



All Theses and Dissertations

2016-12-01

Preventing Vocal Fold Dehydration Using Aerosolized Saline in an Excised Porcine Model

Mallory Lynn Hansen
Brigham Young University

Follow this and additional works at: <https://scholarsarchive.byu.edu/etd>

 Part of the [Communication Sciences and Disorders Commons](#)

BYU ScholarsArchive Citation

Hansen, Mallory Lynn, "Preventing Vocal Fold Dehydration Using Aerosolized Saline in an Excised Porcine Model" (2016). *All Theses and Dissertations*. 6120.

<https://scholarsarchive.byu.edu/etd/6120>

This Thesis is brought to you for free and open access by BYU ScholarsArchive. It has been accepted for inclusion in All Theses and Dissertations by an authorized administrator of BYU ScholarsArchive. For more information, please contact scholarsarchive@byu.edu.

Preventing Vocal Fold Dehydration Using Aerosolized Saline
in an Excised Porcine Model

Mallory Lynn Hansen

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

Kristine Tanner, Chair
David L. McPherson
Christopher Dromey

Department of Communication Disorders
Brigham Young University

Copyright © 2016 Mallory Lynn Hansen

All Rights Reserved

ABSTRACT

Preventing Vocal Fold Dehydration Using Aerosolized Saline in an Excised Porcine Model

Mallory Lynn Hansen
Department of Communication Disorders, BYU
Master of Science

Vocal fold hydration is important for efficient oscillation during voice production. Dehydration of the vocal fold surface is believed to produce adverse effects on the voice. Specifically, low environmental humidity, mouth breathing, and certain medical conditions may contribute to laryngeal and vocal fold dehydration. This dehydration effect may be quantified using the observed pressure and flow at the onset of phonation, operationally defined as phonation threshold pressure (PTP) and phonation threshold flow (PTF), respectively. Previous research has documented that nebulized isotonic saline (0.9% Na⁺Cl⁻) can reduce PTP. Additionally, the topical application of liquid saline increases vocal fold hydration in excised larynx studies. However, no studies have examined the prevention of vocal fold dehydration using aerosolized saline in an excised larynx mechanical model. The purpose of the current investigation was to determine the preventive effects of aerosolized isotonic saline in a physiologically realistic excised larynx model. Using a prospective, mixed experimental design with a control group, five bench-mounted, excised porcine larynges received 4-min doses of aerosolized saline delivered supraglottally for a total of 24 min. Subsequently, larynges received 1-min doses of desiccated air (<1% relative humidity) delivered supraglottally. A control group of five porcine larynges received only desiccated air. Phonation was attempted following each dose of aerosolized saline or desiccated air. The desiccation doses were repeated for both groups until the larynges were no longer able to phonate. The PTP and PTF were measured at baseline and following each dose of aerosolized saline or desiccated air. Analysis of the results indicated that aerosolized saline significantly delayed the adverse effects of vocal fold dehydration based on the total number of desiccation doses required to cease phonation for experimental versus control groups ($p = .002$). Trends demonstrated that PTP decreased after aerosolized saline and increased during desiccation trials. The PTF trends were similar during desiccation. The results from this study indicate that aerosolized saline may be used prophylactically to prevent vocal fold dehydration. These findings offer important advances in vocal fold hydration theory and dehydration prevention in a physiologically realistic excised mechanical model.

Keywords: aerosolized saline, larynx, vocal fold hydration, bench model, phonation threshold pressure, phonation threshold flow

ACKNOWLEDGMENTS

I would like to thank Dr. Kristine Tanner for her mentorship that made this research possible and enjoyable. I am grateful for all the sacrifices she made on my behalf and the time she spent working on this project. I would like to thank my husband for his support and making me believe that I can accomplish anything. I would also like to thank my parents for their constant support. Their support made this project possible. In addition, I would like to thank all the members on my committee for their time and helpful input on this project. Lastly, I would like to thank my Heavenly Father who helped and guided every aspect of our research.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENTS	iii
TABLE OF CONTENTS.....	iv
LIST OF TABLES	vi
LIST OF FIGURES	vii
DESCRIPTION OF THESIS STRUCTURE.....	viii
Introduction.....	1
Systemic Hydration.....	1
Surface Tissue Hydration.....	2
Quantification of Vocal Fold Hydration	4
Excised Larynx Models	6
Prophylaxis	6
Statement of the Problem.....	7
Statement of Purpose	8
Research Questions	8
Method	8
Larynges.....	9
Research Design.....	9
Procedures.....	10
Benchtop setup.....	10
Signal acquisition.....	11
Aerosolization.....	11

Desiccation.....	11
Data analysis.....	12
Statistical analysis.....	12
Results.....	12
Discussion.....	14
Prophylaxis	14
Additional Applications of Aerosolized Saline	15
PTP versus PTF.....	16
Environmental Humidity	17
Fundamental Frequency (F_0).....	17
Experimental Model.....	18
Additional Considerations	18
Conclusions.....	19
References.....	20
APPENDIX A: ANNOTATED BIBLIOGRAPHY	30
APPENDIX B: EXPERIMENTAL PROTOCOL	46
APPENDIX C: FOOD HANDLER’S PERMIT.....	50

LIST OF TABLES

Table 1	<i>Aerodynamic and Acoustic Raw Data at Baseline</i>	25
Table 2	<i>Environmental Humidity During each Experimental Session</i>	26

LIST OF FIGURES

<i>Figure 1</i>	Normalized phonation threshold pressure (PTP) at baseline (0 on the y axis) and following each 4-min aerosolized saline dose.	27
<i>Figure 2</i>	Normalized phonation threshold flow (PTF) at baseline (0 on the y axis) and following each 4-min aerosolized saline dose.	27
<i>Figure 3</i>	Normalized phonation threshold pressure (PTP) at baseline and following the first 10 1-min desiccation doses for prophylactically treated larynges (BA) compared with control larynges, including larynges subsequently treated with aerosolized saline (AB; Stevens, 2016).	28
<i>Figure 4</i>	Normalized phonation threshold flow (PTF) at baseline and following the first 10 1-min desiccation doses for prophylactically treated larynges (BA) compared with control larynges, including larynges subsequently treated with aerosolized saline (AB; Stevens, 2016).	28
<i>Figure 5</i>	The number of desiccation trials required to cease vocal fold vibration for prophylactically treated larynges (BA) compared with control larynges (A), including larynges subsequently treated with aerosolized saline (AB; Stevens, 2016).	29

DESCRIPTION OF THESIS STRUCTURE

This thesis, *Preventing Vocal Fold Dehydration Using Aerosolized Saline in an Excised Porcine Model*, is written in a hybrid format. The hybrid format brings together traditional thesis requirements with journal publication formats. The research presented herein is part of a larger project and has met the guidelines for the university's requirements of a thesis. Furthermore, a portion of this project was presented at the Voice Foundation Annual Symposium and will be submitted for publication in the *Journal of Voice*. Appendix A includes an annotated bibliography related to this work. Appendix B contains operational procedures. Appendix C includes the thesis author's food handler's certification in compliance with Risk Management requirements at Brigham Young University.

Introduction

The vocal folds require adequate internal and surface tissue hydration for efficient voice production (Hanson, Zhang, & Jiang, 2010; Hemler, Wieneke, & Dejonkere, 1997; Sivasankar & Fisher, 2002; Verdolini-Marston, Titze, & Druker, 1990). Changes in airway hydration homeostasis may adversely impact vocal fold health. It is essential to determine how hydration influences voice production in order to prevent vocal fold injury. Two primary contributors to vocal fold hydration—systemic and surface tissue hydration—will be discussed.

Systemic Hydration

Systemic hydration may be defined specifically as the body's state of hydration at a given time (Hartley & Thibeault, 2014). Systemic dehydration of the body, due to inadequate liquid intake or extreme fluid loss, could theoretically cause vocal fold dehydration and corresponding negative changes in voice function (Fisher, Ligon, Sobecks, & Roxe, 2011). Individuals who are believed to be particularly at risk for vocal fold dehydration, such as professional voice users, people with certain diseases such as Sjögren's Syndrome, and those on drying medications are often advised to stay well hydrated by drinking plenty of water (DiRenzo, Tanner, & Thibeault, 2016; Tanner et al., 2013). However, an extensive review of the literature regarding systemic hydration physiology indicated that changes in fluid intake are unlikely to have any influence on voice production (Hartley & Thibeault, 2014). Systemic hydration is so critical for overall health of the body's cells, tissues and systems that it is regulated with physiologic precision; only extreme decreases or increases in fluid intake or loss causes changes in the body's internal hydration state. These changes may cause or result from illness but are unlikely to be linked directly to voice function unless they are extreme and over an extended period of time (DiRenzo et al., 2016). However, the potential for internal dehydration to influence voice production

indirectly (e.g., via glandular secretions, fluid movement from surface cells to internal organs, medication influence on airway surface fluid) is poorly understood.

Surface Tissue Hydration

Surface vocal fold hydration involves the influence of extracellular fluid and airway surface liquid on vocal fold epithelial health. Airway surface liquid consists of a mucous blanket and deeper water layer (Sivasankar & Fisher, 2007). The vocal folds are covered with this fluid that is believed to serve protective and hydration functions. A decrease in the depth of airway surface fluid is believed to alter voice function and associated perceived vocal effort (Roy, Tanner, Gray, Blomgren, & Fisher, 2003; Tanner, Roy, Merrill, & Elstad, 2007). More specifically, the water layer of airway surface liquid is thought to hydrate wet epithelial cells. The presence of water and mucus on the vocal fold surface is also believed to provide lubrication that is important for efficient oscillation (Sivasankar & Fisher, 2002). Studies have identified a direct relationship between the topical exposure to dry or humidified conditions and voice function (Witt et al., 2009). This responsiveness of the vocal folds to surface hydration conditions may also prevent the formation of mass-adding lesions associated with voice disorders (Tao, Jiang, & Czerwonka, 2010).

One key factor associated with vocal fold hydration is the viscosity of fluid both within and on the surface of the vocal folds. Theoretically, because systemic hydration stays relatively constant, changes in the composition of airway surface fluid might be the primary variable that determines vocal fold viscosity. When the viscosity of fluid in the vocal folds increases, vibratory efficiency decreases (Verdolini et al., 1990). Similarly, as the depth of airway surface fluid decreases, its viscosity and tackiness increase, leading to increased friction during oscillation (Hemler et al., 1997). Nakagawa, Fukuda, Kawaida, Shiotani, and Kanzaki (1998)

examined the effects of vocal fold surface fluid viscosity on the amplitude of vibration during oscillation. Two viscosities of physiologic saline—traditional isotonic saline (0.9% Na⁺Cl⁻) and dissolved chondroitin sulfate sodium salt—were applied to the vocal folds in an excised canine model. The results of the study indicated that dissolving chondroitin sulfate sodium decreased the amplitude of oscillation more than isotonic saline due to its higher level of viscosity. Witt, Taylor, Regner, and Jiang (2011) later found that prolonged exposure to dry subglottic airflow increases surface mucus viscosity and increases phonation threshold pressure (PTP), defined by Titze in 1994 as the minimum amount of pressure required to initiate and sustain vocal fold oscillation. Additionally, this study documented decreases in fundamental frequency (F₀) as surface mucus viscosity increased (Witt et al., 2011). Therefore, evidence exists regarding the adverse effects of thick mucus on the vocal fold surface during voice production.

Another factor that relates to surface hydration and voice function is mucociliary clearance. Airway mucociliary clearance contributes to respiratory defense and health, and may contribute to hydration of the vocal tract (Antunes & Cohen, 2007; Wanner, Salathe, & O'Riordan, 1996). The cilia in the respiratory tract facilitate vertical movement of the mucus toward the oral cavity. This movement of fluid through the vocal tract may have a direct effect on the overall lubrication of the vocal folds (Kawaida, Fukuda, Kano, Shiotani, & Kohno, 1990). Several factors affect the function of mucociliary clearance including ciliary function, the health of the epithelial cells, and the depth and viscosity of the water portion of airway surface (Tanner et al., 2007). Further, the presence of low viscosity airway surface fluid appears to relate to healthy mucosal wave during vocal fold vibration (Kawaida et al., 1990).

Quantification of Vocal Fold Hydration

The most common measure used to quantify the effects of variables manipulated ostensibly to influence vocal fold hydration is PTP (Hottinger, Tao, & Jiang, 2007). The PTP is sensitive to changes in voice function in human and excised larynx benchtop animal models. Changes in PTP are associated with the external manipulation of variables used to study their independent effects on voice function in excised larynges. For example, Zhang, Reynders, Jiang, and Tateya (2007) found a significant relationship between vocal fold elongation and PTP. This study examined different levels of elongation such as 0%, 5%, 10%, and 15% in excise canine larynges. As the level of elongation increased, PTP increased. The PTP may also be used to infer changes in the amount of effort required to produce phonation (Titze, 1994). Hoffman et al., (2012) also documented decreases in PTP with increased vocal fold abduction in an excised canine larynx model. Numerous studies have documented increased PTP associated with decreased hydration; similarly, increased vibratory efficiency is generally associated with decreased PTP (Jiang, Ng, & Hanson, 1999; Roy et al., 2003; Tanner et al., 2007).

A second, more recent measure used in excised larynx studies is phonation threshold flow (PTF), or the observed airflow level at the threshold of phonation (Jiang & Tao, 2007). The PTF has been observed to increase when excised larynges are exposed to dry air subglottally (Witt et al., 2009). Additionally, PTF might offer an advantage over PTP because the airflow at the level of the mouth is nearly equivalent to that at the level of the vocal folds, whereas PTP may only be estimated without subglottic measurement (Jiang & Tao, 2007). Jiang and Tao applied a body-cover mass-spring computational model to estimate the vocal fold viscoelastic properties associated with PTF. The results indicated that PTF should decrease with corresponding decreases in tissue viscosity, mucosal wave velocity, and glottal area (Jiang & Tao, 2007).

Hottinger et al. (2007) found that PTF was more sensitive than PTP in response to changes in posterior glottal width. Mau, Muhlestein, Callahan, Weinheimer, and Chan (2011) conducted the first study of PTP, PTF, and the presence of hysteresis (i.e., a measure of vibratory inefficiency due to a lag in response to a given input) in excised human larynges. This study found offset PTP and PTF values to be lower than onset values in human larynges, which is similar to the results of previous canine studies observing hysteresis in other excised larynx animal work (Mau et al., 2011; Regner, Tao, Zhuang, & Jiang, 2008). It was presumed that bowing effects of the human larynges caused by old age contributed to the lack of a relationship between PTP and PTF for posterior glottal width (Mau et al., 2011). Although PTF is a relatively new measure that shows promise in studies attempting to quantify vibratory efficiency, however, its application to hydration studies is relatively undetermined.

Due to the variations in mucosal wave amplitude, high speed images is another method that has proven effective in detecting and quantifying vocal fold vibration (Murugappan, Boyce, Khosla, Kelchner, & Gutmark, 2010). One study indicated that high speed images proved to be more effective in capturing irregular vocal fold vibration as compared to acoustic measures that rely on peak-picking algorithms (Zhang, Jiang, Tao, Bieging, & MacCallum, 2007). Li, Zhang, Maytag, and Jiang (2015) used high speed imaging to quantify vibratory changes associated with vocal fold dehydration. The authors analyzed the glottal area and mucosal wave using digital videokymography. As vocal fold dehydration was increased from 0% to 50% and then 75%, decreases in mucosal wave and amplitude of vibration, as well as increases in glottal gap, were observed (Li et al., 2015). This study offers methodological support for the use of high speed images to accurately detect vocal fold vibratory patterns.

Excised Larynx Models

Research has identified several animals whose vocal folds have features similar to humans. Studies have documented that porcine vocal folds are a viable model for human comparison due to the presence of similar vocal fold layers, including the presence of a vocal ligament (Alipour & Jaiswal, 2008; Johanes, Mihelc, Sivasankar, & Ivanisevic, 2011; Sivasankar & Fisher, 2007; 2008). Alipour and Jaiswal compared excised porcine, ovine, and cow larynges and their vocal fold vibration characteristics to determine which had similarities to human oscillation. The study concluded that porcine had the most similarities to human phonation. Similar to the human female F_0 , excised porcine larynges had an average oscillation frequency of 220 ± 57 Hz and the porcine PTP was 7.4 ± 2 cm H₂O. Secondly, porcine larynx lamina propria collagen organization is similar to that of humans (Johanes et al., 2011). Thus, porcine models are comparable to humans with respect to vocal fold layers, larynx size, the presence of similar intrinsic laryngeal structures (i.e., cricothyroid, vocal ligament), and vibratory characteristics (Alipour & Jaiswal, 2008; Jiang & Titze, 1993).

Prophylaxis

The capacity of the vocal folds to recover from dehydration has been examined in excised larynx and *in vivo* human models. In an early study of hydration employing an excised animal model, 10 canine larynges received subglottal dry air until the vocal folds ceased to vibrate, followed by immersion in isotonic saline for 30 min to examine the rehydration capacity (Jiang et al., 1999). The authors observed that the vocal folds were capable of rehydration based on pressure and airflow measurements; additionally, values were lower following rehydration compared to those obtained at baseline. Another study examined the biphasic composition theory, which is the interaction between the liquid and solid composition of the laryngeal

structure (Hanson et al., 2010). This study provided an explanation of the interaction between solid and liquid and the stress relaxation behavior of the vocal folds. Hanson, Zhang, and Jiang (2011) conducted an additional study about the biphasic theory and found supporting evidence that the level of dehydration affects the ability of the vocal fold tissue to recover. Additionally, Meyer, McAvoy, and Jiang (2013) observed the capacity of rehydrating on several types of tissue, including muscle, fat, lung, tendon, skin, and cartilage. The study determined that the original severity of vocal fold dehydration affected the final mass when rehydration was completed, as well as the total time needed to reach rehydration.

Human studies have also demonstrated the effects of surface hydration treatments on voice production. Roy and colleagues (2003) found that nebulized mannitol decreased PTP as compared to water and Entertainer's Secret Throat Relief in healthy females with normal voices. Tanner et al. (2007) documented that nebulized isotonic saline reversed the adverse effects of a vocal fold desiccation challenge in a similar cohort of participants. Most recently, the potential for nebulized saline—delivered via an ultrasonic nebulizer—to treat and prevent voice problems was demonstrated in people with Sjögren's Syndrome (Tanner et al., 2015).

Statement of the Problem

Although a growing body of literature exists documenting the effects of surface tissue dehydration on the voice, no studies have quantified the independent effects of a prophylactic hydration treatment on voice function. Due to the physiologic complexities that exist in living animals and humans, it is difficult to quantify surface tissue hydration changes using *in vivo* models. For this reason, excised larynx models offer the opportunity to manipulate isolated variables to determine their influence on voice production. However, existing excised larynx literature has included some methodological procedures, such as the subglottic administration of

dehumidified air and the topical application of a saline drip, which might limit translational application.

Statement of Purpose

This study sought to determine if the prophylactic administration of aerosolized saline reduced the adverse effects of vocal fold dehydration in a more physiologically realistic excised porcine larynx model.

Research Questions

This study addressed the following research questions:

1. How does aerosolized isotonic saline affect PTP and PTF in excised porcine larynges over time?
2. Does prophylactic aerosolized saline change PTP and PTF in excised porcine larynges compared with control larynges?

Method

All procedures were completed in rooms 105 and 106 John Taylor Building at Brigham Young University. Appendix B contains operational procedures used in this work. The study was completed in compliance with regulations from Brigham Young University Risk Management and the Institutional Animal Care and Use Committee; a copy of the author's Utah food handler's permit for purposes of working with food grade porcine larynges is included in Appendix C.

Larynges

Excised porcine larynges were collected from a local butcher house, Circle V Meats, Springville, Utah, within 24 hours of beginning experimentation. All larynges were harvested from healthy, food-grade adult pigs that were at least two years of age. The larynges were kept refrigerated in their own secretions in zipped plastic bags until dissection. Larynges were inspected for injury or damage and were discarded if any anomaly was identified. Immediately prior to each experiment, larynges were dissected to expose the true vocal folds. First, the epiglottis and supraglottic tissues were removed. The thyroid cartilage was transected at the level of the false vocal folds and trimmed in a “v” shape to support the angle of the vocal folds during adduction on the bench apparatus. Next, each false fold was separated from the inner surface of the thyroid cartilage by way of one smooth cut which spared the true vocal folds. The arytenoid cartilage was left intact to aid in subsequent benchtop adduction. The trachea was trimmed to approximately 6 cm in length. Isotonic saline (0.9% Na⁺Cl⁻) was applied liberally to larynges during the dissection process and immediately prior to initiation of the experiment.

Research Design

A prospective, mixed between and within groups design with control group was employed in this study. Larynges were randomly assigned either to the experimental or the control group. The experimental group received six 4-min increments of aerosolized saline for a total of 24 min. Voice function measurement was performed at baseline and following each 4-min dose of aerosolized saline, for a total of seven observations. Subsequently, larynges received 1-min doses of desiccated air (<1% relative humidity); voice function measurement was performed following each 1-min dose of desiccated air until the vocal folds no longer phonated. For the control group, 1-min doses of desiccated air were applied to the larynges until the vocal

folds no longer phonated; voice function measurement was performed at baseline and following each 1-min dose of desiccated air. Comparisons between and within groups were completed. Independent variables included group and time; dependent variables included PTP, PTF, and the number of desiccation doses required to cease vocal fold vibration.

Procedures

Benchttop setup. Jiang and Titze (1993) described a benchtop mechanical model for excised larynx experimentation. The current study employed a similar experimental setup, including an excised larynx mounted on semiflexible plastic tubing passed through a circular hole in a standard stainless steel breadboard tabletop (Thorlabs, Ann Arbor, MI). The larynx was secured by three micropositioners (Model 1460, Kopf Industries, Tujunga, CA). Two of these micropositioners had three custom prongs to perform arytenoid adduction bilaterally; the third micropositioner had a loop for purposes of securing a suture string attached to the thyroid cartilage for retraction. Together, these positioners were used to adduct and lengthen the vocal folds until baseline phonation was obtained. The micropositioners were secured to the tabletop using ¼-20 headless screws via custom bases.

A compressed air tank (<1% relative humidity) and adjustable flow regulator with standard 50 psi was attached to an in-line thermal flow meter (Model GFM47A-VDL6-A0, Alborg Instruments, Orangeburg, NY), which connected to a Thera-heat temperature controlled humidifier (Model RC70000, Smiths Medical, Dublin, OH). Clear plastic tubing connected the humidifier to a 20-cm aluminum pseudolung with interior foam. A custom 1-cm long fitted tube with a custom subtracheal outlet permitted the attachment of a pressure transducer (Model PT-25-S, Glottal Enterprises, Syracuse, NY) attached directly to the trachea. The trachea of each

larynx was secured to the clear plastic tubing with a metal hose clamp and teflon tape to prevent air leaks.

Signal acquisition. The acoustic signal, pressure, and airflow were acquired simultaneously using a DATAQ Instruments A/D converter WINDAQ software (Series Di-720, Akron, OH) set to 10 kHz per channel. The acoustic signal was acquired with a dynamic microphone (Model SM-48, Shure, Niles, IL) positioned at a 45-degree angle 6 inches from the excised larynx. An audio mixer (Samsung MIXPAD 4, New York, NY) was used as a preamplifier for the signal. Prior to each data collection session, the pressure transducer was calibrated to 0 and 10 psi using a pressure calibrator (PC-1H, Glottal Enterprises, Syracuse, NY).

The flow meter was calibrated to 0 and 15 L/min. Vocal fold vibration was verified by two examiners using high speed imaging (Pentax Medical, Montvale, NJ). Files were annotated and saved for subsequent PTP and PTF estimation, using the acoustic signal to verify phonation onset. Environmental humidity was monitored for each data collection session using a HygroSet II Digital Hygrometer (model DHYG-Round; HygroSet, Weston, FL), calibrated using the Humidipak calibration kit.

Aerosolization. Each 4-min aerosolized isotonic saline (0.9% Na+Cl-) dose was delivered supraglottally using an Omron ultrasonic nebulizer (Model NE-U22V, Omron Healthcare Inc., Lake Forest, IL) and custom tubing. A 5 mm shim (Allen wrench) was placed between the arytenoid cartilages and between the vocal folds at the posterior two-third point. The shim was used to permit delivery of saline to the medial edges of the vocal folds while keeping adduction and length constant for subsequent phonation trials.

Desiccation. Each 1-min desiccation dose was delivered via custom tubing attached to a different compressed air tank (<1% relative humidity). Similar to aerosolization treatment

delivery, a 5 mm shim (Allen wrench) was placed between the vocal folds during administration. Phonation trials were acquired after each 1-min dose until the vocal folds no longer vibrated.

Data analysis. Time-aligned acoustic, pressure, and airflow signals were segmented by phonation trial in WINDAQ and imported into MATLAB (MathWorks, Natick, MA) for analysis for custom script analysis. The onset of phonation was identified from the acoustic recording. Both PTP and PTF were calculated as the average of the measured subglottal pressure and flow during the 10 ms before and after the phonation onset. The PTP and PTF values were exported from MATLAB to Excel.

Statistical analysis. Summary statistics for central tendency and variability were calculated for the experimental and control groups at baseline. For purposes of within-group analysis, PTP and PTF were normalized to baseline values. That is, each postbaseline value was subtracted from the baseline value for purposes of time series comparisons. Change in dependent variables was examined using linear and polynomial trend analysis with corresponding formulas and R^2 values; the formula indicated how well the R^2 explained variance in each measure. A criterion of $> .80$ was used to determine the most conservative model that provided a relatively high goodness of fit. The number of 1-min desiccation doses required to cease vocal fold vibration were compared for the experimental and control groups, as well as data from Stevens (2016) using one-way analysis of variance. Statistical analysis was accomplished using SPSS version 23 (IBM Corp., Armonk, NY).

Results

Baseline PTP, PTF, and F_0 for the experimental and control groups are presented in Table 1. Specifically, the experimental group in this study represents larynges that received prophylactic saline (B) prior to the desiccation challenge (A). The control group received the

desiccation challenge (A) only. These data are reported in comparison with data from Stevens (2016), who examined the reversal of vocal fold desiccation (A) using subsequent aerosolized saline (B). In summary, the prophylactic saline (BA) and control (A) groups from the present investigation were compared to the desiccation reversal (AB) study by Stevens (2016). Table 2 reports environmental humidity stability for all data collection sessions.

Trend models for PTP and PTF during prophylactic aerosolized saline trials are presented in Figures 1 and 2, respectively. For PTP, a third order polynomial model indicated an average decrease of 5 cmH₂O following the aerosolized saline treatment administration. A second order polynomial model was used to describe trends in PTF. On average, PTF increased by approximately 5 L/min during aerosolization.

Trend models for experimental and control groups are illustrated for PTP and PTF in Figures 3 and 4, respectively. Linear trend analyses indicated that PTP and PTF both increased over time with desiccation trials; however, the slopes of these increases were lower for the experimental group compared with the control group. The PTP increased by approximately 7 cmH₂O for the prophylactically treated experimental group versus a 12 cmH₂O increase for the untreated control group. Similarly, PTF increased by approximately 15 L/min for the prophylactically treated experimental group versus 25 L/min for the untreated control group.

A one-way analysis of variance was employed to compare the number of desiccation trials required to cease vocal fold vibration for the prophylactic (BA) group, the control (A) group, and the desiccation and subsequent aerosolization data (AB) data from Stevens (2016). The results revealed statistically significant differences among the three groups $F(2, 12) = 10.562, p = .002$; these findings are illustrated in Figure 5.

Discussion

This investigation aimed to determine how aerosolized saline affected PTP and PTF in excised porcine larynges over time; further, the study sought to determine if prophylactic aerosolized saline affected PTP and PTF compared with control larynges. Larynges received 24 min of aerosolized saline followed by 1-min desiccation challenges until the vocal folds ceased to vibrate. These results were compared with data from Stevens (2016), who provided fresh, untreated control larynges with 1-min desiccation challenges until vocal fold vibration ceased. Analysis of the PTP results revealed significant differences between the prophylaxis group from the present investigation and the control larynges from Stevens. Following the administration of aerosolized saline, larynges withstood approximately twice the number of desiccation trials before becoming unable to phonate ($p=.002$). The PTF did not change with aerosolized saline; however, it did follow similar PTP trends during the desiccation trials. Collectively, the results indicated that prophylactic treatment successfully reduced the negative effects of the desiccation challenge.

Prophylaxis

This was the first study to examine possible prophylactic effects of aerosolized saline on voice function in an excised larynx mechanical model. The results demonstrate the potential benefits to individuals with vocal fold dryness, particularly in anticipation of episodic or chronic vocal fold dryness symptoms. The current findings offer important basic science physiologic evidence for aerosolized saline treatments in people with dryness-related voice disorders. In Tanner et al. (2007), individuals with chronic laryngeal dryness received significant benefit from the twice-daily administration of aerosolized saline. However, those findings could have been related to a number of physiologic processes that occur in the respiratory system when an

inhalant is introduced. For example, an inhaled irritant could produce a glandular or other physiologic secretory response, inadvertently lubricating the vocal folds and improving voice production. Therefore, it was essential to complete the current study to examine the effects of aerosolized saline and its independent impact on vocal fold vibration. This study also extends the results from Witt et al. (2009), who demonstrated the continued viability of excised larynx bench models when a saline drip was applied. However, the current study introduced a more physiologically realistic component of aerosolization that is more generalizable to humans.

The current work is also generally consistent with other studies related to vocal fold dehydration and the benefits of prophylactic treatment. Hanson et al., (2011) examined levels of dehydration and found that dehydration severity impacts the ability the tissue has to fully heal. Due to the fact that vocal folds can reach the point of not being able to fully recover, the present study found evidence that prophylactic treatments might postpone potential damage to the vocal folds. The results are consistent with Tanner et al. (2007), who demonstrated that isotonic saline attenuated the adverse effects of vocal fold desiccation. Nakagawa et al. (1998) also used saline as the low viscosity fluid and compared the effects to a high viscosity fluid, chondroitin sulfate sodium salt. The authors suggested that that, because saline had a lower viscosity, the hydration effect of saline had a greater impact on improving voice function.

Additional Applications of Aerosolized Saline

Rehydration of vocal folds that have been exposed to dry air is another important topic of interest in hydration research. The results of this study also inform hydration theory by demonstrating that aerosolized saline might have a rehydration effect. For example, one study examined the principle of rehydration and found that excised laryngeal tissues absorb saline at a declining rate until the tissue can no longer absorb moisture (Meyer et al., 2013). Therefore, a

ceiling effect might exist which limits the overall benefit of aerosolized saline in individuals with optimal vocal fold hydration. Furthermore, Jiang and Hanson (1999) postulated that—to decrease PTP and PTF effectively—the superficial layers of the vocal folds must remain hydrated.

In general, research has demonstrated that PTP decreases as the level of hydration increases. An *in vivo* study examined the effects of oral breathing on PTP, documenting that oral breathing increases PTP whereas nasal breathing reduces PTP (Sivasankar & Fisher, 2002). Additionally, as dehydration increases, glottal area decreases and the vocal folds stiffen (Li et al., 2015). Anecdotal evidence from high speed recordings used to determine voicing onset indicated that the vocal folds became stiffer and failed to fully oscillate as dehydration increased.

PTP versus PTF

The PTF values followed similar trends to PTP, but the results did not reach statistical significance. During the desiccation challenge, PTF values were lower for the prophylactic trials than the control trials, but both PTP and PTF increased steadily. This is consistent with previous literature indicating that PTF increases with desiccation (Witt et al., 2009). Furthermore, in a study examining PTF in a single-mass model, Jiang and Tao (2007) observed that PTF may be decreased by decreasing tissue viscosity. These results can be applied to the current work by assuming that saline decreased the viscosity of the vocal folds, and therefore reduced PTF. Additionally, Mau and colleagues (2011) conducted the first study examining the effects of PTF on excised human larynges and found that PTF was significantly higher than *in vivo* models. The present research also found PTF to have much higher values, which suggested that PTP might be a more reliable measure than PTF. Hoffman and colleagues stated that measuring PTF is advantageous because it is less invasive than PTP and might be measured by simply using an

external airflow transducer and mask (Hoffman et al., 2012). Our research contradicts Hoffman et al., because the sensitivity of PTP to hydration overrules the possible increased comfort during PTF measurement.

Environmental Humidity

Hemler et al., (1997) documented the relationship between environmental relative humidity and the subject's sense of vocal effort and acoustic measures including jitter, shimmer, and harmonic-to-noise ratio. The subjects reported that their voices felt impaired due to the dry air and their voices felt comfortable in the humid air. Because of this evidence, each day of experimentation the level of humidity in the room was measured to ensure it did not affect the PTP and PTF results of the study. High humidity levels in the room could aid the level of hydration of the vocal folds and low humidity levels in the room could decrease the level of hydration of the vocal folds. Importantly, the humidity levels remained stable for the duration of the current study and therefore should not have affected the experimental data.

Fundamental Frequency (F_0)

Alipour and Jaiswal (2008) completed a study on excised porcine larynges and found the average frequency oscillation was 220 ± 57 Hz, which is similar to the approximate oscillation frequency found in human females. The current study had much higher frequency oscillation, reaching an average of 450 Hz. The PTP values were also greater in the current study. This might be due to the fact that the experimental configuration differed from that in Alipour and Jaiswal; they adducted the vocal folds by suturing the muscular processes together and did not elongate the vocal folds, whereas the present methodology included adducting the vocal folds using micropositioners, including vocal fold elongation similar to Witt et al (2009). Zhang et al. (2007) reported that elongating the vocal folds from 0% to 15% significantly increased PTP.

Collectively, the findings from these studies are consistent with the increased PTP values in the present study. However, because the vocal fold configuration did not change during the course of the experiment, and results were normalized to baseline values, the present results have strong internal validity.

Experimental Model

This study expanded on existing excised larynx mechanical models by introducing more physiologically realistic components including the supraglottal administration of dry air and saline, as well as warmed and humidified air during all phonation trials. These procedures are substantially more similar to human respiratory physiology. Kumazawa, Asako, Yamashita, and Ha-Kawa (1997) compared three different methods of nebulization to determine which process is the most effective at depositing aerosol particles onto the larynx and upper airway. The results indicated that quick intake of air combined with intermittent vocalizations increased the laryngeal disposition rate. The current experiment followed these guidelines in the bench model. A shim maintained a midmembranous vocal fold gap to simulate fast inhalation and the porcine larynges were vibrated in between each aerosolized treatment to simulate vocalization using heated, humidified air. These operational procedures maximized the similarity to human respiratory mechanics and thus the study's translational value.

Additional Considerations

Several factors should be considered when interpreting the results of this study. First, the small sample size—though similar to other work in this area—limits the generalizability of the results. The significant results in this study are encouraging with regard to the possibility for prophylactic treatment development to address vocal fold dehydration. Additionally, although porcine larynges are similar to those of humans, there are differences particularly with respect to

the 45-degree angle of the true vocal folds in pigs. Occasional peak-clipping also occurred for a few airflow samples that exceeded 50 L/min, however, any values that exceeded this ceiling likely indicated vocal fold stiffness and dehydration to the point of ceasing vibration. Also inherent to any excised larynx study is the fact that one is working with dead tissue where systemic physiologic mechanisms such as circulation and innervation are absent. That said, tissue-based mechanical models are an important first step when examining specific variables in the absence of the physiologic complexities of *in vivo* studies. Therefore, it is a complimentary approach to *in vitro*, *in vivo*, simulation, and computational models to address critical questions surrounding vocal fold hydration.

Conclusions

This study demonstrated significant effects of prophylactic aerosolized saline in delaying the adverse effects of laryngeal desiccation in an excised porcine larynx model. Ultrasonic nebulizers using isotonic saline are most effective in optimizing voice function prior to a dehydration or dry environment. Future work should continue to examine the effects of aerosolized particle exposure to the vocal folds using complimentary *in vitro* and *in vivo* models. The dose-response relationship between aerosolized saline and various clinical populations remains largely unknown. This work is essential to improve voice function in patients who experience or are at risk for vocal fold dehydration.

References

- Alipour, F., & Jaiswal, S. (2008). Phonatory characteristics of excised pig, sheep, and cow larynges. *Journal of Acoustical Society of America*, *123*, 4572-4581.
doi:10.1121/1.2908289
- Antunes, M. B., & Cohen, N. A. (2007). Mucociliary clearance—A critical upper airway host defense mechanism and methods of assessment. *Current Opinion in Allergy and Clinical Immunology*, *7*, 5-10. doi:10.1097/ACI.0b013e3280114eef
- DiRenzo, E. E., Tanner, K., & Thibeault, S. L. (2016). Voice health conventional wisdom: Unknown, plausible, or confirmed? *ASHA Leader*, *21*.
doi:10.1044/leader.FTR3.21022016.np
- Fisher, K. V., Ligon, J., Sobecks, J. L., & Roxe, D. M. (2011). Phonatory Effects of Body Fluid Removal. *Journal of Speech, Language, and Hearing Research*, *44*, 354-367.
- Hanson, K. P., Zhang, Y., & Jiang, J. J. (2010). Parameters quantifying dehydration in canine vocal fold lamina propria. *Laryngoscope*, *120*, 1363-1369. doi:10.1002/lary.20927
- 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
- Hartley, N. A., & Thibeault, S. L. (2014). Systemic hydration: Relating science to clinical practice in vocal health. *Journal of Voice*, *28*, 652 e651-652 e620.
doi:10.1016/j.jvoice.2014.01.007
- 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.

- 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
- Hottinger, D. G., Tao, C., & Jiang, J. J. (2007). Comparing phonation threshold flow and pressure by abducting excised larynges. *Laryngoscope*, *117*, 1695-1699. doi:10.1097/MLG.0b013e3180959e38
- Jiang, J., Ng, J., & Hanson, D. (1999). The effects of rehydration on phonation in excised canine larynges. *Journal of Voice*, *13*, 51-59.
- Jiang, J. J., & Tao, C. (2007). The minimum glottal airflow to initiate vocal fold oscillation. *Journal of Acoustical Society of America*, *121*, 2873-2881.
- Jiang, J. J., & Titze, I. R. (1993). A methodological study of hemilaryngeal phonation. *Laryngoscope*, *103*, 872-882. doi:10.1288/00005537-199308000-00008
- 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
- 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.
- Kumazawa, H., Asako, M., Yamashita, T., & Ha-Kawa, S. K. (1997). An increase in laryngeal aerosol deposition by ultrasonic nebulizer therapy with intermittent vocalization. *Laryngoscope*, *107*, 671-674.

- Li, L., Zhang, Y., Maytag, A. L., & Jiang, J. J. (2015). Quantitative study for the surface dehydration of vocal folds based on high-speed imaging. *Journal of Voice*, *29*, 403-409. doi:10.1016/j.jvoice.2014.09.025
- Mau, T., Muhlestein, J., Callahan, S., Weinheimer, K. T., & Chan, R. W. (2011). Phonation threshold pressure and flow in excised human larynges. *Laryngoscope*, *121*, 1743-1751. doi:10.1002/lary.21880
- Meyer, J. P., McAvoy, K. E., & Jiang, J. (2013). Rehydration capacities and rates for various porcine tissues after dehydration. *PLoS One*, *8*(9), e72573. doi:10.1371/journal.pone.0072573
- Murugappan, S., Boyce, S., Khosla, S., Kelchner, L., & Gutmark, E. (2010). Acoustic characteristics of phonation in "wet voice" conditions. *Journal of Acoustical Society of America*, *127*, 2578-2589. doi:10.1121/1.3308478
- Nakagawa, H., Fukuda, H., Kawaida, M., Shiotani, A., & Kanzaki, J. (1998). Lubrication mechanism of the larynx during phonation: An experiment in excised canine larynges. *Folia Phoniatrica et Logopaedica*, *50*, 183-194.
- 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
- Roy, N., Tanner, K., Gray, S. D., Blomgren, M., & Fisher, K. V. (2003). An evaluation of the effects of three laryngeal lubricants on phonation threshold pressure (PTP). *Journal of Voice*, *17*, 331-342.
- Sivasankar, M., & Fisher, K. V. (2002). Oral breathing increases P_{th} and vocal effort by superficial drying of vocal fold mucosa. *Journal of Voice*, *16*, 172-181.

- Sivasankar, M., & Fisher, K. V. (2007). Vocal fold epithelial response to luminal osmotic perturbation. *Journal of Speech Language Hearing Research, 50*, 886-898.
doi:10.1044/1092-4388(2007/063)
- 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
- Stevens, M. (2016). *An excised model examining the reversal of vocal fold dehydration using aerosolized saline*. (Unpublished master's thesis). Brigham Young University, Provo, UT.
- Tanner, K., Nissen, S. L., Merrill, R. M., Miner, A., Channell, R. W., Miller, K. L., ... Roy, N. (2015). Nebulized isotonic saline improves voice production in Sjögren's Syndrome. *Laryngoscope, 125*, 2333-2340. doi: 10.1002/lary.25239
- 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 Hearing Research, 50*, 635-646.
doi:10.1044/1092-4388(2007/045)
- Tanner, K., Roy, N., Merrill, R. M., Kendall, K., Miller, K. L., Clegg, D. O., ... Elstad, M. (2013). Comparing nebulized water versus saline after laryngeal desiccation challenge in Sjögren's Syndrome. *Laryngoscope, 123*, 2787-2792. doi:10.1002/lary.24148
- Tao, C., Jiang, J. J., & Czerwonka, L. (2010). Liquid accumulation in vibrating vocal fold tissue: A simplified model based on a fluid-saturated porous solid theory. *Journal of Voice, 24*, 260-269. doi:10.1016/j.jvoice.2008.09.005
- Titze, I. R. (1994). *Principles of voice production*. Englewood Cliffs, NJ: Prentice Hall.

- Verdolini-Marston, K., Titze, I. R., & Druker, D. G. (1990). Changes in phonation threshold pressure with induced conditions of hydration. *Journal of Voice*, *4*, 142-151.
doi:10.1016/S0892-1997(05)80139-0
- Wanner, A., Salathe, M., & O'Riordan, T. G. (1996). Mucociliary clearance in the airways. *American Journal of Respiratory and Critical Care Medicine*, *154*, 1868-1902.
doi:10.1164/ajrccm.154.6.8970383
- 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 Otolology, Rhinology, and Laryngology*, *118*, 154-159.
- Witt, R. E., Taylor, L. N., Regner, M. F., & Jiang, J. J. (2011). Effects of surface dehydration on mucosal wave amplitude and frequency in excised canine larynges. *Otolaryngology Head Neck Surgery*, *144*, 108-113. doi:10.1177/0194599810390893
- 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
- 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 Hearing Research*, *50*, 611-620. doi:10.1044/1092-4388(2007/043)

Table 1

Aerodynamic and Acoustic Data at Baseline

Measure	Mean	SD	Median	Range
Phonation Threshold Pressure (cm H ₂ O)				
A/B*	29.3	13.9	25.6	37.0
B/A	17.4	8.5	17.2	21.0
Control*	24.6	10.9	23.0	30.1
Phonation Threshold Flow (L/min)				
A/B*	11.2	7.0	12.5	17.1
B/A	8.5	7.0	5.8	15.7
Control*	23.4	18.0	17.5	41.3
Fundamental Frequency (Hz)				
A/B*	397.5	25.9	397.5	36.7
B/A	441.6	189.8	437.7	486.9
Control*	352.2	77.4	389.2	141.0

Note. A = desiccation; B = aerosolization; SD = standard deviation; * = from Stevens (2016).

Table 2

Environmental Humidity During each Experimental Session

Group	Session Date	Baseline Percent Relative Humidity (%)	Endpoint Percent Relative Humidity (%)
A/B*			
Pig 1	02/19/16	14	13
Pig 2	02/19/16	14	13
Pig 3	02/19/16	14	13
Pig 4	03/05/16	12	12
Pig 5	03/05/16	12	12
B/A			
Pig 1	02/20/16	13	13
Pig 2	02/20/16	13	13
Pig 3	02/26/16	12	13
Pig 4	02/26/16	12	13
Pig 5	03/05/16	12	12
Control*			
Pig 1	02/26/16	12	13
Pig 2	02/26/16	12	13
Pig 3	03/11/16	13	13
Pig 4	03/11/16	13	13
Pig 5	03/11/16	13	13

Note. A = desiccation; B = aerosolization; * = from Stevens (2016).

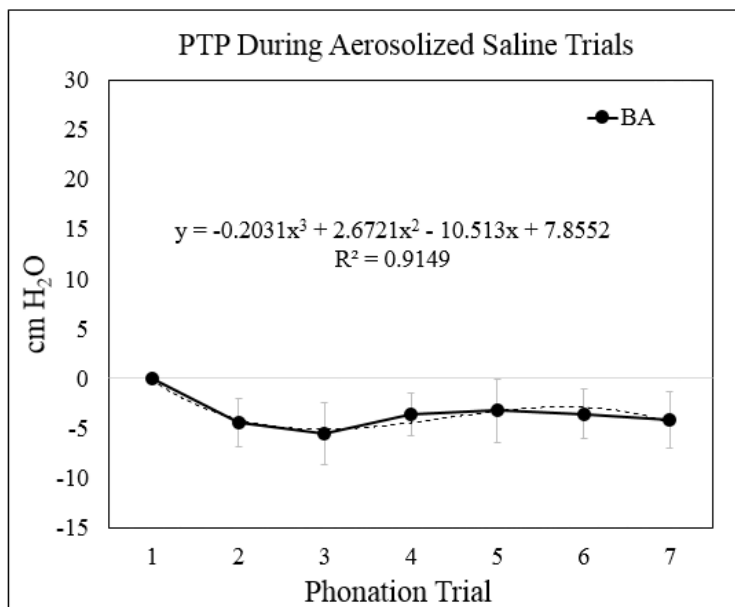


Figure 1. Normalized phonation threshold pressure (PTP) at baseline (0 on the y axis) and following each 4-min aerosolized saline dose. Mean, standard error, third order polynomial model trendline, and R^2 value and formula are provided. BA indicates that this group of larynges received desiccation (A) following saline administration (B). The PTP decreased 5 cmH₂O following the aerosolized saline treatment administration.

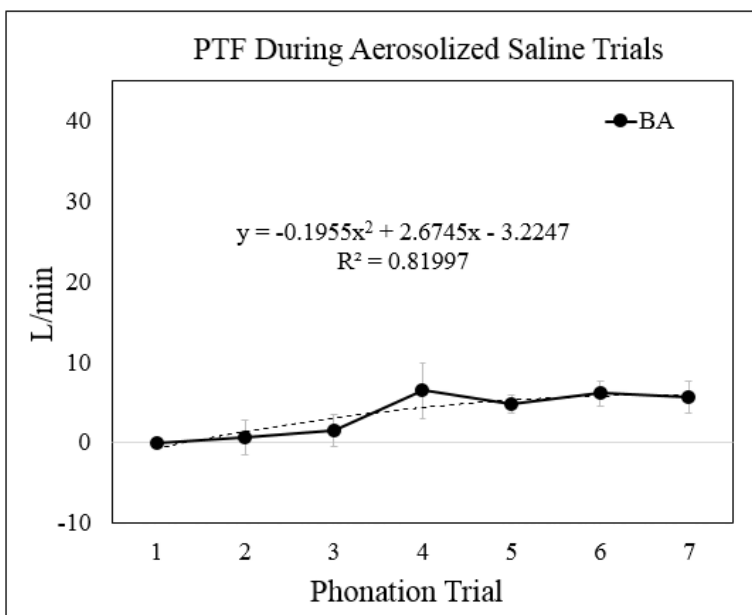


Figure 2. Normalized phonation threshold flow (PTF) at baseline (0 on the y axis) and following each 4-min aerosolized saline dose. Mean, standard error, second order polynomial model trendline, and R^2 value and formula are provided. BA indicates that this group of larynges received desiccation (A) following saline administration (B). The PTF increased by approximately 5 L/min during aerosolization.

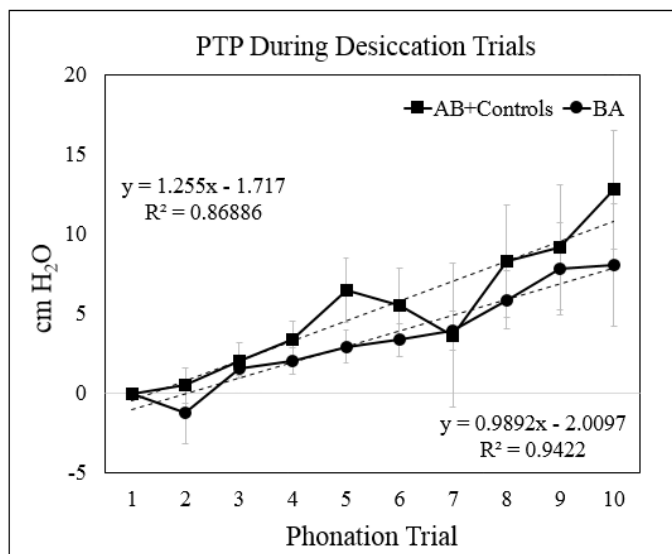


Figure 3. Normalized phonation threshold pressure (PTP) at baseline and following the first 10 1-min desiccation doses for prophylactically treated larynges (BA) compared with control larynges, including larynges subsequently treated with aerosolized saline (AB; Stevens, 2016). Means, standard errors, linear model trendlines, and R^2 values and formulas are provided. The PTP increased by approximately 7 cmH₂O for the prophylactically treated experimental group versus a 12 cmH₂O increase for the untreated control group.

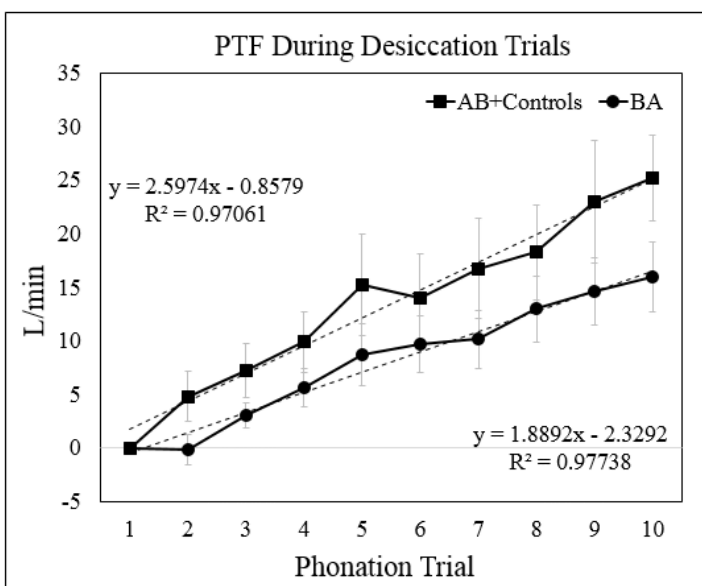


Figure 4. Normalized phonation threshold flow (PTF) at baseline and following the first 10 1-min desiccation doses for prophylactically treated larynges (BA) compared with control larynges, including larynges subsequently treated with aerosolized saline (AB; Stevens, 2016). Means, standard errors, linear model trendlines, and R^2 values and formulas are provided. The PTF increased by approximately 15 L/min for the prophylactically treated experimental group versus 25 L/min for the untreated control group.

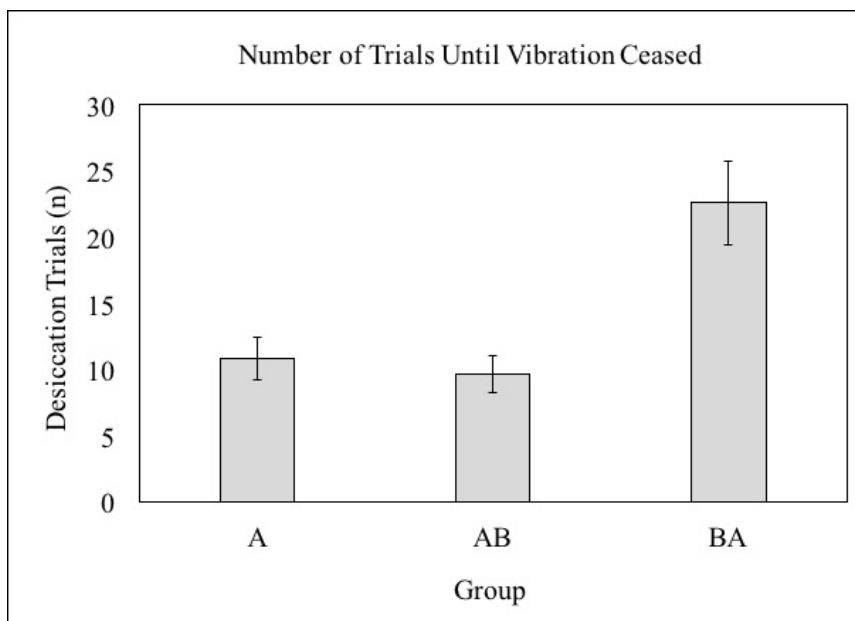


Figure 5. The number of desiccation trials required to cease vocal fold vibration for prophylactically treated larynges (BA) compared with control larynges (A), including larynges subsequently treated with aerosolized saline (AB; Stevens, 2016). Means and standard errors are provided. The results revealed statistically significant differences among the three groups $F(2, 12) = 10.562, p = .002$.

APPENDIX A: ANNOTATED BIBLIOGRAPHY

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

Purpose of the study. This study observed the vibratory characteristics of porcine, bovine, and ovine excised larynges in order to determine which animal was the most similar to human in regard to oscillation. This study also sought to determine whether a relationship between subglottal pressure and fundamental frequency (F_0) of vocal fold vibration existed among each of these three species.

Method. Eight porcine larynges, eight ovine larynges, and six bovine larynges were obtained from a local butcher shop. Each larynx was mounted onto a bench model with heated, pressurized, and humidified air. Next, pressure-flow studies, electroglottograph recordings, mean airflow rate measurements, audio signal acquisition, and sound pressure level measurements were taken on the larynges.

Results. The porcine larynx had a narrow slit-like, long ventricle, which clearly separated its vocal folds. The ovine and bovine larynges lacked this ventricle and their vocal folds were pad-like with no clear boundaries. The porcine inferior vocal folds had a thinner mucosal cover, but the superior vocal folds were more distinct, usually 28 mm long with a thickness of about 3 mm. Ovine vocal folds were soft and pliable with an average length of 17.2 mm and thickness of 6 mm. The bovine vocal folds were longer and stiffer with an average length of 37 mm and a thickness of about 18 mm. The bovine larynx showed a sustained oscillation with steady frequency that was not significantly affected by pressure variations. The typical frequency of the bovine larynx oscillation was about 74 Hz. The porcine larynx had a wider range of oscillation with frequency ranging from 160 to 300 Hz and up to 500 Hz. The ovine larynx had a linear relationship with pressure and frequency at higher pressures, but at lower pressures it was nonlinear. The porcine larynx produced voicing that reached up to 96.1 dB, while the ovine and bovine larynges produced voicing around 78 dB.

Conclusions. The porcine larynx had greater dynamic range of frequency most likely due to having two oscillating vocal folds and a supraglottic structure wall. Additionally, the porcine larynx had the largest range of frequency, a large amplitude, and a higher slope, which made it a good model for the study of pitch control. The bovine larynx had a low phonation threshold pressure (PTP) and almost steady pitch, which is suitable for studies using aerodynamic measurements. The ovine larynx had soft and pliable vocal fold tissue, produced large vibrational amplitudes with big mucosal waves, which could be a useful phonatory model.

Relevance to the current work. This study provided reasoning to use the porcine larynx due to its wide range of frequency, large amplitude, and the presence of two oscillating vocal folds and supraglottic structure.

Finkelhor, B. K., Titze, I. R., & Durham, P. L. (1988). The effect of viscosity changes in the vocal folds on the range of oscillation. *Journal of Voice*, 4, 320-325.

Purpose of the study. This study sought to discover if a change in hydration of the vocal folds could change the oscillation of the vocal folds. To examine this change, PTP was measured in the vocal folds.

Method. This study used four excised canine larynges that were mounted on a custom-designed bench model. The vocal folds were bathed in three different solutions. The larynges were bathed by immersing the glottal and subglottal airway from the surface of the vocal folds in 5 cm of the three solutions. First, the larynges were bathed for 15 min in normal saline, next for 15 min in distilled water, and lastly for 15 min in 2.7% saline. Pressure was measured using a U-tube manometer when vibration began and when vibration ceased.

Results. Higher pressures and less strain improved the vibratory conditions. The trend for each of the larynges was a lower PTP when using distilled water and a higher PTP when using hypertonic saline. There was no significant relationship between vocal fold adduction and PTP. In some cases, as the glottal width increased, PTP increased, however in other cases PTP decreased.

Conclusions. Adduction did not consistently cause an effect on PTP. As dehydration increased, the range of vibration decreased. As hydration increased, the range of vibration increased. Initially, this study tried to inject the different solutions into the vocal folds, but this only disrupted vocal fold vibration. Overall, this study concluded that water was found to permeate the epithelial layer.

Relevance to the current work. This study was the first excised model that looked at the effect viscosity had on the environment of vocal fold vibration. The results from this study are consistent with the findings of the current thesis on how hydration effects the vocal folds.

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 determine if acoustic measures are affected by changes in the humidity level of air inhaled by people with typical voices.

Method. This study used four men and four women. All participants were healthy, nonsmokers with voices within normal limits. Each participant inhaled air by mouth three different levels of humidity including environmental air, medical air with low humidity, and humidified air. The participants were instructed to repeatedly produce a sustained /a/ at a controlled level, with a constant pitch and loudness. This procedure was repeated 12 times for each level of air by each participant. Jitter, shimmer, and noise-to-harmonic measures were recorded and analyzed.

Results. Almost all of the subjects noted, with no prompting, that their airways felt unpleasant and the production of their voice felt impaired due to inhaling the medical air with low humidity. Also, almost all of the subjects noted that after inhaling the humidified air their voice felt comfortable. The effect of humidity had a significant effect on jitter. Perturbation measures increased due to breathing in the medical air with low humidity. There were no significant differences found between humidified air and medical air with low humidity on the noise-to-harmonic ratio.

Conclusions. Due to these findings of increases in perturbation and jitter, the author concluded that the human voice is sensitive to changes in the humidity level of inhaled air. The study showed these effects even after a short time of 10 min with medical air with low humidity. However, humidified air did not have as strong of an effect in decreasing perturbation and jitter as low humidity did in increasing perturbation and jitter. Lastly, the results did not seem to be caused by psychological factors.

Relevance to the current work. This study provided scientific evidence that the humidity level in the air can affect the human voice. The current work recorded the relative humidity level in the room each day the experiment was performed to document any influence humidity may have had on the experiment.

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 measure when vocal fold oscillation began to be irregular, in order to determine if this could help further the understanding of disordered voice production, and also find a new airflow measure.

Method. Seven canine larynges were used in this experiment. Pressure and airflow at phonation onset and chaos onset were measured in three experimental conditions. The three conditions included 0% elongation with no glottal gap, 20% elongation with no glottal gap, and 20% elongation with a 3 mm posterior glottal gap. The experiment began by increasing airflow until PTP and phonation threshold flow (PTF) were achieved and then measured. Airflow then increased until phonation became chaotic and the airflow was decreased until phonation stopped. The larynges were given 5 s of rest and five trials were performed in each of the three experimental conditions.

Results. The results showed both phonation instability flow and phonation flow range were not significant for elongation, but did show significance for abduction. Abduction was more significant for phonation instability flow and flow range than PTP and PTF, but still reached significance for PTP and PTF as well.

Conclusions. Phonation instability flow and phonation flow range could be useful in clinical assessment of voice disorders including vocal fold paralysis and presbylaryngis. There was no relationship found among elongation and PTP and PTF. Limitations that could have influenced the data included vocal fatigue, 20% elongation may have been too extreme, and producing chaos in the larynges could have affected the threshold measures.

Relevance to the current work. These results provided additional information about aerodynamic characteristics. This study found significant results between abduction and PTF, which the current thesis will use constant abduction to not affect PTF. Elongation was not a factor with PTP and PTF in the current thesis.

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

Purpose of the study. This study sought to determine if PTF was a valuable aerodynamic parameter. The glottal width in canine larynges were varied and PTF and PTP were measured to judge their sensitivity to the changes in the vocal folds.

Method. Ten excised canine larynges, which were inspected for any lesions or diseases, were used in this study and were frozen until experimentation. Each larynx was mounted onto a bench model and hydrated with 0.9% saline. The vocal folds were abducted using a shim that varied from 1 to 4 mm. An airflow meter measured airflow and a pressure meter measured pressure. The larynges were tested in five trials at 0, 1, 2, 3, and 4 mm glottal widths.

Results. Significant differences were found between the PTF means. The means from PTP showed no significant differences and were less sensitive than PTF.

Conclusions. The PTF increased as the vocal fold glottis increased to 4 mm, whereas PTP remained relatively stable in the later abduction increments. The PTF proved to be a more sensitive aerodynamic parameter than PTP. PTF also requires a less invasive approach when compared to PTP.

Relevance to the current work. This study provided the rationale for measuring both PTP, a historically reliable aerodynamic measure, and PTF, a new aerodynamic measure in the current work.

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 hydration on vocal fold tissue and to determine if the larynges could be rehydrated after being dehydrated.

Method. This study used 13 excised larynges which were mounted on a bench model and dehydrated with warm, dry air until the vocal folds ceased to phonate. The larynges were bathed in a saline solution to rehydrate the vocal folds. Once the larynges were rehydrated they were remounted on the bench model. The larynges were phonated with pressurized humidified air. A few measurements taken were glottal airflow and amplitude of vibration. A video camera recorded the glottal image, two pressure transducers measured subglottal pressure and airflow, and a microphone was used to measure the acoustic signal.

Results. As dehydration increased, PTP levels increased. As hydration increased, PTP levels decreased. There was not a statistical significance of airflow between the original state and the rehydrated larynges. Hydration caused PTP to decrease and improved vocal fold efficiency.

Conclusions. These results supported that superficially hydrating the vocal folds can lower pressure and airflow needed to begin and maintain oscillation. Additionally, the results support that hydration plays a major role in typical oscillation.

Relevance to the current work. This experiment confirmed that hydration is critical to phonation and that larynges can phonate after rehydration. This study supported the current study and provided rationale for examining both PTP and PTF with hydration.

Jiang, J. J., & Titze, I. R. (1993). A methodological study of hemilaryngeal phonation. *Laryngoscope*, 103(8), 872-882. doi:10.1288/00005537-199308000-00008

Purpose of the study. The purpose of this study was to compare hemilaryngeal vibration with full larynx vibration.

Method. This study used nine healthy, excised, canine larynges twice. First the larynges were phonated as full larynges and then the same larynges were dissected and phonated as hemilarynges. Larger larynges were used and the length of the vocal folds were on average greater than 16 mm. The larger larynges were chosen because they vibrated better and vibrated at a lower PTP. It was easier to place the transducers on larger larynges. The larynges were mounted on a bench model.

Results. The hemilarynges perceptually sounded similar to the full larynges. The hemilarynges vibration was similar as well. First, the bottom lip of the vocal fold closed and then the top lip of the vocal fold closed. The hemilarynges' average phonation instability pressure was 20%

greater. The average airflow in the hemilarynges was scaled 2:1 and acoustic power was scaled 4:1, or 6 dB less compared to the full larynx. The F_0 of the hemilarynx was 25 Hz lower and the amplitude of the lower subglottal pressure was 10% greater when compared to the full larynx. However, the hemilarynx and full larynx had the same higher subglottal pressure range.

Conclusions. The hemilarynx was very similar to the full larynx. Less than 10% of differences were found in vibrational amplitude, phonation instability pressure, PTP, and F_0 . Overall, a hemilarynx can be used instead of a full larynx to complete experiments.

Relevance to the current work. This experiment showed that there is a reasonable substitute for a full excised larynx for experimental purposes. There was great variability among larynges. This bench model was used in the current work.

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

Purpose of the study. This study used an x-ray stroboscope to look at the relationship between vocal fold oscillation and air tract fluid movement.

Method. This study used two kinds of excised canine larynges. Half of the larynges were within functional limits and the other half had a stiff lesion on one of the vocal folds. The lesions were made by attaching a surgical adhesive agent to one of the vocal folds. A contact microphone was attached to the trachea, a light was placed supraglottally to illuminate the surface of the vocal folds, and a nebulizer was placed in the air-blowing circuit. An x-ray stroboscope and an x-ray device observed the vocal fold oscillation.

Results. The typical vocal fold images observed a column of air tract fluid in a short amount of time onto the lateral, upper surface of the vocal folds. The images of the vocal folds with a stiff lesion revealed that fluid accumulated onto the upper surface of the vocal folds as well. However, it did not form a column of fluid; instead it formed a flat layer.

Conclusions. The wave of the mucous membrane brings the fluid in the subglottis to the supraglottis. The fluid columns are located by this mixed fluid where the movement is on the vocal folds. When vocal folds are damaged, then these fluid columns do not form due to a lack of flexibility. This study explained that the rotating column function was to lubricate and cool the vocal folds during oscillation.

Relevance to the current work. These results could be applied to clinical work with patients with stiff lesions. This was important for the current project because the results could be affected if the larynges used in the study had any stiff lesions on the vocal folds.

Kumazawa, H., Asako, M., Yamashita, T., & Ha-Kawa, S. K. (1997). An increase in laryngeal aerosol deposition by ultrasonic nebulizer therapy with intermittent vocalization. *Laryngoscope*, 107, 671-674.

Purpose of the study. This study sought to determine if using an ultrasonic nebulizer provided improvement for pharyngitis or laryngitis and to determine what the best form of inhalation is. In order to answer these questions, the particles that reached the upper airway tract were measured after using an ultrasonic nebulizer.

Method. The subjects used in this study consisted of six healthy males with a mean age of 26 years and six months. Isotonic saline was nebulized for 5 min through the participant's mouth with a mouthpiece using three different methods. The first method was deep and slow inhalation, the second method was fast inhalation, and the third method was fast inhalation with intermittent vocalization. In 5-s increments, images were taken of the participant's mouth and chest.

Results. The deep and slow inhalation method after 15 min had no significant change in the larynx, lung, and pharynx. The second method with fast inhalation caused the deposition rate in the larynx to rise and lowered the deposition rate in the lungs from 3 min to 15 min. The third method with intermittent vocalizations significantly improved the deposition rate in the larynx and decreased the deposition rate in the lungs within 1.5 min and lasted for 15 min. Accumulation of isotonic saline was found in the larynx and not very much accumulation in the pharynx and lung.

Conclusions. This study found that the most effective form of nebulization that reached the upper airway was with fast inhalation and intermittent vocalization. The vocalizations caused the saline to impact the vocal folds on both sides. The inhalation and vocalization caused the saline particles to remain on the vocal folds.

Relevance to the current work. This study provided the rationale for why the current work nebulized the porcine larynges with fast inhalation and intermittent vocalization.

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

Purpose of the study. The purpose of this study sought to quantify how varying levels of dehydration on the surface of the vocal folds could make changes to laryngeal movement during vibration. The study examined how dehydration affected glottal area and mucosal wave.

Method. The dehydration experiment used 10 excised canine larynges with three degrees of dehydration levels including 0%, 50%, and 75%. High-speed images were used to judge any changes in the glottal area and mucosal wave. The glottal area was examined by comparing differences in amplitude directly and non-directly. In order to look at the amplitude and frequency of mucosal wave, digital videokymography was used. Each level of dehydration was then examined and compared to find statistical significance.

Results. These results showed that between every two different dehydration levels there were significant differences of direct and non-direct glottal areas. As dehydration levels increased, the glottal area decreased, meaning the vocal folds were not moving as much and the vocal folds were becoming stiffer. However, no significant differences in frequency were found.

Conclusions. Dehydration resulted in a change of the biphasic structure causing increased stiffness, decreased viscoelasticity, and a large glottal gap, which could be caused from the need to increase vocal effort in order to move the stiff vocal folds. Further study of dehydration on vocal fold oscillation can help improve clinical practices in areas with client populations that include nodules and hoarseness.

Relevance to the current work. This study related to the current thesis because it helped explain why the glottal gap increased during the desiccation trials. As the vocal folds became more dehydrated, then the vocal folds stiffened and required more effort to phonate.

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

Purpose of the study. This study sought to find the PTP and PTF values of human larynges. Additionally, it sought to determine if posterior glottal width, glottal area, and gender caused changes to PTP and PTF. Lastly, this study looked to see if hysteresis was present in human laryngeal phonation and compared these results to previous human PTP and PTF values, as well as to previous canine PTP and PTF values.

Method. Nine excised human larynges were attained within 24 hours of post-mortem. A bench model was used to acquire PTP and PTF at phonation onset and offset. The effects of posterior glottal width, glottal area, and gender were obtained. MATLAB and ImageJ were both used for data analysis.

Results. There was a large amount of variability between subjects in PTP and PTF. The PTP was similar to previous in vivo studies, but PTF was significantly higher than previous studies. Posterior glottal width was not dependent upon PTP and PTF. Hysteresis was seen in human larynges. Both PTP and PTF offset values were lower than onset values. Onset measurements were more variable than offset measurements. The PTP did not have gender differences, but PTF increased in males with glottal width. There was a decrease in airflow after phonation onset began in multiple conditions.

Conclusions. This study found similar PTP and PTF values that have previously been found in canine studies and confirmed aerodynamic parameters. The high PTF observed may be a reflection of a large direct current flow because of vocal fold bowing. The high PTP may be a reflection of the aged population having decreased vocal fold vertical thickness and decreased hydration. Offset PTP and PTF values may be more reliable measurements than onset values. The large inter-subject variability in PTP and PTF may have implication for the clinical application such as the fact that there is no relationship with posterior glottal width. Lastly, human larynges were better than animal larynges for examining tissue effects.

Relevance to the current work. This study showed a large inter-subject variability in human larynges that occurred in PTP and PTF, which was relevant to the current work. The results were consistent with previously reported canine larynx data and were compared to the current thesis.

Meyer, J. P., McAvoy, K. E., & Jiang, J. (2013). Rehydration capacities and rates for various porcine tissues after dehydration. *PLoS One*, *8*(9), e72573. doi:10.1371/journal.pone.0072573

Purpose of the study. This study examined the ability and rate of rehydration among various tissues after desiccation. Also, the study examined different amounts of time of dehydration to determine if overexposure could permanently dehydrate the larynges, making rehydration impossible.

Method. Prior to completing the procedure, the tissues were sectioned into pieces and frozen in a 0.9% saline solution. Twenty-four tissue samples were used in this experiment. The six different types of tissue were cartilage, tendon, fat, skin, muscle, and lung. The tissues were randomly selected from each tissue type and weighed before dehydration. The dehydration procedure included putting the tissue into a vacuum oven until the tissue reached 40% to 100%

of its original size. The rehydration procedure consisted of placing the tissue in 0.9% saline solution for 5 hours. The tissues were weighed three times in 10-min intervals to make sure that the tissue reached its maximum hydration level. This protocol of dehydrating followed by rehydrating was repeated. When looking at the rate of rehydration, four samples from each of the six tissue types were dehydrated to 60% and the rate was measured at 15-min intervals for 1 hour.

Results. Cartilage and fat samples had a significantly greater loss in size after rehydration than tendon, skin, and muscle at the lower dehydration interval. Only muscle had a lower change in size at the 30% to 40% interval compared to skin and fat. The tissue types responded differently to prior dehydration. Muscle had the ability to recover from higher levels of dehydration. Lung, tendon, fat, and muscle tissues showed a significant decrease from the 20% to 30% group to the 30% to 40% group. Each type of tissues absorbed fluid nonlinearly except for fat tissue. After 30 min of soaking, each tissue type made significant increases in size. Additionally, cartilage made an increase in hydration after 1 hour of soaking.

Conclusions. This study found that there was a relationship among initial dehydration level and the tissue's ability to be rehydrated. Lung, tendon, fat, and muscle tissue had a decrease in the liquid mass fraction when the tissues were rehydrated. Each tissue seen in this study successfully rehydrated after some level of dehydration and rehydrated nonlinearly. The tissues absorbed the saline at a decreasing rate until the tissue could no longer absorb anymore saline. Dehydrated tissue samples needed an adequate time to rehydrate, which varied between tissue types.

Relevance to the current work. This study showed that dehydrating and rehydrating tissues changed depending on the tissue type. The current thesis used different time intervals for dehydration and rehydration. A limitation of this study was that lacerations to the tissues may have occurred from the freezing and thawing processes. In the current thesis, the tissue was not frozen or thawed.

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

Purpose of the study. The purpose of this study was to determine if mucous viscosity levels had an impact on vocal fold oscillation.

Method. Twelve excised canine larynges were obtained and eight larynges were used in the trials due to the fact that four of the larynges could not synchronize to the strobe light. Two fluids with different levels of viscosity were applied to the excised canine larynges and then the larynges were phonated. The low viscosity fluid was saline and the high viscosity fluid was dissolving chondroitin sulfate sodium salt in saline. The experiment protocol consisted of the following steps, beginning with the mucous being removed with a cotton swab, followed by 0.1 ml of the fluid applied to the upper surface of the vocal folds supraglottally and phonated, and then the fluid was removed with a cotton swab when finished. Images were taken at 30 frames per second.

Results. The high viscosity fluid produced larger opening and closing phases and when compared to the low viscosity fluid had increased and shorter closed phases. Additionally, the high viscosity fluid also decreased the normalized peak glottal area. Lastly, the high viscosity fluid decreased the amplitude horizontally and vertically. There were no differences found

between the high viscosity fluid and the low viscosity fluid regarding the trajectory of the inferior vocal fold.

Conclusions. This study concluded that high viscous levels in the respiratory mucus of the larynx may cause voice disorders. Increasing the viscosity of the mucus on the vocal fold may change how the vocal folds oscillate. Increased viscosity levels may increase the shear stress, which caused the mucosa velocity to decrease. Similarities between these results and the vibrating pattern of head voicing were found because shortening of the closed phase is represented in both. Both of these conditions resulted from changes in stiffness of the vocal folds. There may be a range of viscosity that is preferred for lubricating the larynx, but further research needs to take place to find the most preferred viscous level.

Relevance to the current work: This study showed that finding the optimum level of viscosity is necessary to lubricate the larynx effectively. Additional studies are needed to determine the optimal viscosity. The current thesis observed the effects of saline on vocal fold hydration to try to answer the question if saline is a good choice for lubrication.

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 discover if phonation threshold power is a sensitive measure to changes in the properties of the vocal folds.

Method. Phonation threshold power was measured in three sample populations including glottal gap, vocal fold elongation, and vocal fold lesions with 10 excised canines in each group. The posterior glottal gap was created using a shim ranging from 0.0 to 4.0 mm. Vocal fold elongation was made by varying the micrometer anteriorly 0%, 5%, 10%, 15%, and 20%. Vocal fold lesions were made by burning the vocal fold at midpoint either unilaterally or bilaterally. Each larynx was mounted on the bench model and recordings were taken at phonation threshold.

Results. Phonation threshold power found statistical differences among the posterior glottal width and vocal fold lesion treatment groups, but not in the vocal fold elongation treatment group.

Conclusions. The weak relationship between vocal fold elongation and phonation threshold power may be due to the interaction between vocal fold elongation and other variables involved that were not measured. Phonation threshold power was a new measurement that had not been used before, but was sensitive to glottal gap and lesions. This new measurement could be useful for determining the larynges' general health.

Relevance to the current work. These results provided evidence that phonation threshold power could be used as a broad screening in clinical practice to indicate an abnormal larynx. The current thesis used a similar shim to produce a glottal gap when nebulizing saline and also used a similar form of elongation with the micropositioner elongating the vocal folds anteriorly.

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. This study sought to determine if PTF was greater than the airflow required for stable phonation and also evaluated the ratio of offset divided by onset.

Method. This study included 10 excised canine larynges that were mounted on a bench model and took measurements of the onset and offset PTF. The airflow was presented subglottally through the bench model to observe phonation. Once phonation began, the airflow was decreased until vibration stopped. The PTP measurements were also measured along with PTF. This study also specifically looked at PTF with larynges that had elongated vocal folds. This was done to observe if there were any differences between normal vocal folds compared to elongated vocal folds.

Results. The results of this study proved the hypothesis to be correct. The onset values of PTF were always greater than the offset values of PTF. The second hypothesis was correct as well; 80% of the larynges were within the ratio limits.

Conclusions. The hysteresis phenomenon was proven by onset always being higher than offset. Knowing the bounds of what the ratio of offset and onset values are can contribute to being able to identify when a voice has abnormal tension from elongation and could have clinical implications. This study helped further understand the physics of phonation and learning more about the hysteresis phenomenon helps further understand how voice is effected by the vocal folds.

Relevance to the current work. This study proved that the onset-offset PTF ratios are bound between 0.707 and 1.0; these boundaries can be used to ensure that the current work was also within these limits, indicating a reliable bench model.

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

Purpose of the study. The purpose of this study was to determine how three lubricants, which included water, Mannitol, and Entertainer's Secret Throat Relief, affect vocal fold function.

Method. This study included 18 healthy female students with typical voices. For baseline measures, each individual had their PTP measured twice before using each nebulized treatment. After each nebulized treatment of 2 ml, the individual's PTP was estimated four times. Nebulized treatments were given on consecutive weeks. The PTP was acquired at 80% of the individual's maximum F_0 during soft phonation.

Results. Mannitol is a substance that facilitates the movement of water onto the surface of the airway, resulting in an immediate decrease in PTP. However, this effect began 5 min into the treatment and only lasted for 20 min. Mannitol only approached statistical significance. Water and Entertainer's Throat Relief did not decrease PTP.

Conclusions. This study included subjects that are within functional limits, which could be the reason for the absence of significant results. These nebulized treatments may work better on patients with laryngeal dryness and other vocal pathologies. Due to the fact that Mannitol only approached significance, the results must be interpreted cautiously. Because water had no effect on PTP, adding additional water to the overall human system will not improve vocal quality or affect PTP.

Relevance to the current work. This study showed that Mannitol did cause a short lived change and that the vocal folds can be hydrated topically. The current work attempted to determine if saline would have a longer effect than Mannitol and reduce PTP.

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

Purpose of the study. The purpose of this study was to compare how oral breathing and nasal breathing can affect PTP and vocal effort. This study also looked at how the sol layer of the vocal fold aided in regulating PTP.

Method. This study used 20 normal and healthy female students. These subjects were split into two groups, either oral breathing or nasal breathing. The subjects wore a vented pneumotachograph mask that included low-bandwidth and wide-bandwidth to generate the glottal volume velocity airflow. After pretreatment measures, subjects in their respective groups participated in oral or nasal breathing for 15 min.

Results. After the participants performed 15 min of oral breathing, 6 out of the 10 participants noted that vocal effort increased at comfortable and low pitch. The PTP increased from oral breathing. After the participants performed 15 min of nasal breathing, 7 out of 10 noted that vocal effort decreased. The PTP decreased in all three levels of pitch from nasal breathing.

Conclusions. Oral breathing caused even healthy voices to be at risk for increasing their vocal effort and eventually caused vocal pathologies. These findings showed that the sol layer, which is a main contributor to maintain topical hydration on the vocal folds, does have a role in supporting PTP. There should be strategies developed to help prevent negative effects of oral breathing.

Relevance to the current work. This article found that superficial hydration does aid in regulating PTP. Also this article emphasized the fact that there was a large population of people who are mouth breathers that will need a strategy to help prevent against the adverse effects of oral breathing. The current work looked at how saline will affect superficial hydration.

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

Purpose of the study. This study sought to discover if the surface of the vocal folds would create a water reflux when exposed to Mannitol or sham challenges.

Method. This study used 36 native ovine vocal folds that either had Mannitol, hyperosmotic perturbations, applied to the surface of the vocal folds or sham, isosmotic perturbations, applied to the surface of the vocal folds. Each larynx was dissected into two hemilarynges and the epithelium was separated from the rest of the vocal folds. Next, the epithelium was mounted onto an Ussing system and a system to measure its water flux and ions. First each vocal fold water flux and each vocal fold viability was measured at baseline and then again 30 min after each challenge.

Results. The vocal fold perturbation with the Mannitol and sham challenge was silent and did not have a significant effect of potential differences. The Mannitol challenge increased water on the epithelium for 60%, however the sham challenge did not increase water on the epithelium. This increase was not statistically significant and was not long lasting. The sham challenge decreased the water flux on the epithelium. Ovine tissue lasted for more than 2 hours.

Conclusions. The ovine vocal fold was able to detect the perturbations and respond to these changes. This ability of the vocal folds is important to maintain regulation. The perturbations represent phonation and respiration that affect vocal folds. Mannitol could be used in treatment

to increase water and hydration on the surface of the vocal folds to reduce negative effects of dehydration.

Relevance to the current work. This study received positive, but not statistically significant results from Mannitol on hydration. The current thesis furthered this research by examining if saline could increase hydration. Factors that could have influenced these results that should be considered in the current work include the dissection protocol, state of the tissue, and gender.

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 Hearing Research, 50*, 635-646. doi:10.1044/1092-4388(2007/045)

Purpose of the study. This study wanted to discover if PTP and self-perceived vocal effort were affected by nebulized isotonic saline, hypertonic saline, and water after the larynx underwent a desiccation challenge.

Method. This study included 60 healthy women that had voices within functional limits. The subjects underwent a desiccation challenge, which included mouth breathing dry air for 15 min. The women were blindly divided into four groups, which received nebulized isotonic saline, hypertonic saline, sterile water, or no treatment. Immediately after the desiccation challenge the subject's PTP and vocal effort was measured. These measures were taken again after 5 min, 20 min, 35 min, and 50 min post nebulization.

Results. The desiccation challenge caused an increase in PTP by 0.5 cm H₂O for all four groups. The vocal effort ratings decreased significantly following the desiccation challenge. The vocal effort ratings did not have a relationship with the PTP values. None of the treatments reached statistical significance for reducing PTP values. However, isotonic saline did have a short-lived trend showing a reduction in PTP values. The adverse PTP effects lasted for at least 60 min.

Conclusions. This study showed that there is a relationship between PTP and vocal fold dehydration. No statistical trends were found from the treatments to cause a recovery from the temporary dehydration. Although there is not a correlation between PTP and vocal effort, questions arose about why these measures might address different constructs.

Relevance to the current work. This study showed that isotonic saline was the most promising at reducing the negative effects of dehydration. The current work tested isotonic saline in an excised model to see if there was a relationship with saline on PTP to reduce the adverse effects of the desiccation challenge.

Tanner, K., Roy, N., Merrill, R. M., Kendall, K., Miller, K. L., Clegg, D. O., . . . Elstad, M. (2013). Comparing nebulized water versus saline after laryngeal desiccation challenge in Sjögren's Syndrome. *Laryngoscope, 123*, 2787-2792. doi:10.1002/lary.24148

Purpose of the study. The purpose of this study was to examine PTP, throat dryness, and vocal effort after participants with Sjögren's Syndrome underwent a desiccation challenge followed by two different hydration treatments using nebulizers.

Method. This study included 11 participants, 10 females and 1 male, with Sjögren's Syndrome. Each individual participated in a 15-min laryngeal desiccation challenge, which included breathing dry air orally. Next, the individuals received nebulized isotonic saline or sterile water and were instructed to use the nebulizer for two consecutive weeks. Before the desiccation trial the participants were assessed for baseline PTP, vocal effort, and laryngeal dryness, and then

they were assessed after the desiccation trial. At 5 min, 35 min, and 65 min during their nebulized treatment the same measures were taken.

Results. Vocal effort, PTP, and laryngeal dryness reached statistical significance for the desiccation trials, but only approached significance for the nebulized saline treatment. The nebulized saline reduced the effects of the desiccation challenge better than water did, but both did not reach statistical significance. The PTP was more correlated with throat dryness.

Conclusions. The desiccation challenge showed that Sjögren's Syndrome patients had phonatory changes following the experiment. Nebulized saline showed some effect on reducing the desiccation effect, but the improvement was short-lived. Continued research should examine if there is a relationship between the dosages of nebulized treatments.

Relevance to the current work. This study showed that dry air does affect hydration and that saline decreases PTP. The current work examined the effects of nebulized saline using an excised model instead of an in vivo model.

Tao, C., Jiang, J. J., & Czerwonka, L. (2010). Liquid accumulation in vibrating vocal fold tissue: A simplified model based on a fluid-saturated porous solid theory. *Journal of Voice*, 24, 260-269. doi:10.1016/j.jvoice.2008.09.005

Purpose of the study. The purpose of this study was to find a set of equations that could describe the vibration of vocal folds. This study compared this new fluid-saturated porous tissue model with the previous continuous, elastic model.

Method. This study analyzed the fluid-saturated porous tissue model using a set of mathematical equations. It also compared previous models to the new model and included an extensive literature review. This study analyzed and solved the problem of the one-dimensional vibration using the assumption of a small-amplitude.

Results. This study found that vibration of the vocal folds caused an excess of liquid to form on the midmembranous section of the vocal fold based off of the fluid-saturated tissue model. The amount of excess liquid that rests on the vocal fold was related to the amplitude and frequency it vibrates at. This is a positive relationship so as amplitude and frequency increased, the amount of liquid increased.

Conclusions. The location of liquid accumulation at the midmembranous section of the vocal fold provided theoretical evidence of why vocal nodules form in the same location. However, vocal nodules are more complex and most likely have other contributing factors. Edema from the accumulation of liquid may also contribute to nodules. The fluid-saturated porous solid theory is the best model to describe vocal fold tissue.

Relevance to the current work. This study provided a good theoretical description of vocal fold tissue and rationale for vocal fold vibration patterns. The current thesis should have an understanding of vocal fold tissue and vibration.

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

Purpose of the study. This study sought to determine the least amount of airflow needed to start stable vocal fold vibration and how dry air effects PTF.

Method. The PTF data was collected from 11 excised canine larynges mounted on a bench model. The protocol involved cycles of phonating for 10 s followed by 3 s of rest. The experimental trials consisted of blowing dry airflow subglottally until phonation was sustained for 10 s and then the airflow was decreased until phonation stopped. Saline was not involved in the experimental trials. The control trials had humidified airflow blown subglottally and had saline applied to the vocal folds.

Results. As dry airflow increased, PTF also increased in the experimental trials. However, in the control trials, the relationship between PTF and dehydration did not reach significance. The dehydrated larynges found that the initial PTF values were between 133.9 to 661.8 ml/s, and the final PTF values were between 196.5 to 1,219.2 ml/s. The vocal folds could no longer produce phonation after 543 s. Lastly, PTF increased between 62.65 ml/s and 735.61 ml/s.

Conclusions. The data produced from this study indicated that PTF increased from surface vocal fold dehydration. Clinically, this information about the effect of dry air was useful in assessing dehydrated vocal folds and in preventing dehydration of the vocal folds. The relationship among PTF and dehydration in human trials should be looked at further and would benefit clinical practices.

Relevance to the current work. This study showed that dehydration affects PTF and provided a reason for further testing the relationship between hydration and PTF for clinical purposes. However, the drying effects seen in excised experiments are not directly comparable to dehydration observed in living organisms.

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

Purpose of the study. The purpose of this study was to discover if the mucosal wave frequency and amplitude are affected by vocal fold surface hydration and particularly dehydration of the lamina propria of the vocal folds.

Method. Ten excised canine larynges were attained immediately postmortem and checked to make sure no diseased tissue or lesions were on the larynges. Eight excised canine larynges were used in the desiccation trial and exposed to dry air at a constant pressure of 20 cm H₂O until the larynges stopped phonating. No saline solution was given to the larynges in the desiccation trials. Each larynx was recorded using high-speed video. The control group included two larynges that were exposed to humidified air and recorded on high-speed video. The larynges were kept hydrated for 30 min of phonation by applying 0.9% isotonic saline solution in 30 s intervals.

Results. The mucosal wave was analyzed from the high-speed videos. Videokymography was used to quantify the mucosal wave characteristics. As dehydration levels increased, the larynges' amplitude and frequency decreased. Statistically significant differences of mean slopes and differences between mean percent changes were found between the control group and the dehydration treatment for both amplitude and frequency.

Conclusions. Vocal fold surface dehydration had a negative relationship with mucosal wave amplitude and frequency. This study determined that extreme voice deterioration is associated with voice deterioration. This study had a clinical impact, and can be an indicator of laryngeal dysfunction.

Relevance to the current work. This study showed that dehydration affects the wave amplitude and frequency of our vocal folds. The current thesis followed similar dehydration and hydration trials. Saline solution was used; however, the current work used nebulized saline.

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. This study used high-speed imaging to look at the biomechanical applications of spatiotemporal vocal fold oscillation and nonlinear dynamics of glottal area analysis to quantitatively describe typical and atypical vocal fold oscillation.

Method. This study used 12 typical vocal fold oscillations that had an average of 16 cm H₂O subglottal pressure along with 12 atypical vocal fold oscillations that had an average of 55 cm H₂O subglottal pressure. The high-speed camera was placed directly above each larynx and had a sampling rate of 4000 frames per s.

Results. A statistical difference between the typical and atypical oscillations was found from the correlation length and global entropy in spatiotemporal analysis and the correlation dimension from nonlinear dynamic analysis. The atypical oscillation had higher global entropy and higher correlation dimension. However, the atypical oscillation had statistically significant lower correlation length.

Conclusions. Simple temporal periodicity, spatial symmetry, temporal periodicity, and discrete frequency spectra were produced by typical oscillations. Complex aperiodic glottal area series, spatiotemporal vibratory patterns, and broadband spectra were produced by atypical oscillations. This study proved that spatiotemporal analysis and nonlinear dynamic analysis can be used to describe oscillation and are reliable.

Relevance to the current work. This study is related to the current work because it provided an example of using a high-speed camera to aid in analysis. It also provided pictures and information about what typical oscillation looks like.

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 Hearing Research*, *50*, 611-620. doi:10.1044/1092-4388(2007/043)

Purpose of the study. This study revealed the mechanisms of phonation instability pressure and used phonation pressure range to assess the normal pressure range for vocal fold vibrations.

Method. This study used bifurcation analysis from acoustic signals of 10 excised canine larynges. The canine larynges were stored in saline solution within 48 hours of being mounted on the bench attached to a pseudolung. As subglottal pressure progressively increased, PTP, phonation pressure range, and phonation instability pressure were measured at the bifurcation pressure points on a spectrogram. The elongation of the vocal folds was measured at 0%, 5%, 10%, and 15% to identify how elongation effects PTP, phonation pressure range, and phonation instability pressure.

Results. The results showed that the bifurcation analysis was effective at determining PTP, phonation pressure range, and phonation instability pressure. Increasing vocal fold elongation significantly increased PTP and phonation pressure range significantly decreased. Phonation instability pressure showed no significant change.

Conclusions. This study showed that important parameters to determine phonation instability include phonation instability pressure and phonation pressure range. A valuable procedure for determining PTP, phonation pressure range, and phonation instability pressure was the bifurcation analysis. These three factors are valuable when investigating vocal fold biomechanical parameters.

Relevance to the current work. The current study used PTP, one of the three parameters tested, which this study proved was valuable at assessing phonation in vocal folds. Additionally, the current thesis elongated the vocal folds, which was found to increase PTP.

APPENDIX B: EXPERIMENTAL PROTOCOL

Materials for Dissection:

1. scalpels (2 different types)
2. apron
3. gloves
4. green dissection paper (to be laid on the dissection table)
5. saline spray bottle
6. 1 Ziploc bag
7. hemostats
8. sutures (1 for each larynx)
9. protective goggles
10. dissection table
11. red hazard box (rinse scalpels and then place them in this box)
12. tub-fridge drawer (to hold un-dissected larynges)
13. Clorox wipes (for clean-up)
14. Paper towels (to hold your larynx steady)

Dissection:

1. Remove all surrounding tissues of the larynx such as the esophagus, thyroid gland, fat, excess tendons, innervation, and vascularization. Make sure the trachea and thyroid cartilage are intact and without any abnormal openings or damage.
2. Use the largest tracheas-these are best for phonation and mounting onto custom tubing
3. Tracheas should be cut superiorly of the true vocal folds
4. The true vocal folds should not be punctured (this will prevent air leakage)
5. The shape should be a smile formed from the anterior commissure to the lateral posterior ends of the thyroid cartilage
6. The arytenoid cartilages should be left intact (this will aid in adduction)
7. The epiglottis should be removed by cutting a triangle posterior and in between the arytenoid cartilages.
8. Remove the false folds completely (may use a hemostat for better precision)
9. Remove any left-over tissue superficial and superior to the vocal folds (this prevents flopping of tissue during vibration of true vocal folds)
10. 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)
11. 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 the suture is tight and tug at it to observe its strength

Materials for Experiment:

1. 4 LED lights (make sure fresh batteries are in place)
2. macropositioners
3. micropositioners

4. nozzle for desiccated air
5. 2 mesh type nebulizers (filled with saline) (MicroAir OMRON NE-U22)
6. Teflon tape (used to seal edges of trachea onto the custom tubing which is attached to the pseudolung)
7. Flow meter (Aalborg mass flow meter GFM-47)—flow should be calibrated at 0 and 15 cmH₂O
8. Medical Flow Meter- attached directly to the air tank and to the Aalborg mass flow meter GFM
9. 2 Air tanks (one will attach to the flow meter and the humidifiers; the other will be for desiccated air)
10. pressure transducer (should be plugged in from computer to inferior lateral portion of larynx or the custom tubing)
11. pressure calibrator box (should be used only to calibrate pressure transducer) calibration occurs at 0 and 10 PSI
12. check all plugs
13. Windaq should be turned on and 4 different waves should be showing (wave 1: microphone signal; Wave 2: pressure; Wave 3: Flow; Wave 4: High Speed Trigger)
14. Humidifier (make sure tubing is plugged in to pseudolung and air tank)
15. High Speed video camera: Trigger should be on and plugged into the sound board
16. 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.)
17. High Speed-make sure trigger is plugged in
18. Hose clamp (secure trachea onto the custom tubing)
19. 4 Metal clamps (hold flashlights & Microphone)
20. Vinegar and distilled water (for cleaning nebulizers)- follow instructions for cleaning at the end of the day of all experiments
21. Clorox Wipes
22. Paper towels
23. Metal shim (Allen wrench- diameter 5 mm)

Measuring Flow

1. Make sure the 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: hsv
- open
- record

If working at the Windaq monitor, you are in charge of triggering high speed and inserting comments for each recording.

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

Procedure for Prophylaxis Trials (prevention)

- 5 pigs were included in this section
- for each larynx, baseline was collected (larynges were vibrated without aerosolization trial) (if the larynx did not vibrate, then it was removed from the study)
- after baseline was collected, pigs were aerosolized for 4-minute increments using a nebulizer with custom tubing attached to the mouthpiece
- each larynx was then vibrated following 4-minute aerosolization
- data was collected after each aerosolization trial, 6 in total
- desiccation trials began immediately after the final aerosolization trial
- each larynx was desiccated for 1-minute increments using custom tubing which was attached to one of the air tanks

- data was collected after each desiccation trial
- desiccation trials were continued until vocal folds ceased to vibrate or phonation ended

Procedure for Control Trials

- 5 pigs were included in this section
- For each larynx, baseline was collected (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 1-minute increments (a shim was held in place posterior to the true vocal folds in the interarytenoid region)
- each larynx was then vibrated following 1-minute desiccations (data was collected after each desiccation trial)
- Desiccation trials were continued until vocal folds ceased to vibrate or phonation ended

Measuring Humidity

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

Humidifier

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

Flashlights:

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

APPENDIX C: FOOD HANDLER'S PERMIT

