



Investigating the Common Loon Syrinx

A Major Qualifying Project

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Abstract

Although the Common Loon has unique vocalizations, there is a lack of research on how its syrinx structure contributes to these calls. Additionally, there is one vocalization that only males produce, so it is worth investigating if this has a structural underpinning. This project aims to lay the foundation for future research on the structural underpinnings of the Common Loon's unique vocalizations, by offering a μ CT imaging methodology to observe and analyze loon syrinx morphology. With the protocol offered in this project, gender differences and other interests can be explored.

Acknowledgements

Special thanks to Dr. Karen Troy for her generosity in training me on and letting me utilize her μ CT machine. This helped propel the course of this project into an exciting venture with many future directions. Thank you to Mark Pokras and Jay Mager for their advice and enthusiasm throughout the course of this project. Additionally, I would like to recognize the graduate students that I consulted with along the way, especially Sydney Packard and Andrew Wilzman.

1. Introduction

While terrestrial vertebrates produce vocalizations using a larynx, avian species have a vocal organ that is unique to them called a syrinx (Goller, 2022). Birds are not missing a larynx. It is at the top of the trachea in the back of mouth and plays an important role in keeping food or water from getting into the respiratory system and in starting and stopping sound production (Goller, 2022). But the true sound-producing organ, the syrinx, is located deep within their chest near the tracheobronchial junction (Goller, 2022). The specific syrinx structure varies greatly among avian species, which makes it a particular area of interest in research regarding bird vocalizations (King & McLelland, 1984).

Although loon calls are iconic sounds in the northern United States and Canada, there are no current studies documenting the gross anatomy of Common Loon (*Gavia immer*) syringes, a structure shown in Figure 1. Therefore, little is known of how their syrinx actually produces the sounds of their calls. Additionally, no work has been done to examine how the syringes might differ between male and female Common Loons. This is a research interest as there are gender-specific calls.

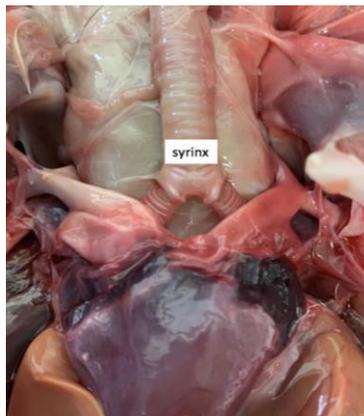


Figure 1: How the syrinx appears in the body of a Common Loon (Image taken by Mark Pokras).

The goal of this project is to investigate the anatomy of the Common Loon syrinx, mainly through micro-computed tomography (μ CT) imaging techniques. This method has been utilized for similar studies, such as in researching Zebra Finch (*Taeniopygia guttata*) syringeal morphology (Düring et al., 2013). In the Zebra Finch study, both μ CT and magnetic resonance imaging (MRI) were utilized in combination with histology and dissection to get a more complete picture (Düring et al., 2013). The researchers were able to make conclusions about how muscle organization and activity, skeletal structure, and cartilage framework contribute to the sounds of these highly vocal songbirds, as well as compare the structure of male and female syringes (Düring et al., 2013). The methodology used in the Zebra Finch study can be adapted to investigate the anatomy of the Common Loon syrinx and determine how it contributes to their vocalizations. A staining procedure for Common Loon syringes has been developed in a previous project completed by past Worcester Polytechnic Institute undergraduates, Emily Gordon and Elisheba Rowland. This procedure included methods for syrinx storage as well as steps for staining particular tissues like muscle, bone, and cartilage. Therefore, imaging will allow for the advancement of this research.

1.2 Types of Common Loon Vocalizations

Hoots are short and quiet calls made by both males and females to maintain contact between parents and offspring (Evers et al., 2010). Wails are calls of a medium length made by both sexes, used as a contact call between adults and young or mates (Evers et al., 2010). Tremolos are short calls used by both males and females when they are threatened or defending their territory (Barklow, 1979). Yodels are long, complex calls made only by males and when defending their territory or fighting with other loons (Mager et al., 2007).

1.3 General Syrinx Anatomy

A typical syrinx structure is shown in Figure 2, which is labeled with muscular, skeletal, and membrane features.

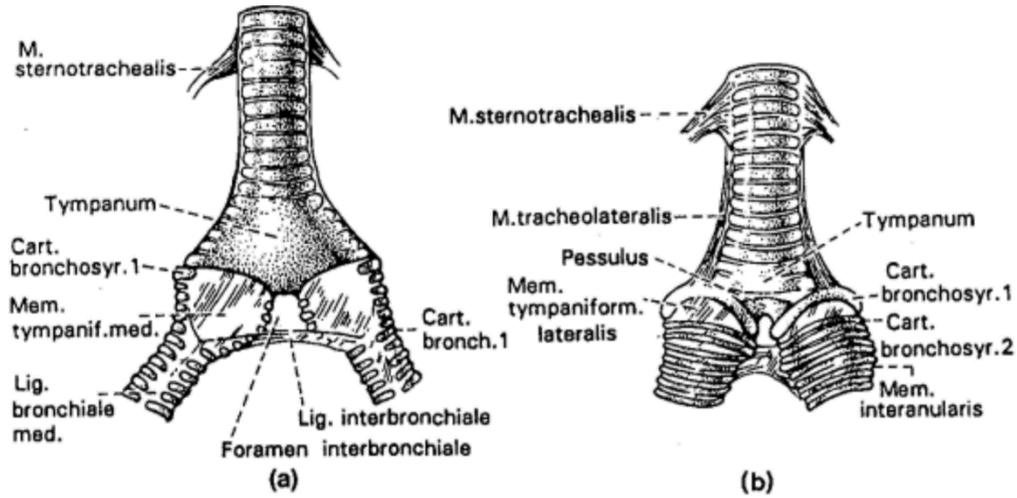


Figure 2: Typical syrinx structure labeled, (a) dorsal view, (b) ventral view. From King (1989).

There are three classifications of syringes (Figure 3) based on their location: tracheal, bronchial, and tracheobronchial, which is the most common (King & McLelland, 1984).

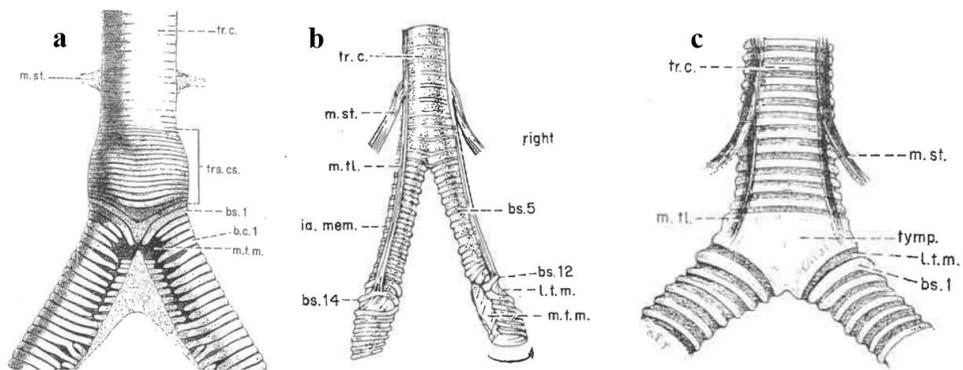


Figure 3: The three different classifications of syringes, (a) tracheal, (b) bronchial, (c) tracheobronchial. Abbreviations: *m. st.* = *m. sternotrachealis*, *trs. cs.* = *tracheosyringeal cartilages*, *tr. c.* = *tracheal cartilage*, *bs.* = *bronchosyringeal cartilage*, *b. c.* = *bronchial cartilages*, *m. t. m.* = *medial tympaniform membrane*, *m. tl.* = *m. tracheolateralis*, *ia. mem.* =

interannular membrane, l. t. m. = lateral tympaniform membrane, m. t. m. = medial tympaniform membrane, tymp. = tympanum. From King (1989).

The tympanum, a direct continuation of the trachea, consists of a cylindrical structure that results from the fusion or close joining of two or more complete tracheal syringeal cartilages (King & McLelland, 1984). A tympanum is usually present in tracheobronchial syringes, sometimes present in tracheal syringes, and usually absent in bronchial syringes (King & McLelland, 1984). Thin, flattened C-shaped tracheal cartilages can be found caudal to the tympanum, which can be attached to the pessulus at both ends or attached at one end and free on the other (King & McLelland, 1984). At the bifurcation of the syrinx, there are bronchial cartilages that are typically paired, incomplete, and C-shaped (King & McLelland, 1984). The medial tympaniform membrane, located on the medial surface of the bifurcation, is considered the primary vibrating membrane (King & McLelland, 1984). Syringeal muscles vary among species, as there are some species with five muscle pairs and some with none (King & McLelland, 1984). The sound from syringes is thought to be due to the vibration of the tympaniform membranes, as the vibrations cause changes in pressure between the clavicular air sac and the lumen of the syrinx (King & McLelland, 1984).

2. Methods

2.1 Sample Collection, Preparation, and Storage

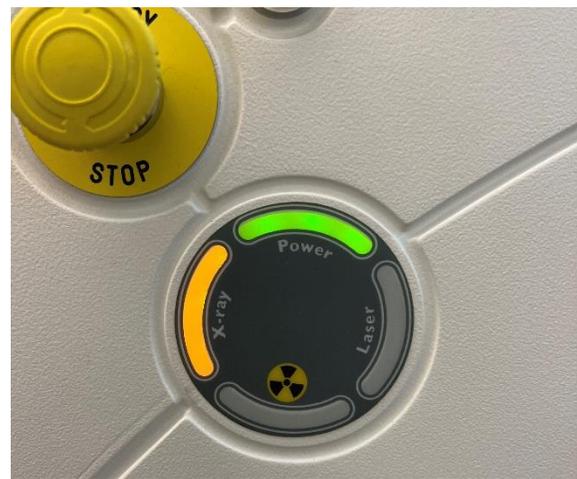
The syringe samples were collected from Common Loons that were euthanized for quality-of-life concerns or found dead by wildlife rehabilitation centers. No loons were euthanized for the sole purpose of this project. The vocal tracts were dissected from the thawed species during necropsy procedures. The samples were stored in formalin and left out to dry for 10-20 minutes prior to imaging. Following imaging, the syringes were put back into formalin.

2.2 μ CT Imaging

The SCANCO Medical XtremeCT (Figure 4a) was utilized for μ CT imaging of the Common Loon syringe samples. Before any samples can be scanned, the x-rays in the machine must warm up, which takes about 30 minutes. The x-ray indicator light on the scanner, seen in Figure 4b, will blink when warming up and then stay solid when fully warmed up.



(a)



(b)

Figure 4: (a) The SCANCO Medical XtremeCT with no sample loaded. (b) The indication lights found on the scanner. The orange light indicates the x-rays are warmed up and ready for

scanning. When the x-rays are warming up, the orange light will blink. When a sample is being scanned, the indication light for radiation will turn on.

The SCANCO Medical software that works with the μ CT machine starts as a small tab with different menu options, seen in Figure 5. To start a scan, click on the menu that looks like the scanner (second from the left). This opens the μ CT tomography page, the main menu of the program in which scan parameters are set up and then scans are taken.

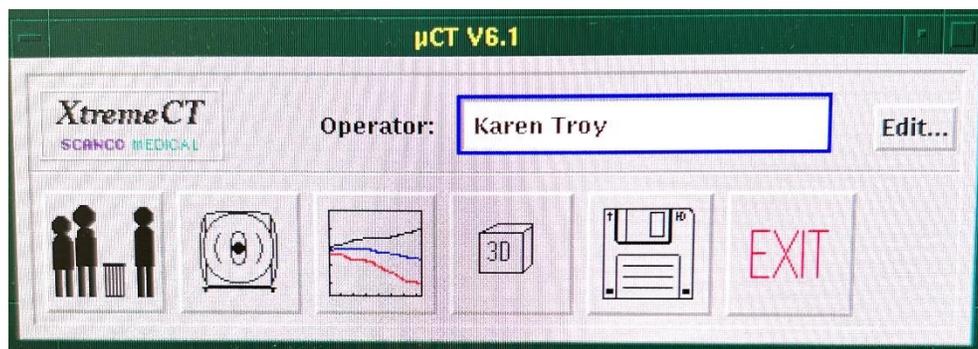


Figure 5: SCANCO Medical software menu options.

At the top of the μ CT tomography page, the measurement sample can be selected. The measurement sample for this project was named “Loon” and had a sample number of 678, as shown in Figure 6. Below that, the measurement history for the selected sample is displayed, which includes the measurement number, control file used, number of slices in the measurement, and the date and time of the scan. Towards the bottom of the page is where the control file applied to the scan is selected. The control file used determines imaging parameters like resolution, size, and number of slices. To get more details on control files, select any of them and click modify. Doing so will show voxel size and number of slices, which gives a better sense of the resolution and size of the image that would result from using that particular control file. The

values that remain constant between all control files is the peak kilovoltage (60 kVp) and intensity (900 μ A).

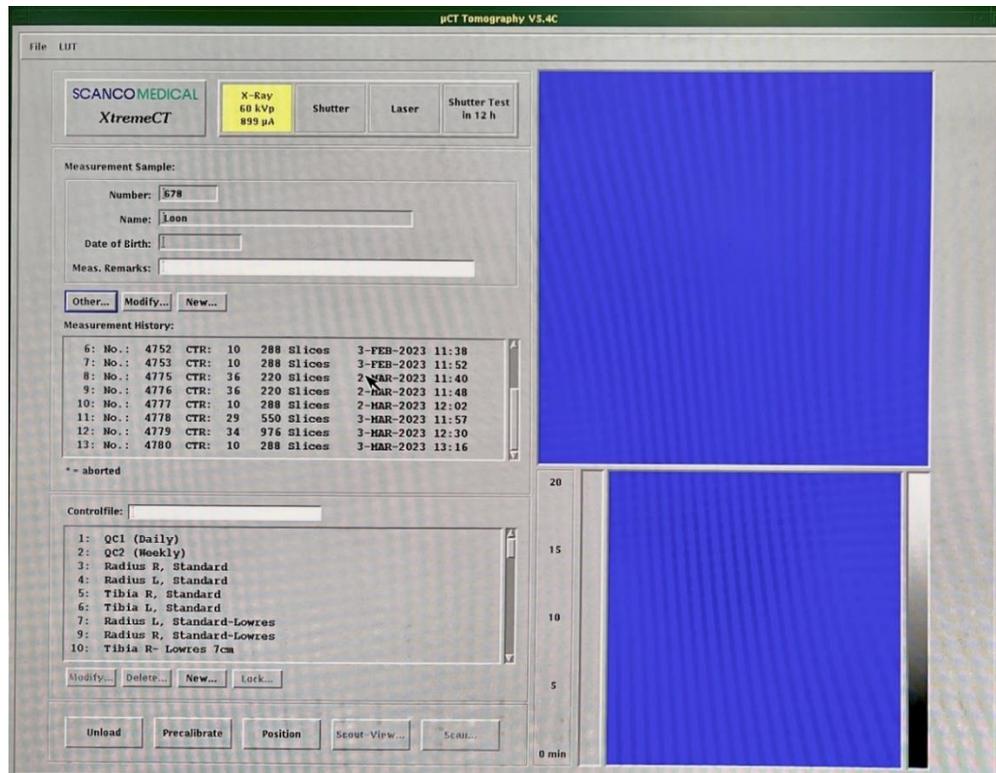


Figure 6: μ CT Tomography page.

Before any scans can be done, the machine will need to be precalibrated, which is done by pressing “Precalibrate” and then “Start Calibration”. During this time, there should be nothing loaded in the scanner. Precalibration takes about 90 seconds and lasts 20 minutes, when it will need to be done again if scanning more samples is desired. After precalibration, place the tray in the machine and position the sample. In order to have some space between the tray and the sample, a plastic zipper bag can be utilized by blowing just enough air into the bag to get the sample off the tray. Avoid blowing up the bag to maximum capacity, as it is not necessary and will likely move the sample out of view when scanned. When sample is positioned, push the tray

all the way into the machine. The software will not let you scan anything until this step is completed. An example of what a loaded sample looks like is displayed in Figure 7.



Figure 7: (left) The SCANCO Medical XtremeCT with tray loaded on machine and pushed in all the way. (right) Example of how a sample can be loaded with a plastic zipper bag to create a gap between the tray and the sample.

Once the sample and tray are properly positioned, the desired control file can be selected. Then, press “Scout-View...” at the bottom of the tomography page. The scout view will allow a visualization of what the image will look like and what size the scan will cover. Pressing “Scout-View” again will scan the sample quickly, which allows the ability to see if the entire sample or desired section will be in the frame when scanned. It is important to stay away from the front of the machine during the scout-view scan to avoid radiation exposure, even if it may be small. The angle at which this scan is done can be changed, with 0 degrees providing a side view and 90 degrees providing a view from the top. After the scout-view scan is taken, the screen will resemble the appearance seen in Figure 8. The green line seen at the top of the image can be moved around using the cursor. The green rectangular block on the right side of the image shows

how much of the scan will be in the final, and often more detailed, scan. The whole sample in Figure 8 would be scanned, as shown by the block covering its whole length. Once the desired scan size is determined by moving the cursor, set the reference line by clicking. The number of slices, the length of the image, and the scan time appear at the bottom of the scout-view.

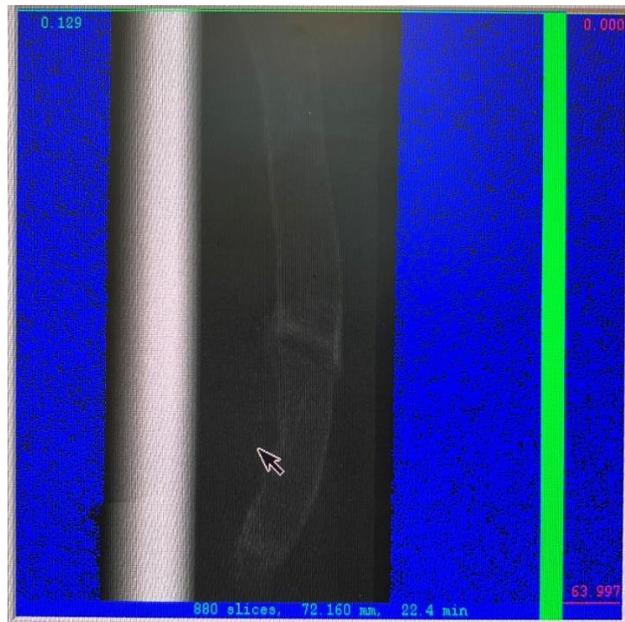


Figure 8: An example of a scout-view scan. The green reference line can be seen at the top of the image. The green rectangular block along the right side of the image provides a visual of how much of the sample will be shown in the final scan. In this case, the whole sample fits in the scan. The slices, image length, and scan time are provided at the bottom of the scan.

After taking the scout view, the “Scan...” button can be pressed on the main tomography page. Press start measurement and avoid standing directly in front of the machine for the duration of the scan. Staying located to the side of the machine during this time will allow safety from radiation exposure.

In order to export image files as DICOM files, start by pressing the menu option called μ CT Evaluation Program (Figure 9). It is the third icon from the left and looks like a line graph. Select the sample number (in this case, 678). Then, select the particular measurement to export. If analyzing a high-resolution file, it might take some time for all the slices to load into the evaluation program. To begin file export, press the “T...” button in the bottom left of the window. Where it says “Task,” press select. Select “Task 15: Convert to DICOM”. A white box will show up on the picture slices. Go through the slices and ensure the entirety of the sample or area of interest is inside the box and the tray is not inside of it. The white box size and position can be adjusted by changing the x and y coordinates. Once the box is adjusted, press “Start Evaluation.” The slices would now be successfully converted into DICOM files, which can be used in 3D reconstruction software for analysis. The program FileZilla is used to transfer the DICOM files to a personal computer.

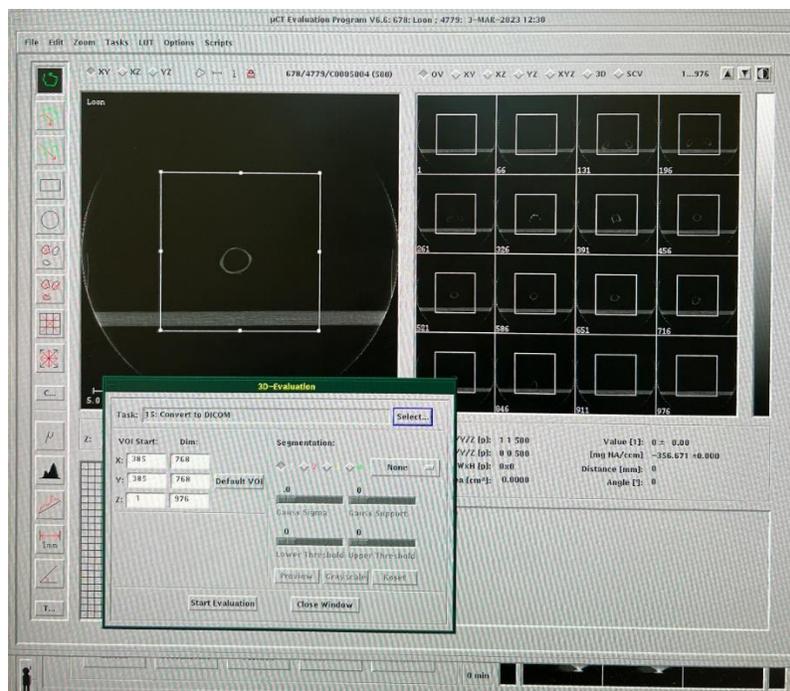


Figure 9: The μ CT Evaluation Program window. The white box around the slices shows what part of the scan will be exported as DICOM files.

2.3 3D Reconstruction and Analysis

The DICOM files extracted from the XtremeCT program are simply image stacks, and there are programs that can stack these images on top of each other to create 3D visualizations of the scans. The higher the voxel size used in the scan, the higher the resolution. In the same way, the larger the image, the more stacks (or files) when voxel size is kept constant.

A program used to generate 3D reconstruction is 3Dicom Patient, which requires a yearly subscription, but acts as a great resource for quick 2D to 3D data conversion. No data analysis or measurements can be done using 3Dicom without purchasing further upgrades. To generate a 3D image from the slices using this program, press “Display” and adjust the lower window and low threshold (Figure 10).

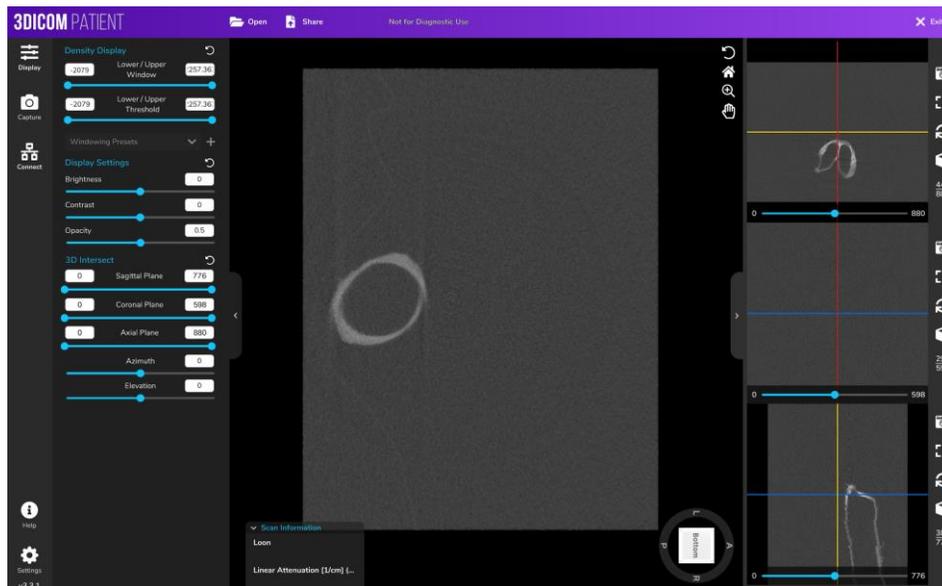


Figure 10: 3Dicom Patient with scan uploaded but no adjustments made to the window or threshold.

These adjustments allow the background noise from the scan to be removed, leaving just the syringe sample. The result of changing the lower window and threshold is shown in Figure 11.

This program also allows adjustments to be made to brightness, contrast, and opacity. Additionally, the three windows on the right can be used to slide through axial, coronal, and sagittal planes of the scan.

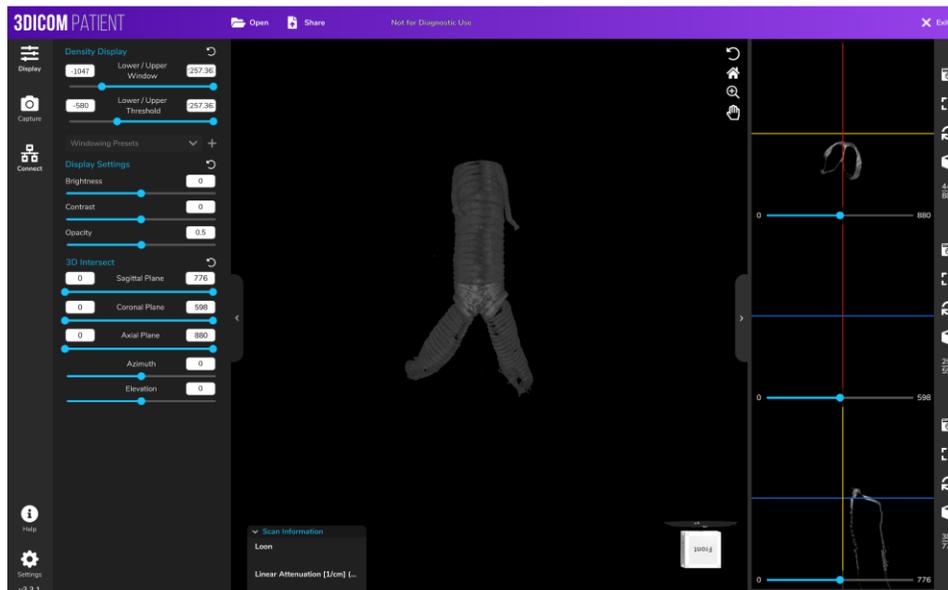


Figure 11: 3Dicom Patient with window and threshold adjusted for a scan. The noise in Figure 10 is removed, leaving just the sample.

A free software used to turn DICOM file stacks into 3D images is Fiji, a distribution of ImageJ that includes useful plugins that aid in image analysis. The ImageJ toolbar is in Figure 12. To import DICOM files, Press “File”, “Import”, and then “Image Sequence...”. Browse for the DICOM folder of sample you want analyze, check off “Use virtual stack”. Click “OK”. To threshold the image, press “Image”, “Adjust”, and then “Threshold...”. To change brightness or contrast, press “Image”, “Adjust”, and then “Brightness/Contrast”. Then to view the image in 3D, press “Plugins” and then “3D Viewer”. Click OK through all the windows, and the 3D rendering will appear.

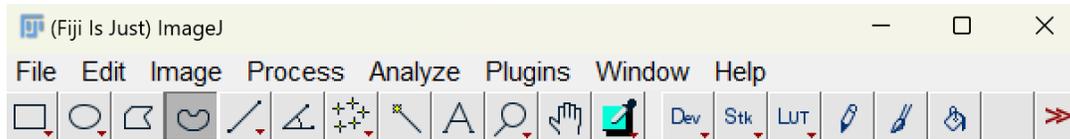


Figure 12: ImageJ (FIJI) toolbar.

3D Slicer is another free software for 3D reconstruction of DICOM file stacks. To generate the 3D image, use the “Segment Editor” from the dropdown menu. Then, press “Add” to start a segment. Then, adjust the threshold range to include just the sample and no background noise. Press “Show 3D” to generate the 3D, which appears in the top right window (Figure 13).

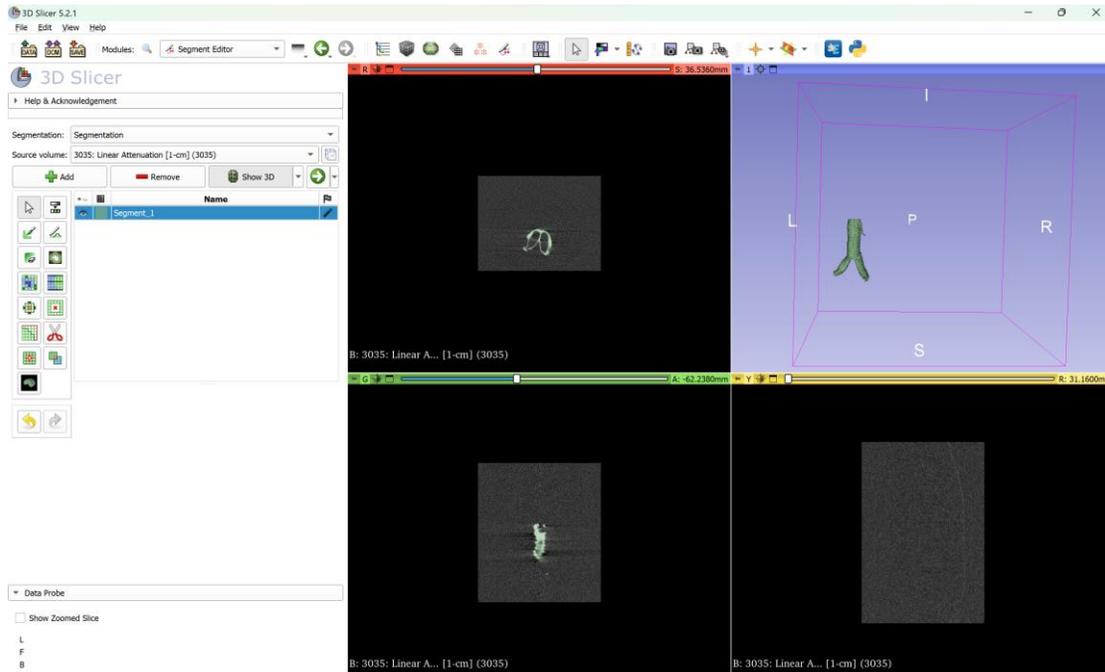


Figure 13: 3D Slicer program window with a 3D reconstruction generated using the Segment Editor.

3. Results

The first few μ CT scans done were purely experimental, with the only goals being to get used to how the machine works and how scans turn out when using different control files. The information about the different control files used is listed in Table 1.

Table 1: Control file information used in XtremeCT program.

Control File	E (kVp)	I (μ A)	Voxel Size	Slices
10	60	900	246.000	288
28	60	900	82.000	880
29	60	900	82.000	550
34	60	900	82.000	976
36	60	900	82.000	220

Figures 14-17 are scans done to test out what the μ CT images look like when reconstructed in a 3D space, using four different samples. The samples do not have a lot of definition; however, it is evident that the bifurcation is the densest area of the syrxinx. This likely indicates that this part of the structure is made of bone. Another issue that arose with these scans is that when adjusting the window and threshold to remove the noise, some of the sample was removed as well. This caused the samples to have gaps in the image where there were no imperfections on the actual sample. The control file (10) used for these scans was a low-resolution setting, so this could be the reason for these observations. Lastly, with the technique used for these samples, there was no way to remove the tray the samples sat on during the scan without removing parts of the sample from the image.



Figure 14: 3D Reconstruction using 3Dicom Patient. Control file 10 used on scan. Specimen information unknown.

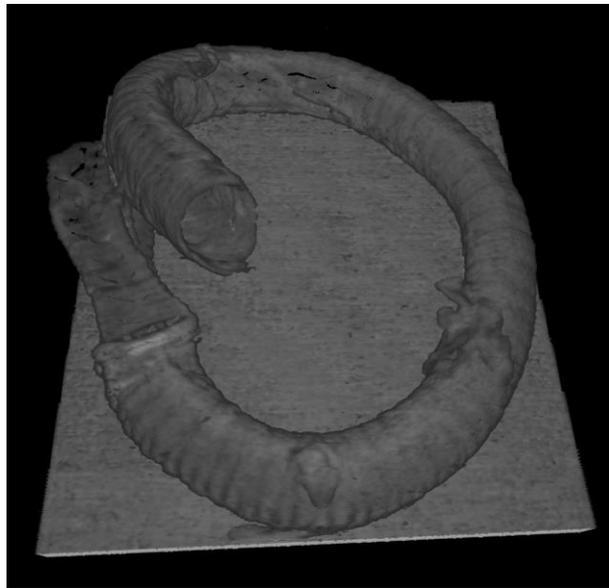


Figure 15: 3D Reconstruction using 3Dicom Patient. Control file 10 used on scan. Specimen information unknown. Sample Number: TV2200098.



Figure 16: 3D Reconstruction using 3Dicom Patient. Control file 10 used on scan. Specimen was an adult male. Sample Number: TV220106.



Figure 17: 3D Reconstruction using 3Dicom Patient. Control file 10 used on scan. Specimen was an adult female. Sample Number: TV220108.

Figure 18 shows a more high-resolution image of the syrxinx, with a focus on the structure of the bifurcation. Compared to the scans done with control file 10, the image is much more defined. The cartilage rings can be counted and the dense area before the bronchi is shown more clearly. The tray is still shown in this scan.

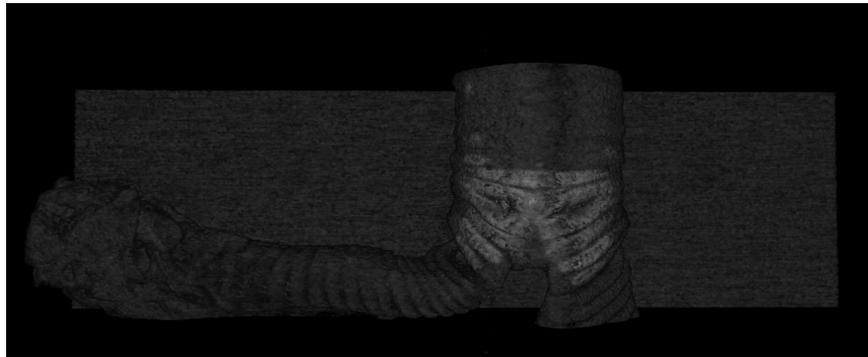


Figure 18: 3D Reconstruction using 3Dicom Patient. Control file 36 used on scan. Specimen information unknown. Sample Number: TV220102.

Figure 19 is another small image with a high resolution, with the focus being the bronchi. This scan allowed for a closer viewing of the cartilage rings.



Figure 19: 3D Reconstruction using 3Dicom Patient. Control file 36 used on scan. Specimen information unknown. Sample Number: TV220102.

Figure 20 is the first attempt at using a plastic zipper bag during scanning to create some space between the tray and the sample. This lower-resolution image acted as a test to determine if filling a plastic zipper bag with air and laying the sample of that during imaging would create enough space between the tray and the syringe that the tray can be cropped out of the scan when files were exported. This attempt was considered successful.



Figure 20: 3D Reconstruction using 3Dicom Patient. Control file 10 used on scan. Specimen information unknown. Sample Number: TV220102.

Once the plastic zipper bag method and different resolutions and scan sizes were tested, a high-resolution, floating imaging could be generated. The results are seen in Figure 21. This scan took about 45 minutes and resulted in a large file due to it being composed of 880 slices. However, the product is an image with high definition, which is fitting for analysis using 3D reconstruction.

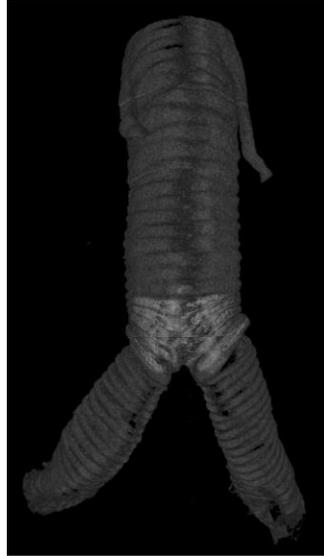


Figure 21: 3D Reconstruction using 3Dicom Patient. Control file 28 used on scan. Specimen information unknown. Sample Number: TV220101.

Figure 22 uses the same scan in Figure 21 but with the 3D reconstruction being completed using ImageJ (Fiji).

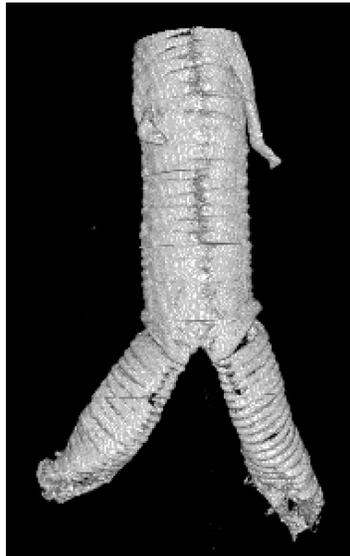


Figure 22: 3D Reconstruction using ImageJ (Fiji). Control file 28 used on scan. Specimen information unknown. Sample Number: TV220101.

Figure 23 is a 3D reconstruction of the same high-resolution image, but this time using 3D Slicer for visualization.

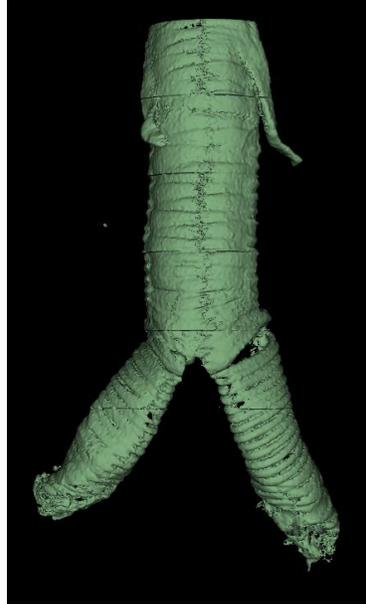


Figure 23: 3D Reconstruction using 3D Slicer. Control file 28 used on scan. Specimen information unknown. Sample Number: TV220101.

Through testing different imaging settings, it was determined that a higher resolution (smaller voxel size) scan can create more noise during imaging. Using 3D visualization software, a density window can be selected that removes the background noise caused by air surrounding the sample. However, when this is done, some parts of the sample may get removed as well if they are of a similar density as the noise. Therefore, windows must be adjusted slowly.

Although the intended hypothesis regarding Common Loon syrinx variation between genders was not addressed, a methodology to begin this research has been established.

4. Recommendations

Although the initial objective of determining morphological differences between male and female Common Loon syrinxes was not completed, a road map now exists for getting this work and future investigations done, such as tracking Common Loon syrinx structure across developmental stages. A preliminary step forward would be to determine the general anatomy of a loon syrinx. This could include determining syrinx type, trachea length, and bronchi length, and counting cartilage rings and observing their shape.

Fiji can be utilized for taking measurements. The measurements are taken directly on scan slices, not on the 3D-rendered image. To measure, select the line tool from the toolbar. Then, drag across the desired area of the sample and click CTRL + M (or alternatively, press “Analyze” then “Measure”). This will bring up a window with the measurement listed, including the units of the measurement. The correct units are predetermined from information transferred from the machine to the image files. Fiji also allows the ability to put a scale bar on an image using the following steps: Analyze – Tools – Scale Bar. This scale feature could be beneficial in putting image slices in a report.

With the guidelines provided in this report, more images can be taken to get a closer look at each of these specific areas. For example, if cartilage rings are the area of interest, a high resolution and smaller scan could be taken of just that area, ensuring that a high-quality image is produced but it will not take an excess of time that would occur from scanning the entire sample.

3D Slicer is a free 3D visualization software that warrants some utilization in this research. It has the ability to assign colors to specific densities (or density ranges). In collaboration with histological work, the densities in the scan can be associated with certain

tissues (nerve, muscle, cartilage, etc.). The result of these two efforts could result in a 3D image that is color-coded and provides a detailed and comprehensive makeup of a Common Loon syrinx.

In order to take advantage of this feature of 3D slicer, open up the DICOM files of a scan with high resolution and then select “Segment Editor” from the dropdown menu. Then, press “Add” to start a segment. The color of the segment can be changed by double clicking on the colored box next to the segment name. To pick the specific density range, adjust the threshold range of the segment using the tool at the top right of the menu that appears on the left side of the page. As the range is adjusted, take note of what is being selected on the three windows that display coronal, axial, and sagittal plane slices. Repeat this step for as many densities ranges necessary, ensuring that each range is small enough to only select one tissue type. Once the segments are set, press “Show 3D” and the color-coded 3D image will appear in the top right window, which can take a few minutes depending on the file size.

Both ethanol and formalin were used in storing the syrinx sample, but formalin was determined to be less harsh on the tissues. However, it might be worth investigating if any shrinkage of tissue occurs from long-term storage of such samples in formalin. If this ends up being the case, then a conversion factor could be applied to each measurement taken for more accurate estimations.

When completing this project, there are some things to keep in mind. For example, it is important to keep track of the gender and age of samples as they are received. Staying on top of this allows for better conclusions to be made when observing the scanned samples. Additionally, it would be important to take note of control files used for each scan. Having this data on-hand will allow determination of what resolution is necessary to get a good image. The lower the

resolution, the less time it takes to scan, so maximizing time while still getting a good image is crucial. Lastly, conducting some further research of other species' syrinxes and how they differ between genders would be useful. This could provide some features to look for when conducting the research.

There were limitations to the amount of imaging and analysis that was able to be completed during the course of this project. However, it is my hope that the methods and future directions presented in this paper provide a roadmap for all the paths this research can take in order to discover more about how the unique vocalizations of Common Loons are produced.

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