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Effects of Larynx Preservation Method on Phonation Threshold Flow in an Excised Porcine Benchtop Model

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ABSTRACT

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An excised animal larynx model has been used in many studies to better understand the physiological and anatomical properties of the human larynx. One difference between an ex vivo model and an in vivo model is that ion loss occurs postmortem. To compensate for this in the excised model, researchers most commonly use a preservation method that includes completely submerging the specimen in isotonic saline (0.9% $Na^{+}Cl^{-}$) and then flash freezing it in liquid nitrogen. The flash freezing method allows researchers to maintain the integrity of the structures while also being able to gather specimens as they become available. Not enough research has been done to understand the effects of a preservation method on the outcomes of the study. Additionally, no common method has been established for preservation across studies to ensure that results are not being influenced by this variable. This prospective, mixed experimental design study includes three groups, a control group and two experimental groups. The control group consisted of 10 bench-mounted porcine larynges that were soaked in isotonic saline and flash frozen with liquid nitrogen. Prior to the experiment, the frozen larynges were thawed overnight before trials. The other two groups consisted of 10 bench-mounted porcine larynges each; these larynges were soaked in either isotonic saline or Ringer's solution, a balanced fluid used in vivo to counteract dehydration. Larynges from these two groups were kept fresh and stored in a refrigerator overnight before trials. On the day of experimentation, each larynx was mounted on a bench top setup including three micropositioners to stabilize, adduct, and elongate the vocal folds. All the larynges were connected to a pseudolung via the trachea and humidified air was passed through to the vocal folds until phonation was achieved. Phonatory trials consisted of brief phonation followed by 5-minute desiccation intervals until phonation was no longer achieved. Phonation threshold flow (PTF), defined as the flow observed at the onset of phonation, was observed during each phonation trial; and flow values were compared within and between groups. Statistically significant differences were found between the Ringer's group and the fresh saline group as well as between the Ringer's group and the frozen saline group, indicating that PTF is influenced by the larynx preservation method.

Keywords: larynx preservation, larynx storage, bench model, phonation threshold flow, laryngeal desiccation

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DESCRIPTION OF THESIS STRUCTURE

The following thesis, *Effects of Larynx Preservation Method on Phonation Threshold Flow in an Excised Porcine Benchtop Model,* is written in a hybrid format. That is, the format of this thesis merges journal publication formats and classic thesis requirements. An annotated bibliography of a comprehensive literature review is found in Appendix A. Appendix B contains the study's experimental check list and Appendix C contains the Food Handler's Permit, obtained in compliance with Risk Management requirements at Brigham Young University.

Introduction

Animal models have a long history in the literature of being complementary to human studies, particularly for the examination of individual components of complex behaviors, processes, or sequences. In vivo animal studies often permit the isolation of specific variables that would be otherwise impossible in humans. Similarly, animal organ and tissue studies have offered alternatives to human research. Prior to the widespread use of a particular animal model, generalizability to humans must be established. Furthermore, different animal models may be more or less suited to address particular research questions or to be generalizable to humans.

Animal models have translational value in understanding human speech. However, specific study procedures must be established so that the quantity of samples and resources can be minimized and impact maximized for animal models. Standardized operational procedures are essential to programmatic research lines. For the past 25 years, procedures for the examination of voice function in excised larynx models have been well-established (Jiang & Titze, 1993; Regner, Tao, Zhuang, & Jiang, 2008). Research has been undertaken to determine which animals best translate to humans for different experimental questions (Alipour & Jaiswal, 2008; Berry, Herzel, Titze & Story, 1996; Finkelhor, Titze, & Durham, 1988; Hanson, Zhang, & Jiang, 2011; Howard, Mendelsohn & Berke, 2015; Johanes, Mihelc, Sivasankar, & Ivanisevic, 2011; Regner et al., 2008). For example, to answer the question of what animal model is most similar to humans, studies have found that the porcine larynx has the closest anatomical features. As the traditional benchtop setup has evolved, however, the methods for tissue storage and later retrieval have varied. Because preservation methods for excised tissue may influence its integrity for future experimentation (Stevens, 2017), it is important that the operational procedures for these steps also be standardized. The current work examined the effects of established and novel excised larynx storage methods on vocal fold vibratory function in traditional benchtop models.

Excised Larynx Models

The use of excised animal larynges has been a critical methodology in the realm of voice research. Excised larynges have been used for a variety of voice studies that have examined laryngeal structure and function. Specific to the current investigation, prior excised larynx studies have contributed to the understanding of vocal fold hydration, mobility, and vibratory mechanics. For example, there is seminal work in excised larynx research documenting the influence of hydration and dehydration states on vocal fold vibration (Finkelhor et al., 1988). Other work has been performed to quantify aerodynamic parameters of normal vocal fold vibration to serve as a comparison for excised animal models of disordered phonation (Zhang, Reynders, Jiang, & Tateya, 2007). Excised larynx work has also been extended to human phonatory and tissue studies to maximize translational impact (Chan & Titze, 2000; Mau, Muhlestein, Callahan, Weinheimer, & Chan, 2011).

Depending on the aims of the research study, an excised animal laryngeal model can be preferred over in vivo data collection for several reasons. First, in vivo data collection lacks the ability to control for many extraneous variables while excised often allows for examination of the main and interaction effects for each variable (Berry et al., 1996). For example, it is difficult to control and manipulate pitch during in vivo studies, whereas, in an ex vivo model pitch can be manipulated and controlled easily. Because animals have the same basic physiological complexities as humans it can be difficult when using in vivo animal studies to distinguish direct relationships between variables. Another issue is that procedures with animals that are awake are difficult to perform and behavioral training is expensive and time consuming. Often this means the in vivo animal work that involves behaviors such as phonation requires that experiments be done while the animal is asleep. The limitation that comes with excised models is that there are more steps to experimentation and therefore, more possibilities for discrepancies to happen

within the steps. For example, ex vivo experimentation requires dissection steps that may or may not be held constant depending on the person performing the dissection. A cut made a few millimeters away from another could potentially make a difference in the study outcome. This, however, is compensated by the fact that an excised model can produce clear results and better control for certain variables than an in vivo laryngeal animal study.

Research groups have completed studies to determine the similarities of animal and human larynges. Many different animals have been used for research and there is some variability in the literature regarding the rationale behind which animal to use. The basis for some studies is to compare two or more ex vivo animal types to each other for greater understanding of how each then compares to human phonation. Even though studies use different animals for experimentation, a norm is needed for larynx model work. One study was completed in an attempt to find a suitable ex vivo laryngeal model that would compare to natural human phonation. The findings indicated that phonation is possible in a porcine ex vivo larynx and that the results could be compared to human phonation (Howard et al., 2015). All findings of the study were replicated to allow for a solid conclusion to be drawn about porcine phonation being comparable to human phonation.

Additional research toward the optimization of animal-to-human translation includes a study completed by Alipour and Jaiswal (2008). These investigators compared excised pig, sheep, and dog larynges to human larynges to find the most comparable model to use for phonation research. The conclusion was that the porcine larynges were the most similar in regard to physical characteristics, while also having a wide range of frequency like the human phonation abilities. Additionally, they found the average fundamental frequency (F_0) of the porcine larynges to be 220 +/- 57 Hz and the onset pressure to be 7.4 +/-2 cm H_2O (Alipour, Jaiswal, & Finnegan, 2007); these values are similar to human female measurements. Another

study found that the lamina propria collagen in porcine larynges was similar to that of humans (Johanes et al., 2011). Collectively, these findings indicate that using porcine larynges for an ex vivo study is a suitable choice for comparing to humans because of the physical features and size, the phonation characteristics, and the similar laryngeal structure (Alipour & Jaiswal, 2008; Jiang & Titze, 1993).

Computer models based on excised larynx experiments have also been tested and compared to in vivo vibratory patterns. One such study found that using an excised model for phonation research is superior to a computer-based model (Berry et al., 1996). This is because an excised larynx model more closely approximates human patterns of having a large amplitude of vibration, complete closure of the vocal folds, and a clearly defined mucosal wave. Additionally, the excised model is important because it can be manipulated to find new information in a way that is impossible in human folds. As applied to phonatory benchtop work, the literature supports the idea that a porcine model may be optimal for excised larynx studies. Furthermore, it is currently more advanced and generalizable than computer-based models (Birk et al., 2017).

Phonation Threshold Flow

A traditional aerodynamic measure used to determine the condition and function of the vocal folds is phonation threshold pressure (PTP), the minimum amount of pressure at the onset of phonation (Hottinger, Tao, & Jiang, 2007; Titze, 1994). This measure has been used to discriminate between typical and pathologic phonation by assessing laryngeal function (Hottinger et al., 2007). Although this measure has been extensively reported, another measure, phonation threshold flow (PTF), has been used as an aerodynamic measure in laryngeal studies, including a study by Jiang and Tao (2007). The operational definition for PTF is the flow level at the onset of phonation. It has been suggested in the literature that PTF might be a stable and complementary measure to PTP for benchtop excised larynx work. For example, Mau and

colleagues (2011) conducted a study using excised human larynges to observe the effects of hydration on PTP and PTF (Mau et al., 2011). They found offset PTP and PTF values to be lower than onset values in human larynges. These findings were similar to the results of past studies observing hysteresis in canine larynges (e.g., Regner et al., 2008).

To establish the viability of PTF as research measure, a number of studies were conducted examining the effects of several independent variables on PTF in excised larynges. In 2011, Witt and colleagues concluded that PTF increased as the hydration of the vocal folds decreased (Witt, Taylor, Regner, & Jiang, 2011). Another study found that the following factors cause PTF to decrease: decreasing the glottal area, decreasing tissue viscosity, and vertically increasing the glottal duct length (Hottinger et al., 2007). Direct in vivo measurement of PTP requires a tracheal puncture, whereas it can only be estimated intraorally (Hoffman et al., 2012; Regner et al., 2008). PTF on the other hand is obtained in a noninvasive manner by using an external flow transducer or a circumferentially-vented pneumotachograph mask. This benefit may make PTF an attractive and practical choice for researchers examining voice function (Jiang & Tao, 2007). For these reasons, the present study will use PTF to examine the differences between experimental groups.

Relationship of Pressure and Flow

Pressure and flow do not exist independently of each other. In fact, their measures can be used to compute a resistance measurement by dividing pressure by flow. In a living model as pressure changes so would flow and vice versa. This natural relationship between the two measures makes it difficult to parse out one factor from another in vivo. In order to understand the impact these measures have on each other, an ex vivo model can be used because it allows for manipulation of individual factors. For example, the researchers can change flow while keeping pressure constant. The current study capitalizes on the ability to manipulate pressure and

flow independently to determine the unique effects of the independent variables on flow during phonation.

Human Studies

As discussed, human studies have the greatest external validity for clinical voice research but are limited by design and measurement constraints. In a recent article regarding hydration and vocal loading, the investigators discussed some of the needs for future research on these same topics, given the current limitations of human studies (Fujiki, Chapleau, Sundarrajan, McKenna, & Sivasankar, 2017). Others have described the complexities of examining vocal fold vibratory characteristics, length, and adduction during human phonation (Kunduk, Vansant, Ikuma, & McWhorter, 2017). Due to measurement limitations, to completely understand the results, many human studies incorporate a perceptual measure to help further quantify laryngeal function (Verdolini, Sandage, & Titze, 1994). Unfortunately, this type of measurement is also inherently subjective, and therefore, can leave some disparities between perceptual and instrumental measures. One study that examined the relationship between hydration and vocal acoustics reported several difficulties with their perceptual findings due to many variables (Franca & Simpson, 2011). These variables included parameters such as lifestyle, voice use patterns, and instruction compliance (i.e., not eating or drinking; not using their voice for a certain period).

In vivo human studies have produced data that in part answer basic questions regarding human phonation. They are not enough, however, to provide both a basic and complete understanding of laryngeal physiology. Currently, no model fully addresses this need, but it is clear that excised larynx models can offer an alternative method for examining certain research questions on many areas including phonation and vocal function. Unfortunately, the wide variability in experimental methodologies and setups in excised larynx research is a critical

barrier to phonation research advances (Birk et al., 2017). The lack of a specific protocol introduces variability into research and causes study outcomes to not be as comparable to each other as they could be. A potentially contaminating variable to the use of excised benchtop and tissue studies is the variability in preservation and storage practices. Freezing, refrigeration, and liquids used for storage purposes may influence the vibratory features of excised larynges. It is currently unknown how these storage variations influence translational value to human phonation or which storage practices are optimal. The current investigation addresses which storage practices are optimal for research.

Ringer's Solution

Ringer's lactate solution is a commonly used fluid in veterinary clinics and hospitals to help counteract dehydration. Each 100 mL of Ringer's contains 860 mg of sodium chloride (Na^+Cl^-), 30 mg of potassium chloride (K^+Cl^-), 33 mg of calcium chloride ($CaCl_2$), and 33 mg of dihydrate ($2H_2O$). By comparison, each 100 mL of isotonic saline contains 900 mg of sodium chloride (Na^+Cl^-). Ringer's is considered a balanced fluid, which approximates the normal bicarbonate concentration in a living organism (Schwarz, 2015). Although isotonic saline has the same particle concentration as the extracellular fluid in the human body, it is not considered balanced. There is a robust literature on the applications of Ringer's, predominantly in veterinary medicine for purposes of maintaining adequate systemic hydration. One recent human study compared the effects of Ringer's versus isotonic saline in patients recovering from dehydration due to choleriform diarrhea. They found that Ringer's produced a superior clinical response because it brought about a more rapid physiological correction (Cieza, Hinostroza, Huapaya, & Leon, 2013). Another study examined the effects of Ringer's on mucociliary clearance using an excised rat airway model. Based on the analysis of mucus extracted from the trachea, the investigators concluded that Ringer's was effective in improving movement of mucus from the

lower to upper airways (Okuyucu et al., 2009). Given the precedence for using Ringer's in both animal and human research, including excised tissue models, there is a strong theoretical rationale for using Ringer's for tissue storage in excised larynx models.

Statement of Problem

Significant variability exists in the literature regarding experimental methodologies for excised larynx studies. Some studies have been undertaken to optimize parameters such as the type of animal model, dissection techniques, experimental setup, and measurements. Unfortunately, substantial variability exists regarding the method of tissue storage prior to data collection. The most common method in recent years has been flash freezing individual containers in liquid nitrogen after immersing each larynx in saline. Freezing is a necessary reality for many studies due to the need to store tissue when it becomes available. However, even the methods for flash freezing vary and therefore, the effects of freezing on vocal fold vibration in benchtop models also varies. Recent studies by Hansen (2016) and Stevens (2017) documented significant differences in PTP and PTF for freshly obtained porcine larynges versus those values previously reported in the literature. Another concern with tissue storage is the significant ion loss that occurs quickly postmortem. A storage option that minimizes this ion loss would also be an important step toward a more physiologically realistic excised larynx model. Therefore, it would be valuable to examine the effects of tissue storage on phonation in excised larynx models.

Statement of Purpose

The purpose of the present investigation was to determine how tissue preservation methods influence vocal fold vibration in an excised larynx mechanical model. This work examined flash freezing in saline versus refrigerator storage in saline and in Ringer's solution. The aim of this study was to quantify the differences, if any, that these preservation methods had

on phonation. The results of this study will contribute significantly to the literature by helping to standardize tissue storage and reduce variability in excised larynx studies.

Research Questions

The following questions were addressed using an excised porcine larynx mechanical model:

1. What are the effects of the following tissue preservation methods on PTF?
    a. flash freezing in 0.9% isotonic saline
    b. refrigerator storage in 0.9% isotonic saline
    c. refrigerator storage in Ringer's lactate solution
2. How does preservation method affect PTF during exposure to dry air?

Method

The procedures and experiments for the present investigation were completed on the Brigham Young University campus in the John Taylor Building, rooms 105 and 106, and the Joseph K. Nicholes Building, room 126. The porcine larynges were obtained from Circle V Meats, a local butcher shop in Spanish Fork, UT, and were extracted from animals sacrificed for other non-research purposes. The author of this thesis obtained a Utah food handler's permit for working with food grade animal tissue, a copy of which is included in Appendix C. All experimental procedures were completed in compliance with Brigham Young University Risk Management and the Institutional Animal Care and Use Committee.

Larynges

Thirty healthy adult excised porcine larynges of less than two years of age were obtained from the local butcher shop within 24 hours postmortem. After pickup, the larynges were taken back to the lab and underwent a rough dissection to remove additional tissue that was unnecessary for the experiment. During rough dissection, the larynges were scrutinized for any

structural abnormalities or punctures and discarded if any were present. The larynges were then divided randomly into three equal groups and stored. One group of 10 larynges was assigned to the 0.9% $Na^{+}Cl^{-}$ isotonic saline group and each individual larynx was submerged in a plastic zip closure bag of this solution. Another group of 10 larynges was assigned to the Ringer's lactate solution group and underwent the same process as the saline group described above. Both groups of larynges were then stored in a refrigerator for approximately 15 hours prior to data collection. The final 10 larynges were submerged in a plastic zip closure bag containing 0.9% $Na^{+}Cl^{-}$ isotonic saline and then flash frozen by immersing each bag in liquid nitrogen for approximately eight minutes. The flash frozen larynges were stored in a freezer and removed the evening prior to experimentation to slowly thaw in the refrigerator overnight.

Immediately prior to data collection, the larynges underwent fine dissection to remove the supraglottal cartilages and tissue including the false vocal folds. The arytenoid cartilages were left intact to facilitate vocal fold adduction while on the benchtop setup. The upper trachea was transected at precisely 6 cm below the glottis for placement on the airflow tubing. Disposable scalpels and metal hemostats were used to facilitate fine dissection. The thesis author completed all fine dissection procedures for purposes of consistency within and across experimental groups. After dissection, the following parameters were measured for each larynx: vocal fold length, vocal fold width, distance from the lateral margin of the vocal fold to the medial surface of the thyroid cartilage, and the vertical distance from the thyroid prominence to the superior surface and inferior surfaces of the thyroid cartilage, respectively. A suture was then added to the thyroid cartilage at approximately 0.25 cm above the anterior commissure to allow for vocal fold elongation during the trials. Each larynx was then placed in a bag of the corresponding storage solution (i.e., saline or Ringer's) for five minutes before being mounted on the benchtop.

Research Design

The study utilized a between and within groups mixed experimental design, with group and time serving as independent variables. Dependent variables were PTP and PTF, with PTF being used for this current work and PTP measures being taken for a larger ongoing project. After fine dissection, all larynges were once again bathed in their respective solutions for five minutes. The time variable consisted of the number of 5-minute desiccation trials required until each larynx ceased to vibrate. Subglottal pressure and airflow were recorded at baseline and following each desiccation trial for purposes of PTP and PTF analysis.

Procedures

Benchtop setup. The benchtop mechanical model was designed after the setup originally devised by Jiang and Titze (1993). A stainless-steel breadboard tabletop (Thorlabs, Ann Arbor, MI) served as the experimental work surface. Through a circular hole in the table, semiflexible plastic tubing was passed through for purposes of attaching the trachea of each excised larynx. Below the table, the tubing was surrounded by a custom 20-inch pseudolung constructed from aluminum and insulated with acoustic foam. The trachea was held in place with an adjustable metal hose clamp and Teflon tape. Each larynx was positioned and stabilized with three micropositioners (Model 1460, Kopf Industries, Tujunga, CA) that were attached to the benchtop table via custom bases with ¼-20 headless screws. Two of the micropositioners had three prongs attached for purposes of arytenoid positioning. One of the micropositioners was looped with the suture from the anterior portion of the thyroid cartilage to elongate the vocal folds. Once a laryngeal position was established that resulted in phonation, no further modifications to the micropositioners or laryngeal position were performed.

Airflow for phonation. Compressed air tanks with <1% relative humidity supplied subglottal air for purposes of phonation. The air tanks were capped with an adjustable flow

regulator at the standard 50 psi. Plastic tubing running from the flow regulator was equipped with an in-line thermal flow meter (Model GFM47A-VDL6-A0, Alborg Instruments, Orangeburg, NY). After passing through the flow meter, the tubing was connected to a Theraheat temperature controlled humidifier (Model RC70000, Smiths Medical, Dublin, OH) before reaching the pseudolung. Finally, the tubing exited the pseudolung superiorly, passed through the table, and was fitted with a pressure transducer (Model PT-25-S, Glottal Enterprises, Syracuse, NY) before reaching the trachea of each larynx.

Desiccation trials. Once the bench setup was arranged and the larynx was properly positioned, data collection commenced. Baseline pressure and flow values were acquired. Subsequently, 5-minute desiccation trials were undertaken. Supraglottal dry air (<1% relative humidity) was administered to the vocal folds via plastic tubing attached to a second tank of compressed air. To maximize air exposure to the medial and inferior surfaces of the vocal folds, a 5-mm shim (Allen wrench) was placed at the posterior two-thirds of the vocal folds to allow air flow to reach the infrasurface of the vocal folds, as well as the medial edges. After each 5-minute desiccation trial, phonation was attempted. Trials were concluded when each larynx ceased to phonate.

Signal acquisition. Air pressure, flow, and the acoustic voice signal were acquired each time phonation was sampled. Signals were digitized with a DATAQ A/D (DI-720 Series) converter and WinDaq software (Series Di-720, Akron, OH) at a sample rate of 10 kHz per channel. A dynamic microphone (Model SM-48, Shure, Niles, IL) was used to gather the acoustic signal and was positioned six inches from the larynx at a 45-degree angle. The signal was preamplified with an audio mixer (Samsung MIXPAD 4, New York, NY). Calibration was undertaken prior to each data collection session. The pressure transducer was calibrated to 0 and 10 cmH_20 with a pressure calibrator (PC-1H, Glottal Enterprises, Syracuse, NY) while the flow

meter was calibrated at 0 and 15 L/min using the compressed air tank. Windaq files were coded and saved for subsequent analysis. A HygroSet II Digital Hygrometer (Model DHYG-Round; HygroSet, Weston, FL) was used to measure environmental humidity at each data collection session and was calibrated with a Humidipak calibration kit.

Data analysis. Time-aligned acoustic, air pressure, and airflow signals were segmented in WinDaq and imported into Matlab (MathWorks, Natick, MA) for analysis. Phonation onset was identified using the acoustic signal. Pressure and flow for the 10 ms prior to and following the acoustic onset were averaged to quantify PTP and PTF, respectively. Nearly all WinDaq files were segmented automatically using a custom Matlab script that identified the acoustic onset of phonation. The remaining files were hand segmented by the thesis author.

Statistical analysis. Data from each of the three groups were evaluated for central tendency and variability at baseline. Differences between the two experimental groups and the control group were examined using one-way analysis of variance and Tukey's HSD post-hoc tests (alpha = .05) for each dependent variable. Interjudge and intrajudge reliability were determined for the small subset of PTP and PTF values derived from the hand segmented Windaq files; 10% of these files were resegmented by the thesis author and a second examiner and Pearson correlations calculated. All analyses were performed using SPSS, version 24 (IBM Corp., Armonk, NY).

Results

The anatomical dimensions for each larynx specimen are reported in Tables 1, 2, and 3. Table 1 includes vocal fold length, width, and distance from the medial edge to the inner surface of the thyroid cartilage in mm. Table 2 provides the dimensions of the thyroid cartilage, specifically from the protuberance to the superior surface, the protuberance to the inferior surface, and the maximum width. Trachea dimensions are provided in Table 3. Environmental

humidity was recorded at the beginning and end of data collection for each larynx except for the first day. The average environmental humidity was 31.3% (*SD* = 1.46) at baseline and 28.5% (*SD* = 1.35) at the end of data collection. Similarly, temperature was also recorded at baseline for each larynx with an average of 23.7 degrees Celsius (*SD* = 0.96).

Groups at Baseline

Average PTF for the flash frozen group at baseline was 12.13 L/min (*SD* = 5.70). For the fresh saline group, average PTF was 12.06 L/min (*SD* = 6.32); for the Ringer's, average PTF was 26.15 L/min (*SD* = 19.16). The results from a one-way ANOVA indicated significant differences among groups, $F(2, 27) = 4.493$, $p = .021$. Post hoc Tukey's HSD tests indicated significant differences between Ringer's and frozen, $p = .0.038$, and Ringer's and fresh saline, $p = .0.039$.

Phonation Trials

Normalized flow values were calculated for each of the three groups. Specifically, for each larynx specimen, baseline flow was subtracted from each subsequent observation to account for any baseline differences. For each of the three groups, PTF demonstrated a sharp initial increase followed by steady increases over time until phonation ceased. At each observation, the greatest PTF variability was observed for the Ringer's group and the least for the flash frozen group. Postbaseline PTF values were plotted until the majority of larynges ceased to vibrate. Second order polynomial model trend lines provided an adequate fit for each group. Figure 1 illustrates normalized PTF values for the fresh saline group across nine postbaseline observations. Figure 2 illustrates normalized PTF values for eight postbaseline observations for Ringer's and Figure 3 illustrates 11 observations for the flash frozen group. Polynomial regression formulas and percent explained variance are indicated for each of the three trend lines.

Figure 4 illustrates the average number of desiccation trials required to cease vocal fold vibration. For the flash frozen group, the average number of trials until vibration ceased was 8.3

(*SD* = 6.93, range = 2 to 22). For the fresh saline group, average number of trials was 6.8 (*SD* = 4.54, range = 2 to 17); for the Ringer's, average number of trials was 6.4 (*SD* = 5.08, range = 1 to 19). The results from a one-way ANOVA indicated no significant differences at the .05 alpha level, $F(2, 27) = .297$, $p = .745$. Of note, the PTF sample ceiling was set at 50 L/min *(*Alipour & Jaiswal, 2008).

Discussion

This study sought to establish a standardized method of preservation for excised animal studies by comparing freshly used versus flash frozen larynges, as well as the type of storage solution. All specimens in three groups were challenged with desiccation trials in 5-minute increments until vibration ceased; PTP and PTF were measured after each trial. Previous studies have concluded that an excised model can be informative for in vivo work, but no preservation method has been cited as the standard and variability exists in preservation methods across studies. As hypothesized, this study found that the solution type used in preservation greatly influenced the PTF in the dehydration challenges at baseline and over time. The statistically significant differences in PTF were observed for the Ringer's and the fresh saline group and the Ringer's and the frozen saline group. Additionally, the Ringer's group presented with the most variability in PTF over observations while the least variability in PTF was found in the frozen saline group.

Phonation Threshold Flow

Phonation threshold flow is an aerodynamic measure that has become more common in recent years to use when evaluating vocal function. This measure is dependent on pressure and glottal resistance in human phonation. When there are vocal pathologies affecting vocal fold abduction, adduction, or any physiological changes affecting the posterior glottal width, PTF is thought to be a more sensitive measure than PTP. Hottinger et al. (2007) conducted a study

involving 10 excised canine larynges and found that when the posterior glottal width changed between 1mm and 4 mm there was an effect on the PTF. Overall, they reported that as the glottal width increased the PTF increased as well. This increase in PTF could be due to the gap between the folds growing larger as the trials progressed and causing a greater amount of air flow to pass through the folds.

Research has also used PTF to quantify changes in voice function associated with dehydration. One early work reported that changes in flow were associated with poor vocal fold closure following a desiccation challenge in an animal benchtop model (Finkelhor et al., 1988). Another study found that the PTF increased as the larynges were exposed to dry air. The same effect did not occur in the control trials who received no desiccation, indicating that the dry air and corresponding dehydration were the cause of the higher PTF (Witt et al., 2009). Like past research, the current work saw dehydration influencing the groups as PTF increased with desiccation trials.

It is important to note that the current work held elongation and adduction constant during all trials. A study was conducted that looked at the impact of abduction and elongation on PTF (Hoffman et al., 2012). This study found significant differences in the PTF values when abduction was changed, but no change in PTF with elongation differences. This information indicates that PTF is sensitive to abduction. Due to this information, the current work established elongation and adduction positions that were sufficient to create good phonation for each larynx, and then no adjustments were made as the trials went on. Even though no changes were made to the positioning of the larynges, and regardless of the preservation method used, the dehydration factor influenced the vocal folds and caused the PTF to be higher within and across groups.

Histology

Even though no significant statistical difference was found in the number of trials required to cease phonation between the groups, the group that was flash frozen in saline required the most trials on average and had the most variability in the number of trials. This could be for many reasons, including histological changes that occur when a specimen is flash frozen and then thawed. Allenspach and Kramer (1989) examined ice crystal patterns after slow freezing and flash freezing artificial gels of extracellular matrix molecules and found that slow freezing resulted in relatively large crystals compared to the crystals formed with flash freezing. Additionally, Chan and Titze (2002) observed that these large crystals formed on the tissue of the vocal folds when they were slow frozen, but that flash freezing resulted in smaller ice crystals on the vocal fold tissue. These researches determined that flash freezing and the resulting smaller ice crystals, were much less likely to disturb the histological properties of the vocal folds. This research is a contributor to why many use a flash freezing method of preservation.

The method used for freezing is an important factor to examine; however, the method in which the tissue is thawed can also contribute to a histological change. It has been noted that a slow rate of thawing can lead to larger ice crystals forming on the tissue (Young, Armitage, Bowerman, Cook, & Easty, 1994). This work used a refrigerator to thaw the larynges overnight for 24 hours. This process of thawing before experimentation could have led to larger ice crystals forming and the pliability of the vocal folds being compromised. The actual change that occurs in the tissue from this process could be different from subject to subject which could then lead to more variability in the data collected from these subjects. This may be a partial cause of the larger variability this study encountered with the frozen group. Additionally, the freezing and thawing process might change the histology of the tissue by creating small tears from the ice crystals forming that loosen the tissue and make the vocal folds more pliable. This could result in

higher PFT values for this groups. Further research should be conducted to determine the effects of different thawing methods on vocal fold histology and phonation.

Ringer's Solution

As previously mentioned, all three groups in the study showed an increase in PTF over time, but it is important to note that the Ringer's group had the lowest PTF values across all trials on average. For example, when looking at the average PTF required for trial 8 across the groups the Ringer's group average was 14.9 L/min, whereas the fresh saline group was 27.3 L/min and the frozen saline group was 35.7 L/min. This is a notable difference in flow values that was consistently found for trials when comparing trend lines from the three groups. Of note, the data for all the groups was normalized and because the Ringer's group had such a high baseline average to normalize from, the normalized data for Ringer's might appear even lower than the other groups with normalized information. This lower PTF average could be due to the solution type used in the preservation method. Research suggests that Ringer's solution is better able to counteract the ion loss that occurs postmortem (Cieza et al., 2013) and, therefore, could be responsible in part for the specimens in the Ringer's group having lower PTF values. The two groups that used saline as the solution for preservation had very similar average PTF values as seen with the trend analysis, with the fresh saline group having slightly lower values then the frozen saline group. Overall, these two groups performed similarly over time and no statistical difference was found between them. This information strengthens the idea that the solution type could affect PTF more than the specimen being stored fresh versus frozen. It also explains why the group with the different solution type would have average values that were so different than the other two groups stored in the same solutions.

The greatest variability in values at each observation was found in the Ringer's group and the least amount of variability was found in the frozen saline group. This information may mean

that there are more factors influencing how Ringer's solution influences the tissues than previously understood. One of these factors could be how many hours pass by from the time the specimen was sacrificed to the time it is submerged in the Ringer's solution. The length of time could affect how well the Ringer's solution can reverse the ion loss in the tissue.

Limitations

There were a few limitations that occurred during this study. First, as is the case with many studies, there was a steep learning curve for the procedural operations, and the researchers may have improved in both their dissection and mounting of the larynges by the end of the study. This limitation was minimized by having training for consistency of dissection prior to the initiation of the experiment and by having one person conducting the dissections, but the learning curve still exists. Next, the overall dissection process involved multiple steps and this leaves room for error and variability. An example of this is that in order to run the study, the false folds of the larynges needed to be removed, which could have minutely damaged the true vocal folds in the process in some cases. Another instance of possible variation is with the flash freezing procedure. Although all the larynges were immersed for approximately eight minutes in the liquid nitrogen, it was challenging to tell if the very center of the larynx was completely frozen in that time. This issue was noted and great care was taken to examine each larynx to see if additional freezing time was necessary, but error could still exist in this process.

The specific larynges and their size, age, and gender could add to some variability that was not accounted for in the study. The size of the larynges was recorded after dissection, but the age of the pig and its gender were unknown to the researchers. Additionally, the precise time of death for each pig was unknown, with the researchers only being able to know sacrifice time within five hours. The porcine larynx has many similarities to the human larynx structurally; however, porcine vocal folds are at a 45° angle within the larynx whereas human vocal folds are

at a 0° angle. This kind of difference could influence data outcomes because the two angles could have different outcomes with PTF measures over time. Some equipment limitations may have included the microphone being too close to the larynx, which caused peak clipping in a few instances and a few instances of peak clipping with the flow meter. Additionally, because the microphone had to be adjusted due to peak clipping, there may be variability in the audio recording due to variable distance of the microphone from the larynx. The phonatory trials took place over three different seasons of the year; therefore, the ambient humidity levels may have influenced the pliability of the folds. The humidity levels were recorded for information purposes, but they were not controlled for. Lastly, this experiment did not take place in a sound booth, therefore reverberations and ambient noise may have affected the detection of phonation onset in some cases.

Implications for Future Research

In the future studies, researchers should limit the porcine larynges to a specific gender, preferably male due to their larger size, and continue to reduce variability with dissection and mounting practices. Larger sample sizes should be sought out to have more reliable data. Since there was less variability in the frozen group, future studies should examine the effects of Ringer's solution on tissue during different durations of storage. These studies should examine many time lengths including a flash frozen group for comparison on tissues response to being frozen in various solutions. Additionally, future studies may also look at flow measurements for sustained phonation and at phonation offset. Lastly, future work may consider histology comparisons between larynges or tissue types that were stored fresh verses frozen.

Conclusion

The results from the present investigation indicate that larynx storage in Ringer's solution resulted in flow values that were significantly different than those from larynges stored in

isotonic saline. Although Ringer's solution storage resulted in lower PTF with more modest increases during desiccation, greater variability was observed in this group as well. These findings suggest that Ringer's solution might be a better alternative to storage in saline, perhaps due to reduced excised larynx ion loss. Future work should consider the inclusion of ionically balanced solutions toward the generation of a gold standard for the storage of experimental specimens.

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Table 1

Dimensions of the Vocal Folds

Group	Session Date	Length of Vocal Folds	Width of Vocal Folds	Width from Vocal Folds to Thyroid Cartilage
Fresh IS				
Pig 1	06/17/16	23 mm	7 mm	15 mm
Pig 2	06/17/16	20 mm	5 mm	17 mm
Pig 3	06/22/16	20 mm	7 mm	15 mm
Pig 4	06/22/16	20 mm	6 mm	14 mm
Pig 5	06/22/16	20 mm	7 mm	14 mm
Pig 6	06/22/16	17 mm	7 mm	15 mm
Pig 7	12/09/16	19 mm	7 mm	16 mm
Pig 8	12/09/16	23 mm	4 mm	19 mm
Pig 9	12/09/16	21 mm	4 mm	14 mm
Pig 10	12/09/16	26 mm	7 mm	19 mm
Ringer's				
Pig 11	07/08/16	24 mm	12 mm	20 mm
Pig 12	07/08/16	19 mm	8 mm	18 mm
Pig 13	07/08/16	22 mm	6 mm	15 mm
Pig 14	10/29/16	17 mm	4 mm	12 mm
Pig 15	10/29/16	17 mm	5 mm	13 mm
Pig 16	10/29/16	24 mm	6 mm	19 mm
Pig 17	10/29/16	28 mm	6 mm	17 mm

Pig 18	10/29/16	23 mm	6 mm	14 mm
Pig 19	10/29/16	30 mm	7 mm	12 mm
Pig 20	12/09/16	21 mm	5 mm	11 mm
Frozen IS				
Pig 21	01/05/17	22 mm	4 mm	13 mm
Pig 22	01/05/17	19 mm	3 mm	15 mm
Pig 23	01/05/17	21 mm	5 mm	17 mm
Pig 24	01/05/17	23 mm	5 mm	19 mm
Pig 25	03/10/17	19 mm	8 mm	13 mm
Pig 26	03/10/17	21 mm	6 mm	15 mm
Pig 27	03/10/17	23 mm	6 mm	17 mm
Pig 28	04/07/17	21 mm	4 mm	14 mm
Pig 29	04/07/17	23 mm	6 mm	14 mm
Pig 30	04/07/17	20 mm	5 mm	19 mm

Table 2

Dimensions of Thyroid Cartilage

Group	Session Date	Height (protuberance to top)	Height (protuberance to bottom)	Width of Thyroid Cartilage
Fresh IS				
Pig 1	06/17/16	58 mm	30 mm	43 mm
Pig 2	06/17/16	59 mm	25 mm	44 mm
Pig 3	06/22/16	56 mm	27 mm	47 mm
Pig 4	06/22/16	57 mm	27 mm	44 mm
Pig 5	06/22/16	59 mm	21 mm	44 mm
Pig 6	06/22/16	64 mm	36 mm	44 mm
Pig 7	12/09/16	55 mm	23 mm	42 mm
Pig 8	12/09/16	56 mm	25 mm	50 mm
Pig 9	12/09/16	61 mm	24 mm	41 mm
Pig 10	12/09/16	53 mm	31 mm	44 mm
Ringer's				
Pig 11	07/08/16	69 mm	34 mm	60 mm
Pig 12	07/08/16	59 mm	26 mm	50 mm
Pig 13	07/08/16	62 mm	25 mm	47 mm
Pig 14	10/29/16	48 mm	28 mm	46 mm
Pig 15	10/29/16	55 mm	27 mm	42 mm
Pig 16	10/29/16	56 mm	32 mm	44 mm
Pig 17	10/29/16	57 mm	26 mm	44 mm

Pig 18	10/29/16	56 mm	23 mm	41 mm
Pig 19	10/29/16	53 mm	21 mm	41 mm
Pig 20	12/09/16	50 mm	31 mm	40 mm
Frozen IS				
Pig 21	01/05/17	60 mm	31 mm	49 mm
Pig 22	01/05/17	61 mm	27 mm	49 mm
Pig 23	01/05/17	67 mm	39 mm	53 mm
Pig 24	01/05/17	53 mm	28 mm	43 mm
Pig 25	03/10/17	54 mm	22 mm	49 mm
Pig 26	03/10/17	53 mm	34 mm	51 mm
Pig 27	03/10/17	56 mm	34 mm	43 mm
Pig 28	04/07/17	62 mm	33 mm	44 mm
Pig 29	04/07/17	52 mm	31 mm	45 mm
Pig 30	04/07/17	54 mm	31 mm	49 mm

Table 3

Length and Width of Trachea

Group	Session Date	Trachea Length	Trachea Width
Fresh IS			
Pig 1	06/17/16	77 mm	20 mm
Pig 2	06/17/16	77 mm	16 mm
Pig 3	06/22/16	77 mm	20 mm
Pig 4	06/22/16	77 mm	19 mm
Pig 5	06/22/16	77 mm	21 mm
Pig 6	06/22/16	77 mm	22 mm
Pig 7	12/09/16	77 mm	20 mm
Pig 8	12/09/16	77 mm	19 mm
Pig 9	12/09/16	77 mm	21 mm
Pig 10	12/09/16	77 mm	22 mm
Ringer's			
Pig 11	07/08/16	77 mm	25mm
Pig 12	07/08/16	77 mm	20 mm
Pig 13	07/08/16	77 mm	23 mm
Pig 14	10/29/16	77 mm	18 mm
Pig 15	10/29/16	77 mm	19 mm
Pig 16	10/29/16	77 mm	22 mm
Pig 17	10/29/16	77 mm	20 mm
Pig 18	10/29/16	77 mm	22 mm

Pig 19	10/29/16	77 mm	22 mm
Pig 20	12/09/16	77 mm	22 mm
Frozen IS			
Pig 21	01/05/17	77 mm	21 mm
Pig 22	01/05/17	77 mm	22 mm
Pig 23	01/05/17	77 mm	23 mm
Pig 24	01/05/17	77 mm	22 mm
Pig 25	03/10/17	77 mm	20 mm
Pig 26	03/10/17	77 mm	19 mm
Pig 27	03/10/17	77 mm	15 mm
Pig 28	04/07/17	77 mm	22 mm
Pig 29	04/07/17	77 mm	22 mm
Pig 30	04/07/17	77 mm	21 mm

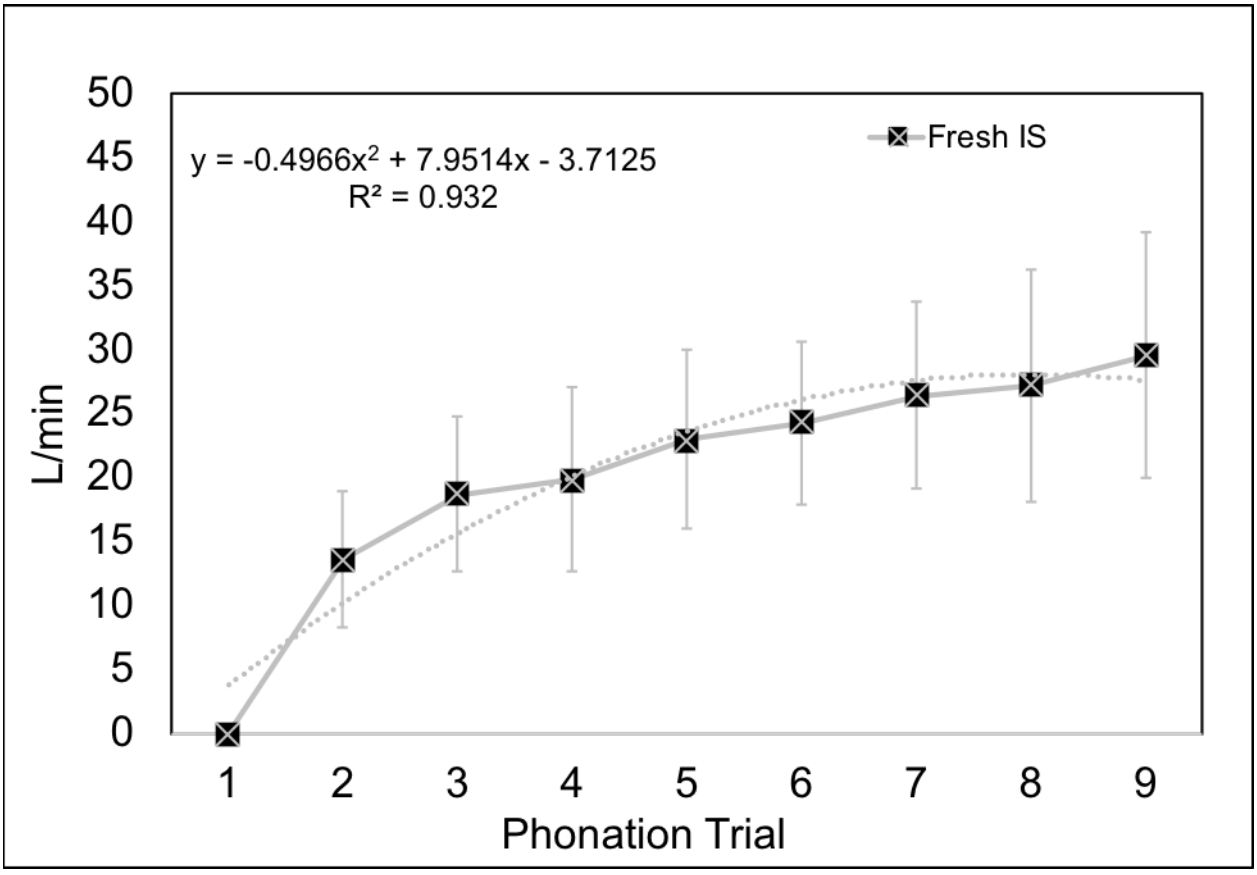

Figure 1. PTF during desiccation for the Fresh IS group. Normalized phonation threshold flow (PTF) at baseline (0 on the y axis) and following each 5-min desiccation dose for the Fresh IS group. Mean, standard error represented by vertical bars, linear trend lines with a > .80 criterion for fit, and R^2 values with formulas are provided.

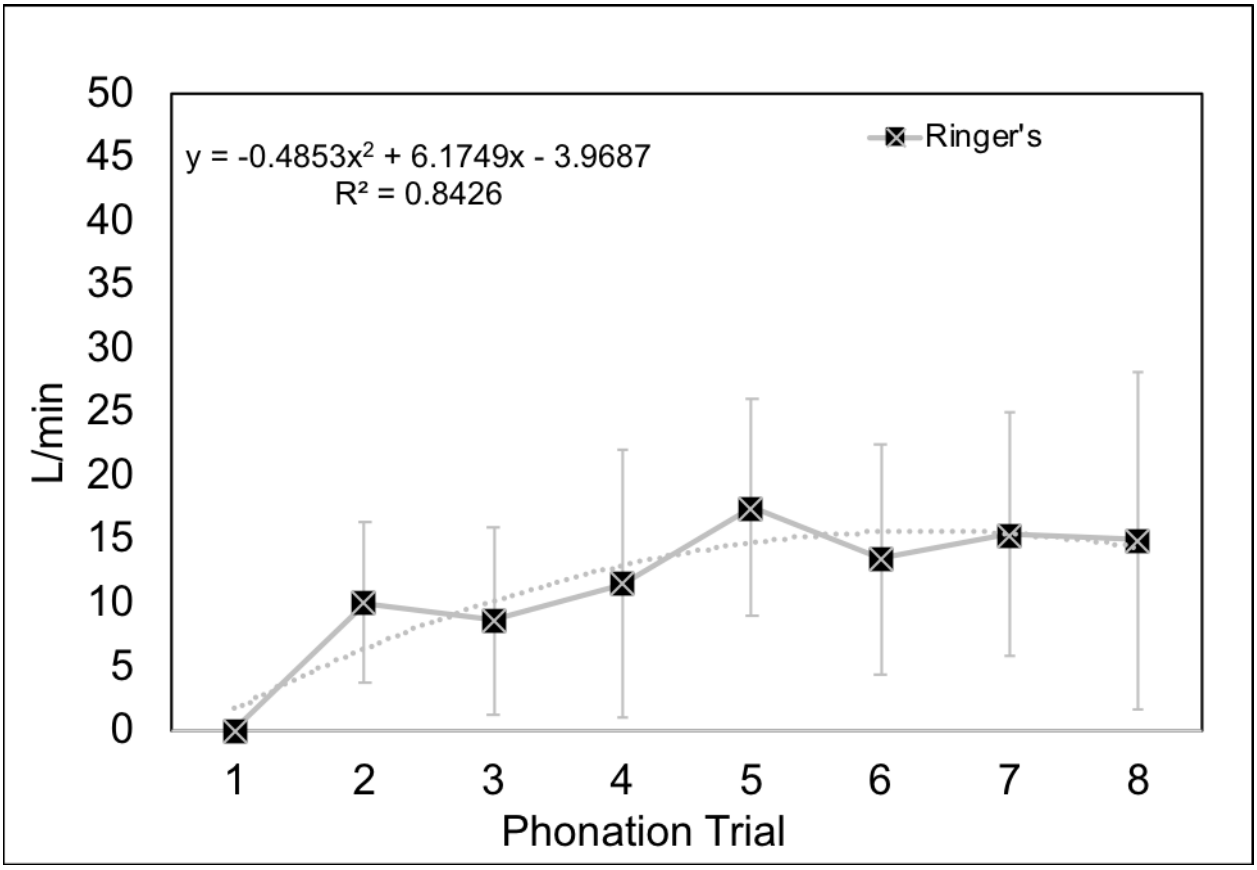

Figure 2. PTF during desiccation for the Ringer's group. Normalized phonation threshold flow (PTF) at baseline (0 on the y axis) and following each 5-min desiccation dose for the Ringer's group. Mean, standard error represented by vertical bars, linear trend lines with a > .80 criterion for fit, and R^2 values with formulas are provided.

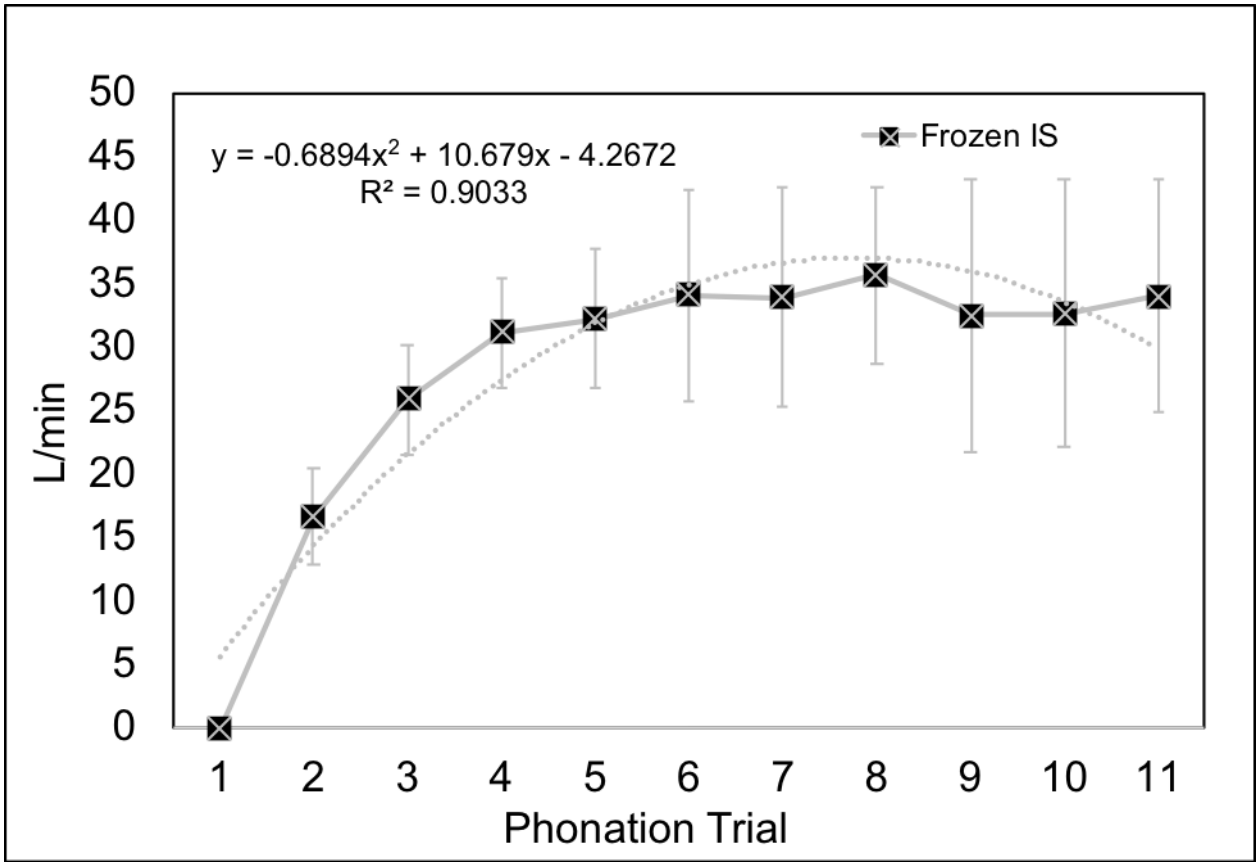

Figure 3. PTF during desiccation for the Frozen IS group. Normalized phonation threshold flow (PTF) at baseline (0 on the y axis) and following each 5-min desiccation dose for the Frozen IS group. Mean, standard error represented by vertical bars, linear trend lines with a > .80 criterion for fit, and R^2 values with formulas are provided.

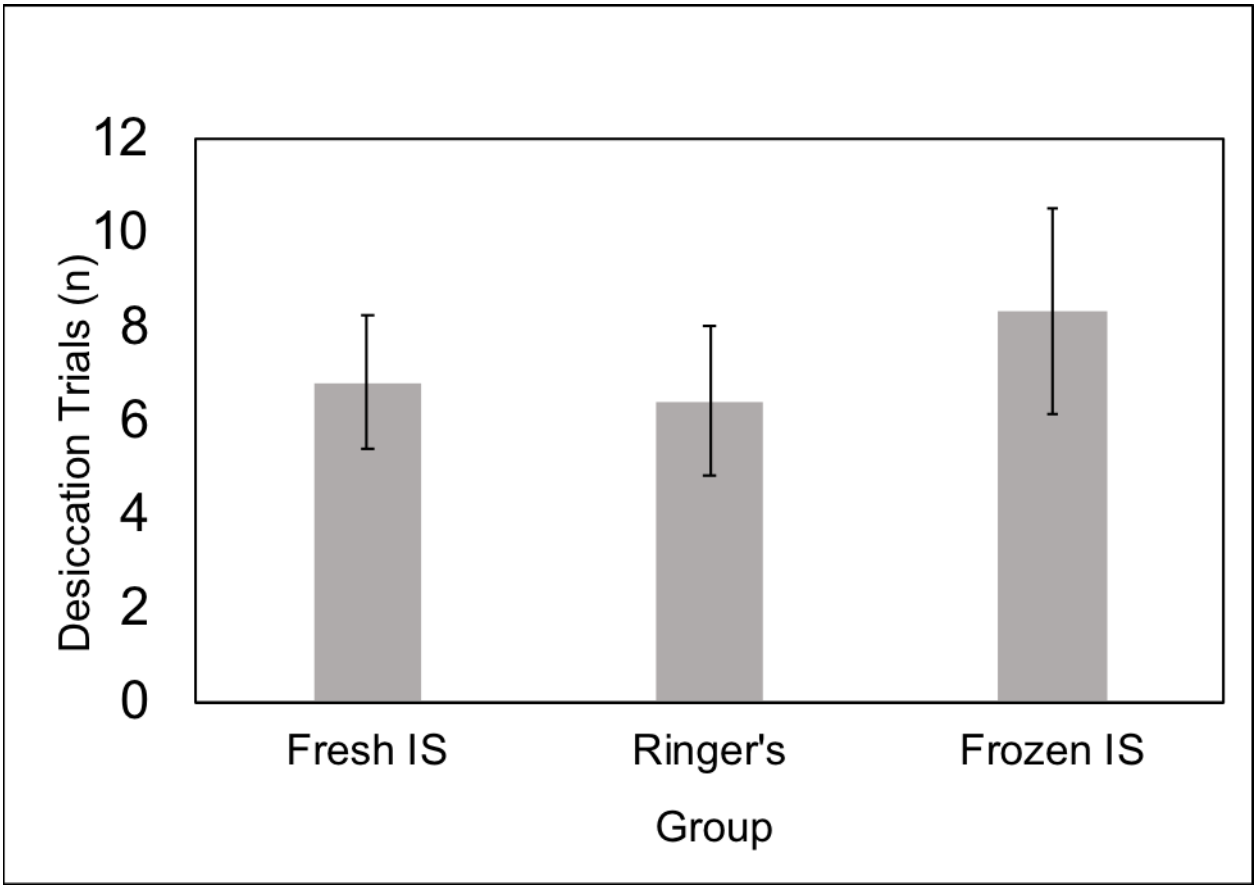

Figure 4. Number of trials until vibration ceased. The number of desiccation trials required to cease vocal fold vibration for larynges in the Fresh IS, Ringer's, and Frozen IS groups. Standard error is represented by vertical bars.

APPENDIX A: Annotated Bibliography

Alipour, F., & Jaiswal, S. (2008). Phonatory characteristics of excised pig, sheep, and cow larynges. *Journal of the Acoustical Society of America, 123*, 4572-4581. doi:10.1121/1.2908289

Purpose of the study. The purpose of this study was to determine the similarities and differences among porcine, bovine, and ovine larynges and human larynges, particularly with regard to vocal fold vibratory characteristics and oscillation patterns. Additionally, the investigators examined the relationships between subglottal pressure and fundamental frequency in each of the three species.
Method. A local butcher shop provided eight porcine larynges, eight ovine larynges, and six bovine larynges, which were cleaned and slowly frozen using traditional freezing methods. Prior to the experiment, the larynges were removed from the freezer and thawed overnight in 0.9% saline. Next, the larynges were mounted using a benchtop model that provided humidified, pressurized, and heated air. The following variables were sampled during vibration for each larynx: acoustic signal, mean flow rate, subglottal pressure, electroglottograph, and sound pressure level.
Results. Compared to the ovine and bovine larynges, porcine larynges were found to have the greatest F_0 range and largest sound pressure level. The bovine larynges had an oscillation frequency around 74 Hz that was steady and was not impacted by a change in pressure. The ovine larynges demonstrated a linear relationship between frequency and pressure at higher but not lower pressures. The porcine larynges produced voicing that was as loud as 96.1 dB and the ovine and bovine reached approximately 78 dB. Structurally, the porcine larynx was judged to be most like humans due to the presence of ventricles.
Conclusions. This study provided evidence for the use of porcine larynges as a useful model to examine phonatory variables that influence human phonation.
Relevance to the current work. Information from this study supports the rationale behind conducting experiments with porcine larynges and comparing them to human counterparts. It shows that porcine is the most similar in regards to physical characteristics, as well as having a wide range of frequency.

Berry, D. A., Herzel, H., Titze, I. R., & Story, B. H. (1996). Bifurcations in excised larynx experiments. *Journal of Voice, 10*, 129-138.

Purpose of the study. This study was conducted to examine the bifurcations found in excised canine vocal folds and the corresponding relationships with vibration.
Method. Five reportedly large canine larynges were obtained postmortem and dissected. The larynges were then mounted on a benchtop and vibrated using subglottal humidified heated air. The folds were elongated with a suture attached to the anterior arch of the cricoid cartilage. Throughout the experiment, the folds were elongated asymmetrically via the arytenoids to gather various positional data. The variables sampled included phonation onset, vibratory patterns, and various bifurcations.
Results. The study determined that the canine larynges could produce voice at different registers. The vibratory pattern for chest-like phonation had a large amplitude of vibration, complete closure, and a clearly defined mucosal wave. The pattern for falsetto-like phonation consisted of small amplitude, incomplete closure, and some phase asymmetry. Relationships were found

among asymmetry of adduction, asymmetry of elongation, and vibration that were similar to those in humans.
Conclusions. Comparison of bifurcations in the excised larynges to a computer-based model and found that the excised models more closely approximated human patterns. The excised larynx model is important because it can be manipulated to find new information in a way that is impossible in human folds. The bifurcation diagrams generated in this study successfully modeled vocal fold dynamics.
Relevance to the current work. This offered additional evidence that excised models are an important mechanical model to examine variables that influence phonation in humans. This work did not include excised larynx storage methodology, which was the focus of the current study.

Chan, R. W., & Titze, I. R. (2003). Effect of postmortem changes and freezing on the viscoelastic properties of vocal fold tissues. *Annals of Biomedical Engineering, 31*, 482-491.

Purpose of the study. The aim of this study was to determine if there are adverse effects on the biomechanical physiology of vocal fold tissues after having been frozen and stored for later experimentation.
Method. Investigators harvested 16 canine larynges from animals being euthanized for other purposes. Tissue harvest occurred within the first 10 minutes postmortem. Vocal fold lamina propria and muscle samples were dissected from each larynx and wrapped in 0.9% saline-soaked gauze; samples were stored in a plastic zip storage bag at room temperature. Following the 24 hour postmortem testing, samples were either slow frozen or flash frozen in liquid nitrogen and stored for one month. Following this storage period, samples were slowly thawed in a refrigerator and then viscoelastic tests were undertaken again. Viscoelastic shear properties were assessed at approximately 20 minutes and 24 hours postmortem. All measurements were collected with a controlled-stress torsional rheometer.
Results. The results from elastic shear modulus and dynamic viscosity tests indicated that viscoelastic properties remained nearly unchanged in the first 24 hours using the above protocol. Additionally, the data showed that the viscoelastic shear properties did not significantly change after one month of frozen storage with quick freezing. The only exception was some change in the higher frequencies, which increased very slightly. Slow freezing did have an impact on the properties, showing significant decreases across all measurements.
Conclusions. This study concluded that using fresh tissues within 24 hours postmortem did not alter viscoelastic properties. Additionally, quick freezing better preserves the integrity of the tissues then slow freezing. Flash freezing may be a feasible option for future studies to ensure that tissue viscoelastic properties are not altered for experiments.
Relevance to the current work. The current work includes quick flash freezing as a control group, compared to fresh larynges in various solutions. This study gives reason to use the quick frozen as a control.

Chan, R. W., & Titze, I. R. (1999). Viscoelastic shear properties of human vocal fold mucosa: measurement methodology and empirical results. *Journal of the Acoustical Society of America, 106*, 2008-2021.

Purpose of the study. This study was undertaken to determine the viscoelastic shear properties of human mucosal tissues, in particular the superficial layer of the lamina propria.

Method. Ten male and five female excised human larynges were either collected and studied within 24 hours postmortem or quick frozen for later study within 18 hours postmortem. The vocal folds were dissected from each larynx and viscoelastic assessment was accomplished via rheometry. The tissues were examined while placed between two rotating circular plates that created shear deformation. The rheometer measured the shear deformation, the elastic shear modulus, and the viscous shear modulus.
Results. Results showed that there was no mechanical asymmetry in the shear properties between the mucosal tissues of the contralateral vocal folds, which was expected. Overall, the male mucosal tissue was more stiff than the female mucosal tissue. Many of the differences found were attributed to the age and gender of the larynges. For instance, the average elasticity and viscosity of the male vocal fold mucosa was between three and five times greater than females. Results also showed that the damping ratio of human musical tissue was relativity constant across all frequencies.
Conclusions. Large differences were found for between larynges, which could be due to age and gender. Limitations of the study included the fact that data were collected at or below 15 Hz and that the sample size was small. Future work is needed to compare age and gender effects on tissue viscoelasticity.
Relevance to the current work. This work included human samples that were both fresh and flash frozen. The current work will examine tissue storage specifically.

Finkelhor, B. K., Titze, I. R., & Durham, P. L. (1988). The effect of viscosity changes in the vocal folds on the range of oscillation. *Journal of Voice*, *1*, 320-325.

Purpose of the study. This study quantified PTP in excised animal vocal folds to determine if there was a relationship between the hydration and vocal fold onset.
Method. The study included four excised canine larynges. Each larynx was then mounted on a custom benchtop including micropositioners to adduct and lengthen the vocal folds. The glottal and subglottal area of each larynx was bathed in a sequence of 0.9% saline, distilled water, and 2.5% saline for 15 minutes, respectively. The vocal folds were subsequently lengthened in a uniformed manner and measurements were taken for three different glottal widths. A u-tube manometer was used to measure pressure when vibration began and ceased.
Results. This study found that a higher vocal fold onset threshold occurred when the larynges were bathed in hypertonic saline and a lower oscillation threshold occurred when the larynges were bathed in distilled water. Additionally, it was noted that less strain and higher pressures improved vibratory characteristics.
Conclusions. This study demonstrated that the oscillation threshold is impacted by hydration state of the vocal folds. The investigators also suggested that water could permeate the epithelial layer and influence vocal fold viscosity.
Relevance to the current work. This was the first study to examine vibratory patterns in excised larynges in relation to viscosity factors. The findings are generally consistent with later studies of hydration effects on the vocal folds. Additionally, the investigators suggested that increased vocal strain caused by dehydration may lead to vocal pathologies.

Hanson, K. P., Zhang, Y., & Jiang, J. J. (2010). Parameters quantifying dehydration in canine vocal fold lamina propria. *The Laryngoscope, 120*, 1363-1369. doi:10.1002/lary.20927

Purpose of the study. The purpose of this study was to determine the permeability of excised canine vocal fold lamina propria. The investigators examined these properties during different levels of dehydration. They approached this work from the viewpoint that vocal fold lamina propria is biphasic and is defined by both solid and liquid dynamics and sought data to support this hypothesis.
Method. A total of 15 excised canine larynges were collect and both vocal folds were dissected, for a total of 30 samples. These samples were then divided into three groups and underwent either 30%, 50% or 70% dehydration. Tissue samples were mounted and the permeation of 0.9% saline through the tissue was measured over time.
Results. Ratios of were computed to compare liquid-to-solid mass and volume ratios, as well as liquid-to-solid fractions. These calculations indicated that the 30% and 50% levels were not significantly different for the volume ratio, but all other comparisons were significant. An inverse linear relationship was also found for the liquid-to-solid fraction. Additionally, it was found that at permeability increased incrementally after an initial 20% strain.
Conclusions. The study indicated that the permeability increased along the anterior-posterior axis. This, along with the discovery of the increased permeability with the >20% strain, showed that some phonation tasks may influence vocal fold dynamics. The results also supported the biphasic theory.
Relevance to the current work. The awareness that permeability is influenced by both the location along the lamina propria and the strain percentage gives information for future experiments that examine hydration factors. These data are also relevant to studies that aim reverse, offset, or prevent the effects of vocal fold dehydration.

Hanson, K. P., Zhang, Y., & Jiang, J. J. (2011). Ex vivo canine vocal fold lamina propria rehydration after varying dehydration levels. *Journal of Voice, 25*, 657-662. doi:10.1016/j.jvoice.2010.06.005

Purpose of the study. This study examined lamina propria recovery from several levels of vocal fold dehydration.
Method. Ten healthy porcine larynges were collected and then immersed in 0.9% saline immediately postmortem. The vocal folds were removed and initial measurements were taken of the thickness of the samples. The vocal folds were divided into two groups, ten folds in each group, and underwent a dehydration process. One group was 30% dehydrated from its original mass and one was 70% dehydrated from its original mass. Lastly, the mass was restored with 0.9% saline until mass stabilized.
Results. Approximately half of the specimens in the 30% dehydration group regained their original mass with hydration, while only one specimen from the 70% dehydration group regained its original mass. This proved that there is a large difference in ability to regain original mass after dehydration between 30% and 70% despite uniform hydration processes.
Conclusions. These results support the biphasic theory and suggest that the potential amount of rehydration could be directly linked to the original dehydration severity. Specifically, it is possible that there could be a point at which dehydration is at least partially irreversible.
Relevance to the current work. This work supports the idea that vocal tissue could be desiccated to a point where complete rehydration cannot be achieved and thereby phonation is permanently impacted. Additionally, it lays foundation for work aimed at preserving vocal fold hydration in mechanical model and tissue experiments.

Hartley, N. A., & Thibeault, S. L. (2014). Systemic hydration: Relating science to clinical practice in vocal health. *Journal of Voice, 28*, 651-652. doi:10.1016/j.jvoice.2014.01.007

Purpose of the study. The aim of this study was to better understand vocal health with regard to systemic hydration.
Method. The paper was a literature review spanning multiple disciplines. Some of the disciplines included in the search were: speech language pathology, dietetics, medicine, exercise science, physiology, and biomechanics.
Results. Many different disciplines are interested and invested in the relationship between hydration and tissue function. The literature that has currently been published points to the fact that there is indeed a relationship between hydration and voice production, but further research must be conducted to understand underlying mechanisms and to further develop evidence-based treatment interventions. In particular, work needs to be done to research reversing vocal dehydration and to find a more effective way to prevent vocal dehydration from occurring.
Conclusions. Many areas lack complete research in dehydration. The work needs to be multidisciplinary and provide a clear analysis for future studies so that the impact can span disciplines.
Relevance to the current work. This study states the need for further studies on vocal fold dehydration and the need to work across disciplines to expand the knowledge base.

Hoffman, M. R., Rieves, A. L., Budde, A. J., Surender, K., Zhang, Y., & Jiang, J. J. (2012). Phonation instability flow in excised canine larynges. *Journal of Voice, 26*, 280-284. doi:10.1016/j.jvoice.2011.03.007

Purpose of the study. This study quantified phonation instability flow (i.e., when vocal fold oscillation becomes irregular) to estimate the range in which vocal fold vibration begins.
Method. Seven excised canine larynges were mounted on a benchtop. Air pressure and flow were measured at phonation onset and subsequent chaos onset under three conditions: 0% elongation with no glottal gap, 20% elongation without a glottal gap, and 20% elongation with a 3-mm posterior glottal gap. The experimenters increased airflow until they achieved PTP and PTF and then continued to increase until they achieved chaotic phonation. At that time, airflow was decreased until phonation ceased. A paired *t*-test was used to measure the effects of elongation and the posterior glottal gap.
Results. The results indicated that phonation instability flow and flow range findings were significant for abduction, but not for elongation. Phonation instability pressure on the other hand was not significant for either elongation or abduction. Phonation instability flow and flow range showed more significant differences for vocal fold abduction compared to PTP and PTF.
Conclusions. The results showed that phonation instability flow and phonation flow range could enhance clinical assessment for pathologies such as vocal fold paralysis, vocal nodules, and presbylaryngis. Additionally, the study found that there was not a relationship between elongation and PTP or PTF.
Relevance to the current work. This study provided further insight into aerodynamic measures in an excised larynx benchtop model. This information could be useful for observing vocal fold biomechanics. The study also concluded that abduction influences PTF, which was held constant for the current study.

Hottinger, D. G., Tao, C., & Jiang, J. J. (2007). Comparing phonation threshold flow and pressure by abducting excised larynges. *Laryngoscope, 117*, 1695-1699. doi:10.1097/MLG.0b013e3180959e38

Purpose of the study. This study manipulated the glottal width in excised canine larynges to determine if PTP and PTF were sensitive to these mechanical changes.
Method. Ten excised canine larynges were examined for lesions and then tested in a benchtop model measuring subglottal pressure and airflow at phonation onset. The ranges of glottal abduction were from 0 to 4.0 mm and were achieved with a metal shim. Airflow was increased in each case until vibration was achieved. Onset airflow and onset pressure data was taken from each trial.
Results. Significant differences in the PTF were observed when the glottal width was greater than 1.0 mm. PTF increased as glottal width increased. Alternatively, PTP was found to be insignificantly impacted by glottal width at all values.
Conclusions. These findings indicate the PTF is more sensitive to glottal width then PTP is. In diagnosing and assessing vocal diseases, PTF may be a better indicator of a problem with adduction then PTP.
Relevance to the current work. This study supports the measurement of both PTP and PTF in vocal fold vibration experiments. It provided a framework for understanding how glottal width impacts these measures, as well as a rationale for why they should be examined in hydration experiments.

Howard, N. S., Mendelsohn, A. H., & Berke, G. S. (2015). Development of the ex vivo laryngeal model of phonation. *The Laryngoscope, 125*, 1414-1419. doi: 10.1002/lary.25149

Purpose of the study. The purpose of the study was to find a suitable ex vivo laryngeal model that would compare to in vivo human phonation. This would allow for greater research without the design limitations that come with human studies.
Method. The study included 19 excised canine larynges that were dissected and then reperfused to oxygenate the tissues. Electrodes were placed on the recurrent laryngeal nerve and the superior laryngeal nerve. An endotracheal tube was also placed inside the trachea. Stimulation was then administered to the nerves while humidified air was passed through the trachea to initiate phonation.
Results. The findings indicated that full neuromuscular stimulation and phonation are possible in an ex vivo larynx. Heparinized whole blood had the most robust neuromuscular response. Additionally, the best long-term phonation was achieved in larynges that received reperfusion in a pulsatile flow. All findings were replicated to demonstrate reliability.
Conclusions. Phonation in an ex vivo model can be compared to in vivo human phonation. This model can be adapted to research other information regarding the human larynx. Some applications of this line of research include neuromuscular reinnervation, transplant preservation, and effects of organ ischemia.
Relevance to the current work. This work identified and demonstrated an appropriate use of animal ex vivo experiments to compare to in vivo human phonation. Similarly, the current work uses porcine larynges and is also applicable to human larynges.

Jiang, J. J., Raviv, J. R., & Hanson, D. G. (2001). Comparison of the phonation-related structures among pig, dog, white-tailed deer, and human larynges. *Annals of Otology, Rhinology, and Laryngology, 110*, 1120-1125.

Purpose of the study. This study was designed to identify the best possible animal larynx to use in place of a human larynx for experimental purposes. To accomplish this objective, the study included examination of the anatomical structures and biomechanical features of pig, dog, white-tailed deer, and human larynges.
Method. Each of the different species of larynges were obtained and dissected to produce a clear view of the true vocal folds. Each larynx was then measured to obtain data on vocal fold height, range of motion of the cricothyroid joint, vocal fold stiffness, and glottal configuration.
Results. Results indicated that all species were of similar vocal fold length. The human, pig, and dog larynges were similar in the size of their cricothyroid muscles, had greater amounts of mobility in their cricothyroid joint, and had a cartilaginous framework that allowed for more specific movement. The deer larynx had less range in the cricothyroid joint and less precise movement. The pig vocal folds had the closest physiology to human vocal fold because they had the most similar vocal fold cover and vocal fold stiffness.
Conclusions. Although all the samples had similar vocal fold length, the pig larynx proved to be superior as a substitute for human phonation research. Additionally, pig larynges are generally widely available.
Relevance to the current work. This study provides additional rationale for the use of pig larynges in the current study. The current study also builds on this work by including phonatory variables.

Jiang, J. J., & Tao, C. (2007). The minimum glottal airflow to initiate vocal fold oscillation. *Journal of the Acoustical Society of America, 121*, 2873-2881.

Purpose of the study. The purpose of this study was to introduce PTF as a new aerodynamic measure. The study was designed to identify the minimum airflow required to elicit vocal fold vibration.
Method. This work used one mass model and quantified airflow at the onset of phonation in excised canine larynges.
Results. Glottal shape significantly influenced PTF measurements. Additionally, factors such as viscosity of the tissue also impacted PTF. A factor observed to decrease PTF was minimizing vocal tract resistance.
Conclusions. The investigators determined that PTF is a more practical and stable measurement then PTP to use in assessing laryngeal function with application to clinical vocal disorders assessment.
Relevance to the current work. This work documented that influence PTF in an excised larynx model. Additionally, it provided a rationale for the use of PTF in excised larynx experiments that study phonation.

Jiang, J. J., & Titze, I. R. (1993). A methodological study of hemilaryngeal phonation. *The Laryngoscope, 103*, 872-882. doi:10.1288/00005537-199308000-00008

Purpose of the study. This study examined the differences between hemilaryngeal phonation and full larynx phonation.

Method. Nine excised canine larynges were used and refrigerated in 0.67% saline before being thawed and mounted on a benchtop model. Data was collected for whole laryngeal phonation. Next, the left vocal fold was removed from each specimen and a vertical plexiglass plate was put in its place. Phonation trials were repeated. The following measurements were collected: PTP, sound pressure level, average glottal flow, F_0, and amplitude of vibration.
Results. Similarities in full larynges and hemilarynges included PTP, amplitude and frequency of vibration, and perceptual sound quality. Major differences included the finding that airflow in the hemilarynx was approximately half of the airflow in the full larynx. The sound pressure level in the hemilarynx was found to be 25% less as well.
Conclusions. The hemilarynx is generally very similar to the full larynx model. Therefore, a hemilarynx may be a suitable substitute in experiments that wish to examine sagittal views during phonation. This model could also be useful in studies that apply to partial vertical laryngectomy. Notably, PTP was relatively stable in both models.
Relevance to the current work. This experiment used a similar benchtop model to the current study and documented the relative stability that is possible in excised larynx PTP measurement.

Johanes, I., Mihelc, E., Sivasankar, M., & Ivanisevic, A. (2011). Morphological properties of collagen fibers in porcine lamina propria. *Journal of Voice, 25*, 254-257. doi:10.1016/j.jvoice.2009.09.006

Purpose of the study. The purpose of this study was to quantify the morphological properties of collagen in vocal fold propria because it influences the biomechanical properties of the folds. Additionally, investigators sought to discover the effects of pepsin exposure on the form and structure of collagen.
Method. For this study, 26 vocal fold samples were collected from porcine larynges, 13 of which were dissected and then immediately imaged using atomic force microscopy. The other 13 samples were exposed to pepsin prior to measurement. Parameters that were quantified included d-periodicity, diameter, and the roughness of the collagen fibers.
Results. The samples with pepsin exposure did not demonstrate significantly different structure or form with the exception of a slight difference in the thickness of collagen fibers. The other subset of samples that were immediately imaged demonstrated results that were consistent with those previously reported in the literature.
Conclusions. Morphological properties of collagen fibers were not altered with exposure to pepsin. Understanding the collagen makeup in healthy vocal tissue is important for determining when changes occur. The investigators indicated that this work will inform future creation of biomaterials that match the native vocal fold lamina propria.
Relevance to the current work. This study supports the premise that porcine samples are the closest in form to humans. Knowing more about the collagen makeup of the tissue will aid in future studies, particularly those associated with vibratory physiology during phonation.

Kawaida, M., Fukuda, H., Kano, S., Shiotani, A., & Kohno, N. (1990). Dynamic movement of air tract fluid in lubrication of the larynx during phonation: A basic study using excised canine larynges and experimental air tract fluid by means of x-ray stroboscope system. *Auris Nasus Larynx, 16*, 237-243.

Purpose of the study. This study utilized an x-ray stroboscope to determine the relationship between air tract fluid and vocal fold vibration. Additionally, investigators sought to understand

the wave motion of the mucosal membrane within the vocal folds.
Method. This study used excised canine larynges in two different groups, including a group of normal vocal folds and a group with a unilateral stiff vocal fold lesion. The group with the stiff vocal lesions were constructed with surgical adhesive applied to a fold. The stroboscope was placed to sample the frontal plane and the folds were sutured at the posterior portion to increase adduction. A nebulizer was located in the air blowing circuit and a microphone was attached to the trachea; a light was placed to illuminate the vocal folds from above. Using two cameras, the frontal and superior views were captured concurrently.
Results. Significant differences were observed between the two groups. Those with the lesions accumulated fluid buildup on the upper surface of the vocal fold in a flat layer. The normal group accumulated fluid as well, but it was in a column instead of a flat layer. The vocal folds with the lesions also had less surface fluid then the normal vocal folds. Vertical displacement was observed for both groups.
Conclusions. The fluid moving from the subglottis to the supraglottis may be controlled by the mucosal membrane wave pattern. The lack of flexibility in the lesioned vocal folds contributed to the lack of a fluid column and caused it to be flat. Investigators concluded that the function of the rotating column was to lubricate and cool the vocal folds as they vibrate.
Relevance to the current work. This work can be directly applied to patients with stiff vocal lesions. It is also relevant to the current study because any kind of lesion on the vocal folds in our experiment can change the outcome. Lesions must be noted and considered with the end results and conclusions of the current study.

Li, L., Zhang, Y., Maytag, A. L., & Jiang, J. J. (2015). Quantitative study for the surface dehydration of vocal folds based on high-speed imaging. *Journal of Voice, 29*, 403-409. doi:10.1016/j.jvoice.2014.09.025

Purpose of the study. The purpose of this study was to quantify how three different levels of dehydration on the surface of the vocal folds affected the mucosal wave and glottal area.
Method. Ten excised canine larynges were studied at three hydration levels, first at 100% and then dehydrated to 50% and 0%. Using high speed imaging and digital videokymography, data on the mucosal wave and glottal area was collected. To understand the difference in glottal area better, both direct and indirect amplitude of vibration components were compared. The mucosal wave data included amplitude and frequency and was collected from the imaging sequences. The dehydration process included one minute of blowing dry air and then two minutes of rest.
Results. There were significant differences for direct and indirect components of glottal area when each dehydration level was compared. The results indicated that, as the folds became more dehydrated, glottal width increased and mucosal wave amplitude decreased.
Conclusions. The dehydration impacted the folds by changing the biphasic structure. This caused increased stiffness, a larger glottal gap, and decreased viscoelasticity. This could be because the stiff fold requires more vocal effort for vibration. This study may suggest a relationship between dehydration and hoarseness. The mucosal wave frequency merits further investigated.
Relevance to the current work. This study is important for the development of treatments to address dehydration-induced laryngeal pathologies. It also increases understanding for why the glottal gap could increase with dehydration.

Nakagawa, H., Fukuda, H., Kawaida, M., Shiotani, A., & Kanzaki, J. (1998). Lubrication mechanism of the larynx during phonation: An experiment in excised canine larynges. *Folia Phoniatrica et Logopaedica, 50*, 183-194.

Purpose of the study. The study sought to determine if the viscosity of the laryngeal mucosal layer was influential on vocal fold vibration.
Method. The study used 8 excised larynges divided into two groups. Each group was assigned a different solution with varying viscosities and applied to the larynges. The low viscosity solution was saline and the high viscosity solution was chondroitin sulfate sodium salt. Increased adduction was facilitated by sealing the arytenoid cartilages. The mucosal layer was swabbed off and then the solution was applied in a random order. A laryngostroboscope and an x-ray stroboscope were used.
Results. The high viscosity solution required a greater number of frames to capture the opening and closing phases. A decreased normalized peak glottal area was also noted with high viscosities. While viscosity did not affect the trajectory of the inferior vocal fold, horizontal and vertical amplitude was reduced in the high viscosity group.
Conclusions. High viscosity vocal fold surface fluid affects mucosal wave. Further research is needed to determine the preferred level of viscosity in the mucosal layer. In clinical cases of increased vocal fold surface fluid viscosity, such as upper respiratory infection, voice disorders may result.
Relevance to the current work. The current work involves the impact of certain lubricants on the vocal folds in regards to vibration. The tissue storage and dehydration methods being used in the study may impact the observed vibratory patterns.

Regner, M. F., Tao, C., Zhuang, P., & Jiang, J. J. (2008). Onset and offset phonation threshold flow in excised canine larynges. *The Laryngoscope, 118*, 1313-1317. doi:10.1097/MLG.0b013e31816e2ec7

Purpose of the study. This study examined the hypothesis that the airflow required for phonation onset is greater than the airflow required during sustained phonation. The ratio of offset flow divided by onset flow was also examined.
Method. Ten excised canine larynges were mounted on a benchtop after having been stored in 0.9% saline. The larynges were attached to a pesudolung to provide airflow. As soon as phonation began in the specimens, the airflow was decreased until the phonation ceased. Some specimens had elongated vocal folds to determine effects on flow threshold; this methodology was used to simulate the increased tension that can occur with vocal fold pathology.
Results. The study found that the offset airflow was less than the onset in all cases. The ratio was found to be between 0.515 and 0.972 which was a larger range than expected. This being said, 80% of the ratios did fall between the numbers originally hypothesized.
Conclusions. Phonation threshold flow could be useful when diagnosing voice disorders particularly with respect to laryngeal resistance. These results added to the knowledge base involving the physics of phonation. The range of expected ratios could be helpful clinically in future.
Relevance to the current work. This relates to the current study because the bench setup and procedures are similar. Additionally, this study provides airflow data that served as a comparison during the pilot phase of the current study.

Roy, N., Tanner, K., Gray, S. D., Blomgren, M., & Fisher, K. V. (2003). An evaluation of the effects of three laryngeal lubricants on phonation threshold pressure (PTP). *Journal of Voice, 17*, 331-342.

Purpose of the study. The purpose of this study was to determine the effects of three laryngeal lubricants, mannitol, water, and a glycerin-based lubricant, on PTP.
Method. The study included 18 healthy females ranging from 20 to 35 who had normal voices and no history of voice disorders. The participants received 1.5 mL of each nebulized lubricant on three consecutive weeks. PTP was estimated during two baseline and four posttreatment observations. PTP was sampled at 80% of the individual's maximum F_0 during soft phonation.
Results. They found that water and the glycerin product did not decrease PTP. Mannitol on the other hand decreased PTP at 5 minutes posttreatment, but the effect did not exceed 20 minutes in duration.
Conclusions. All subjects were within normal limits and this could have led to no major results or differences being observed. A human system that is properly hydrated may not receive any additional benefit from having more lubrication. The only lubricant found to offer any potential decrease vocal effort was mannitol.
Relevance to the current work. This study showed that a large molecule agent could influence water balance on the surface of the vocal folds. Other agents such as Ringers solution or saline, with their ionic properties, might also influence vocal fold vibration.

Sivasankar, M., & Fisher, K. V. (2002). Oral breathing increases P_{th} and vocal effort by superficial drying of vocal fold mucosa. *Journal of Voice, 16*, 172-181.

Purpose of the study. This study examined oral versus nasal breathing effects on PTP and perceived vocal effort.
Method. The study included 20 females with typical voices and in overall good health. Participants received rigid videolaryngostroboscopy and each had a normal larynx. Participants were divided into two groups, 10 who breathed transorally for 15 minutes and 10 who breathed transnasally for 15 minutes. PTP was estimated at high pitch, low pitch, and habitual pitch. Vocal effort ratings were performed after singing happy birthday.
Results. In the nasal breathing group, 7 of 10 participants reported that vocal effort decreased. Additionally, PTP at all three pitch levels for nasal breathing decreased. In contrast, 6 out of 10 of the subjects in the oral breathing group reported that vocal effort increased; PTP at all pitch levels increased.
Conclusions. Even women with healthy, strong voices may experience increased effort with oral breathing. This could put individuals at risk for vocal pathologies. The water layer on the vocal fold surface likely explained the observed changes in PTP. Adverse effects could prevail in vocal users with oral breathing patterns unless strategies are implemented.
Relevance to the current work. This study demonstrates the clinical relevance of the current study. In particular, it highlights the need for alternate methodologies for studying the main effects of variables that influence phonation but are not easily measured in humans.

Sivasankar, M., & Fisher, K. V. (2007). Vocal fold epithelial response to luminal osmotic perturbation. *Journal of Speech, Language, and Hearing Research, 50*, 886-898. doi:10.1044/1092-4388(2007/063)

Purpose of the study. This study sought to determine and measure bidirectional transepithelial water fluxes in vocal folds when they were exposed to physiologically representative luminal osmotic perturbations in vitro. Additionally, it sought to discover if the vocal folds would create a water reflux when exposed to mannitol or sham challenges.
Method. This study included 35 excised ovine larynges. Each fold either had mannitol hyperosmotic or sham perturbations applied to the surface of the folds, or they had isosmotic perturbations applied to the surface of the folds. The measurements taken were the vocal fold viability and the water fluxes toward the lumen. These measurements were taken at baseline and after the 30-minute challenge.
Results. The vocal folds that were exposed to the mannitol and sham challenge did not produce any difference across all time. The mannitol challenge did increase the water fluxes in 60% of the vocal folds, but no change was seen with the sham challenge. The increase in water fluxes was very short and did not change the osmotic gradient. The resistance of the folds exposed to the sham verse the osmotic challenges were not different. The tissues used in the study remained viable for more than two hours.
Conclusions. Mannitol was successful in increasing water fluxes and could be a useful tool for treatment. It could help with dehydration and decreasing mucus in the vocal tract. The ovine vocal folds were able to sense the perturbations and respond to lumen changes. This detection ability is important for the vocal folds because it help them to regulate during vocalizing and respiration.
Relevance to the current work. This study examined the effects several solution exposures on hydration in an in vitro tissue model. The current study extends this methodology to phonatory parameters in an excised animal model and adds the variable of time.

Tanner, K., Roy, N., Merrill, R. M., & Elstad, M. (2007). The effects of three nebulized osmotic agents in the dry larynx. *Journal of Speech, Language, and Hearing Research, 50*, 635-646. doi:10.1044/1092-4388(2007/045)

Purpose of the study. This study examined the effects of three different solutions, hypertonic saline, isotonic saline, and sterile water, on PTP and self-perceived vocal effort following a desiccation trial.
Method. Sixty healthy women with normal voices were included in the study, with 15 in each group. All participants completed a 15-minute desiccation challenge involving oral breathing of medical-grade dry air. One group received no treatment while the other groups each received one of the nebulized treatments. PTP and the self-perceived effort level measurement were sampled immediately after the desiccation trial and then at 5, 20, 35, and 50 minutes.
Results. All groups demonstrated an average increase in PTP of 0.5 cmH_2O following desiccation. Nebulized treatments produced no statistically significant effects; only transient reductions in PTP occurred and only for the 0.9% saline group. Overall, the adverse effects on PTP lasted for at least 60 minutes. The self-perceived ratings decreased after the desiccation trials and were not strongly correlated with PTP.
Conclusions. Desiccation trials greatly increased PTP and none of the treatments had a great impact on reversing this impact. Additionally, the self-perceived measure was poorly correlated to PTP measurements.
Relevance to the current work. This study showed that 0.9% saline was the most viable in decreasing PTP. The current study will build on the routine use of 0.9% saline to store and hydrate excised larynx tissue.

Witt, R. E., Regner, M. F., Tao, C., Rieves, A. L., Zhuang, P., & Jiang, J. J. (2009). Effect of dehydration on phonation threshold flow in excised canine larynges. *Annals of Otology, Rhinology, and Laryngology, 118*, 154-159.

Purpose of the study. This study was designed to determine if PTP increases with dry air exposure in excised larynges.
Method. Eleven excised canine larynges were used and mounted on a benchtop setup. The larynges were divided into three groups: eight for desiccation trials, two for control trials, and one was used for both groups. The trials involved cycles of 10 seconds of phonation and then three seconds of rest. Subglottal dry air was supplied until phonation started. The control trials receive humidified subglottal air and topical 0.9% saline applied to the vocal folds supraglotally.
Results. PTP increased with the duration of dry air exposure. The same effect did not occur in the control trials, indicating that the dry air and corresponding dehydration were the cause. Ultimately, the vocal folds ceased to vibrate, perhaps indicating that the tissue can reach a point of dryness that does not allow it to vibrate due to extreme dehydration.
Conclusions. This work demonstrated a clear relationship between vocal fold dehydration and PTP values. This is important for clinical assessment of vocal dehydration and preventing the effects in humans. Additional studies need to be conducted to further understand the impact and relationship of vocal fold health and dehydration. In particular, these studies need to be done in vivo for discovery of hydration therapy techniques.
Relevance to the current work. The current thesis is focused on understanding the effects of different solutions and settings on vocal fold hydration. This study demonstrated that additional studies on this topic are warranted.

Witt, R. E., Taylor, L. N., Regner, M. F., & Jiang, J. J. (2011). Effects of surface dehydration on mucosal wave amplitude and frequency in excised canine larynges. *Otolaryngology Head Neck Surgery, 144*, 108-113. doi:10.1177/0194599810390893

Purpose of the study. This study sought to understand the effects of surface dehydration on mucosal wave amplitude and frequency in vocal folds.
Method. Ten excised canine larynges were placed in 0.9% saline and quickly frozen. They were then thawed and mounted on a bench model with a pesudolung. Trials were completed in a triple-walled sound-attenuated room that had controlled humidity and temperature. Eight of the 10 larynges were exposed to dehumidified air and high speed video was taken. The other two larynges were exposed to humidified air.
Results. Overall, it was found that the increased amounts of dehydrated air correlated with a decreased amplitude and frequency. This was found by examining videokymography data. Additionally, statistically significant differences were found between the control group and experimental group in regards to mean slopes and mean percent changes.
Conclusions. A decrease in mucosal wave amplitude and frequency was observed with vocal fold dehydration. This can be useful as a clinical indicator of impaired voice function.
Relevance to the current work. This work showed a relationship between dehydration and a decreased in musical wave amplitude and frequency. The current study expanded on this work using several tissue storage agents that address hydration-related theoretical constructs.

Zhang, Y., Reynders, W. J., Jiang, J. J., & Tateya, I. (2007). Determination of phonation instability pressure and phonation pressure range in excised larynges. *Journal of Speech, Language, and Hearing Research, 50*, 611-620. doi:10.1044/1092-4388(2007/043)

Purpose of the study. This study used bifurcation analysis to understand phonation instability pressure and used phonation pressure range to assess the pressure range of a normal vocal fold vibration.
Method. Ten excised canine larynges were used within 48 hours of sacrifice. The larynges were attached to a pesudolung and secured with metal prongs. Regulated air was blown through subglottally and measurements were taken at the bifurcation points on a spectrogram. The measurements taken included PTP, phonation pressure range, and phonation instability pressure. The vocal folds were then elongated and measured at 0%, 5%, 10% and 15%. The purpose of this was to determine what impact elongation has on all the above measurements taken. To ensure the data was accurate, the measurements were each taken three times at each elongation percentage.
Results. The study found that PTP, pressure range, and instability pressure can be successfully found using bifurcation analysis. Elongation at 15% had an impact on the measurements causing PTP to be significantly increased, range to be significantly decreased, while instability remained unchanged.
Conclusions. This study showed that instability pressure and pressure range can be influential measurements when assessing phonation. Additionally, the bifurcation analysis is helpful in collecting data on phonation measurements. Knowing this will help with clinical assessment and treatment for patients with laryngeal pathologies.
Relevance to the current work. This work used a similar experimental setup and measurements as the current work. The investigators also studied the impact of elongation on PTP measurements.

APPENDIX B: Experimental Checklist

Materials for Dissection:

1. scalpels (2 different types)
2. apron
3. gloves
4. green dissection paper (to be laid on the dissection table)
5. 1 Ziploc bag
6. hemostats
7. sutures (1 for each larynx)
8. protective goggles
9. dissection table
10. Distilled water
11. 0.9% saline or Ringer's solution
12. red hazard box (rinse scalpels and then place them in this box)
13. tub-fridge drawer (to hold un-dissected larynges)
14. Clorox wipes (for clean-up)
15. Mini fridge
16. Freezer

Additional Notes:

- Remove all surrounding tissues of the larynx such as the esophagus, thyroid gland, fat, excess tendons, innervation, vascularization. Make sure the trachea and thyroid cartilage are intact and without any abnormal openings or damage.
- Use the largest tracheas-these are best for phonation and mounting onto custom tubing
- Tracheas should be cut superiorly of the true vocal folds
- The shape should be a smile formed from the anterior commissure to the lateral posterior ends of the thyroid cartilage
- The true vocal folds should not be punctured (this will prevent air leakage)
- The arytenoid cartilages should be left intact (this will aid in adduction)
- The epiglottis should be removed by cutting a triangle posterior and in between the arytenoid cartilages
- Remove false folds completely (may use a hemostat for better precision)
- Remove any leftover tissue superficial and superior to the vocal folds (this prevents flopping of tissue during vibration of true vocal folds)
- Trim the trachea leaving the trachea about 8-10 cm in length. (verify the inferior end of the trachea fits around the custom tubing connecting to the pseudolung)
- Suturing: should be placed above the anterior commissure on the thyroid cartilage. First tie the end of the string attached to the suture in a knot (make several knots in the same location in order to prevent the string from going through the cartilage). Hold the sharp

end of the suture using a hemostat to provide support to puncture the anterior end of the thyroid cartilage (located just above and in front of the anterior commissure) (repeat this 4 times) make sure suture is tight and tug at it to observe its strength

Materials for Quick Freezing:

1. dissected larynges in Ziploc bags filled with saline
2. extra saline
3. extra Ziploc bags
4. protective goggles
5. 2 styrofoam boxes
6. sharpie
7. cold resistant gloves
8. liquid nitrogen
9. freezer
10. refrigerator

Additional Notes

- Fill a styrofoam box with liquid nitrogen
- While wearing cold resistant gloves and eye protection, submerse 2 larynges in the bags with saline in the liquid nitrogen. Leave the Ziploc bags open
- Leave the larynges submersed for approximately 8 minutes. Check and make sure they are completely frozen by tipping the bag on its side and observing if any liquid is still moving
- Once frozen, place each larynx in a second Ziploc bag
- Write the date on each Ziploc bag
- Place each frozen larynx in the second styrofoam box to keep them from melting
- Once all the larynges are frozen, put them in a freezer until the day before the experiment
- Move the larynges from the freezer to a refrigerator the night before the experiment to allow them to thaw. Ensure that they are in an enclosed space, such as a drawer, because the bags tend to leak as they thaw

Materials for Experiment:

1. 4 LED lights (make sure fresh batteries are in place)
2. macropositioners
3. micropositioners
4. nozzle for desiccated air
5. Teflon tape (used to seal edges of trachea onto the custom tubing which is attached to the pseudolung)
6. Flow meter (Aalborg mass flow meter GFM-47)—flow should be calibrated at 0, 10 and 15 cmH_20

7. Medical Flow Meter- attached directly to the air tank and to the Aalborg mass flow meter GFM
8. 2 Air tanks (one will attach to the flow meter and the humidifiers; the other will be for desiccated air)
9. Pressure transducer (should be plugged in from computer to inferior lateral portion of larynx or the custom tubing)
10. pressure calibrator box (should be used only to calibrate pressure transducer) calibration occurs at 0, 10 and 15 PSI
11. check all plugs
12. WinDaq should be turned on and 4 different waves should be showing (wave 1 measures: microphone signal; Wave 2: pressure; Wave 3: Flow; Wave 4: High Speed Trigger)
13. Humidifier (make sure tubing is plugged in to pseudolung and air tank)
14. High Speed video camera: Trigger should be on and plugged into the sound board
15. Microphone (SHURE SM-48) should be on and plugged in (before starting experiment make sure the wave shows up on WinDaq by tapping the mic lightly) (position microphone about 4 inches away from the larynx.)
16. High Speed-make sure trigger is plugged in
17. Metal clamps (secure trachea onto the custom tubing which attaches to the pseudolung)
18. Metal clamps (hold flashlights & Microphone)
19. Clorox Wipes
20. Paper towels
21. Metal shim (diameter 5mm)

Measuring Flow

1. Make sure flow meter (Aalborg mass flow meter GFM) is plugged into outlet
2. Verify computer is turned on and the WinDaq window is opened
3. Verify flow signal is not peaking (max should be 100 liters/min)
4. Should be directly attached to WinDaq box which is attached to the computer
5. Record when flow is at 0 (mark exact number ~ -.6)
    a. Shift space-to make a comment
6. Record when flow is at 15 (mark exact number)
7. System is ready to record
    a. Hit F4 to record
    b. Hit shift F4 to standby
    c. Hit shift space to apply comment (comment does not appear until you hit enter)

Measuring Pressure

1. Make sure pressure transducer is plugged into the WinDaq box which is connected to the computer

2. PSI or cm H2O
3. Insert pressure transducer directly into PC-IH box
4. Verify WinDaq is picking up pressure signal by observing wave 2
5. Calibrate pressure at 0 and 10 PSI
    a. Record F4 at 0 PSI
    b. Hit shift space to apply the comment (insert press_cal_0)
    c. Do the same for 10 PSI
6. Remove pressure transducer from PC-IH box
    a. Press button before releasing syringe
    b. There should not be any tension when releasing the syringe
7. Insert pressure transducer into opening inferior to the mounted trachea
8. Ready to record
    a. Record F4
    b. Hit shift space to apply the comment (e.g., D3P01) (trial type and pig number along with trial number)
    c. Do the same for all trials

Recording High Speed

- Unit should be plugged in and on
- Verify all components are turned on in order (high-speed, computer, monitor)
- Login to computer, open Kay Pentax software
- Verify camera and waveform signals are on, and ensure settings are to record "END"
- Click record, wait for camera to lock
- Click trigger when ready to record (records 4 seconds prior to trigger)

Microphone signal

- SHURE SM-48
- Make sure the microphone is plugged into an outlet.
- The microphone should be about 4 inches away from the glottis
- Make sure there is not peak clipping in the recording. If there is, move the mic away from the larynx

Procedure for Phonatory Trials

- For each larynx, baseline measures were collected and were named trial 1 (larynges were vibrated without desiccation trial) (if the larynx did not vibrate, then it was removed from the study)

- After baseline was collected, pigs were desiccated for 5- minute increments using custom tubing which was attached to one of the air tanks (a shim was held in place posterior to the true vocal folds in the interarytenoid space)
- each larynx was then vibrated following 5- minute desiccations (data was collected after each desiccation trial)
- Phonatory trials were continued until vocal folds ceased to vibrate or phonation ended

Measuring Humidity

- Record % humidity at the beginning of the experiment and at the end
- Make sure hygrometer is calibrated.

Humidifier

- (Thera-Heat Heated Humidifier-Portex) by Smiths Medical:
- Make sure this is plugged into an outlet
- Use standard settings
- Should be plugged in directly to the flow meter (clear tube) and into the custom tubing of the pseudo lung. (blue tube should be attached to the pseudolung)

Flashlights:

- UltraFire XML-T6
- Verify these have fresh batteries and are working prior to beginning the experiment
- Should be equidistant from the glottis.
- Position one directly anterior to the glottis
- 2 will be positioned laterally equidistant from the glottis
- 1 should be positioned posteriorly
- Use as many as are necessary (check prior to beginning experiment)

APPENDIX C: Food Handler's Permit

Utah Food Handler
Certificate of Training Completion

StateFoodSafety.com

Presented to: Emily Huber

6f233-h175ji3
Certificate Verification Number
Verify at www.foodhandlerverification.com

May 17, 2016
Date of Completion (**valid 30 Days**)

Employers: According to the new Utah food handler law, this certificate allows your employee to handle food for 30 days until they receive their official permit from the local health department.

Training approved by Utah Department of Health

UTAH DEPARTMENT OF HEALTH

Christie H. Lewis
President, StateFoodSafety.com