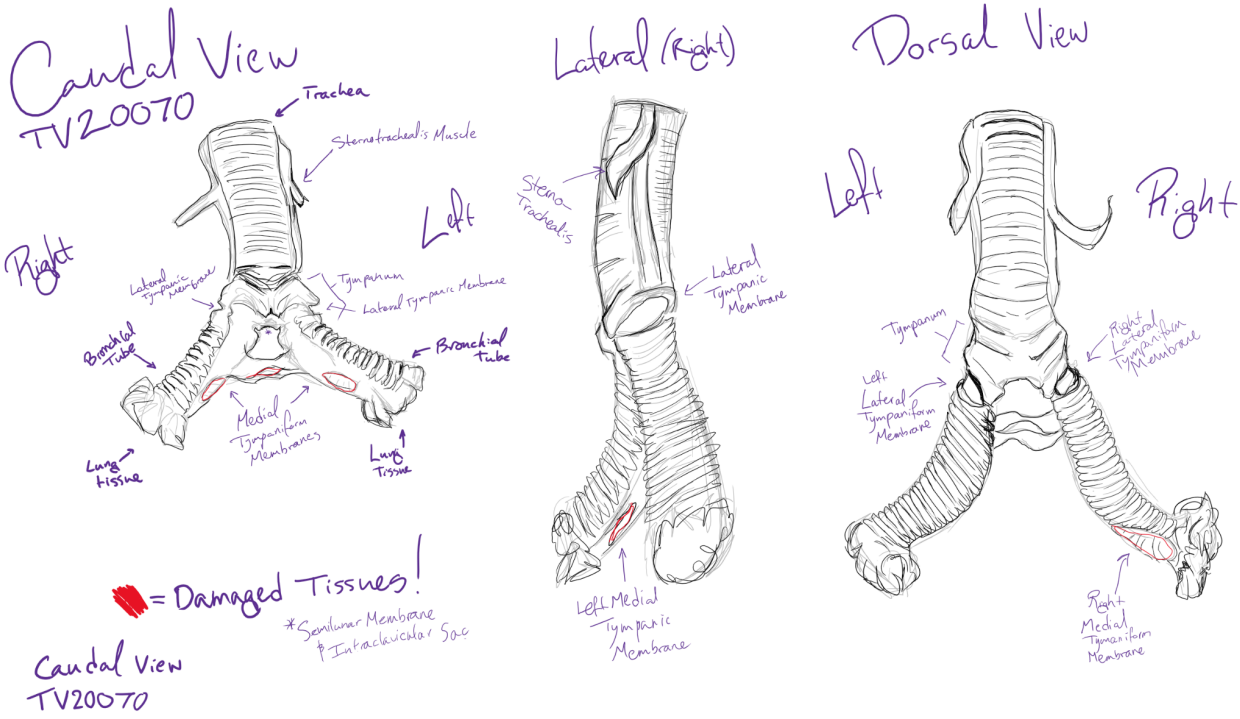

Figure 13. Diagram of damages to sample TV20070 outlined in red.

This damage was suspected to be due to long-term formaldehyde exposure, as the Cannell protocol only dictates for syringes to be in formaldehyde for a few days prior to staining whereas those used in this project were in formaldehyde for three to six months prior to staining. To test if this finding was in fact due to long term formaldehyde exposure, one syrinx was prepped in 10% methylamine in ethanol solution for one week prior to staining. The sample was initially in very poor shape - quite literally disintegrating in the formaldehyde - but upon removal from the methylamine solution it had appeared to repair itself. The syrinx was rehydrated and the membranes were no longer peeling apart. Furthermore, the sample remained in this preserved condition after muscle and cartilage staining. This confirmed that long-term storage in a formaldehyde solution resulted in negative effects on the syringes when submerging them in the potassium iodide-iodine solution. Unpredicted molecular interactions between the

iodine and the formaldehyde resulted in dry, stretched, brittle tissue that ultimately caused tears along target membranes shown in Figures 12 and 13.

IV. Discussion and Conclusion

Ultimately, the processes developed in this experiment outline proper staining techniques as well as some troubleshooting remedies for studying the syrinx of the common loon. Although the initial goal of documenting the morphological differences across gender and developmental life stage was not able to be achieved here, the staining guide that was finalized can provide insight to future researchers on how to stain syringes for imaging and measuring purposes. Major structures - such as the bony or cartilaginous rings, the pessulus, and the tympanic membranes - can be minutely measured. Thus, images generated through these protocols can serve as comparative models for the anatomical and morphological differences of loon syringes across not only sex and age lines, but also across species lines. As a note, the staining protocols may require further adjustment when handling adolescent syringes as the delicate tissues may be more sensitive to the solutions utilized. Additionally, in order to preserve the health of the tissues formaldehyde should not be used for long-term storage. We can not say definitively what storage solution should be used as this will require additional experimentation, but based on the healthy condition in which the final storage solution from this protocol kept the syringes in after staining it is likely that a glycerin-ethanol based solution would be effective for long-term storage of samples prior to staining as well as post-staining.

Acknowledgments

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*This source is a peer-reviewed secondary source containing information and data from out-of-print sources that we were unable to find online.

All diagrams/drawings in the materials and methods and results sections were generated by Elisheba Rowland.

Appendix I: Staining Guide

Emily Gordon and Elisheba Rowland

Worcester Polytechnic Institute (WPI)
Biology & Biotechnology Department - Major Qualifying Project
Advisors: Jill Rulfs (WPI), Michael Buckholt (WPI), Mark Pokras (Tufts University Cummings School of Veterinary Medicine), Jay Mager (Ohio Northern University)

Loon Syrinx Staining Guide

Academic Year 2020-2021

Purpose Statement:

There is one standard protocol for staining avian syringes that has been used successfully for decades (Cannell, 1988). However, this protocol must be adapted specifically to the tissue of each species. This procedure has yet to be adapted to loon syringes previously, so this MQP aimed to test and modify the procedure by Cannell to specifically adapt it to loon syringes. The majority of samples provided for this project were from adult females, so this protocol is specific to adult female common loon syringes. We believe it is likely that this protocol will also work for syringes from male common loons, though the protocol may need to be modified when staining syringes from younger birds.

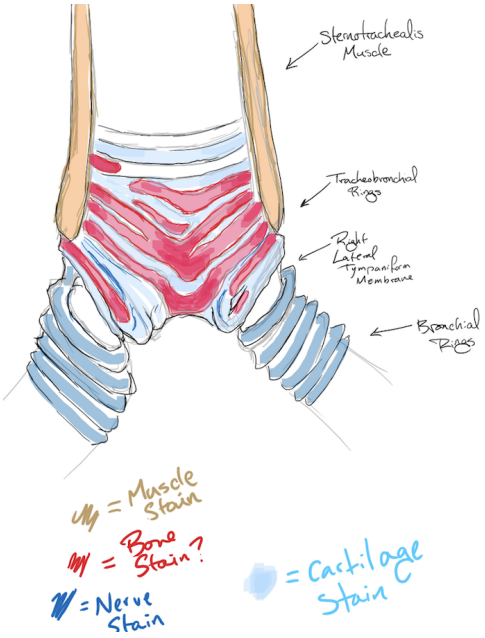

Figure 1. *Diagram showing how typical stains uptake in a loon syrinx.*

Syrinx Preparation for Staining

This staining guide focuses specifically on syringes that have already been excised, cleaned thoroughly, and stored long-term in 10% formalin for preservation.

The Cannell procedure dictates for syringes to be submerged in “10% formalin for several days (Cannell, 1988)”. Long-term storage in formalin did adversely affect the staining process, so it is not recommended to store syringes in formaldehyde prior to staining. The cartilage and bone stain require that any samples currently stored in formalin or isopropyl alcohol be transferred to 70-75% ethanol for one week prior to staining. However, it was determined

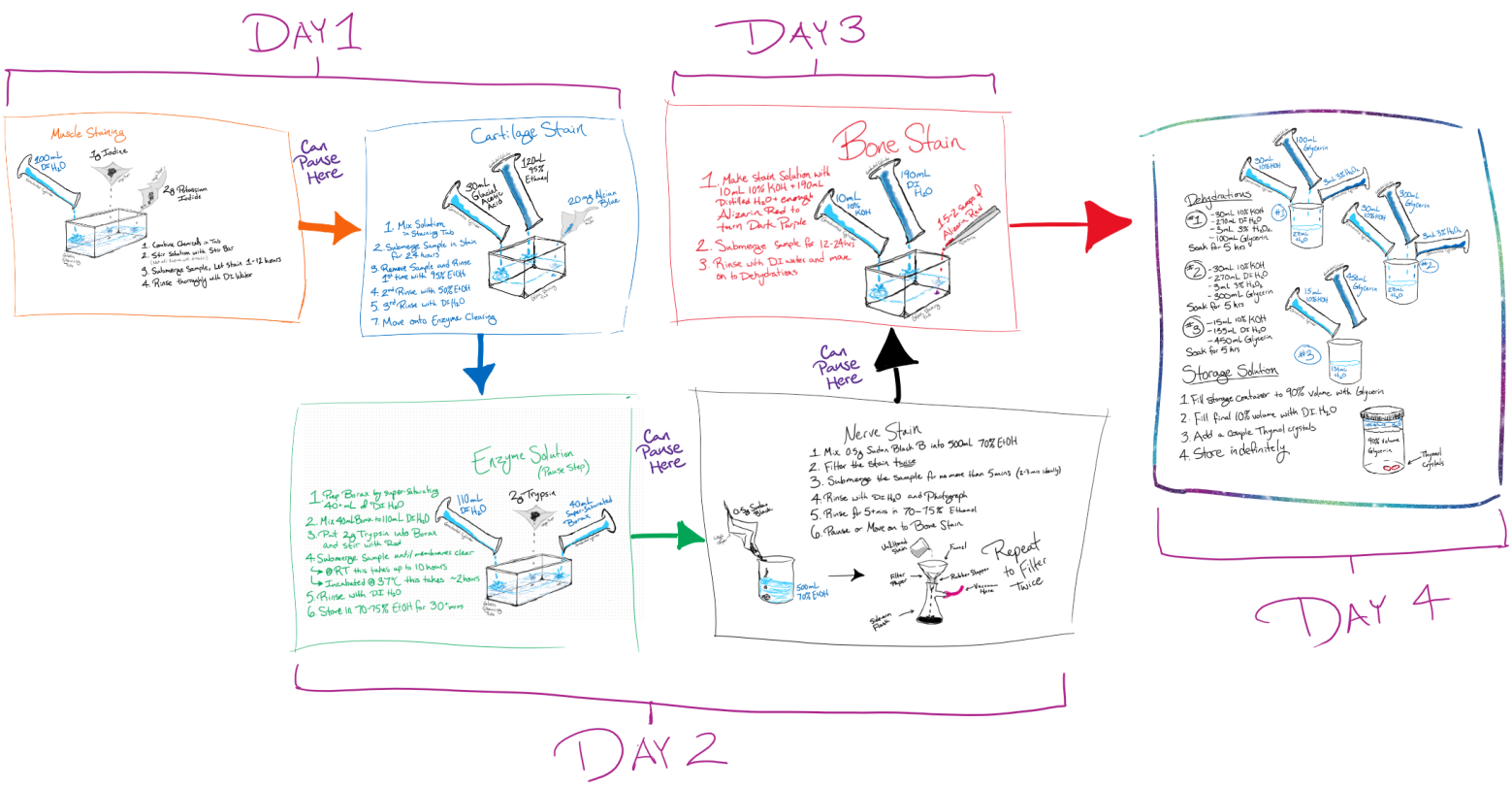

Figure 2. *Flow chart depicts an overview of the staining process. Details of each stain, as well as information regarding troubleshooting various issues that may arise in the process, are discussed at length below. This was drawn by Elisheba Rowland using the adjusted Cannell, 1988 protocols.*

experimentally that soaking the syrinx in ethanol was not sufficient to leach out all of the formaldehyde. Therefore, methylamine should be diluted to 10% with ethanol and the syrinx should be removed from the formaldehyde and placed into the methylamine-ethanol solution for one week prior to staining. The methylamine reacts with the formaldehyde to form a Schiff base, which blocks the action of the formaldehyde. This reaction is reversible, however, so borohydride can be used to make this reaction non-reversible. While this preparatory step is not necessarily required prior to muscle staining, it is not believed to be destructive to the tissue in any way since the sample is already fixed. Therefore, it does not have any adverse effects on the muscle stain. After one week in ethanol (for syringes not previously stored in formaldehyde) or

the methylamine-ethanol solution (for syringes that had previously been stored long-term in formaldehyde), the staining process may begin in the following order over the course of 3 to 4 days.

Muscle Staining

"By immersing specimens in a solution of distilled water (100 ml), potassium iodide (2.0 g), and iodine (1 .O g) for several minutes, muscles become a dark reddish-brown… This stain, fading under light, is reversible and nondestructive (Cannell, 1988)".

When initially staining the samples, it was found that the volume of solution above was insufficient to submerge the sample in the larger glass containers used. To remedy this, the recipe was doubled. This can also apply to the cartilage, nerve, and bone stains as the amount of each component can be scaled up or down depending on how much is needed to fully submerge the syrinx. For solution compositions in percentages, see Appendix III.

This solution can be prepared in advance, but it must be stored in the dark and wrapped in tinfoil. This is because the color of the iodine will fade with light exposure. One problem that was encountered with this stain was that "several minutes" was not sufficient to see significant uptake of the iodine stain on the tissue. The first round of staining, the sample was submerged in the solution for approximately 30 minutes. When microscopy was performed after completion of all four stains, the muscle stain was visible though it was lighter than expected. For this reason, it was hypothesized that a longer period of time submerged in the staining solution would result in a darker stain. Based on other studies utilizing iodine stains, it was decided to attempt submerging the sample in the muscle stain for 48 hours during the next round of staining.

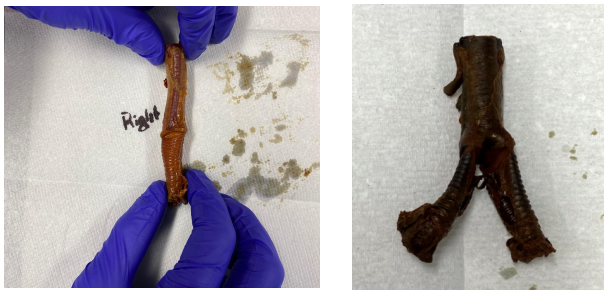

Figure 3. *Muscle stain uptake in first (left) and second (right) attempts at staining.*

After 48 hours the iodine did stain the muscle darker, but the sample became very rigid and the membranes were rendered like tissue paper. This is concerning because the Cannell protocols state that iodine staining is not destructive to tissues. Given the absence of data on how the stain interacts with loon syringes, it is possible that this stain did damage the tissue. However, it is also possible that the

syrinx adversely reacted with the formalin, ethanol, or another chemical it came into contact with before coming to WPI.
After a few more trials, it was determined that 4-6 hours was sufficient to get proper stain uptake without causing extensive damage to the common loon syringes.

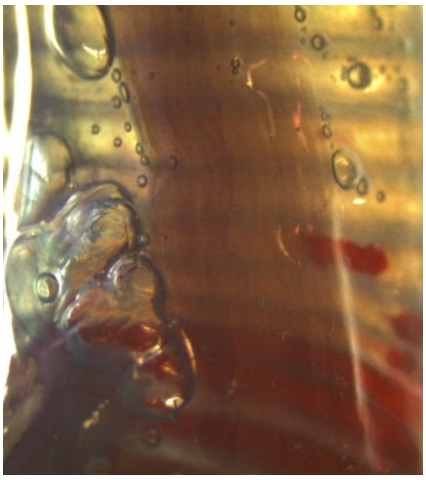

Figure 4. *The outcome of muscle staining, as can be seen under a dissecting microscope after the first round of staining.*

After completion of muscle staining, pictures should be taken immediately because the stain will fade with light. Next, the sample must be cleared, but how to do so was not provided in the Cannell paper. It was determined experimentally that agitating the sample in ethanol for approximately 5 minutes followed by agitating the sample in deionized water for 2 minutes should be sufficient. This is because when the sample was submerged in ethanol for less than 5 minutes not all of the excess iodine had been removed, but after five minutes no more was coming off anymore. Without rinsing, the iodine will all wash off when placed into the cartilage stain, which could adversely affect the outcome. Lastly, the sample should be completely dried with paper towels before proceeding to the cartilage stain to remove any excess liquid.

Cartilage Staining

"Stain cartilage with alcian blue for 24 h… Alcian blue stain: 30 ml glacial acetic acid; 120 ml 95% ETOH; 20 mg alcian blue powder… [After staining for 24 hours] Rinse in 95% ETOH for several minutes (until specimen sinks)… Rinse in 50% ETOH for several minutes (until specimen sinks)… Rinse in distilled water… Clear in enzyme solution for 5-6 h, or longer, until membranous parts are clear… This solution is temperature dependent; activity can be increased by mild heating (27°C is ideal) or virtually halted with refrigeration. Enzyme solution: 40 ml saturated aqueous sodium borate (distilled water saturated with borax); 110 ml distilled water; 2 g trypsin… [After the enzyme soak is completed] rinse gently in tap water… (Cannell, 1988)."

The alcian blue stain cannot be prepared in advance due to instability in solution for extended periods of time. However, a solution of glacial acetic acid and ethanol was prepared in advance. When it is time to

Figure 5. *View of the sample immediately after cartilage staining (left) and after soaking in the trypsin (right).*

do the cartilage stain, simply add the appropriate amount of alcian blue powder into the solution to a final concentration of 0.013%.

From here, the protocol worked well and did not require any modifications. However, the only issue encountered was speeding up the activity of the trypsin. The Cannell protocol states that it will take five to six hours for the trypsin to clear the alcian blue stain off the membranes at room temperature. To speed up the process during the first round of staining, it was placed in a 27°C incubator. At this slightly elevated temperature it took approximately nine hours for the membranes to shed their blue tint. The optimal temperature for trypsin is 37°C, so during the next round of staining the sample was placed into a 37°C incubator, clearing the membranes in 90 minutes. This is likely because 37°C is the optimal temperature for trypsin activity. Therefore, it can be established that this is the optimal temperature and length of time for this step.

The Cannell protocol states that there can be a "pause step" after soaking in the trypsin if needed. It was experimentally determined that after rinsing the sample with water, it can be stored in deionized water at 4°C until the nerve stain could be completed. Regardless, soaking the sample in water before nerve staining is recommended by the Cannell procedure.

Nerve Staining

"Immerse in 70-75% ETOH for 30 min or more… Immerse in Sudan Black B stain. Check frequently at first, then every 5-10 minutes; remove when nerves are a dense blue-black. Gentle agitation facilitates staining. Sudan Black B stain: dissolve 0.5 g Sudan Black B in 500 ml of 70% ETOH with the aid of a warm water bath. Filter solution before use… Destain in 70-75% ETOH for 5 min or more, then rinse gently in tap water (Cannell, 1988)."

This stain can be prepared in advance, and is stable at room temperature indefinitely.

This protocol makes it sound as if the nerve stain will take a substantial amount of time, but this was not found to be the case with loon syringes. In the first round of staining the sample was submerged in the stain and checked every few minutes. However, each time the sample was assessed there were no discernible nerves and the entire sample appeared to be uniformly blue. After 35 minutes in this solution, it was decided to remove the sample and try to clear the excess stain off of the sample using the ethanol and water rinses. This was not particularly successful, so it was

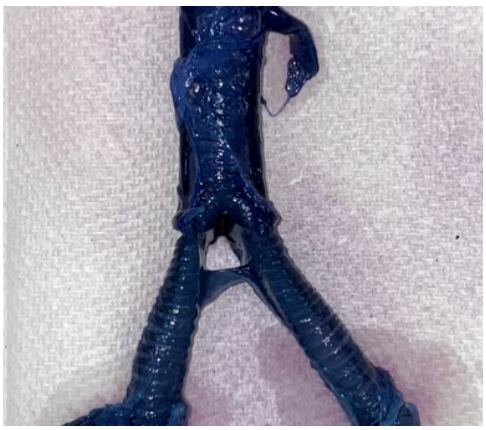

Figure 6. *Result of leaving the syrinx in nerve stain for 35 minutes. The stain was unable to be efficiently cleared from the sample and did not reveal any distinct nerves.*

hypothesized that the sample was submerged for far too long in the nerve stain. In the next trial, the staining solution was double filtered and diluted with approximately 100 mL of 70% ethanol to make it less concentrated. The sample was only submerged in the solution for two minutes. When removed, the sample was uniformly coated in the nerve stain, but clearing the stain with ethanol and water worked. Unlike the first round of staining, the nerves were visible on post-staining microscopy the second time. Therefore, in this step the nerve stain should be diluted with an extra 100mL of 70% ethanol, filtered twice, and the sample should not be left in this solution for longer than two minutes because the loon syringeal tissue binds the dye rapidly.

As with the muscle stain, it is also important to take pictures before proceeding to the bone stain because the nerve stain can fade with time.

Bone Staining

"Stain for calcium phosphates with alizarin red S for 12-14 h (no danger of overstaining up to 24 h). Alizarin red stain solution: 10 ml 10% KOH stock solution…; 190 ml distilled water; enough alizarin red S powder to color solution deep purple… Rinse gently in tap water (Cannell, 1988)."

This solution can be prepared in advance, but it will begin to fade and not stain as well over time. For this reason, it is recommended to prepare it fresh for each round of staining. Additionally, the color change happens very suddenly when adding the alizarin red to the liquid components, so only add a little bit at a time using a small spatula to avoid wasting materials. This stain worked very well on the loon syringes following the Cannell protocol without any modifications.

Figure 7. *Outcome of bone staining, which proceeded without issue according to the Cannell protocol.*

Post-Staining Dehydrations and Storage

"...Dehydrate through a series of KOH/glycerin solutions… Leave syringes in each step for 5 or more hours… [Dehydration #1] 3: 1 KOH/glycerin: 30 ml 10% KOH; 270 ml distilled water; 3 ml 3% hydrogen peroxide (bleaches pigments);

100 ml glycerin… [Dehydration #2] 1: 1 KOH/glycerin: 30 ml 10% KOH; 270 ml distilled water; 3 ml 3% hydrogen peroxide; 300 ml glycerin… [Dehydration #3] 1:3 KOH/glycerin: 15 ml 10% KOH; 135 ml distilled water; 450 ml glycerin… Store syringes in solution of: 90% glycerin, 10% distilled water; a few thymol crystals (inhibits mold and bacteria) (Cannell, 1988).”

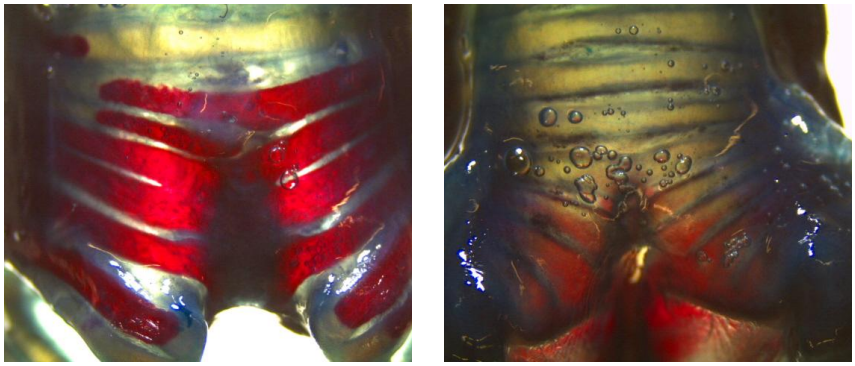

Figure 8. *Examples of images able to be captured on a dissecting microscope after the staining process. These specific images are of the bifurcation, with the left image being a dorsal view of the bifurcation and the right image being a caudal view.*

These dehydrations were very straightforward and did not require any modifications. After these dehydrations and being placed into the final storage solution, the sample can undergo microscopy. The dehydration solutions can be reused for multiple samples.

Microscopy

After the completion of all four stains and dehydrations the samples were imaged using a dissecting microscope. Location of all four stains enabled the composition of loon syringes to be determined and documented.

The only issue encountered when imaging was the presence of air bubbles trapped under the outermost layer of tissue. At this time, this is believed to be an unavoidable consequence of the staining process and any attempts to remove the bubbles were deemed to cause more harm than good.

Though not done in this project, these microscopy images can be used to take measurements of structures using computer programs, such as ImageJ. These measurements can be used to make comparisons between common loons of the same or different sexes and developmental stages, as well as to compare the common loon syrinx to that of other species.

Source: Cannell, P. F. (1988). *Division of Birds,* National Museum of Natural History, Smithsonian Institution, Washington, D.C. 20560. Received I6 June 1987, accepted 3 Nov. 1987.

Appendix II: Preparatory Solution, Enzyme Solution, and Dehydration and Storage Solutions

Preparatory Solution: In this experiment, 33% methylamine in ethanol was used and diluted further to 10% with 70% ethanol. This was done because the formaldehyde solution that the syrinx was initially in had a concentration of 10%. Any concentration of methylamine solution can be used so long as it can be diluted to a final concentration of 10%, though it is recommended that it is diluted only with ethanol since the original protocol called for the syrinx to be submerged in ethanol for one week. It is unknown at this time if any other diluents would adversely affect the tissue. The syrinx was submerged in this solution for one week prior to staining.

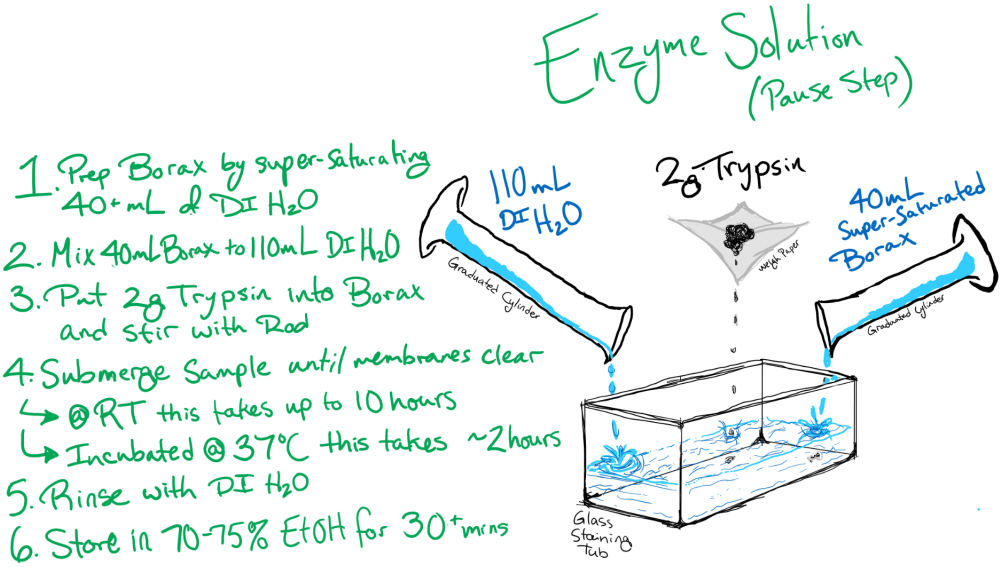

Figure a. Enzyme solution, membrane clearing protocol with modifications to temperature and length of time.

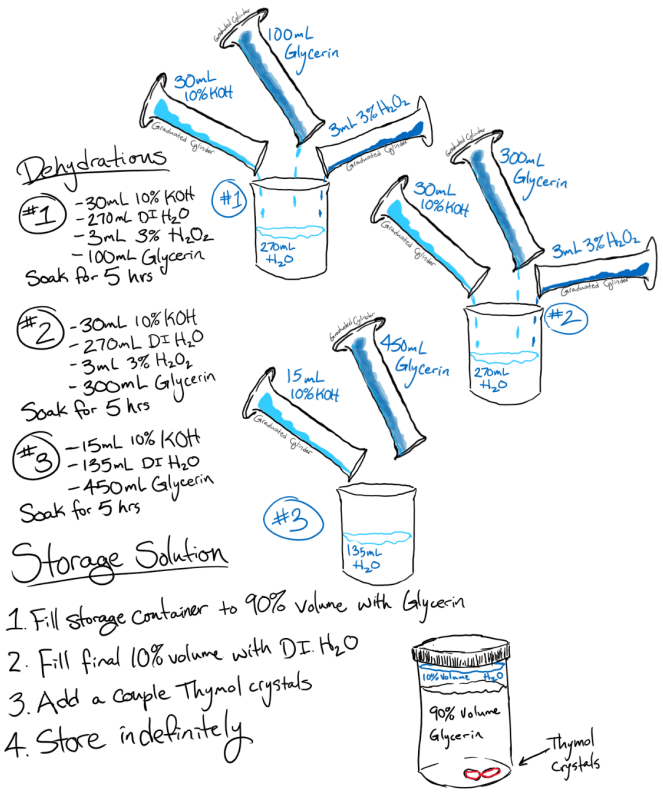

Figure b. Dehydration and storage solution preparation protocols according to Cannell, 1988.

Appendix III: Staining Solution Composition

Muscle Stain: 2% (m/v) potassium iodide + 1% (m/v) iodine in DI water

Cartilage Stain: 20% (v/v) glacial acetic acid + 80% (v/v) 75% ethanol + 0.013% (m/v) alcian blue powder

Trypsin Solution: 26.27% (v/v) saturated aqueous sodium borate solution + 73.3% (v/v) DI water + 1.3% (m/v) trypsin

Nerve Stain: 0.1% (m/v) sudan black B powder in 75% ethanol

Bone Stain: 5% (v/v) 10% potassium hydroxide solution + 95% (v/v) DI water + enough alizarin red powder to turn the solution dark purple