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Results: Baseline shear modulus was calculated between 1076 to 1307 Pascals. In the ex vivo human larynx, the shear modulus increased gradually to a maximum of 1.6 times baseline value with graded arytenoid adduction and 3.7 times baseline value with manual cricothyroid approximation. In the in vivo larynx, the shear modulus increased to a maximum of 1.6 times baseline value with RLN stimulation and 2.5 times baseline value with SLN stimulation.

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CONTROL OF VOCAL FOLD COVER STIFFNESS BY LARYNGEAL MUSCLES

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INTRODUCTION

The ability to control the fundamental frequency (F0) of voice is critical to human communication, expression, and singing. Hirano (1) laid the groundwork for understanding of F0 control when he introduced the "body-cover" theory of phonation. He proposed that the histology of the vocal fold lends itself to division into two distinct layers: the "body" layer consisting of the thyroarytenoid (TA) muscle and the adjacent deep collagen fibers, and the "cover" layer consisting of the superficial lamina propria and the epithelium. The body layer is the "active" layer as it is able to shorten with neuromuscular stimulation while the cover layer is the "passive" layer whose tension is affected by the actions of the intrinsic laryngeal muscles. The cover layer has elastic properties necessary for the propagation of mucosal waves that is ultimately responsible for the quality of the generated sound.

The body-cover model of phonation facilitated an explanation of F0 control based on tension or stiffness of the vocal fold. In this model, cover layer stiffness is primarily responsible for F0 control and the TA and the cricothyroid (CT) muscles change the stiffness of the cover layer by altering its length. Contraction of the CT muscle elongates and stiffens the cover layer, thus increasing F0, while activation of the TA muscle shortens the body layer while concurrently creating a slack in the cover layer, thus decreasing F0. This model provides antagonistic roles for TA and CT muscles, and laid the groundwork for F0 control based on variable levels of TA and CT muscle contraction. Interestingly, this also allows for the theoretic possibility to obtain the same F0 at various combinations of TA and CT activation level. While other parameters such as subglottic pressure also affect F0, these other factors are considered minor compared to the activity of the TA and CT muscle (2).

The body-cover model has been further expanded upon using mathematical models where relative contributions of the TA and CT in F0 control have been assigned (2-4), particularly in regards to explaining how TA activity could lead to both increase and decrease in F0. Whereas computational models of F0 control have become more complex and sophisticated, *in vivo* data supporting these models is lacking. While current computational models are based on assumptions derived from measurements of the anatomic, histologic, acoustic, aerodynamic and biomechanical properties of the larynx, and consider the overall stiffness of the cover layer the most important factor in controlling F0, there have been no *in vivo* measurements of cover stiffness with concurrent laryngeal muscle activation. Such *in vivo* investigations have been hampered by lack of a reliable tensionometer to measure stiffness.

Study of vocal fold viscoelasticity has applications beyond the study of F0 control. A reliable quantitative method of measuring vocal fold pliability is necessary to understand vocal fold changes induced by diseases such as vocal fold edema, scar, and neoplasm. A reliable methodology is also necessary to objectively assess the results of vocal fold treatments such as laryngeal reinnervation, lamina propria replacement therapy, and vocal fold replacement with tissue engineering. This study is a preliminary report on the measurement of vocal fold viscoelasticity with laryngeal muscle activation. While the ultimate goal is a systematic and detailed measurement of *in vivo* vocal fold stiffness with individual and combinations of intrinsic muscle activation, we report preliminary results evaluating the feasibility of obtaining reliable measurements of the shear modulus using the LSR during intrinsic laryngeal muscle activation.

MATERIALS AND METHODS

Ex vivo larynx: An adult human larynx was harvested from an autopsy case less than 48 hours post-mortem and kept quick-frozen at -80^{o}C until the day before the experiment. The larynx was then removed from deep freeze and allowed to thaw overnight at -4^{o}C in the refrigerator then kept soaked in isotonic saline in the morning of the experiment until it was thawed soft. The supraglottic structures, including the epiglottis and the false vocal cords, were excised. Arytenoid adduction sutures (3-0 nylon) were then placed through the left muscular processes and brought out through the anterior inferior thyroid lamina to adduct the vocal fold, thus simulating lateral cricoarytenoid (LCA) muscle contraction. A 3-0 nylon suture was placed circumferentially through the anterior cricoid and the anterior inferior border of the thyroid cartilages for manual cricothyroid approximation, thus simulating CT muscle contraction. The larynx was then mounted horizontally on a custom designed laryngeal holder for experimental measurements (Figure 1). Increasing weights in 10 gram increments were placed on the adduction sutures using a pulley mechanism to simulate increasing vocal fold adduction. The cricothyroid approximation suture was tightened to simulate CT muscle action and the shear modulus measured at baseline (no suture tension), medium (CT approximation midway between baseline and maximum), and maximum tension (maximum CT approximation possible with tightening of the CT approximation sutures). The larynx was periodically sprayed with saline to keep the surface moist.

In vivo canine model: A mongrel canine (25 kg) was used. The animal study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act. Our institutional Animal Research Committee approved the research protocol.

After anesthesia was induced with intravenous thiopental the animal was orally intubated and placed under halothane general anesthesia.

A vertical midline skin incision was then made on the anterior neck to widely expose the larynx and the trachea. Bilateral recurrent laryngeal nerves (RLNs) and superior laryngeal nerves (SLNs) were isolated. A low tracheotomy was performed for intra-operative ventilation and the oral endotracheal tube was removed. The larynx was exteriorized into the neck by first performing a suprahyoid pharyngotomy and then by dividing the pharynx circumferentially at this level. This allowed the larynx to be slightly lifted off the neck and fixed in placed using a custom designed laryngeal holder. This exposure allowed placement of the LSR probe on the vocal fold externally and unhindered by oral and pharyngeal structures. Custom designed monopolar electrodes with silicone insulation were applied to the isolated nerves bilaterally. The electrodes were attached to a constant current nerve stimulator (WR Medical Electronics Co., Model 2SLH, St. Paul, Minnesota). The nerves were stimulated at 80 Hz, 1.5 msec pulses, at approximately 0.06 mA increments.

The Linear Skin Rheometer: Measurements of the vocal fold shear modulus were obtained using a modified Linear Skin Rheometer (LSR) (5). This device was originally developed to measure the biomechanical properties of the stratum corneum of skin, and was identified (6) as a potential method to quantify the viscoelastic properties of the human vocal fold, and to quantify the effectiveness of tissue augmentation therapy. This device has been used to measure vocal fold viscoelasticity in a variety of reports (7-14) and the device concept has also been successfully adapted to measure human vocal folds in-vivo (15-16).

The LSR (Figure 2) is a programmable tensionometer capable of measuring displacement to a resolution of 4 μm using a linear variable displacement transducer (LVDT), and force to a resolution of 20 mg using a built-to-order force sensor with a full-scale reading of 50 g. The force sensor can be attached to the tissue under test using a variety of special probes. For this study, a suction cannula probe with a right angle tip and 2 mm diameter was used and the left vocal fold was selected for measurement. The LSR with the probe was aligned at right angles to the longitudinal axis of the vocal fold such that the there was no gap between the tip of the suction probe and the superior medial vocal fold epithelium, at which time 50mbar of suction was applied. The suction force was then released and the instrument zeroed prior to measurement. While some stress is applied to the vocal fold during this maneuver, this arrangement minimizes the effect and also maintains the probe in position. Previous experimentation with various probe designs suggested that the suction probe was the most reliable at maintaining position during vocal fold stimulation (11). Also the LSR operates by applying a cyclical force and any DC offset due to initial loading is removed. The force sensor and suction attachment are gently cycled in a sinusoidal manner such that a cyclical shear force of one gram is applied to the vocal fold. Five measurements were performed at each muscle contraction level.

Calculation of the Shear Modulus: The LSR applies a known amount of force (one gram in this study) and measures the displacement achieved, and this force/displacement data is used to derive the "Dynamic Spring Rate" (DSR). In mechanical engineering the term DSR defines the amount that a spring changes in length when a unit of force is applied to it. It is not a time dependant term. By applying knowledge of the probe geometry, it is possible to estimate the stiffness, or shear modulus, of the vocal fold. Using a simple shear model

(modulus (G) = stress / strain) the geometry of this setup was defined as follows. The shear stress is the shear force (F) applied by the LSR transmitted to the vocal fold cover over the area determined by the probe diameter (A). The shear strain is the resultant displacement (X), which is tangential to the epithelial surface and is measured by the LSR, over the thickness of the lamina propria layer (H). The lamina propria layer thickness (H) was assigned a value of 1 mm for the human larynx and 2 mm for the canine larynx. Thus an estimate for the shear modulus (G) is derived as further elaborated in the appendix.

RESULTS

In the *ex vivo* human larynx, with manual cricothyroid approximation, the shear modulus increased from a baseline value (1307 Pa) to 3.7 times baseline value (4786 Pa) at maximal CT approximation (Figure 3). With gradual increase in the force of arytenoid adduction with graded increase in weights, the vocal fold shear modulus gradually increased from a baseline value (1076 Pa) to a maximum of 1.6 times baseline value (1723 Pa) at an adduction force of 60 grams, and thereafter remained relatively unchanged with increasing weight (Figure 4).

In the *in vivo* canine larynx, with graded neuromuscular stimulation of bilateral SLNs, the vocal fold shear modulus remained stable around the baseline values (1134 Pa) until stimulation level reached 0.23 mA, at which point a hint of cricothyroid activity was noted. Cricothyroid approximation was seen with further stimulation. The shear modulus increased from 0.23 mA to 0.31 mA to a maximum of 2.5 times baseline value (2818 Pa), and thereafter remained stable or slightly decreased with further stimulation (Figure 5). Similarly, when graded neuromuscular stimulation was applied to the RLN, the vocal fold shear modulus remained stable around the baseline value (1077 Pa) until stimulation level reached 0.21 mA, at which point some twitching of the vocal fold without adduction was observed. Vocal fold adduction commenced with further stimulation, and the shear modulus increased to a maximum of 1.6 times baseline (1762 Pa) at 0.32 mA, and thereafter remained stable or slightly decreased with further stimulation (Figure 6).

DISCUSSION

The results of this study indicate that while both RLN and SLN stimulation increase cover stiffness, cricothyroid muscle activity results in the most dramatic increase. In the human ex vivo larynx, CT approximation was applied manually and an almost four fold increase in shear modulus was achieved between baseline and maximal CT approximation. In the canine larynx, *in vivo* SLN stimulation could achieve only a 2.5 fold increase in the shear modulus despite maximal stimulation. The canine response is more physiologic than the manual CT approximation *ex vivo,* because with manual approximation it is possible to nearly completely appose the cricoid and the thyroid cartilages whereas this cannot occur *in vivo*. The cricothyroid muscles insert at the edges of the thyroid and the cricoid cartilages and during physiologic muscular contraction the CT muscles cannot shorten to the degree that the cricoid and the thyroid cartilages would approximate completely.

Control of vocal fold stiffness via RLN stimulation appears more complex. Adductor muscles innervated by the RLN include the interarytenoid, LCA, and the TA muscles. In the *ex vivo* human larynx, gradual increase in the force of arytenoid adduction lead to gradual increase in shear modulus to a maximum increase of 1.6 times baseline value. This result appears physiologically consistent because during arytenoid adduction the vocal process rotates medially and posteriorly, thus adducting and lengthening the vocal fold, which would account for the increase in shear modulus. However, once the limits of the cricoarytenoid joint rotation is reached no further vocal fold lengthening can take place and further force of adduction does not lead to additional increase in the shear modulus.

Interestingly, the increase in shear modulus *in vivo* with RLN stimulation alone also reached a maximal value of 1.6 times baseline value. The *ex vivo* experiment would suggest

that it would be possible to reach this level of increase in the shear modulus with arytenoid adduction (LCA activation) alone. This would suggest that the generally excellent voice outcome after arytenoid adduction surgery for vocal fold paralysis may occur not only through improved closure but better stiffness match with the opposite vocal fold as well (17). However, patients who undergo laryngeal reinnervation in addition to arytenoid adduction for unilateral vocal fold paralysis also report an additional improvement in vocal quality three to six months after surgery, presumably when the laryngeal reinnervation kicks in (17). Therefore, increase in body (TA) stiffness plays an important role in modulating vocal quality. While it would be reasonable to assume that both TA and LCA are contributing to cover stiffness the nature of their individual contributions are unknown. Previous *in vivo* canine studies in our laboratory showed that at high levels of CT stimulation increasing TA activity *decreased* F0, presumably by slackening the cover layer and consistent with the classic cover-body theory (18). However, in the absence of CT activity and in the presence of posterior glottic closure was maintained by isolated activation of the LCA muscles, increasing TA activity resulted in gradual *increase* in F0. Therefore, it seems plausible that at low CT activation levels the medial bulging of the vocal fold during TA activity affects phonation via mechanisms separate from significant viscoelastic changes of the cover layer, such as by improving closure, facilitation of improved entrainment of the vocal folds, and control of the effective depth of vibration as proposed by Titze (3). Future studies systematically measuring cover stiffness with isolated stimulation of adductor branches as well as combinations of adductor muscles could delineate the role of each muscle. Studies are also needed to evaluate and correlate the acoustic and aerodynamic effects of TA activity

with cover stiffness, and experimental strategies to measure the effective depth of vibration need to be developed.

The baseline shear modulus was found to be similar in both the human and the canine larynx. The canine larynx is the closest match to the human larynx, both in its overall dimensions as well as histopathologic characteristics (18), and the canine F0 is also similar to that of the human larynx. Therefore, the similarity in the shear modulus between these larynges likely results from these common characteristics. It is also possible that all laryngeal vibratory systems require similar tissue stiffness to effectively produce sound. Futures studies measuring vocal fold shear modulus from various species of animals and correlating their F0 would be potentially illustrative.

CONCLUSION

Both RLN and SLN stimulation lead to increased viscoelasticity of the vocal fold. However, a more dramatic change in stiffness is seen with CT stimulation. Therefore, CT appears to play a greater role in control of F0 range. The role of the individual laryngeal adductors in changing cover stiffness needs further studies. However, it seems plausible that the TA in particular acts as fine modulator of F0 control.

Appendix

Mathematical model for Derivation of Shear Modulus

A sinusoidal force F is applied to the material under test and the resultant displacement X is logged.

(1) $F = F_{max}Sin(t)$

(2) $X = X_{max}Sin(t+\tau)$

Where
F = instantaneous force
F_{max} = the maximum force
t = time
X = instantaneous displacement
X_{max} = the maximum displacement
τ = the phase shift in radians.

The DSR of the tissue is defined as F max / X max, and is expressed in g/mm. As we are not using the time dependant information associated with the sinusoidal nature of the applied force we can substitute F for F_{max} and X for X_{max}. DSR can then be used to estimate the shear modulus of the displaced vocal fold tissue using knowledge of the geometry of the test site, as follows:

The stress σ is the applied force F_{max} per unit area A given by

(3) $\sigma = F_{max} / A$

The resultant strain ε is given by tangential displacement X_{max} per material thickness H.

(4) $\varepsilon = X_{max} / H$

Shear modulus G is defined as stress per unit strain

(5) $G = \sigma / \varepsilon$

(6) $G = (F_{max} / X_{max}) * (H / A)$

As DSR = F_{max} / X_{max} then

(7) $G = DSR * H / A$

It is important to note that this simple shear model does not make any allowance for the attached tissue, which is also subjected to shear stresses due to displacement of the tissue directly underneath and surrounding the suction attachment. This effect drops off rapidly as the force transmitted through a solid is inversely related to distance. However, a rigorous mathematical solution to describe the elastic processes involved has not been published. In the absence of a mathematical solution for the shear modulus of tissue attached to other tissue, we incorporated a simple correction derived experimentally based on a widely accepted mathematical model developed by W.C. Hayes (19). This model derives shear modulus from indentation data. This correction methodology has been evaluated using data collected from 40 human hemilarynges [10], which were tested using both the Hayes indentation method and the LSR. The data sets from the two methods correlated well when the surface area of attachment used to analyse the LSR data was increased by 0.75 mm in all dimensions. Based on these results, we employed a comparable correction to the data in this study by increasing the diameter of the area of attachment (A) from 2 mm by 1.5 mm to 3.5 m

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FIGURE LEGENDS

Figure 1 Ex vivo larynx set-up for measurement of shear modulus. The suction probe is attached to the left vocal fold.

Figure 2 Schematic of the LSR device for measurement of vocal fold shear modulus.

Figure 3 Shear modulus of the vocal fold (ex vivo human larynx) with manual cricothyroid approximation.

Figure 4 Shear modulus of the vocal fold (ex vivo human larynx) with graded increase in the force of arytenoid adduction

Figure 5 Shear modulus of the vocal fold (in vivo canine larynx) with graded bilateral SLN stimulation

Figure 6 Shear modulus of the vocal fold (in vivo canine larynx) with graded ipsilateral RLN stimulation

Figure 1

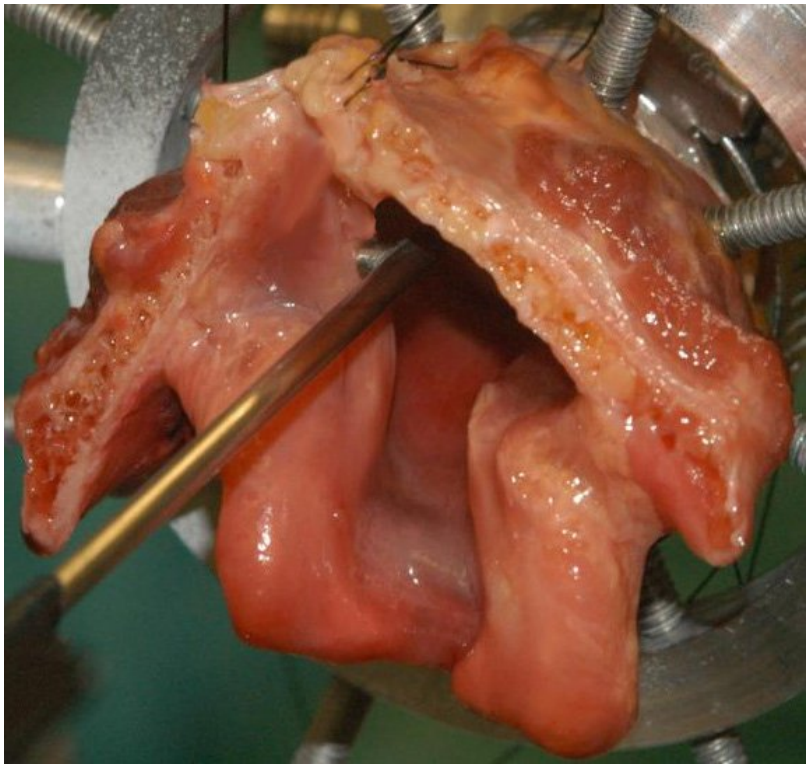
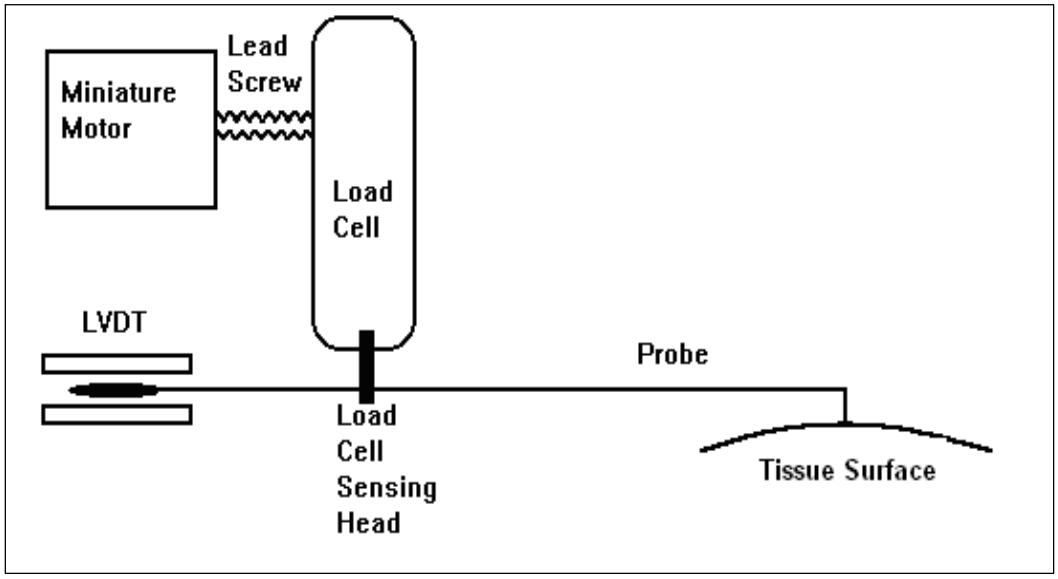


Figure 2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Lead Screw
Load Cell
LVDT
Probe
Load Cell Sensing Head
Tissue Surface

Figure 3

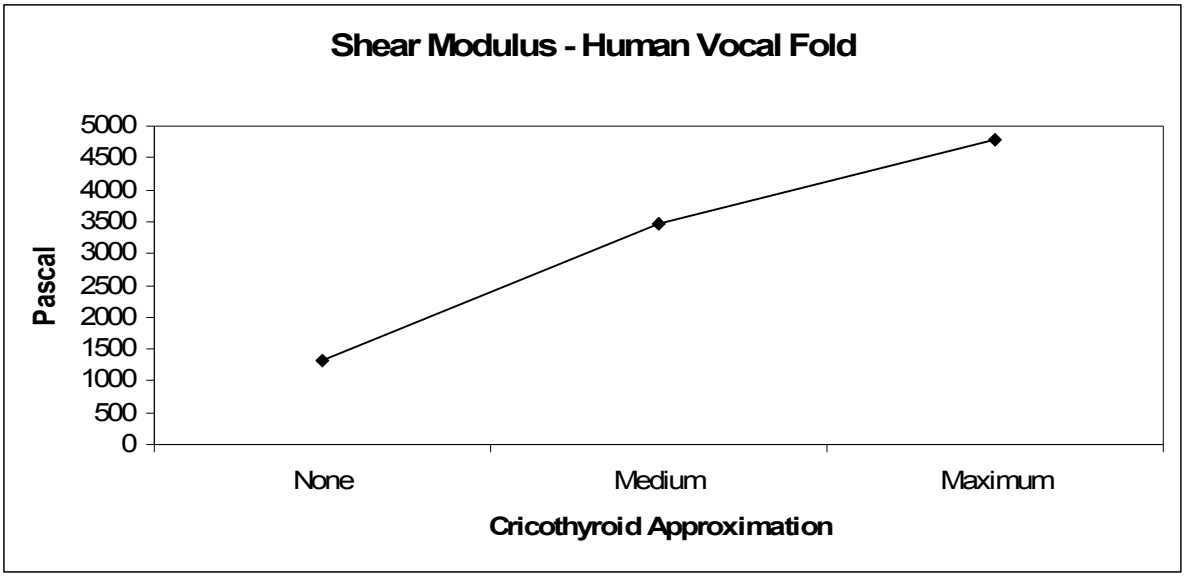


Figure 4

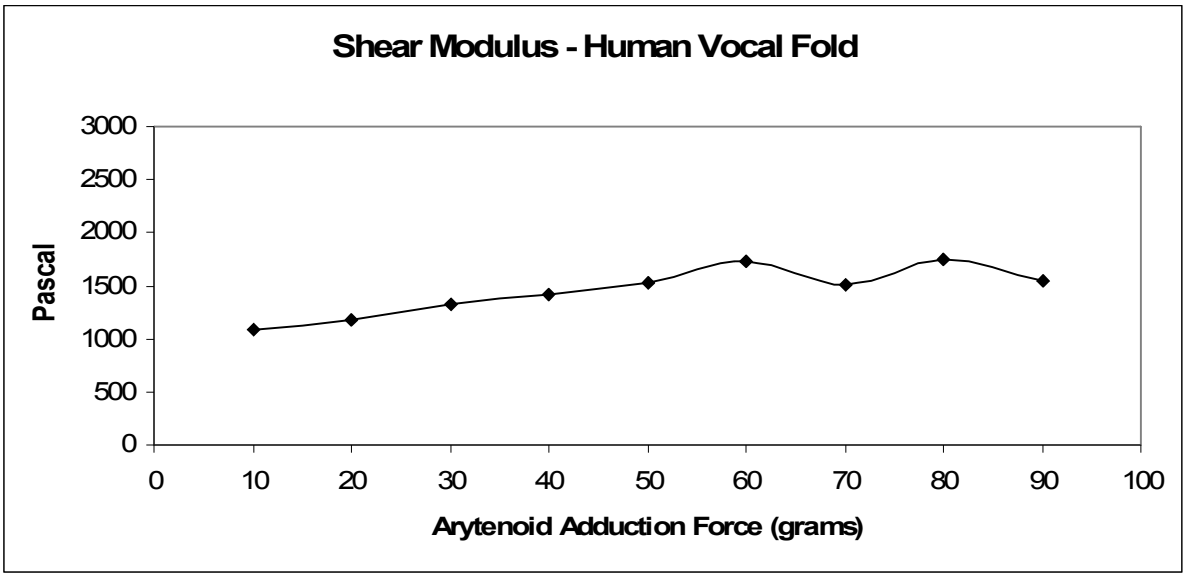


Figure 5

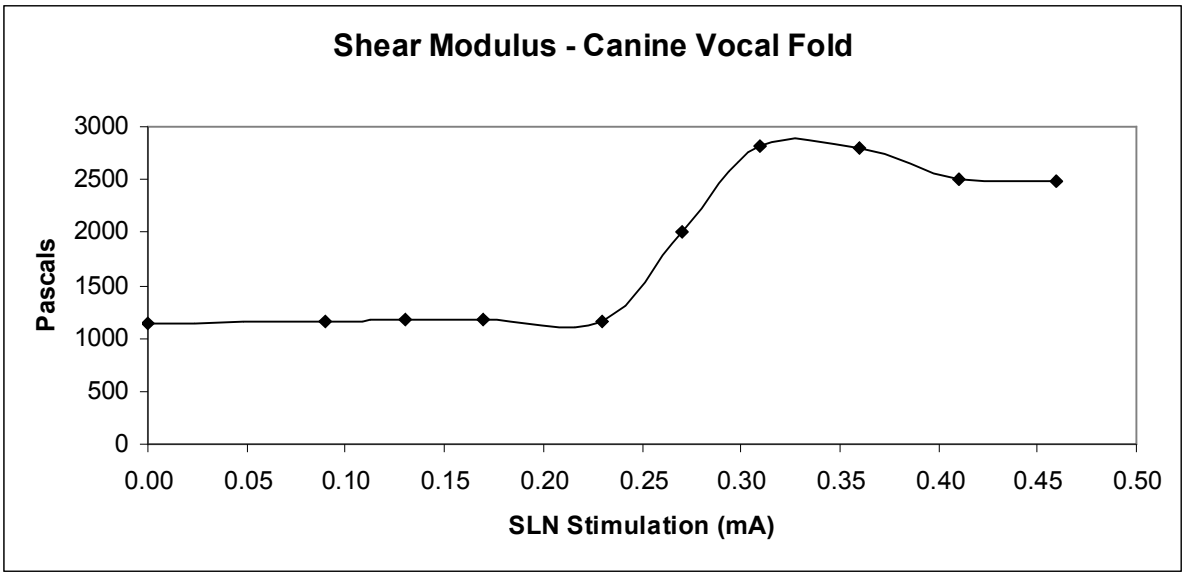
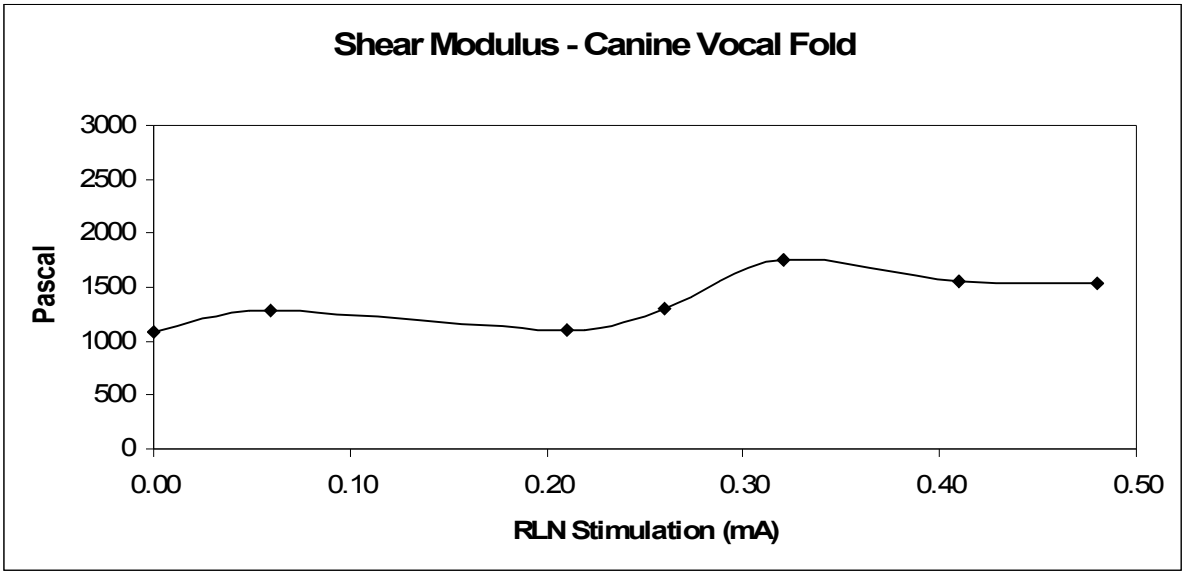


Figure 6



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