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

Chelsea Savannah Pipkin Litster

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

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ABSTRACT

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

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Several studies involving excised animal larynges have been performed to simulate the structural and physiological properties of the human larynx. The most common way to preserve the laryngeal tissue being studied is by immersing it in a 0.9% isotonic saline solution and then flash freezing it. Isotonic saline is used empirically to replenish the potential ion loss that occurs postmortem. Each larynx is flash frozen so it can be used at a more convenient time while still maintaining the integrity of the tissue. However, the preservation methods found in previous studies tend to vary and no consensus had been reached about which method of preservation is ideal. This study sought to investigate the effects of solution and storage on phonation threshold pressure (PTP). Phonation threshold pressure is commonly used to investigate mucosal wave of the vocal folds, prephonatory glottal width, and vocal fold cover.

This study involved a prospective, mixed experimental design with three groups, including a control group and two experimental groups. Each group consisted of 10 bench-mounted porcine larynges. The control group was immersed in 0.9% isotonic saline, flash frozen with liquid nitrogen within 24 hours postmortem, and thawed overnight before the experiment. The second group was immersed in 0.9% isotonic saline and the third group was submersed in Ringer's solution. Each of these groups was kept in their solution in a refrigerator for approximately 15 hours and was used for the experiment within 24 hours postmortem. Each larynx was mounted on a bench on a tabletop with three micropositioners to adduct and elongate the vocal folds. A pseudolung connected to the trachea directed humidified air to the vocal folds subglottally until phonation was achieved. The larynges in all three groups underwent these phonatory trials with 5-minute desiccation trials between each until phonation could no longer be achieved. Phonation threshold pressure was then observed and compared within groups and between groups. The signals were obtained using MATLAB.

The results indicated that PTP was lowest for the frozen versus fresh groups. PTP values increased slightly for the frozen group, but the frozen group demonstrated less variability across specimens as compared to the fresh groups. Collectively, these results indicate that there are substantial differences between fresh and frozen specimens. These differences should be considered when designing tissue studies for purposes of generalization to human phonation.

Keywords: larynx preservation, bench model, phonation threshold pressure, Ringer's solution, laryngeal desiccation

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DESCRIPTION OF THESIS STRUCTURE

The following thesis, *Effects of Larynx Preservation Method on Phonation Threshold Pressure in an Excised Porcine Benchtop Model*, is written in a hybrid format. The format of this thesis combines classic thesis requirements with journal publication formats. A comprehensive literature review is included in Appendix A. The study's experimental checklist is found in Appendix B. The Utah Food Handler's Permit, which indicates compliance with Risk Management requirements and Brigham Young University, is found in Appendix C.

Introduction

Excised larynx benchtop models represent a valuable research methodology for examining phonation. Many researchers have used excised larynx models to study and measure the structural and physiological properties of the larynx. These models usually involve a larynx that has been excised from an animal within 24 hours postmortem (Alipour, Finnegan, & Jaiswal, 2013; Jiang, Raviv, & Hanson, 2001; Zhang, Jiang, Tao, Bieging, & MacCallum, 2007). To maintain the structural integrity of the larynx, multiple steps are taken to preserve it. These preservation methods vary widely between studies. Most researchers immerse the tissue in a 0.9% isotonic saline to prevent the ion loss that occurs after death (Chan & Titze, 2003; Hottinger, Tao, & Jiang, 2007); however, there are a handful of others who have chosen to use Ringer's solution instead (Hemler, Wieneke, van Riel, Lebacqz, & Dejonckere, 2001; Quirinia & Viirdik, 1991). Additionally, most researchers flash freeze excised larynges in liquid nitrogen within 24 hours postmortem to preserve their integrity to be able to use them at a more convenient time (Alipour & Karnell, 2014; Hoffman et al., 2012; Regner & Jiang, 2011). Very little research has been performed to evaluate whether this flash freezing method has an effect on the structural, biological, or mechanical characteristics of excised larynges. Because so little has been studied about preservation methods, currently there is no consensus within the field about which preservation method is best for which types of investigations. The current investigation addresses this important methodological question.

Excised Larynx Models

Excised animal and human larynges allows researchers to study a wide variety of laryngeal structures and functions. Commonly, researchers opt for this model to examine vocal fold hydration, vibratory mechanics, and vocal fold adduction and abduction abilities. For

example, one study measured the effects of hydration on phonation threshold flow (PTF) using an excised canine larynx model (Witt, Taylor, Regner, & Jiang, 2011). Another study by Alipour, Jaiswal, and Finnegan (2007) used an excised larynx model to examine how the false vocal folds and epiglottis had an effect on aerodynamic and acoustic measures of the voice. Hottinger, Tao, and Jiang (2007) conducted a study to compare the effects of abduction on PTF and phonation threshold pressure (PTP). One study even measured the parameters of straw phonation therapy using excised canine larynges (Conroy et al., 2014).

An *in vivo* animal larynx model is also an option; however, the excised larynx model can be more beneficial to certain lines of research for several reasons. First, *in vivo* experimentation does not always allow for precise control or manipulation of independent variables, such as length and adduction, whereas the excised larynx benchtop setup can (Berry, Herzel, Titze, & Story, 1996). Because animals' larynges have a similar structure and physiology to human larynges, it can also be difficult to isolate main effects of variables and remove covariates. For example, in measuring frequency, the length of the vocal folds cannot be known or taken into account. Additionally, animals cannot comply with instructions, which creates limitations that do not exist in either *in vivo* human studies or excised larynx benchtop investigations. The excised larynx model, however, requires multiple steps of excision, dissection, and preservation, all of which can cause variability that cannot be accounted for; this type of variability is introduced by the examiner, but it can be minimized with consistent operational procedures.

Computer-based models have also been used to simulate *in vivo* phonatory behavior. In an early work, Berry et al. (1996) concluded that the excised larynx model is superior to a computer-based model. However, more recent advances in computer simulation suggest that computer models, particularly those based on data from mechanical research, hold great promise

in the future. The excised larynx model behaves more like an in vivo larynx and it can be manipulated in a variety of ways, which allows for greater understanding of the effects of extraneous variables on the phonatory characteristics of the in vivo larynx, such as PTP, PTF, and vocal fold pliability. This literature supports the idea that the findings from an excised larynx model are more generalizable than a computer based model to an in vivo larynx and may serve as a complementary methodology to computer models.

Several studies have been conducted to determine the similarity between various animal larynges and the human larynx. Alipour and Jaiswal (2008) examined the phonatory characteristics of excised pig, sheep, and cow larynges and how they compared to the human larynx. The results indicated that each larynx had distinct qualities from each other that made them appropriate models for studying the human larynx. The pig larynx had a supraglottic duct that was similar in size to the human larynx, a frequency variability closest to that of the human larynx, similarly angled vocal folds, and similar fundamental frequency (F_0) and PTP. A later study conducted by Alipour et. al (2013) used the measurements of an excised human larynx to compare to the results of their previous study. They found that the pig larynx also had similar oscillation behavior to the human larynx. Another study conducted by Howard, Mendelsohn, and Berke (2015) confirmed that the phonation of the ex vivo porcine larynx is comparable to the phonation of an in vivo human larynx. These findings support the method used in the current work to use porcine larynges to study the effects of preservation on PTP.

Phonation Threshold Pressure/Flow

For the purposes of benchtop setups, PTP may be defined as the least amount of pressure needed to start phonation (Titze, 1994). Hottinger et. al concluded that PTP is useful in assessing the aerodynamic vocal fold properties and laryngeal function, which is useful in

discriminating between typical and atypical phonation. PTP is commonly used to assess vocal fold function because it directly relates to properties of the vocal fold cover, mucosal wave, and prephonatory glottal geometry (Mau, Muhlestein, Callahan, Weinheimer, & Chan, 2011). Vocal fold pathology often causes disruption in these properties, which usually results in an increased PTP. To better understand the functions of a normal and disordered voice and to generalize the results of this study for the use of future works, the current work will be analyzing PTP. PTF, defined in this study as the least amount of flow needed to start phonation (Hottinger, Jiang & Tao, 2007), is also of value in quantifying effects of experimental parameters on phonation. Although measured separately, PTP and PTF are related and affect each other. For purposes of stability in measuring PTP, vocal fold elongation can be controlled. One study conducted by Hoffman et al. (2012) found that no significant relationship existed between vocal fold elongation and PTP. Another study, however, found that vocal fold elongation resulted in a significant increase in PTP (Zhang, Reynders, Jiang, & Tateya, 2007). Further studies should be conducted to conclude whether vocal fold elongation has a significant effect on PTP.

Relationship of Pressure and Flow

Although the current work is measuring pressure and flow independently, these two measures are interdependent. Ohm's Law states that subglottic pressure divided by airflow is equal to laryngeal resistance. As pressure changes, so does flow, and vice versa. This relationship makes it difficult to parse out these measurements from one another during in vivo phonation. However, it is easier to understand and measure pressure and flow in an ex vivo model because it allows for manipulation of individual factors. For example, researchers can keep pressure constant while changing flow. Although this study will mainly be comparing

pressure within and between groups, flow will still be taken into consideration when analyzing the results.

In Vivo Human Studies

An alternative to studying the mechanisms of the human larynx through a mechanical larynx model is measuring vocal function while a person phonates. Many previous researchers have opted to use human participants to study variables such as vibratory characteristics (Kunduk, Vansant, Ikuma, & McWhorter, 2017) and the effects of hydration on the voice (Fujiki, Chapleau, Sundarrajan, McKenna, & Sivasankar, 2017). Using human subjects allows researchers to obtain real-time feedback from their participants, which can be helpful information for generalizing to voice treatment. However, using this type of a model does present with some limitations. Many of these studies rely heavily on perceptual measures due to the significant variability often observed on aerodynamic parameters (Verdolini-Marston, Sandage, & Titze, 1994). Although perceptual measures are important in this type of study, these measures are subjective, which can create unaccounted for variation between and within participants. Additionally, behavioral practices of participants in these studies can introduce variability across individuals. Franca and Simpson (2009) conducted a study to understand the effects of hydration on voice acoustics and reported that there were certain variables for the individual participants that could not be adequately controlled. For example, they instructed each participant to avoid eating and drinking before the experiment; however, there was no way to control whether each participant followed these instructions. There are a number of variables such as these in human studies that can pose methodological challenges. Therefore, alternative study methods and designs are needed to lay sufficient foundation for subsequent human

investigations. Excised larynx benchtop studies offer a strong and viable alternative to human studies.

Ringer's Solution

Once a larynx is excised, it goes through a significant ion loss, which is a limitation of using an excised larynx model. As mentioned previously, the majority of researchers have used saline as a method to counteract this loss (Khosla, Murugappan, Lakhamraju, & Gutmark, 2008; Regner & Jiang, 2011). However, very little research has been done to know whether there is a more effective solution available to preserve the tissues of the larynx.

According to results obtained in previous studies, Ringer's solution could also be a viable method for preserving ex vivo tissue. Each 100 mL of Ringer's solution is composed of 860 mg of sodium chloride, 30 mg of potassium chloride, 33 mg of calcium chloride, and 33 mg of dihydrate. Each 100 mL of isotonic saline contains 900 g of sodium chloride. Ringer's solution is considered a balanced fluid, whereas saline is not. A fluid is considered a balanced fluid if it approximates the normal bicarbonate of a living organism (Schwarz, 2015). Because Ringer's is more comparable to fluid found in living organisms, it is possible that using it for preservation rather than saline could result in a larynx model that is more similar to the living environment.

Several studies have used Ringer's solution for tissue preservation. Steiner and Ramp (1988) performed a study to examine the effectiveness of Ringer's solution when compared with isotonic saline and distilled water. The results showed that both Ringer's solution and isotonic saline were effective in preserving the physiological properties of bone grafts. A study conducted by Yessenow and Maves (1988) also concluded that Ringer's, when injected into porcine skin samples, was effective in preserving its physiological properties. Additionally, Ringer's is often used in a medical setting for the purpose of hydration; therefore, various studies

have also been performed to examine its effectiveness in recovering tissue from dehydration. One study found that Ringer's brought about a more rapid physiological correction than isotonic saline in hydrated patients (Cieza, Hinojosa, Huapaya, & Leon, 2013). Another study examined the effects of multiple solutions on mucociliary clearance in excised rat tracheas and concluded that Ringer's was the most effective option (Okuyucu, Akoglu, Oksuz, Gorur, & Dagli, 2009). Collectively, research studies involving Ringer's suggest that this might be a viable storage alternative to saline in excised larynx research.

Statement of Problem

The lack of standardized methods for excised larynx preservation poses significant limitations to benchtop mechanical models of phonation. It is unknown how much storage variability might contribute to differences between research laboratories and individual studies. Although it is more common to flash freeze larynges, previous work (Stevens, 2017) indicates that larynges studied immediately after excision had different baseline pressure and flow values than those reported in the literature. Most frequently, excised larynx preservation is accomplished through immersion in isotonic saline, flash freezing using liquid nitrogen, and then freezer storage until needed for data collection. This methodology poses several problems, however. Freezing the tissue of the larynx and subsequently thawing may change the structural integrity of the larynx, which would result in a larynx model which is less representative of in vivo tissue and thus less generalizable to human phonation. Secondly, because there are more steps involved in the process of freezing a larynx, there is a greater chance of variability in the operational procedures of preserving it. Without standard preservation methods, excised larynx models are limited and have less translational value to human phonation. Furthermore, significant ion loss occurs shortly after death. Therefore, preserving excised larynges in a

solution that preserves ions, such as Ringer's, might reduce or offset this loss, whereas isotonic saline is unlikely to have this effect.

Statement of Purpose

This study was undertaken to examine the effects of standard excised larynx storage via flash freezing versus fresh tissue storage using isotonic saline or Ringer's solution. The purpose of this study was to quantify differences related to the methodologies used to store excised larynges prior to their use in experiments. The outcome of the current work will lay the groundwork for more physiologically realistic excised larynx mechanical models for phonation.

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 PTP?
 - 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 PTP during exposure to dry air?

Method

All research activities for the current study were performed in accordance with Risk Management and the Institutional Animal Care and Use Committee at Brigham Young University. Excised porcine larynges for this study were obtained at a local butcher shop (Circle V Meats, Spanish Fork, UT). All of the procedures described in this section were performed in rooms 105 and 106 of the John Taylor Building and the Chemistry Central Stockroom, room 126, of the Joseph K. Nicholes Building at Brigham Young University.

Research Design

The study utilized a prospective mixed experimental design with a control group, including within group and between group comparisons. All larynges were randomly placed into either the control group or one of two experimental groups. Larynges in the control group were immersed in isotonic saline (0.9% Na⁺Cl⁻) and flash frozen. The larynges in the two experimental groups were immersed in either isotonic saline or Ringer's solution and studied within 48 hours postmortem. For all three groups, five minutes of desiccated air was administered between each phonatory trial until phonation ceased. Pressure and flow were recorded during each phonatory trial. The independent variables were group and time. The dependent variables were PTP (cmH₂O) and PTF (L/min), although the current work focused on PTP.

Larynges

Thirty porcine larynges were obtained from the local butcher shop within 24 hours postmortem. Larynges were from adult food-grade pigs less than two years in age. Upon retrieval, each larynx underwent rough dissection. Specifically, the external tissue was removed and each larynx was thoroughly inspected for any structural abnormalities; those with structural abnormalities or tissue perforations were discarded. The larynges were then placed in individual zip top plastic bags and fully submerged in either Ringer's solution or isotonic saline, 10 larynges per group. These larynges were stored in a refrigerator for approximately 15 hours before the experiment commenced. Ten additional larynges were immersed individually in bags of isotonic saline and flash frozen in liquid nitrogen for approximately eight minutes each. Subsequently, these larynges were stored in a freezer until the night before the experiment, when they were removed and left to thaw in a refrigerator. Immediately prior to each experiment, the

larynges underwent a fine dissection. Disposable fine scalpels were used to complete the dissection. A larger scalpel was used to transect the thyroid cartilage and remove the epiglottis. The thyroid cartilage was transected at an upward angle anterior to posteriorly starting anteriorly at approximately 0.5 cm above the true vocal folds. This angle was created to leave additional structural support for arytenoid adduction during the phonatory trials. A smaller scalpel was used to carefully remove the false vocal folds. Metal hemostats were used to aid in abducting the false folds so that they could be removed without causing damage to the true vocal folds. Each trachea was then cut to approximately 6 cm in length. A suture was added to the thyroid cartilage about 0.25 cm above the anterior commissure. This was done for elongation purposes during phonation. Each larynx was bathed again in either isotonic saline or Ringer's solution, respectively, for five minutes and then mounted immediately on the benchtop.

Procedures

Benchtop setup. The benchtop experimental setup for excised larynx experimentation was based on Jiang and Titze (1993). The present study used a similar mechanical model, including each larynx mounted vertically on plastic tubing. A foam-insulated custom pseudolung surrounded the plastic tubing below the breadboard benchtop (Thorlabs, Ann Arbor, MI). The trachea of each larynx was situated on the vertical tubing and secured in place with an adjustable metal hose clamp and $\frac{3}{4}$ inch Teflon tape. Three micropositioners (Model 1460, Kopf Industries, Tujunga, CA) were secured to the tabletop by $\frac{1}{4}$ -20 headless screws via custom bases. The two lateral micropositioners had three prongs; these gently pierced the lateral surface of each arytenoid cartilage to adduct the vocal folds. The third micropositioner was positioned near the anterior commissure so that the suture thread could be tied to it. This allowed the micropositioner to be used to adduct and lengthen the vocal folds until phonation was achieved.

The following describes the measurement of pressure and flow during the phonatory trials. An adjustable flow regulator at 50 psi and compressed air tank (<1% relative humidity) were attached to an in-line thermal flow meter. This was attached to a tube connected to a Theraheat temperature controlled humidifier (Model RC70000, Smiths Medical, Dublin, OH), which was attached to clear plastic tubing that passed through a 20-cm aluminum pseudolung insulated with foam. This tube passed through a hole in the table top and had a subtracheal outlet where a pressure transducer (Model PT-25-S, Glottal Enterprises, Syracuse, NY) was connected perpendicular to the direction of the flow.

Phonatory trials. Once positioned on the bench, each larynx underwent a series of phonatory trials. Compressed air was passed through the humidifier and pseudolung to reach the vocal folds subglottally. Using the anteriorly placed micropositioner, the length of the vocal folds was adjusted until phonation was achieved. This length remained stable for the rest of the trial. As soon as phonation was achieved the compressed air was turned off. Compressed air from a separate tank (<1% relative humidity) was then administered to the vocal folds supraglottally via custom tubing. These desiccation trials lasted for five minutes. While keeping vocal fold length and adduction constant, a 5-mm shim (Allen wrench) was placed between the vocal folds at the posterior two-thirds position to ensure the air reached the infrasurface and medial edges of the true vocal folds. This pattern of trials was continued until audible phonation was no longer obtained.

Signal acquisition. The acoustic signal, air pressure, and airflow signals were obtained at baseline and following each five-minute desiccation trial. A DATAQ A/D (DI-720 Series) converter and Windaq software (Windaq Pro+, Akron, OH) were used to acquire signals at 10 kHz per channel. A dynamic microphone (Model SM-48, Shure, Niles, IL) was positioned about

6 in above the true vocal folds and an audio mixer (Samsung MIXPAD 4, New York, NY) preamplified the signal. Prior to data collection, a pressure calibrator (PC-1H, Glottal Enterprises, Syracuse, NY) was used to calibrate the pressure transducer to 0 and 10 cmH₂O and the flow meter was calibrated at 0 and 15 L/min. Prior to the study, a HygroSet II Digital Hygrometer (model DHYG-Round; HygroSet, Weston, FL) was calibrated using the Humidipak calibration kit and was then used to monitor environmental humidity during the experiment. Each Windaq file was coded and saved for subsequent pressure and flow analysis.

Data analysis. Windaq files were segmented and then imported into MATLAB (MathWorks, Natick, MA); phonation onset was identified using the acoustic signal via a custom Matlab program. Phonation threshold pressure and PTF were obtained by identifying the subglottal pressure and flow of 10 ms prior to and after the onset of phonation and averaging it.

Statistical analysis. The data collected from the control group and two experimental groups were assessed for central tendency and variability at baseline. One-way analysis of variance and Tukey's HSD post hoc tests ($\alpha = .05$) were used to evaluate differences between the experimental groups and the control group. All analyses were performed using SPSS, version 24 (IBM Corp., Armonk, NY).

Results

At the beginning and end of each data collection session, the relative humidity of room air was recorded. The average humidity at baseline was 31.3% ($SD = 1.46$) at baseline and 28.5% ($SD = 1.35$) after each session. Temperature at baseline averaged 23.7 degrees Celsius ($SD = 0.96$). On the first day of data collection, humidity and temperature measurement was inadvertently admitted.

For each larynx, the vocal fold length, vocal fold width, and the distance from the medial edge of each vocal fold to the thyroid cartilage inner surface are provided in Table 1. The length, width, and height of the thyroid cartilage are reported in Table 2. Tracheal width and height are included in Table 3. All measurements were collected in mm prior to initiation of each data collection session.

Groups at Baseline

For the fresh saline group, average PTP was 21.52 ($SD = 14.25$). For the Ringer's group, average PTP was 24.28 ($SD = 12.56$). Average PTP for the flash frozen group at baseline was 8.39 ($SD = 3.78$). The results from a one-way ANOVA indicated significant differences among groups, $F(2, 27) = 5.762, p = .008$. Post hoc Tukey's HSD tests indicated significant differences between frozen and fresh saline, $p = .036$, and frozen and Ringer's, $p = .010$.

Phonation Trials

Baseline pressure was subtracted for each subsequent observation for each larynx to generate normalized values. These values are graphed for the fresh saline, fresh Ringer's, and frozen saline groups in Figures 1, 2, and 3, respectively. Second order polynomial trend lines provided the best fit for data presented for each of the three groups. Observations were presented graphically until less than three pigs remained per group; corresponding standard deviations increased steadily for each subsequent observation due to pigs dropping from each group.

The frozen group demonstrated the lowest pressure values and the lowest variability across larynges as compared to the other two groups. A slight increasing trend was observed during desiccation. Baseline values were fairly similar for both the fresh saline and Ringer's groups. Fresh saline larynges did not demonstrate much change from baseline prior to ceasing to

vibrate. Ringer's larynges also demonstrated a fairly flat pattern, decreasing slightly before ceasing to vibrate; however, significant variability was observed. Second order polynomial trendlines provided the best fit to the data; trendline formulas and associated percent variance explained are provided in Figures 1, 2, and 3, respectively.

The mean number of desiccation trials required to cease vocal fold vibration are displayed in Figure 4. 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). For the flash frozen group, the average number of trials until vibration ceased was 8.3 ($SD = 6.93$, range = 2 to 22). The results from a one-way ANOVA indicated no significant differences at the .05 alpha level, $F(2, 27) = .297, p = .745$.

Discussion

The present study sought to determine if tissue preservation methods had an effect on PTP in an excised porcine larynx. The purpose was to establish a standardized method of preservation for excised animal larynges. The preservation methods examined included immersing larynges in Ringer's solution and performing trials within 24 hours postmortem, immersing larynges in 0.9% saline solution and performing trials within 24 hours postmortem, and immersing larynges in 0.9% saline solution and quick freezing them until the day of the experiment.

The results of this study showed a significant difference in average PTP between the frozen group and fresh saline group and the frozen group and fresh Ringer's group. No significant differences were found between the fresh saline group and the fresh Ringer's group. The frozen group demonstrated the lowest PTP across groups with a slight increasing trend from baseline during desiccation. Both fresh saline larynges and Ringer's larynges did not

demonstrate much change from baseline; however, significant variability was observed for Ringer's larynges. There were no significant differences between groups in the number of trials until vibration ceased.

Phonation Threshold Pressure

Phonation threshold pressure is a measurement used to quantify vocal fold integrity. Specifically, PTP can be influenced by mucosal wave, prephonatory glottal width, and the vocal fold cover. The present study examined the effect of three different preservation methods on PTP. The results indicated that the frozen group had a significantly lower average PTP than those of the fresh saline and Ringer's groups. The average PTP for the frozen group was 8.39 while the average PTP for the fresh saline group was 21.52 and the average for the Ringer's group was 24.28. The average PTP in humans ranges from 3 to 5 cmH₂O. These findings indicate that immersing the larynx in saline and then flash freezing it may result in a PTP that is more similar to human PTP than immersing the larynx in saline or Ringer's solution and using it within 24 hours postmortem.

A significant difference existed between the frozen group and the fresh groups, regardless of solution. This observation could lead to the conclusion that when examining PTP, the frozen larynx model is more comparable to a human larynx than a fresh larynx model, which may be an artifact of the flash freezing process. Allenspach and Kraemer (1989) examined ice crystal patterns after slow freezing and quick freezing artificial gels of extracellular matrix molecules. Their results showed that slow freezing resulted in relatively large crystals. Chan and Titze (2003) observed that these large crystals formed on the tissue of the vocal folds when they were slow frozen and found that quick freezing resulted in smaller ice crystals on the vocal fold tissue. They concluded that these small ice crystals were much less likely to disturb the composition of

the vocal folds. Their study found that there was no significant difference between the shear modulus of larynges that were flash frozen and those that were used within 24 hours postmortem. The present study, however, examined PTP as an indication of vocal fold function and did find a significant difference between flash frozen and fresh larynges. The present study also used porcine larynges instead of canine larynges, which may have caused differing results. It should also be noted that it has also been observed that a slow rate of thawing may cause larger ice crystals to form on the tissue (Young, Armitage, Bowerman, Cook, & Easty, 1994). The current work left the larynges to thaw overnight in a refrigerator for approximately 15 to 24 hours, which could affect the pliability of the vocal folds. Further research should be conducted to determine the effects of different thawing methods on vocal fold histology and phonation.

It is also possible that the flash freezing process delayed the rigor mortis process, which is a chemical change that happens in the body postmortem that causes the muscles to stiffen. This process occurs when adenosine triphosphate (ATP) levels, which help maintain a chemical balance in the muscles, are lowered due to a lack of oxygen. When ATP levels are low, the body leaks calcium around muscle fibril bundles, which causes a contraction of the muscles in their current position. This process of rigor mortis begins to set in around six to eight hours after the time of death (Bucholtz, 2014). Therefore, it is possible that freezing the larynx before this process begins may slow down the rate at which it happens. The fresh groups are left in a refrigerator for 24 hours, so it is possible that rigor mortis set in before the experiments began and made the tissue less pliable than the tissue of the frozen group.

The results also indicated that the frozen group demonstrated the least variability in PTP values from baseline to cessation of phonation. This may indicate that we can expect larynges that are flash frozen in saline to provide a more stable PTP over time, which provides a more

realistic model of a healthy human larynx. Mau et al. (2011) examined the PTP in excised human larynges and found that PTP was relatively stable between trials, which confirms the conclusion that PTP values over time in a flash frozen larynx is more similar to human PTP. The fresh saline and Ringer's groups both showed more variation than the frozen group across trials, which supports the idea that the process of flash freezing the larynx is the best option for consistency across trials. However, there was a slight increase in PTP values over time for the frozen group during desiccation. Tanner, Roy, Merrill, and Elstad (2007) performed a study where PTP was measured before and after each participant inhaled dry air for 15 minutes. The results showed that desiccation resulted in a significantly higher PTP. For the frozen group, PTP may have increased due to the desiccation trials performed between each phonatory trial. Since the frozen group was the only group that demonstrated an increasing trend across desiccation trials, it could be assumed that the frozen group behaved most like a human larynx. These results suggest that the type of storage could have a greater effect on vocal fold integrity than the type of solution.

Laryngeal Desiccation

Although there were no significant differences between groups for the number of trials needed to cease phonation, the frozen group had the highest average amount of trials before phonation could no longer be achieved. As mentioned earlier, this could be influenced by the flash freezing process that occurs to preserve the larynges. The changes that occur during this process could be a factor in why most of the variables examined in this study are significantly different between the frozen and fresh larynges.

Limitations

This study may have had several limitations. 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 their dissection and mounting of the larynges by the end of the study. However, this limitation was minimized having the same researcher perform the same task each day of experimentation. Additionally, there were many steps involved in the dissection of the larynx, which leaves room for error and variability. For example, while removing the false folds, the true folds may have been minutely damaged, which could cause a change in PTP and PTF values. There may have also been variability in the operational procedures of quick freezing each larynx. For example, although each larynx was immersed in liquid nitrogen for approximately eight minutes, it was difficult to tell whether or not the larynx and all of its internal structures were completely frozen. The researchers were aware of this limitation and were careful in examining the larynx thoroughly to note any additional need for freezing, but error could still exist in this process.

There was also variability between each larynx that could not be accounted for. Although the size of each porcine larynx was accounted for, the age and gender of the larynx at the time of death was unknown. The porcine larynx has many similarities to the human larynx; however, porcine vocal folds are at a 45° angle within the larynx whereas human vocal folds are at a 0° angle. This type of difference could influence the outcome of the aerodynamic measures taken for the study. Equipment limitations may have included the microphone being too close to the larynx, which caused peak clipping in a few instances. Finally, this experiment did not take place in a sound booth, therefore reverberations and ambient noise may have affected the detection of phonation onset.

Implications for Future Research

Future studies should conduct research with larger samples sizes and consider age and gender to reduce variability between trials and groups. The large size of the male larynx makes

it preferable for use. It may also be beneficial to examine the histology of the tissue that future researchers may use in their experiments. Specifically, a study examining the differences in histology between tissue that has been frozen then thawed, versus fresh tissue, would be informative. Additionally, it may be of benefit to examine the effects of different thawing methods on the histology of the tissue. To gain a bigger picture, researchers may also consider examining the pressure offset. Future research should also be conducted to examine the effects of freezing on the rigor mortis of ex vivo tissue. When considering future research involving excised larynges, the results of this study and the limitations in access to and variability in using fresh larynges likely make the flash freezing technique the most viable methodology in the majority of studies. However, it is essential to develop a standard methodology in the literature so that individual variability related to tissue preservation and variability between studies may be minimized.

Conclusion

The results from the present investigation indicate that flash freezing a larynx in 0.9% saline solution resulted in pressure values that were significantly lower than those from larynges that were used within 24 hours postmortem. Freezing the larynx also resulted in the least variability between groups. These findings suggest that quick freezing each larynx may result in a model that more closely resembles human phonation, which could be an artifact of the quick-freezing process. Future work involving excised larynges should continue to implement flash freezing methods when examining PTP to create a more realistic model of phonation.

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Table 1

Vocal Folds Anatomical Size and Dimension

Group	Session Date	Length	Width	Width from Vocal folds to Thyroid Cartilage
Fresh Isotonic Saline				
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 Isotonic Saline				
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

Thyroid Cartilage Anatomical Dimensions

Group	Session Date	Height (protuberance to top)	Height (protuberance to bottom)	Width
Fresh Isotonic Saline				
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 Isotonic Saline				
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

Trachea Anatomical Dimensions

Group	Session Date	Trachea Length	Trachea Width
Fresh Isotonic Saline			
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 Isotonic Saline			
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

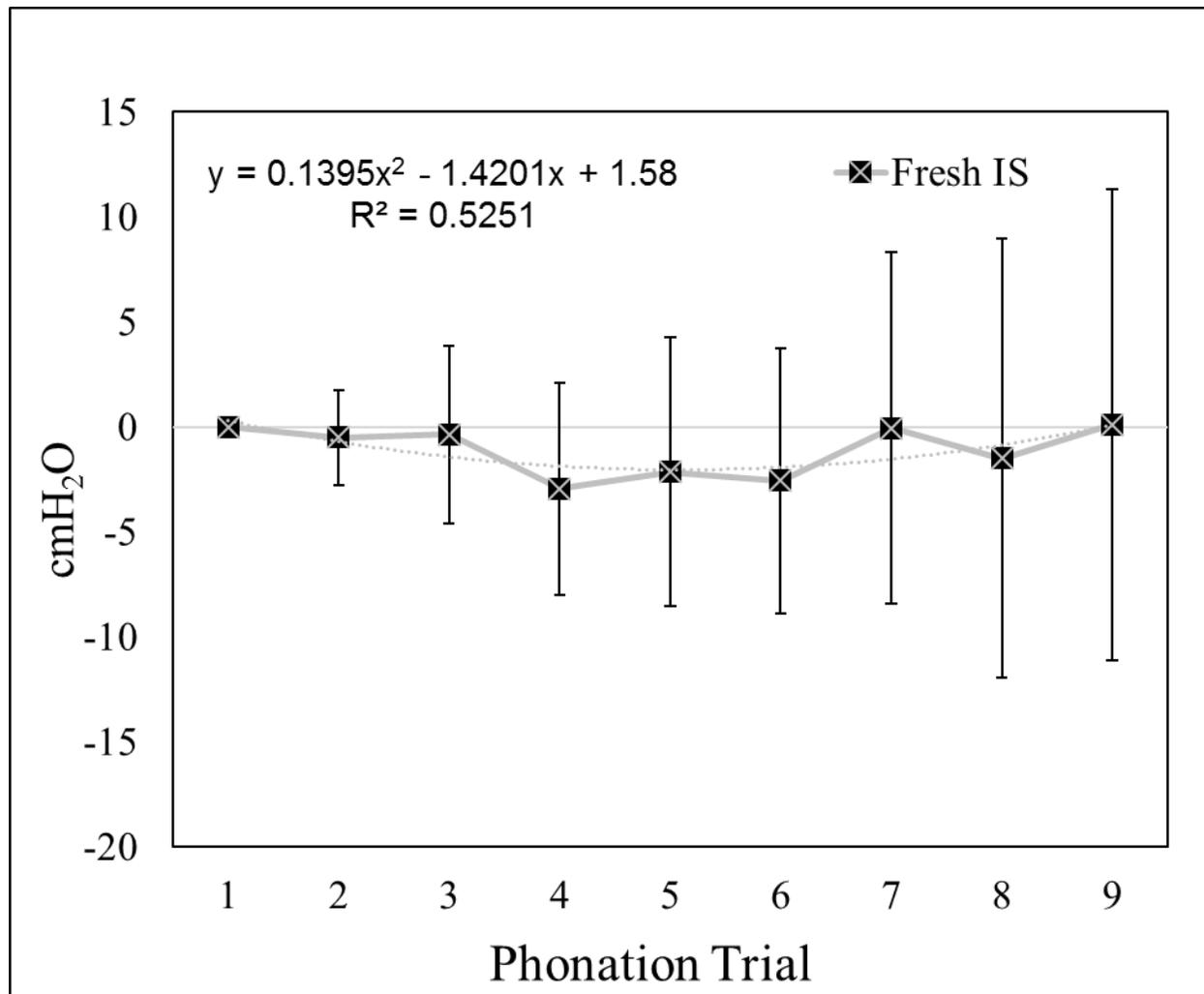


Figure 1. PTP during desiccation for fresh saline. Normalized phonation threshold pressure (PTP) for the fresh saline group at baseline (0 on the y axis) and following each 1-min desiccation dose. Mean, standard error represented by vertical bars, linear trendlines with a $> .80$ criterion for fit, and R^2 values with formulas are provided.

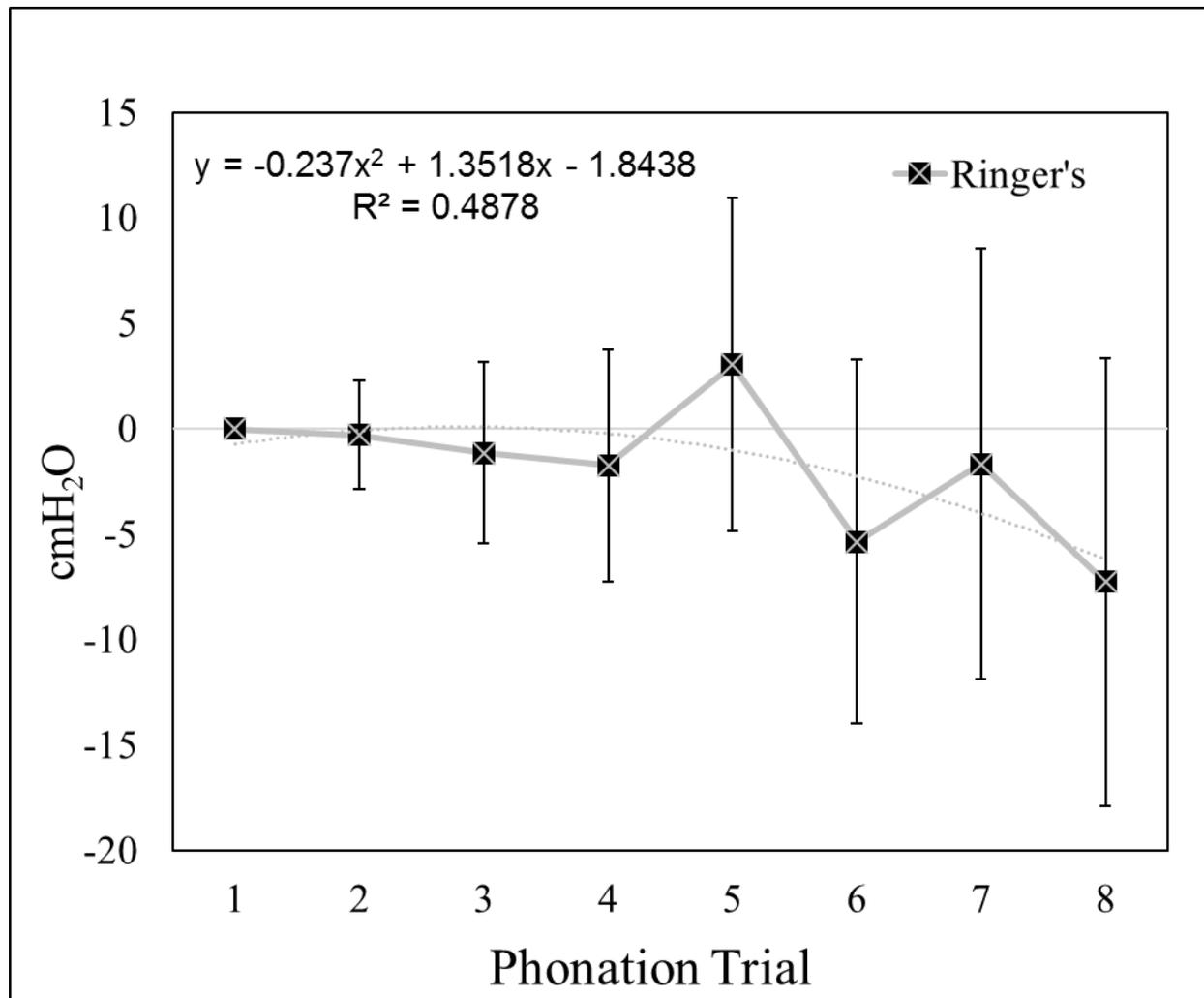


Figure 2. PTP during desiccation for Ringer's. Normalized phonation threshold pressure (PTP) for the Ringer's group at baseline (0 on the y axis) and following each 1-min desiccation dose. Mean, standard error represented by vertical bars, linear trendlines with a $> .80$ criterion for fit, and R^2 values with formulas are provided.

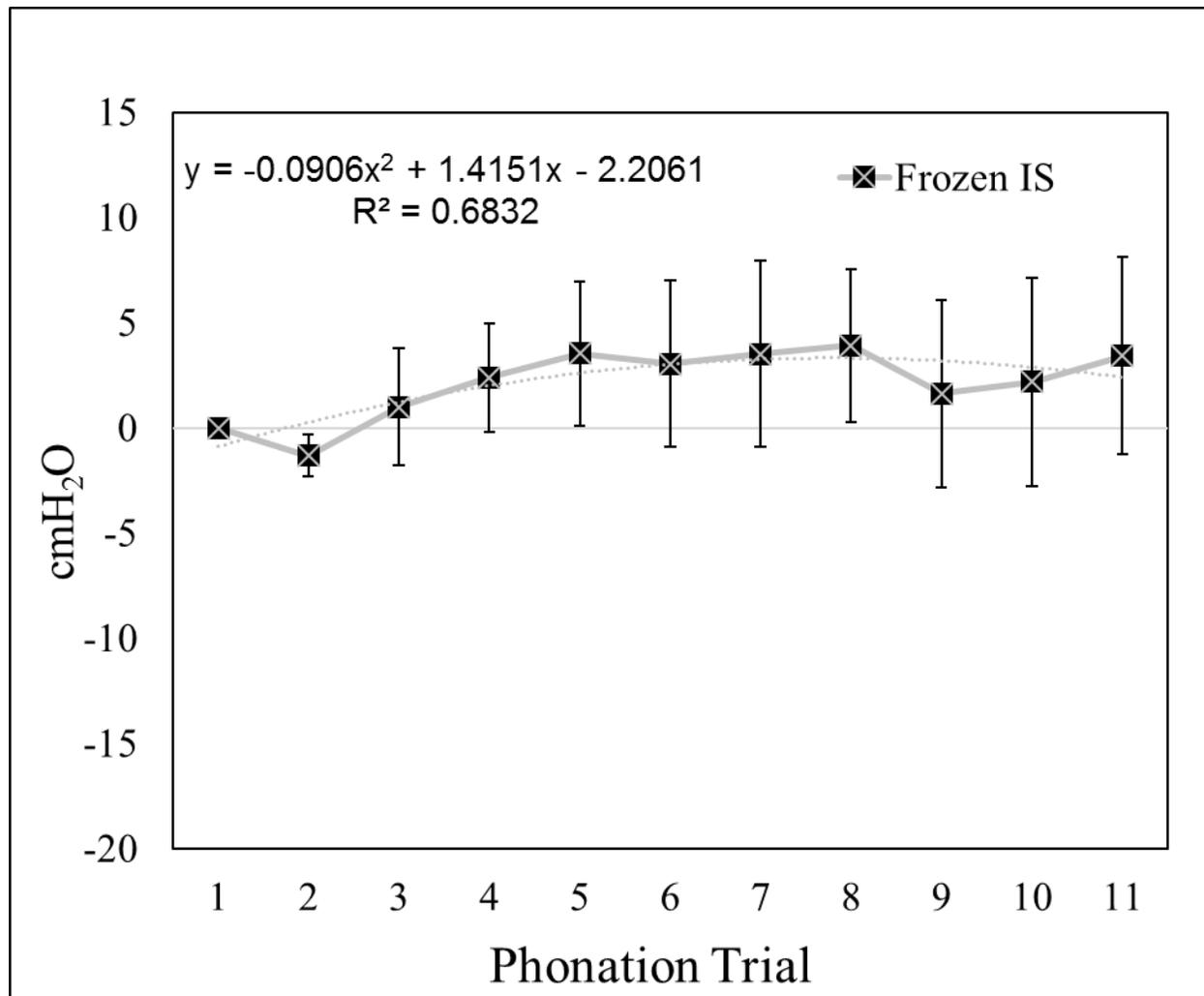


Figure 3. PTP during desiccation for frozen saline. Normalized phonation threshold pressure (PTP) for the frozen group at baseline (0 on the y axis) and following each 1-min desiccation dose. Mean, standard error represented by vertical bars, linear trendlines with a $> .80$ criterion for fit, and R^2 values with formulas are provided.

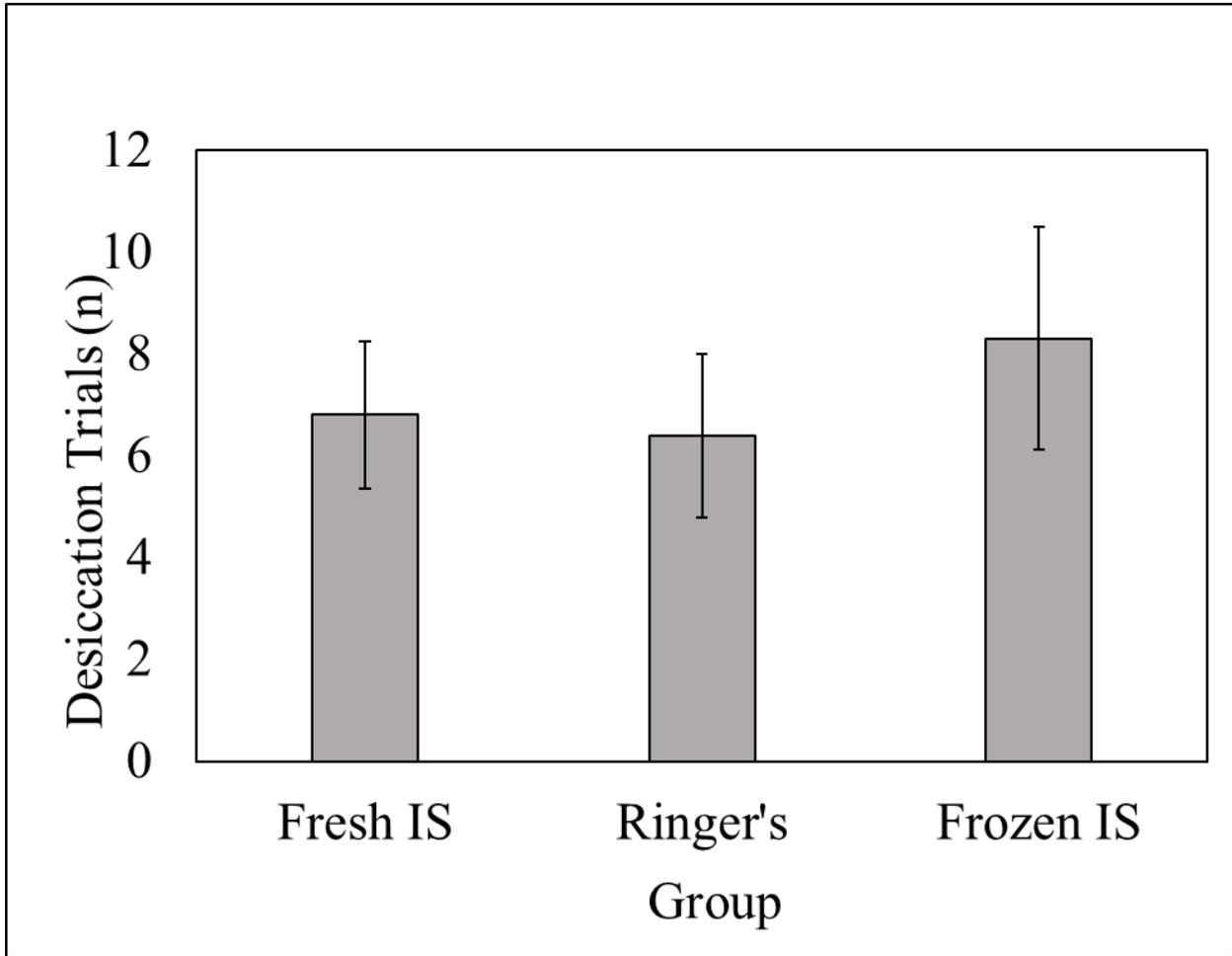


Figure 4. Number of trials until vibration ceased. The number of desiccation trials required to cease vocal fold vibration for larynges in the Fresh IS, Ringer's, and Frozen IS groups. Standard error is represented by vertical bars.

APPENDIX A: ANNOTATED BIBLIOGRAPHY

Alipour, F., Finnegan, E. M., & Scherer, R. C. (2009). Aerodynamic and acoustic effects of abrupt frequency changes in excised larynges. *Journal of Speech, Language, and Hearing Research*, 52, 465-481. doi: 10.1044/1092-4388(2008/07-0212)

Purpose of the study. The purpose of this study was to examine how a sudden change in frequency affects the aerodynamics and acoustics of excised larynx phonation.

Method. Ten excised canine larynges were used as specimens. For the experiment, each larynx was mounted on a benchtop with the trachea connected to a tube housed within a pseudolung. Sutures placed on the larynx were used to stabilize and manipulate adduction and elongation. To initiate phonation, humidified air was passed through the tube into the trachea. For each experiment, flow was manually increased from the phonation threshold flow to the highest level of flow that could maintain phonation. In some of the trials, adduction and elongation were manipulated to test the effects of these physiological changes. Electrodes placed on the thyroid lamina measured F_0 . A pressure transducer measured subglottal pressure, a flow meter measured flow, and a microphone measured acoustic signals. MATLAB was used to collect and analyze the data.

Results. The F_0 and mode of vibration changed abruptly during the phonation trials where adduction and elongation were not manipulated. The F_0 also changed abruptly during the trials where adduction and elongation were manipulated. Lower frequencies were characterized by greater adduction, higher intensity, and large amplitude oscillation. The higher frequencies were characterized by little to no adduction.

Conclusions. This study demonstrates that these mode changes are possible in the canine larynx and without a vocal tract. The finding that the manipulation of adduction and elongation was not necessary for the mode change indicates that breath management plays a significant role in pitch and register control.

Relevance to the current work. The current work also used excised larynges to study certain characteristic of phonation, as well as used a similar method to stimulate phonation, including mounting the larynx on a base connected to a pseudolung and passing hydrated air through the trachea. The instruments used to measure pressure, flow, and the acoustic signal were also the same. The current work also used MATLAB to collect and analyze the data.

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

Purpose of the study. The purpose of this study was to find a feasible substitute for studying the human larynx by measuring the phonatory characteristics of sheep, porcine, and cow larynges.

Method. Excised larynges of sheep, pigs, and cows were obtained from a local butcher shop and then dissected further to remove any excess structures from the larynges. They were then slow frozen in a saline solution and left for several days to several weeks. The larynges were then thawed overnight in a saline solution in preparation for each experiment. To test the phonatory characteristics of the larynges, each larynx was mounted on a base and sutures were placed on the larynx to simulate varying degrees of adduction. An EEG device was also placed on the

larynx to track frequency. To manipulate phonation, filtered and humidified air was passed through a tube connected through the trachea. During vocal fold oscillation, flow was measured by a flow meter connected to the tube and subglottal pressure was measured by a manometer attached below the larynx. Time-varying flow rate was measured by pneumatic flow meter; low-range pressure transducer and time varying subglottal pressure was measured by a pressure transducer. Intensity was measured by a sound level meter. Oscillation of the vocal folds was monitored and recorded by a stroboscopic light source. Signals recorded during phonation were quantified by MATLAB software and analyzed.

Results. Results showed that sheep, porcine, and cow larynges have distinct qualities from each other that make them appropriate models for studying human larynges. The porcine larynx has the greatest variability in frequency and steeply angled vocal folds. The cow larynx had the lowest PTP, the steadiest pitch, and largest dimensions. The sheep larynx is anatomically similar to the human larynx in vocal fold length, laryngeal dimensions, and tissue histochemistry. One characteristic that they all had in common was a similarly sized supraglottic duct, which aided in stabilizing phonation.

Conclusions. The variability in frequency of the porcine larynx makes it the most appropriate model for studying pitch control, although its steeply angled vocal folds make it less preferable for studying vocal amplitude. The cow larynx would be an appropriate model for studying aerodynamic measurements because of its low phonation threshold pressure and steady pitch. Its large dimensions and steady pitch also make it a good model for measuring oscillation amplitude and intraglottal pressure. The sheep larynx is a good model for studying the physiology of the larynx because of its anatomic similarity to the human larynx. Their similarly sized supraglottic duct made all of them good phonatory and aerodynamic models.

Relevance to the current work. The results of this study validate the use of the porcine larynx in the current work as a phonatory model. The methods to mount the larynx and manipulate its phonation are also relevant to the current work, whose methods included mounting the larynx on a base and manipulating its phonation with humidified air. The method to collect data in this study is also relevant because the current work, which also used a flow meter to track the flow of air and used MATLAB to record and analyze the results. There are a few differences, however, between this study and the current work. For example, this study used slow frozen larynges, whereas the current work used fast frozen larynges as to not damage the cellular makeup of the larynx. Also, this study used a stroboscopic light to be able to capture oscillation in slow motion, whereas the current work used a high-speed camera to capture a more accurate depiction of the oscillation in slow motion.

Alipour, F., Jaiswal, S., & Finnegan, E. (2007). Aerodynamic and acoustic effects of false vocal folds and epiglottis in excised larynx models. *Annals of Otology, Rhinology, and Laryngology*, 116, 135-144.

Purpose of the study. The purpose of this study was to examine how the false vocal folds and the epiglottis affect the aerodynamics and acoustics of excised larynx phonation.

Method. Canine larynges were excised and the excess muscles and tissue were dissected. Each larynx was frozen for preservation and then thawed before the experiment. For each experiment, the larynx was mounted on a base with the trachea connected to a tube which was surrounded by a pseudolung. Sutures placed on the larynx were used to stabilize it and manipulate adduction and elongation. To stimulate phonation, hydrated air was passed through the tube into the

trachea. Phonation was measured with the false vocal folds and the epiglottis and then again with them removed. Electrodes placed on the thyroid lamina measured the F_0 , jitter, and closed quotient. A pressure transducer measured subglottal pressure and a flow meter measured flow, which were used to calculate glottal flow resistance. A microphone measured acoustic signals. **Results.** Values for glottal flow and sound intensity were higher in phonation that included the false vocal folds and the epiglottis. The pressure-flow relationship remained the same for both types of phonation.

Conclusions. The false vocal folds and the epiglottis have a significant impact on the aerodynamic and acoustic effects of phonation in the excised canine larynx.

Relevance to the current work. The current work removed the false vocal folds and part of the epiglottis on each larynx before conducting phonation trials. This study supports that practice because if the false vocal folds and epiglottis had not been removed the results of the trials would be significantly different. Although the current work used porcine larynges, it can be concluded that the false vocal folds and the epiglottis would have had a similar effect on phonation. Also, the methods used to mount the larynx, stimulate phonation, and record data were similar, although the current work did not use electrodes.

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. The purpose of this study was to examine the bifurcations found in vocal fold vibration.

Method. Several canine larynges were excised and the excess muscles and tissue were dissected. For each experiment, the larynx was mounted on a base with the trachea connected to a tube which was connected to a pseudolung. Sutures placed on the larynx were used to stabilize it and manipulate adduction and elongation. To stimulate phonation, hydrated air was passed through the tube into the trachea. Phonation experiments were conducted under multiple asymmetric conditions of the vocal folds. Phonation onset, vibratory patterns, and various bifurcations were measured and recorded.

Results. Bifurcations of symmetric folds, asymmetric adduction, and elongation asymmetry were all produced through the manipulation of the excised larynges.

Conclusions. The ability to manipulate and measure the bifurcations of phonation demonstrate that excised larynges more accurately represent the human vocal folds than computer models. Also, the bifurcation diagrams used in this study were effective in analyzing vocal fold dynamics. Bifurcation diagrams may be useful in studies examining vocal fold oscillation.

Relevance to the current work. This study supports the current work's use of excised larynges as a model for human phonation. The current work, however, used a slightly different apparatus to stimulate phonation, although the current work also used humidified air. Also, this study did not specify any type of method used to preserve the larynges, which was the focus of the current work.

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. The purpose of this study was to quantify the viscoelastic shear

properties of the superficial layer of the lamina propria of the human vocal folds.

Method. The laryngeal samples were obtained fresh within 24 hours postmortem or quick frozen within 18 hours postmortem. The epithelium and superficial layer of the lamina propria were dissected from each larynx. The samples were placed between two rotating circular plates to create shear deformation. A rheometer collected and analyzed the shear deformation of the tissue samples. The rheometer also recorded the elastic shear modulus and the viscous shear modulus.

Results. Results showed that the elastic shear modulus and the damping ratio of human vocal fold mucosa were relatively constant across the range of frequencies observed, while the dynamic viscosity decreased monotonically with frequency.

Conclusions. The results of this study may reflect the part that age and gender may play in the viscoelastic shear properties of the vocal folds. The tissue samples of male specimens were stiffer than those of female specimens; the tissue samples of older specimens were stiffer than younger specimens.

Relevance to the current work. This study supports the current work's method of preserving the larynges within 24 hours postmortem.

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 purpose of this study was to examine the effects of postmortem changes and freezing on the viscoelastic shear properties of the vocal fold mucosa.

Method. Sixteen canine larynges were excised immediately postmortem and the complex shear modulus of one vocal fold from each larynx was measured. Measurement was repeated for 10 mucosal specimens after 24 hours postmortem storage in a saline solution at room temperature. Eleven of the 16 larynges were stored and frozen at -20°C. Six of them were frozen slowly in a normal freezer and five of them were frozen quickly with liquid nitrogen prior to storage in a freezer. After one month in the freezer the larynges were slowly thawed overnight and then the viscoelastic shear properties of the contralateral vocal fold mucosa were measured by subjecting the tissue to precisely controlled oscillatory shear stress. The measurements were quantified by a controlled-stress torsional rheometer.

Results. Elastic shear modulus and dynamic viscosity were the two viscoelastic shear properties calculated to show the effect of postmortem tissue changes on the larynx. These properties were measured at a range of 0.01-15 Hz using an upward frequency sweep. Results showed that the viscoelastic shear properties of the vocal fold mucosa did not significantly change after 24 hours postmortem storage in a saline at room temperature nor after one month of frozen storage following quick freezing.

Conclusions. This study showed that quick freezing better preserves the physiological properties of the larynx than slow freezing. The quick freezing may be a feasible strategy for the preservation of vocal fold connective tissues for various research applications.

Relevance to the current work. The methods used to quick freeze the larynx, the effects of quick freezing on the larynx, and knowing that freezing the larynx quickly is preferable to freezing the larynx slowly are relevant to the current study, which is also measuring the physiological properties of quick frozen larynges.

Fang, Q., Wang, Y., Zhao, X., Cao, L., Sun, N., Tan, X., . . . Sun, G. (2012). Vocal fold composition and early glottic carcinoma infiltration. *World Journal of Surgical Oncology*, 10, 178. doi: 10.1186/1477-7819-10-178

Purpose of the study. The purpose of this study was to determine the average depth that laryngeal carcinomas infiltrate the tissue of the vocal folds.

Method. Larynges were collected from 30 males who had a history of smoking and had undergone a laryngectomy. All samples were collected immediately following the surgery. The vocal folds were removed, divided lengthwise into three equal parts, and then divided into two equal halves. Half of the sections were frozen and the other half was dehydrated and preserved in paraffin. The samples preserved by paraffin were deparaffinized and treated prior to observation. The frozen samples were frozen using a Shandon Frozen Microtome and then thawed to room temperature and treated prior to observation. Each sample was observed and measured with a light microscope.

Results. For the normal vocal folds, the average depth of the mucosa was 0.15 mm, of the submucosa 2.30 mm, and of the muscular layers 2.87 mm. T1 tumors infiltrated 1.62 mm deep into frozen samples and 1.32 mm deep into paraffin-embedded samples. T2 tumors infiltrated 2.87 mm deep into frozen samples and 2.58 mm deep into paraffin-embedded samples. T1 tumors occupied 24.8% of vocal fold depth and T2 tumors occupied 48.5% of vocal fold depth.

Conclusions. These measurements are an important reference for surgeons removing carcinomas from vocal fold tissue. This data may be used to assess how much a tumor has infiltrated the vocal folds, which will allow for more accurate surgical procedures.

Relevance to the current work. The methods of preservation are relevant to the current work. Although the types of preservation used in this study are different, the types of preservation used is important to the current work, which focused on the best form of preservation.

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 measure the effects of dehydration on the function of the vocal folds. The biphasic theory of vocal fold physiology was also tested to measure the effect of dehydration on the mobility of the vocal folds.

Method. Fifteen larynges were excised from laboratory dogs, yielding 30 samples of the lamina propria of the vocal folds for the study. Larynges were placed in saline solution, frozen, and then thawed the day of each experiment. The lamina propria were dissected out of each larynx and the volumes and masses were recorded. The tissue samples were then dehydrated in a vacuum oven heated to 40°C. The samples were measured every two minutes during the process of dehydration until the desired amount of dehydration at either 30%, 50%, or 70% dehydration. Using this data, the liquid mass and volume fractions and the solid:liquid mass and volume ratios were calculated.

Results. At each dehydration level, all measurements were significantly different. Liquid:solid volume ratios of 30% dehydration compared to 50% dehydration were the only measurements that were not significantly different. The linear, inverse relationships measured for all data suggested that dehydration levels can predict solid and liquid parameters of the lamina propria.

Conclusions. The biphasic theory, which explains the solid-liquid interaction of the lamina

propria, is supported by the results of the study as well as the quantification of biphasic model parameters by lamina propria tissue dehydration.

Relevance to the current work. The effects and methods of dehydration found in this study are relevant to the current work in which the larynx is hydrated in two different solutions and then dehydrated while assessing phonation.

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. The purpose of this study was to quantify the ability of canine vocal folds to recover from varying dehydration levels.

Method. Tissue samples from canine larynges were obtained and frozen in saline solution. These samples were thawed in a saline solution in preparation for each experiment. The vocal folds were then excised from the larynges and their volume and mass were measured. Dehydration was performed by placing the tissue in a vacuum oven heated to 40°C. Dehydration was monitored by the tissue mass and the samples were dehydrated to 30% or 70% dehydration. Each sample was rehydrated by being submersed in a saline solution. The volume and mass were again measured and compared to the original measurements.

Results. The calculations made showed different results between 30% and 70% dehydration. Significantly fewer vocal folds that were subjected to 70% dehydration recovered to their original volume. Ten of the samples subjected to 70% dehydration recovered, whereas 20 of those subjected to 30% dehydration recovered.

Conclusions. This study supports the theory that rehydration of the vocal folds is a valid treatment and is important to implement before permanent damage to the vocal folds occurs.

Relevance to the current work. The current work also subjected excised larynges to dehydration. Although the methods of dehydration used in this study were different than those of the current work, it supports the theory that dehydration influences the vocal folds, which can adversely affect phonation.

Hemler, R. J., Wieneke, G. H., & Dejonckere, P. H. (1997). The effect of relative humidity of inhaled air on acoustic parameters of voice in normal subjects. *Journal of Voice*, 11, 295-300.

Purpose of the study. The purpose of this study was to demonstrate the effects of humidified air on the voice.

Method. In trials lasting 10 minutes, eight participants inhaled air in three different conditions: a desiccated air trial, a normal air trial, and a humidified air trial. To measure voice quality, each participant produced a sustained /a/ after each trial. The parameters used to measure voice quality were perturbation and noise-to-harmonic ratio.

Results. Perturbation was greater after the desiccated air trials and noise-to-harmonics ratio remained the same between each trial.

Conclusions. It was concluded that inhalation of dry air negatively influences phonation.

Relevance to the current work. The current work simulated inhalation of air by blowing compressed air on the vocal folds for five minutes after every phonation trial. Therefore, it is important to know the effect of air exposure on the quality of phonation.

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. The purpose of this study was to quantify canine phonation flow range, of phonation by subtracting PTP from phonation instability flow.

Method. Larynges from seven canines were excised postmortem and frozen in a saline solution. Before each experiment, the larynges were thawed, excess tissue was removed to expose the true vocal folds, and then each larynx was mounted on a benchtop setup. Air was passed through the tube into the trachea to stimulate vibration. PTP and phonation instability flow were measured at 0% elongation with no glottal gap, 20% elongation with no glottal gap, and 20% elongation with a 3 mm posterior glottal gap.

Results. Elongation did not have significant effects on PTP, phonation flow range, or phonation instability flow, but glottal abduction did produce significant effects on these variables.

Conclusions. Because phonation instability is a characteristic of certain voice disorders, PTP and phonation instability pressure may be important measures for assessing vocal fold pathology. Understanding flow range and instability flow may help to more accurately interpret instability pressure and PTP.

Relevance to the current work. The methods used to phonate the canine vocal folds support the methods used in the current study, which also included freezing larynges in saline and later mounted them on a bench setup. The current work, however, is also comparing the method of using a previously frozen larynx to using a fresh larynx.

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

Purpose of the study. The purpose of this study was to examine how phonation threshold flow and PTP are affected by positional changes in the benchtop larynx setup.

Method. Excised canine larynges were obtained immediately postmortem and then submersed in a saline solution and quick frozen. The larynges were thawed before each experiment and the false folds were dissected away to expose the true vocal folds. The dissected larynx was then mounted on a tube connected to a pseudolung and three-pronged micrometers were laterally inserted into the arytenoids to stabilize the larynx. The vocal folds were abducted by metal shims placed between the arytenoid cartilages. Humidified air was passed through the pseudolung into the larynx to stimulate phonation. The flow of air was gradually increased to the point that initiated phonation. Five phonation trials were performed at five levels of abduction. Airflow was measured by a flow meter and pressure was measured by a pressure meter. Acoustic signals were recorded and digitized by a microphone and preamplifier. Data were recorded and analyzed by Labview 7.1 software.

Results. The data showed that phonation threshold flow is affected by the level of abduction and PTP is not affected by the level of abduction. These findings suggested that phonation threshold flow might be more sensitive to posterior vocal fold abduction than PTP.

Conclusions. These findings indicate that phonation threshold flow can be used as a measure to assess voice disorders that involve insufficient posterior glottal closure.

Relevance to current work. The current work also used excised larynges to PTP. The current

work used a similar method to stimulate phonation, including mounting the larynx on a base connected to a pseudolung and passing hydrated air through the trachea. The instruments used to measure pressure, flow, and the acoustic signal were also the same. Additionally, the current work studied the effectiveness of freezing the larynx as a method of preservation, which was the method used in this study.

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 this investigation was to create a method for studying the phonation of ex vivo larynges in order to better understand the phonation of in vivo human larynges.

Method. Larynges of 19 canines were surgically removed and perfusion delivery was administered to oxygenate them. Once excised, electrodes were applied to the recurrent laryngeal nerve and the superior laryngeal nerve and an endotracheal tube was placed inside the trachea. The laryngeal nerves were stimulated by the electrodes and humidified air was passed through the endotracheal tube to stimulate phonation.

Results. The larynges that produced the most successful long-term phonation were those that received perfusion in a pulsatile flow.

Conclusions. The ex vivo laryngeal model is appropriate for studying human phonation. As well as phonation, this model can be used to study tissue preservation techniques.

Relevance to the current work. This study supports the current work's use of an ex vivo larynx to study preservation techniques. The design used to phonate the larynx was similar, but not the same. The current work used saline to preserve the physical properties of the larynx, whereas this study did not. Also, the current work did not use the superior laryngeal nerve or the recurrent laryngeal nerve to stimulate the muscles of the larynx.

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. The purpose of this study was to examine the anatomic and biomechanical features of the larynges of four species: human, pig, dog, and white-tailed deer.

Method. Excised larynges were obtained and dissected to have a clear view of the true vocal folds. Measurements were taken of the vocal fold height, the range of motion of the cricothyroid joint, the vocal fold stiffness, and the glottal configurations.

Results. All larynges studied presented with similar vocal fold length. Human, dog, and pig larynges all presented with similarly sized cricothyroid muscles. Also, human, dog, and pig larynges presented with greater mobility in the cricothyroid joint than the deer larynx and a cartilaginous framework that allows for more precise movement than that of the deer larynx. The human and pig larynges presented with the most similar vocal fold cover and vocal fold stiffness.

Conclusions. Because of the availability, anatomical features, and structures associated with phonation of the pig larynx, the pig may be the most appropriate substitute for studying phonation of the human larynx.

Relevance to the current work. This study supports the current work's use of porcine larynges

to study phonation. This study, however, could have been more thorough in their research by doing phonation trials in addition to taking measurements of the laryngeal structures.

Jiang, J., Ng, J., & Hanson, D. (1999). The effects of rehydration on phonation in excised canine larynges. *Journal of Voice*, 13, 51-59.

Purpose of the study. The purpose of this study was to examine the effects of rehydration on dehydrated larynges.

Method. Thirteen excised canine larynges were excised immediately postmortem. They were then inspected for possible disease or lesions and then mounted on a base. Once mounted, the larynges were dehydrated by a constant airflow through the vocal folds until phonation ceased. The larynges were then rehydrated using a saline drip on the superior surface of the vocal folds and then being immersed completely in saline for 30 minutes. The larynges were remounted and phonatory measures were recorded. PTP, onset airflow, and acoustic intensity were recorded for both the dehydrated and rehydrated larynges. Vocal efficiency was calculated using the measurements of acoustic intensity and airflow.

Results. There was a significant increase in vocal efficiency and significant decrease in PTP after the methods of hydration were performed.

Conclusions. Because PTP is a measurement of phonatory effort, the results of this study suggest that greater hydration of the larynx results in less effortful phonation.

Relevance to the current work. The current work also used excised larynges to study the effects of hydration on phonation. Methods used to stimulate phonation were also similar, as well as methods of dehydration. However, dehydration in the current work was performed after phonation trials, rather than during phonation. Excluding initial submersion in saline postmortem, no methods of rehydration were used on the larynges of the current work.

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 examine the morphological attributes of collagen in porcine vocal folds to better understand the collagen in human vocal folds, which can be affected by dysphonia associated with aging and scarring.

Method. Vocal folds were excised from thirteen pig larynges and two to three collagen samples were dissected from each one. Images of the samples were obtained through atomic force microscopy imaging and were analyzed. Once analyzed, the samples were divided into a pepsin or sham group. The pepsin group was moistened with pepsin and the sham group was moistened with phosphate buffered solution. The samples were then dried and imaging was collected for each. The vocal fold epithelium went through the same pepsin procedure in order to simulate in vivo physiology.

Results. The physiological and morphological properties of the collagen fibers were found to be comparable with the results of other studies performed to measure the properties of collagen fibers. The morphological properties of collagen were not significantly changed when directly exposed to phosphate buffered solution. Most properties were not significantly changed when directly exposed to pepsin, except for a significant decrease in d-periodicity. There were not significant changes to the morphological properties of collagen after indirect exposure to both

phosphate buffered solution and pepsin.

Conclusions. Atomic force microscopy may provide a more specific measurement than electron microscopy, which has been more commonly used as a measurement tool. Atomic force microscopy will be a useful tool in helping engineer tissue with properties similar to the original topography of the lamina propria.

Relevance to the current work. Understanding the physiological and morphological attributes of the lamina propria of the porcine larynx is important to the current work, which also studied the porcine larynx. It also supports the theory used in the current work that porcine larynges are an appropriate substitute for understanding the properties of the human larynx.

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. The purpose of this study was to examine the movement of the hydrated air that flows through the glottis and its effect on the vocal folds.

Method. Excised canine larynges were used as a substitute for human larynges. Each larynx was sutured at the posterior end of the vocal folds and phonation was stimulated by blowing aerosol-hydrated air through a vinyl tube into the trachea. Crushed iopanic acid tablets were added to the air to create a radiographic marker, which was monitored by an x-ray. A stroboscopic, superior view of the vocal folds was recorded with a video camera and a 16-mm cine-camera. The data was analyzed frame by frame with a film motion analyzer.

Results. The fluid blown through the trachea moved in rotation similar to a waterwheel and formed columns of fluid on the superior surface of the vocal folds within the boundaries of the ventricles.

Conclusions. It was concluded that the movement of the mucosal wave of the vocal folds created the columns of fluid as well as manipulated the rotational movement of the air. It was presumed that this rotational movement lubricated and cooled the phonating vocal folds. The termination of phonation brought about the decomposition of the columns of fluid, which caused most of the fluid to flow into the subglottis. Some of the fluid remained on the vocal folds and lubricated them.

Relevance to the current work. This study offers insight into what is happening to the vocal folds as hydrated air passes through them, which is relevant to the current work that used hydrated air to phonate the vocal folds. The current work, however, had a greater focus on the effects of preservation on the physiological components of the larynx postmortem, whereas this study did not expound upon their preservation methods.

Mau, T., Muhlestein, J., Callahan, S., Weinheimer, K. T., & Chan, R. W. (2011). Phonation threshold pressure and flow in excised human larynges. *The Laryngoscope*, 121, 1743-1751. doi: 10.1002/lary.21880

Purpose of the study. The purpose of this study was to quantify PTP and PTF of excised human larynges and determine how these measures are affected by posterior glottal width, glottal area, and gender. Also, this study aims to determine whether hysteresis is present in the phonation of excised human larynges.

Method. Excised human larynges were obtained and preserved in a sealed beaker filled with phosphate-buffered saline. Before the experiment, the exterior laryngeal muscles, except for the strap muscles, and the ventricular folds were dissected away to expose the true vocal folds. For the experiment, each larynx was mounted on a base with the trachea connected to a tube which was connected to a pseudolung. Hydrated air was passed through the tube into the trachea to stimulate vibration. The posterior portion of the larynx was stabilized by micrometers and arytenoid adduction sutures were used to manipulate adduction. For each larynx, five phonation trials were performed at different glottal widths and PTP and phonation threshold flow onset and offset values were recorded for each trial. PTP and phonation threshold flow were determined by MATLAB.

Results. PTP and PTF varied between each subject. Glottal width did not show significant differences in PTP and PTF. An increase in glottal area produced an increase in phonation threshold flow but did not show significant differences in PTP. Male larynges showed a greater PTF than female larynges and there was no significant difference in PTP. Hysteresis was present in the phonation of excised human larynges.

Conclusions. PTP measurements were comparable to in vivo measurements, but PTF measurements were much higher than in vivo measurements, which may be due to vocal fold bowing. Because there was no significant variance between glottal width, canine larynges may be a more appropriate tool for determining the effect of adduction on phonation. Offset PTP and PTF measurements may be more accurate than onset measurements. The variability found in PTP and PTF measurements may be important in reevaluating the aerodynamic parameters used in a clinical setting.

Relevance to the current work. The current work used a similar apparatus for phonation trials. The current work also used MATLAB to measure pressure and flow. Although a saline solution was also used to preserve the larynges, the methods of preservation were different in the current work.

Meyer, J. P., McAvoy, K. E., & Jiang, J. (2013). Rehydration capacities and rates for various porcine tissues after dehydration. *PLoS One*, 8, e72573.
doi: 10.1371/journal.pone.0072573

Purpose of the study. The purpose of this study was to examine the capacity of porcine tissues to rehydrate after varying levels of dehydration.

Method. Various porcine tissue was obtained and sectioned off into 1.5 cm pieces. The samples were submersed in a saline solution and frozen. Before the experiment, the tissue was thawed in room temperature water. For the experiment, each tissue's mass was recorded and was then dehydrated in a vacuum oven. Each tissue's mass was recorded again and then submersed in a saline solution for five hours. Each tissue was then weighed three times, being submersed in the saline solution between each time it was weighed. The tissues were then dehydrated a second time, submersed in saline, and then weighed in three intervals. The rehydration capacity and rehydration rate were calculated and the data were analyzed with MATLAB.

Results. After the first experiment of dehydration and rehydration, the tissue gained most of its original fluid volume back. After the second experiment of dehydration and rehydration, the tissue gained part of its original fluid volume back, but not as much as the first experiment.

Conclusions. The results of this study demonstrate that the tissue's capacity to rehydrate is dependent on the level of dehydration. Therefore, to prevent incomplete rehydration in

rehydration experiments, it is necessary to control the dosage and exposure to dehydration.

Relevance to the current work. The current work is also a methodological study involving porcine tissue and used a saline solution to hydrate the porcine tissue. This study also supports the current work's controlled method of dehydration.

Murugappan, S., Boyce, S., Khosla, S., Kelchner, L., & Gutmark, E. (2010). Acoustic characteristics of phonation in "wet voice" conditions. *Journal of the Acoustical Society of America*, 127, 2578-2589. doi: 10.1121/1.3308478

Purpose of the study. The purpose of this study was to examine the effects that liquid material on the vocal folds has on the acoustic characteristics of phonation.

Method. Excised porcine larynges were obtained immediately postmortem and were dissected so that the true vocal folds were exposed. Once dissected, the larynges were submersed in a saline solution. For the experiment, each larynx was mounted on a base with the trachea connected to a tube which was connected to a pseudolung. The vocal fold processes were adducted with a stitch and four prong pins stabilized the larynx. To stimulate phonation, humidified air was passed through the tube into the trachea. Each larynx underwent four phonation trials. Three liquids of different viscosity were used for the last three trials of each larynx. Values for PTP and the acoustic signal were collected and analyzed.

Results. The subglottal pressure for each larynx increased with the viscosity of each liquid. The F_0 for each larynx remained the same between each condition, which suggests that the liquid did not affect the vibratory patterns of the larynges. Irregular and aperiodic phonation was observed when liquid was present on the vocal folds.

Conclusions. The results of this study may provide more information in the diagnostic and treatment process of vocal folds that have excess material on their surface, whether it be liquid or a lesion. The results may also help in future studies analyzing the effects of swallowed material on the vocal folds on lower airway protection and respiratory health.

Relevance to current work. The current work also used porcine larynges in their study. Although the methods of preservation seem similar with the use of saline, the extent of similarity cannot be determined since this study did not include whether the larynges were tested after being frozen and thawed or were tested fresh. The methods used to stimulate phonation were similar in the current work, which mounted the larynx on a base connected to a pseudolung and passed hydrated air through the trachea. PTP and the acoustic signal were also the measurements used to study the physiological properties of the larynx.

Quirinia, A., & Viidik, A. (1991). Freezing for postmortal storage influences the biomechanical properties of linear skin wounds. *Journal of Biomechanics*, 24, 819-823.

Purpose of the study. The purpose of this study was to examine the effects of freezing on the biomechanical properties of linear skin wounds

Method. Sixteen male rats were used as test subjects. After being anaesthetized, the skin of their backs was shaved off and two incisions were made into the skin, which were immediately sutured. The sutures were removed 10 days after the procedures. The skin of the back of the rats was removed a second time and the skin was randomly divided into three groups. One group was tested fresh, the second time and the skin was randomly divided into three groups. One group was tested fresh, the second was tested after two months frozen in gauze soaked in

Ringer's solution, and the third group was tested after two months frozen in a vial filled with Ringer's solution. The biomechanical properties of the three groups were then tested.

Results. Freezing the samples significantly affected the wound's ability to heal. Freezing the samples significantly affected the biomechanical properties, although the biomechanical properties of the samples frozen in Ringer's-soaked gauze were more greatly affected than those frozen in Ringer's. The biomechanical properties of the frozen samples were more greatly affected than those of the fresh samples.

Conclusions. The results show that freezing tissue samples causes the biomechanical properties of healing wounds to be affected, which should be taken into consideration when deciding how to store tissue samples.

Relevance to current work. The current work also tested the effects of freezing on tissue samples. However, the properties that were measured in the current work are different than those that were measured in this study.

Regner, M. F., & Jiang, J. J. (2011). Phonation threshold power in ex vivo laryngeal models. *Journal of Voice*, 25, 519-525. doi: 10.1016/j.jvoice.2010.04.001

Purpose of the study. The purpose of this study was to examine the effect of the biomechanical properties of the vocal folds on phonation threshold power.

Method. Excised canine larynges were obtained immediately postmortem, submerged in a saline solution, and quick frozen. Before each experiment, the larynges were thawed, excess tissue was removed to expose the true vocal folds, and then each larynx was mounted on a base with the trachea connected to a tube which was connected to a pseudolung. Humidified air was passed through the tube into the trachea to stimulate phonation. A three-pronged instrument was laterally inserted into the arytenoids to stabilize them and manipulate vocal fold elongation. The laryngeal prominence of the thyroid cartilage was sutured and connected to a micromanipulator to manipulate vocal fold elongation. The larynges were divided into three groups of 10 to measure the effects of three different manipulations: posterior glottal gap, vocal fold elongation, and vocal fold lesions. A pressure meter measured pressure, a flowmeter measured flow, and a microphone measured the acoustic signal. MATLAB was used to collect and analyze the data.

Results. Data analysis showed that a posterior glottal gap and vocal fold lesions had a significant impact on phonation threshold power and that vocal fold elongation had a significant but weak impact on phonation threshold power.

Conclusions. The results of this study indicate that a posterior phonation threshold power may be used as a measurement in a clinical setting to provide a more accurate diagnostic and treatment information.

Relevance to the current work. The current work also used excised larynges to study a certain characteristic of phonation. The larynges were also obtained immediately postmortem, submerged in a saline solution, and quick frozen. The current work used a similar method to stimulate phonation, including mounting the larynx on a base connected to a pseudolung and passing hydrated air through the trachea. The instruments used to measure pressure, flow, and the acoustic signal were also the same. The current work also used MATLAB to collect and analyze the data.

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

Purpose of the study. The purpose of this study was to discover if more air flow is required to start phonation, known as onset phonation threshold flow, than the minimal flow required to sustain phonation, known as offset phonation threshold flow. Also, this study aimed to test the hypothesis that the ratio of onset and offset threshold flow falls within 0.707 to 1.0.

Method. Excised canine larynges were obtained immediately postmortem and submersed in a saline solution and frozen. The larynges were thawed before the experiment and each larynx was dissected before the phonation trials. For the phonation trials, each larynx was mounted for stability and humidified air was blown through the trachea to stimulate phonation. An airflow meter was used to measure onset and offset flow, a pressure transducer was used to measure PTP, and a microphone was used to acquire acoustic data. All of the data was collected for the normal length and elongated length of the vocal folds.

Results. Onset threshold flow was greater than offset PTF 100% of the time and the ratio of the two fell within 0.707 to 1 80% of the time.

Conclusions. The relationship between onset and offset flow could be important in the diagnosis and treatment of voice disorders and could help to advance what is known of the physics of phonation.

Relevance to the current work. The current work is also a methodology study involving excised larynges. The current work is also used saline as a form of preservation but also tested to compare fresh larynges with frozen larynges. The current study also mounted the larynges on a bench and directed humidified air through the trachea to initiate phonation.

Sivasankar, M., & Fisher, K. V. (2008). Vocal folds detect ionic perturbations on the luminal surface: an in vitro investigation. *Journal of Voice*, *22*, 408-419. doi: 10.1016/j.jvoice.2006.11.005

Purpose of the study. The purpose of this study was to examine the effects of changes in the composition of fluid on the surface of the vocal folds.

Method. Excised ovine larynges were used for the experiment. Once obtained, the larynges were immersed in Hanks Balanced Salt Solution and then bisected into hemilarynges. One hemilarynx from each pair was randomly chosen for the experiment and then the vocal fold epithelium and superficial lamina propria were dissected. The dissected tissue was subjected to one of five different experiment conditions: sham, ionic, osmotic, or combined ionic-osmotic. The potential difference, short circuit current, and tissue resistance were measured. MATLAB was used to analyze the data.

Results. The tissue subjected to sham experiments showed no change in the magnitude of potential difference, no change in short circuit current, and no altered resistance. The tissue subjected to ionic experiments showed a reduced magnitude of potential difference, a reduced short circuit current, and an altered resistance. The tissue subjected to osmotic experiments showed no change in the magnitude of potential difference, no change in short circuit current, and no altered resistance. The tissue subjected to combined ionic-osmotic experiments showed a decrease in the magnitude of potential difference, a reduced short circuit current, and an altered resistance.

Conclusions. The results of this study show that the vocal fold epithelium plays an important role in keeping the surface of the vocal folds hydrated. This hydration is necessary in the phonation of human larynges and excised larynges.

Relevance to the current work. The results of this study support the current work's method of preserving excised larynges through hydration via a saline solution. Also, the current work manipulated the hydration of the larynx by blowing dry air onto the surface of the vocal folds.

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. The purpose of this study was to examine how exposure to nebulized hypertonic saline, isotonic saline, and sterile water affect PTP and how surface laryngeal dehydration affects perceived phonatory effort.

Method. Participants included 60 females, who were assigned to one of four groups, three being treatment groups and one being a control group. Before treatment, each participant inhaled medical-grade dry air for 15 minutes to desiccate the larynx. The three treatment groups were administered either hypertonic saline, isotonic saline, or sterile water orally via a small-particle nebulizer for 8 to 10 minutes. The control group was not administered any type of nebulization, rather each participant was instructed to breathe normally in a comfortable sitting position for 10 minutes following desiccation. Both PTP and perceived phonatory effort were collected immediately prior to and following laryngeal desiccation. PTP was also collected 5, 20, 35, and 50 minutes after nebulization.

Results. There was a significant increase in PTP after desiccation trials but there was no significant increase or decrease following nebulization treatments. There was also no significant difference in PTP across the four groups. Mean PTP values for the control group, however, were consistently lower. Also, the isotonic saline treatment group showed a temporary trend of PTP reduction. There was a significant decrease in perceived phonatory effort ratings immediately following desiccation trials. There was not a significant correlation between perceived phonatory effort ratings and PTP.

Conclusions. The observed results show that nebulized treatments may be an ineffective treatment of desiccation. Although previous studies have found a correlation between PTP and effort, the results of this study demonstrate that a correlation between the two measures is unlikely.

Relevance to the current work. The current work used surface laryngeal desiccation; therefore, knowing the effects of this type of desiccation is relevant to the current work. Although the current work also used a saline solution as a method of hydration, the larynges were excised and submerged in a saline solution, which had a very different effect on the vocal folds than a nebulized administration.

Verdolini-Marston, K., Sandage, M., & Titze, I. R. (1994). Effect of hydration treatments on laryngeal nodules and polyps and related voice measures. *Journal of Voice, 8*, 30-47.

Purpose of the study. The purpose of this study was to assess the effectiveness of hydration treatments in the clinical management of selected voice disorders.

Method. Six females between the ages of 18 and 33 years who all had laryngeal nodules or

polyps participated as volunteers. Each subject received a hydration treatment as a placebo/control treatment to account for all changes in voice and laryngeal measures that were caused by factors that were not of direct interest to the study. Hydration treatment was administered by placing subjects in a high-humidity environment for two hours for five consecutive days.

Results. Results of the treatment were measured by a patient-based questionnaire, three binomial tests assessing the benefits of hydration, and variance analyses of the laryngeal and voice measures. The subjects tended to rate the placebo treatment effects as somewhat improving their voice and the hydration treatment effects as somewhat and quite a bit improving their voice. The results for the binomial tests and the variance analyses showed significant improvements in voice for both the placebo and hydration treatments and significant superior improvements in voice for the hydration treatments.

Conclusions. The results from this study confirmed that general benefits were obtained from hydration treatment.

Relevance to the current work. The current work studied the effects of multiple solutions to hydrate the vocal folds, which is supported by the conclusion found in this study that hydration of the vocal folds can improve phonatory effort.

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 Otolaryngology, Rhinology, and Laryngology*, 118, 154-159.

Purpose of the study. The purpose of this study was to determine the effects of dehydration on phonation threshold flow.

Method. Canine larynges were excised, submerged in a saline solution, and frozen. Before the experiment, each larynx was thawed and the epiglottis, corniculate cartilages, cuneiform cartilages, and ventricular folds were removed. The larynges were mounted on a base with the trachea connected to a tube connected to a pseudolung and the arytenoid cartilages stabilized by lateral micrometers. For the control trials, hydrated air was passed through the tube connected to the trachea to stimulate phonation and the saline solution was applied to the vocal folds between phonation trials. For the dehydration trials, non-hydrated air was passed through the tube connected to the trachea to stimulate phonation and no saline solution was applied. Pressure, flow, and acoustic data were all recorded.

Results. Increased exposure to dehydration caused an increase in phonation threshold flow. There is also an extent of exposure to dehydration that makes the vocal folds unable to initiate phonation.

Conclusions. Because dehydration can be a symptom of vocal fold pathologies, phonation threshold flow can be used as a diagnostic measure in assessing the severity of disordered vocal folds.

Relevance to the current work. The current work also used excised larynges as a method to study the physiological properties of the vocal folds. The methods used to preserve the larynx and to stimulate phonation in this study are similar to the methods used in the current work, including submersing the larynges in a saline solution and freezing them. The methods used to stimulate phonation are also similar, including mounting the larynges on a base by connecting the trachea to a tube and stabilizing the arytenoid cartilages with lateral micrometers. Knowing

the effects of dehydration on the vocal folds is important to the current work which dehydrated the vocal folds in five minute intervals after each trial.

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 and Neck Surgery*, *144*, 108-113. doi: 10.1177/0194599810390893

Purpose of the study. The purpose of this study was to determine the effects of dehydration applied to the surface of the vocal folds on the amplitude and frequency of the mucosal wave.

Method. Canine larynges were excised, submerged in a saline solution, and frozen. Before the experiment, each larynx was thawed and the epiglottis, corniculate cartilages, cuneiform cartilages, and ventricular folds were removed. The larynges were mounted on a base with the trachea connected to a tube connected to a pseudolung and the arytenoid cartilages stabilized by lateral micrometers. For the control trials, hydrated air was passed through the tube connected to the trachea to stimulate phonation and the saline solution was applied to the vocal folds between phonation trials. For the dehydration trials, nonhydrated air was passed through the tube connected to the trachea to stimulate phonation and no saline solution was applied. Air was passed through the vocal folds at a constant pressure until phonation ceased. A high speed camera was mounted superiorly to the vocal folds to record vocal fold vibrations. The recordings were analyzed with MATLAB.

Results. Analysis of the data showed a correlation between dehydration and the amplitude and frequency of the mucosal wave. Both amplitude and frequency were significantly lower with increased exposure to dehydration.

Conclusions. Dehydration of the vocal folds negatively affects the amplitude and frequency of the mucosal wave, which can negatively affect voice quality. Therefore, this relationship may be used to assess the severity of dehydration in disordered vocal folds.

Relevance to the current work. The current work also used excised larynges as a method to study the physiological properties of the vocal folds. The methods used to preserve the larynx and to stimulate phonation in this study are similar to the methods used in the current work, including submersing the larynges in a saline solution and freezing them. The methods used to stimulate phonation were also similar, including mounting the larynges on a base by connecting the trachea to a tube and stabilizing the arytenoid cartilages with lateral micrometers. Knowing the effects of dehydration on the vocal folds is important to the current work, which dehydrated the surface of the vocal folds in five minute intervals after each trial. The current work also used MATLAB to analyze data.

Zhang, Y., Jiang, J. J., Tao, C., Bieging, E., & MacCallum, J. K. (2007). Quantifying the complexity of excised larynx vibrations from high-speed imaging using spatiotemporal and nonlinear dynamic analyses. *Chaos*, *17*, 043114. doi: 10.1063/1.2784384

Purpose of the study. The purpose of this study was to examine the variations of the phonation instability pressure and the phonation pressure range of a normal larynx.

Method. Canine larynges were excised postmortem and submerged in a saline solution for less than 48 hours before being used in the experiment. The larynges were mounted on a tube that was connected to a pseudolung and then stabilized onto the base by two laterally positioned instruments with three prongs. To stimulate phonation, humidified air was passed through the

pseudolung at increments of 1 cm H₂O every seven seconds. Phonation was stimulated at resting vocal fold length (0%) and then elongated to 5%, 10% and 15% of vocal fold length. Subglottal pressure was measured by a manometer, glottal airflow was measured by a mass flow meter, and the acoustic signal was measured by a microphone connected to a computer. Phonation instability pressure and pressure range were measured using bifurcation analysis.

Results. In comparing the results of resting vocal fold length and 15% elongated resting vocal fold length, results showed that phonation instability pressure showed no significant change and pressure range showed a significant decrease.

Conclusions. It was concluded that elongation of the larynx resulted in more stiffness in the vocal folds. Greater stiffness required a higher phonation threshold pressure, which was observed to cause phonation instability. Various vocal fold pathologies, such as scarring, can cause stiffness in the vocal folds, which may change the phonation instability pressure of the larynx. Therefore, it was suggested that range could be indicative of pathologies and a measure for determining the extent of damage to the vocal folds. It was also concluded that bifurcation analysis can be used as an effective measure to determine instability pressure and range.

Relevance to the current work. The current work also used excised larynges to study certain physiological properties of the vocal folds. The methods used to preserve the larynges and stimulate phonation were also extremely similar. The current work also submerged the excised larynges in saline and later mounted them to a tube connected to a pseudolung that blew subglottal hydrated air into the larynx. The current work, however, used porcine larynges and used them in the experiment 24-hours postmortem rather than 48 hours.

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. The purpose of this study was to quantify the regular and irregular phonation of excised larynges.

Method. Excised canine larynges were mounted onto a base with a tube connected to the trachea. The larynx was stabilized with a three-pronged instrument inserted into both sides of the larynx and hydrated air was blown through the trachea to stimulate phonation. Varying subglottal pressure was used to stimulate regular and irregular phonation. A high-speed camera, mounted superiorly to the larynx, acquired video of the vocal folds during vibration.

Spatiotemporal analysis and nonlinear dynamic analysis were used to quantify regular and irregular phonation.

Results. Measures used to quantify phonatory patterns showed consistent differences between the regular and irregular phonation.

Conclusions. Spatiotemporal analysis and nonlinear dynamic analysis are measures that can be used to better understand the physiological properties of normal and disordered larynges and can also help in the diagnosis and treatment of the larynx.

Relevance to the current work. Similar methods were used to perform phonation trials in the current work; however, this study did not outline the methods of preservation used for the excised larynges. The current work also used a high speed camera to conduct spatiotemporal analysis of the vocal folds during phonation, yet many of the other variables measured differed.

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, submerge 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 cmH₂O
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)