Megan J. Williams

Graduate Interdisciplinary Program of
Biomedical Engineering,
University of Arizona,
1130 North Mountain Avenue,
Tucson, AZ 85721
e-mail: mjoy@email.arizona.edu

Urs Utzinger

Department of Biomedical Engineering;
Graduate Interdisciplinary Program of
Biomedical Engineering,
University of Arizona,
Tucson, AZ 85721;
BIO5 Institute,
University of Arizona,
Thomas W. Keating Bioresearch Building,
#131D, P.O. 210240,
Tucson, AZ 85721
e-mail: utzinger@email.arizona.edu

**Julie M. Barkmeier-
Kraemer**

Voice and Swallowing Center,
The University of California—Davis,
2521 Stockton Blvd Suite 6200,
Sacramento, CA 95817
e-mail: jbark@ucdavis.edu

Jonathan P. Vande Geest¹

Associate Professor
Mem. ASME
Department of Aerospace and
Mechanical Engineering;
Department of Biomedical Engineering;
Graduate Interdisciplinary Program of
Biomedical Engineering,
University of Arizona,
Tucson, AZ 85721;
BIO5 Institute,
University of Arizona,
Tucson, AZ 85721
e-mail: jpv1@email.arizona.edu

Differences in the Microstructure and Biomechanical Properties of the Recurrent Laryngeal Nerve as a Function of Age and Location

Idiopathic onset of unilateral vocal fold paralysis (UVP) is caused by damage to the recurrent laryngeal nerve (RLN) and results in difficulty speaking, breathing, and swallowing. This damage may occur in this nerve as it loops around the aortic arch, which is in a dynamic biomechanical environment. The goal of this study is to determine if the location-dependent biomechanical and microstructural properties of the RLN are different in piglets versus adolescent pigs. The neck/distal and thoracic/proximal (near the aortic arch) regions of the RLN from eight adolescent pigs and six piglets were isolated and mechanically assessed in uni-axial tension. Two-photon imaging (second harmonic) data were collected at 5%, 10%, and 15% strain during the mechanical test. The tangential modulus (TM) and the strain energy density (W) were determined at each level of strain. The mean mode of the preferred fiber angle and the full width at half maximum (FWHM, a measure of fiber splay) were calculated from the imaging data. We found significantly larger values of TM, W, and FWHM in the proximal segments of the left RLN when compared to the distal segments ($18.51 \text{ MPa} \pm 1.22$ versus $10.78 \text{ MPa} \pm 1.22$, $p < 0.001$ for TM, $0.046 \text{ MPa} \pm 0.01$ versus $0.026 \text{ MPa} \pm 0.01$, $p < 0.003$ for W, $15.52 \text{ deg} \pm 1.00$ versus $12.98 \text{ deg} \pm 1.00$, $p < 0.001$ for FWHM). TM and W were larger in the left segments than the right ($15.32 \text{ MPa} \pm 1.20$ versus $11.80 \text{ MPa} \pm 1.20$, $p < 0.002$ for TM, $0.038 \text{ MPa} \pm 0.01$ versus $0.028 \text{ MPa} \pm 0.01$, $p < 0.0001$ for W). W was larger in piglets when compared to adolescent pigs ($0.042 \text{ MPa} \pm 0.01$ versus $0.025 \text{ MPa} \pm 0.01$, $p < 0.04$). The proximal region of the left porcine RLN is more stiff than the distal region and has a higher degree of fiber splay. The left RLN of the adolescent pigs also displayed a higher degree of strain stiffening than the right. These differences may develop as a result of the more dynamic environment the left RLN is in as it loops around the aortic arch. [DOI: 10.1115/1.4027682]

1 Introduction

Unilateral vocal fold paralysis/paresis (UVP) occurs when one of the vocal folds becomes paralyzed or has very limited movement, often resulting in difficulty with speaking, swallowing, or breathing. Among those diagnosed with UVP, 38% exhibit aspiration during swallowing due to the impaired closure of the larynx necessary to protect the airway during eating [1]. UVP is caused by damage to the RLN, which is the primary motor input to the muscles of the vocal folds in the larynx [2–6]. On the right side, the RLN branches from the vagus nerve at the level of the subclavian artery. The right RLN loops around the subclavian artery and travels superiorly and medially toward the tracheoesophageal

groove (TEG). On the left side, the left RLN branches from the vagus nerve at the level of the aortic arch. This nerve passes anterior to the aortic arch before looping underneath the arch and traveling superiorly and medially toward the TEG (see Fig. 1) [7].

UVP may occur subsequent to trauma, surgery, or respiratory infection [8–12]. The nerve may become compressed by surrounding structures due to edema resulting in ischemia. It is also possible that the nerve tissues are stretched beyond their limit while retracted from the surgical field resulting in tearing or breakage. In general, the left RLN is impaired more often than the right in humans [13,14]. The more frequent impairment of the left RLN is attributed to its longer path through the thoracic cavity. Thus, pathologies such as tumors involving the lungs, esophagus, or trachea may potentially affect left RLN function [9]. Interestingly, 12–42% of laryngeal paralyses are diagnosed as idiopathic or unknown etiology [15]. Several cases have been published in recent years further elucidating the link between UVP and aortic

¹Corresponding author.

Manuscript received January 22, 2014; final manuscript received April 30, 2014; accepted manuscript posted May 15, 2014; published online June 5, 2014. Assoc. Editor: Barclay Morrison.

Fig. 1 Anatomy of the right and left recurrent laryngeal nerve and surrounding environment. This figure has been reprinted and adapted from Deslauriers, J., 2007, "Anatomy of the Neck and Cervicothoracic Junction," Thoracic Surg. Clin. 17, pp. 529–544. Copyright 2007 by Elsevier, Inc. [50].

or pulmonary artery aneurysm, enlarged atrium, or neoplasm affecting the lower lobe of the left lung [16–19]. Thus, a proportion of those previously diagnosed with idiopathic UVP may have resulted from changes in the aortic arch, pulmonary vein, or atrium resulting in increased mechanical load on the nerve that it was unable to withstand.

Connective tissues of peripheral nerves play an important role in insulation and protection [20–28]. Beginning with the innermost connective tissue layer, nerve fibers are contained within the endoneurium, perineurium, and epineurium. The endoneurium surround individual axons and provide a degree of tensile strength [21,29,30]. Nerve fascicles are contained within several layers of flattened cells that comprise the perineurium which provides tensile strength and elasticity. The most external layer surrounding all of the fascicles within each nerve is the epineurium. This layer contains collagen fibrils, adipose tissue, and elastin to protect nerves from compressive forces to the nerve structure as a whole [21,29]. While it has been hypothesized that the epineurium does provide some protection to the nerve in tension, it does not appear

to be the primary contributor to the tensile mechanical strength of the nerve [25,31–33]. The amount of epineurium present in a peripheral nerve varies relative to the environment through which the nerve travels. For example, peripheral nerves exhibit increased amounts of epineurium where they cross joints [29]. Such increases in epineurium would facilitate improved protection from nerve compression resulting from joint movement [22–24,26–28,34]. Thus, consideration of the composition of the epineurium of peripheral nerves can serve as an important factor when attempting to understand the onset of nerve dysfunction such as described for the RLN.

An investigation of the quantity and composition of epineurium along the length of the RLN was performed in ten juvenile pigs and eight piglets [35]. This study described the composition and relative amount of epineurium in the cross section of the RLN from its origination at the vagus nerve to its insertion into the posterior larynx. This study demonstrated RLN epineurium quantity and composition of three-month-old (i.e., juvenile) pig exhibited more epineurium than found in the piglet, but similar to that found in humans (76–79%). Significantly more epineurium was found in distal segments (neck region) than in proximal segments (thorax region) for both the left and right RLN. However, the right RLN exhibited significantly more epineurium and adipose tissue than the left RLN across all segments. Interestingly, the left RLN exhibited a significant increase in adipose tissue within the distal segments. The proximal segments of the left RLN had more collagen and perineurium than distal nerve segments on either side. These findings support the hypothesis that epineurium and perineurium quantity and composition changes with growth and with anatomical setting. This study supports our hypothesis that due to the anatomical setting of the RLN, the proximal sections of the RLN that loop around arteries, will have different biomechanical properties than the more distal sections in the neck. To further test this hypothesis RLN nerves of piglets were segmented and uniaxially stretched to quantify mechanical differences between nerve segment locations [36]. The general finding from this preliminary study was that biomechanical differences exist along the length of the left RLN and between the left and right RLN. In response to stretch, the left RLN exhibits increased stiffness at the level of the aortic arch. The right RLN exhibits greater stiffness than the left RLN. The differences between the left and right RLN may reflect differences in the anatomical setting that already exist at birth in the piglet [36]. These findings support Sunderland's theory that connective tissues within peripheral nerves may develop in response to environmental forces to which the nerve is exposed [22–25,37,38].

The purpose of this study is to determine whether the biomechanical and microstructural properties of RLN tissues change with growth and development and whether these properties differ between location (thorax versus neck). Outcomes of this research may elucidate if the onset of UVP is associated with deformation of RLN connective tissues due to surgery, disease, and/or diameter/compliance changes in the aortic arch or subclavian artery. The structure–function relationships of the protective components of the nerve may provide a direct link between the structural organization of the RLN, the complex mechanical environment that surrounds it, and the resulting RLN deformations.

2 Methods

2.1 Specimen Preparation. All animal specimens were acquired from the Animal Facility at the University of Arizona Health Sciences Center. None of the animals used in this research underwent prior procedures affecting the central or peripheral nervous system, cardiovascular system, or tissues in the neck or chest. There were six piglets (7–11 days old) and eight adolescent pigs (four months old) utilized in this study. Left and right RLNs from each adolescent pig and each piglet were excised along their

entire length from the origin at the vagus nerve until it inserts into the posterior larynx. Dissections were completed with the RLN—vagus nerve branch in tact so that nerves were coded for their anatomical setting (the branch noting the proximal end). Each nerve was sectioned into a distal and proximal sample. All nerve samples were stored in physiological saline at 4°C until the time of testing within 24 h after tissue harvest. Adolescent pig RLNs were measured to have an average diameter of 1.58 ± 0.06 mm while the piglet RLNs were measured to have an average diameter of 0.74 ± 0.03 mm. All data in this paper is reported as mean $\pm$ standard error of mean the mean.

2.2 Uni-axial Testing. The organization of the collagen fibers within the RLN epineurium and their changes during biomechanical stress were studied using a custom designed uni-axial testing device. This device allows biomechanical testing underneath a multiphoton microscope. This device was designed to conduct uni-axial tensile testing of a specimen through controlled changes in applied strain or load. The assessment of RLN mechanical behavior was performed using a strain-controlled experiment. The resistance to given deformation was quantified using a 5lb in-line load cell. Each nerve sample was mounted in a closed bath filled with phosphate-buffered saline (PBS), pH-7.4 warmed to $37 \pm 0.7^\circ\text{C}$. A minimal load of 3 g was applied to ensure that the nerve was in a state of pretension before testing commenced. The nerve was stretched in increments of 5%, 10%, and 15% of the initial length at a rate of 0.03 mm/min. The average gauge length for all nerve specimens was 24.5 ± 1.41 mm. At each stepwise administration of strain the multiphoton microscope was used to image collagen fibers within the RLN.

2.3 Multiphoton Imaging. Our device has been specifically designed for integration with the Advanced Intravital Microscope (AIM) for multiphoton imaging at the University of Arizona's BIO5 Institute. Multiphoton microscopy offers advantages of superior image quality, deeper optical sectioning, and reduced photo damage for hydrated ex vivo tissues when attempting to visualize the extracellular matrix microstructure [39]. The AIM uses a pulsed Titanium–Sapphire laser (680–1060 nm) for simultaneous two-photon excitation and second harmonic imaging. For this study an Olympus UMPLFLN 10 $\times$ water immersion objective with a numerical aperture of 0.3 was used with a laser power on the sample of 40 mW and excitation wavelength of $\lambda = 780$ nm. Collagen visualization came from second harmonic generation (SHG) collected through a bandpass filter (377/50). Image stacks were taken for each specimen at 0%, 5%, 10%, and 15% strain, with each stack having an acquisition time of approximately 10 min. Image stacks were taken of the specimen using an $800 \times 800 \mu\text{m}^2$ field of view at $3 \mu\text{m}$ steps imaging through the epineurium of the nerve to a depth of approximately $250 \mu\text{m}$. From these images, the preferred orientation of the collagen fibers was determined at each level of applied strain.

2.4 Image Analysis. Image stacks of each specimen contained an SHG channel that was used to determine collagen fiber orientations using a custom written fiber-orientation analysis script for MATLAB (R2013a, MathWorks, Natick, MA) [40]. The software overlays vectors in the direction of fibers, from which histograms were generated of the collagen fiber orientations for each 0%, 5%, 10%, and 15% stacks from each specimen. Vector orientations are included from every image in an image stack giving a view of the cumulative fiber orientation through the thickness. The stretch dependent mean mode and full width at half maximum (FWHM) of the fiber histograms were used as indicators of the preferred fiber alignment and spread of fiber orientation, respectively. From this changes in fiber alignment or fiber dispersion that occur with nerve stretch were quantified.

2.5 Constitutive Model. Data collected from the uni-axial mechanical tests were analyzed using MATLAB. Cauchy stress, T_{11} , was calculated at each time point as

$$T_{11} = \left(\frac{f_1}{A_0} \right) \lambda_1 \quad (1)$$

where f_1 is the instantaneous load, A_0 is the initial cross sectional area (assumed to be circular), and λ_1 is the stretch.

Following the work of Raghavan and Vorp [41], we modeled the nonlinear mechanical behavior of the RLN as a homogeneous, incompressible, isotropic, and hyperelastic material using a strain energy density function as shown in our previous work [36].

Equation (2) was used in this study to model each nerve segment's behavior during uni-axial tensile testing,

$$T_{11} = [2\alpha + 4\beta(\lambda_1^2 + 2\lambda_1^{-1} - 3)][\lambda_1^2 - \lambda_1^{-1}] \quad (2)$$

2.6 Data Analysis. A custom written MATLAB program was used to generate individual specimen T_{11} – λ_1 plots. The program was also used to calculate a TM and a strain energy density (W) at each strain step for each specimen. The constitutive model (2) was fit to each nerve segment's T_{11} – λ_1 data to determine the values of α and β using nonlinear optimization in MATLAB. α and β were constrained to be greater than or equal to zero. Figure 2 shows the Cauchy stress–stretch ratio data from a representative adolescent pig specimen (A) and a representative piglet specimen (B) and corresponding fits using Eq. (2).

2.7 Statistical Analysis. One-sample Kolmogorov–Smirnov tests were used to determine the normality of the data for the end points TM, W , α , β , mean mode, and FWHM. All of the end points

Fig. 2 Cauchy stress–stretch ratio data from a representative piglet specimen (a) and a representative adolescent pig specimen and (b) with corresponding fits with α and β values

rejected the null hypothesis that the data is normal ($\alpha = 0.05$). A Box–Cox transformation was then utilized to normalize the data so that statistical analyses of the independent variables and between interactions of independent variables could be performed using a commercial statistical software package (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0, IBM Corp., Armonk, NY). A repeated measures analysis of variance (ANOVA) was chosen to evaluate the significance of the following independent variables in each end point: side (left and right), age (adolescent and piglet), location (proximal and distal), and strain level (0–3).

All of the graphical data are presented using error bars equal to the standard error of the measurement. The mean values and standard errors are back-calculated from the Box–Cox transformed data (Box–Cox transformed mean and Box–Cox transformed distribution, respectively). Post hoc analyses consisting of student's t -tests with Bonferroni correction were performed to compare specific mean values of significant two-way interactions, since there were no significant three-way or four-way interactions in our results. The Bonferroni correction method was chosen as it is the most conservative method to account for familywise error rate.

3 Results

3.1 Biomechanical Response

Tangential Modulus. In both the adolescent pigs and the piglets there was a strain stiffening effect with the TM being larger at larger values of strain (Fig. 4(a)). A significant main effect ($p < 0.002$) was identified for side with the average TM being larger in the left nerve samples ($15.32 \text{ MPa} \pm 1.20 \text{ MPa}$) than the right ($11.80 \text{ MPa} \pm 1.20 \text{ MPa}$) (Fig. 4(b)). A significant interaction was found for side $\times$ location ($p < 0.001$). Figure 4(c) shows that among the proximal nerve segments those from the left nerve have a higher average TM ($18.51 \text{ MPa} \pm 1.22 \text{ MPa}$) than those from the right nerve ($10.78 \text{ MPa} \pm 1.22 \text{ MPa}$). Figure 4(c) also shows that among left nerve specimens those that are proximal nerve segments have a significantly larger TM ($18.51 \text{ MPa} \pm 1.22 \text{ MPa}$) than distal segments ($12.60 \text{ MPa} \pm 1.22 \text{ MPa}$).

Strain Energy Density (W). The piglet specimens had larger values of strain energy at all stretch increments than the adolescent pigs (Fig. 5(a)). A significant main effect ($p < 0.038$) was identified for age with the average W being larger in the piglets ($0.042 \text{ MPa} \pm 0.01 \text{ MPa}$) than in the adolescent pigs ($0.025 \text{ MPa} \pm 0.01 \text{ MPa}$) (Fig. 5(b)). A significant main effect ($p < 0.0001$) was also identified for side with the average W being larger (0.038 ± 1.07) in left nerve samples than right (0.028 ± 0.01) (Fig. 5(b)). A significant interaction was found for age $\times$ location ($p < 0.022$). Figure 5(c) shows that within the distal segments, the piglets had a significantly larger W ($0.044 \text{ MPa} \pm 0.01 \text{ MPa}$) than the adolescent pigs ($0.021 \text{ MPa} \pm 0.01 \text{ MPa}$). Also, within the adolescent pigs, the proximal segments had a significantly greater W ($0.030 \text{ MPa} \pm 0.01 \text{ MPa}$) than the distal segments ($0.021 \text{ MPa} \pm 0.01 \text{ MPa}$). A significant interaction was found for side $\times$ location ($p < 0.003$). Figure 5(d) shows that within the proximal segments, the left specimens have a larger average W (0.046 ± 0.01) than the right specimens (0.026 ± 0.01). Also among the left specimens, the proximal segments have a larger average W (0.046 ± 0.01) than the distal segments (0.032 ± 0.01).

Constitutive Modeling. α : A significant main effect ($p < 0.006$) was identified for age with the average α being larger in the piglets ($1.55 \text{ MPa} \pm 1.20 \text{ MPa}$) than the adolescent pigs ($0.70 \text{ MPa} \pm 1.17 \text{ MPa}$) (Fig. 6(a)). A significant interaction was found for side $\times$ location ($p < 0.044$). Figure 6(b) shows that among proximal nerve segments the left specimens have a larger average value of α ($1.42 \text{ MPa} \pm 1.21 \text{ MPa}$) than the right specimens ($0.78 \text{ MPa} \pm 1.21 \text{ MPa}$).

β : A significant main effect ($p < 0.014$) was identified for age with the average β being larger in the adolescent pig specimens

($8.36 \text{ MPa} \pm 1.38 \text{ MPa}$) than the piglet specimens ($3.93 \text{ MPa} \pm 1.44 \text{ MPa}$) (Fig. 6(b)).

3.2 Image Analysis. Representative SHG images of a left proximal segment of adolescent pig RLN and a right proximal segment of piglet RLN at 0% strain and 15% strain are shown in Fig. 3.

Full Width Half Maximum. There was no significant main effect identified for FWHM, however there was a significant interaction found for side $\times$ location ($p < 0.0001$). Figure 7(a) shows that among the distal nerve segments the right specimens had a significantly larger FWHM ($15.56 \text{ deg} \pm 1.00 \text{ deg}$) than the left specimens ($13.72 \text{ deg} \pm 1.00 \text{ deg}$) and among the proximal segments the left specimens showed a larger FWHM ($15.52 \text{ deg} \pm 1.00 \text{ deg}$) than the right ($12.98 \text{ deg} \pm 1.00 \text{ deg}$). Additionally, Fig. 7(a) shows that within the left nerve specimens the proximal segments had a significantly larger FWHM ($15.52 \text{ deg} \pm 1.00 \text{ deg}$) than the distal segments ($13.72 \text{ deg} \pm 1.00 \text{ deg}$) and within the right nerve specimens the distal segments had a larger FWHM ($15.56 \text{ deg} \pm 1.00 \text{ deg}$) than the proximal segments ($12.98 \text{ deg} \pm 1.00 \text{ deg}$).

Mean Mode: A significant main effect ($p < 0.004$) was identified for location with the average mean mode being larger in the proximal segments ($89.43 \text{ deg} \pm 4.13 \text{ deg}$) than the distal segments ($88.13 \text{ deg} \pm 4.16 \text{ deg}$) (Fig. 7(b)).

Please refer to Table 1 for a summary of all main effects including the mean differences, standard error and associated p values. Also refer to Table 2 for a summary of all pairwise comparisons completed for all significant interactions including the mean differences, standard error, and associated p values.

4 Discussion

Our data suggests that overall piglet RLN tissues have a larger W and TM than those of the adolescent pigs and would therefore be more resistant to deformation (Figs. 4(b) and 5(b)). However, values of TM at strains above 10% and values of β are larger in the adolescent pigs than in the piglets (Figs. 4(a) and 6(b)). When looking closer at differences in nerve segment location, results in W suggest that in adolescent pigs the proximal segments (near aortic arch) are more stiff than the distal segments (Fig. 5(c)). We found the same result when comparing proximal and distal segments of the left nerves, W , TM, and α were larger in the proximal segment compared to the distal (Figs. 4(c), 5(d), and 6(a)). It was also shown in the results for W , TM, and α that for proximal segments, the left nerves are more stiff than the right nerves (Figs. 4(c) and 5(d)). Our previous study showed that larger values of α result in larger values of stiffness (slope of the Cauchy stress–stretch curve) at low values of stretch and larger values of β result in an increase in stiffness at higher values of stretch [37]. Therefore, the differences in α and β highlight that the adolescent pigs are more resistant to deformation than piglets at higher values of stretch while the piglets are more resistant to deformation at lower values of stretch.

In our previous study of two-day-old piglet specimens we calculated maximum tangential modulus (MTM), instead of a TM at each strain step, and parameters α and β as was done in this study. The average yield stress calculated for each piglet nerve segment group ranged from $1.30 (\pm 0.05) \text{ MPa}$ to $2.52 (\pm 1.25) \text{ MPa}$ associated with 15–25% strain [36]. When comparing to this study we find similar results with maximum stress values ranging from $0.91 (\pm) \text{ MPa}$ to $3.28 (\pm) \text{ MPa}$ associated with 15% strain. The results of the previous study showed the parameters α , β , and MTM to be larger in the left proximal segments compared to the distal segments. This is comparable to the results we found in the current study with values of TM, W , α , and β in the left proximal segments being larger than that for the distal segments. The previous study also found the left nerves to have smaller values of α (proximal and distal segments) and MTM (distal segments only) than the right nerves where β was larger in the left nerves (proximal and distal segments). The results of this study show that for

Fig. 3 Representative SHG multiphoton images of a proximal segment of piglet RLN (left) and a proximal segment of adolescent pig RLN (right) at 0% strain (top) and 15% strain (middle). SHG channel depicts the collagen content of the tissue. These images are taken approximately 100 μm into the adolescent pig segment and 60 μm into the piglet segment. The red arrows indicate the angle at which 90 deg is measured. Histograms (bottom) show the overall collagen fiber orientations throughout the tissue at 0% and 15% strains.

proximal nerves only, the left nerves have larger values of α , β , W , and TM (indicators of stiffness) than the right nerves. However for the distal segments the right nerves have larger values of α , β , W , and TM . The minor differences in these results may be attributed to the differences in age and size of the animals used in the studies or to the differences in testing protocols.

Qualitatively the microstructure of the piglet nerve segments and adolescent pig nerve segments look very different (Fig. 3). It is clear that the adolescent pig specimens have a larger nerve diameter and also appear to have larger individual fiber diameters. Additionally, the collagen fibers of the piglet nerve segments appear to be more densely packed together. Quantitatively there were no significant changes in fiber dispersion (FWHM) or alignment (mean mode) with increased strain within any group of nerve segments. This may be attributed to the fact that nearly all

of the collagen appeared to be aligned and all of the fiber crimp removed as the segments were loaded into the device. There was little to no change in fiber alignment because the preferred direction of the collagen fiber is along the length of the nerve in both adolescent pigs and piglets (Fig. 3). The only difference found in FWHM was that in left nerves the proximal segments have a larger FWHM than the distal segments and the reverse was true for right nerves (Fig. 7(a)). This is the same result that was found for W , TM , and α . This suggests that segments with higher values of stiffness may also have higher degrees of fiber splay. Additionally, only one significant result was found for mean mode, in which we found a mean mode value closer to 90 deg (longitudinal alignment) in the proximal segments than the distal (Fig. 7(b)). This result may not have physiological significance, however, since the relative difference was small (88.1 deg versus 89.4 deg).

Fig. 4 (a) Average TM is reported for adolescent and piglet nerves at each value of stretch; (b) average TM is reported for all adolescent and piglet RLN segments and all left and right RLN segments (* $p < 0.002$); and (c) average TM is reported for proximal and distal segments of the left and right RLN (*, † $p < 0.001$)

Fig. 6 (a) Average values of α and β are reported for all adolescent pig and piglet RLN segments (* $p < 0.006$, † $p < 0.014$); (b) average α is reported for all proximal and distal segments of the left and right RLN (* $p < 0.011$)

Fig. 5 (a) Average W is reported for adolescent and piglet nerves at each value of stretch; (b) average W is reported for all adolescent and piglet RLN segments (* $p < 0.038$) and all left and right RLN segments († $p < 0.001$); (c) Average W is reported for all proximal and distal segments of adolescent pigs and piglets (†, † $p < 0.008$; *, § $p < 0.007$); and (d) Average W is reported for all proximal and distal segments of the left and right RLN (*, † $p < 0.002$)

Fig. 7 (a) Average FWHM is reported for all proximal and distal segments of all left and right RLNs ($\dagger, *p < 0.004$; $\ddagger, §p < 0.001$); (b) average mean mode is reported for all proximal and distal segments ($*p < 0.004$)

Looking back at the results of the histological data previously published by Campbell et al., we can make some interesting comparisons with our mechanical data. They reported a higher percentage of epineurium in the right RLN when compared to the left, regardless of age. They also report a higher percentage of epineurium in the piglets compared to the adolescent pigs. These findings appear to have an inverse relationship in our results with greater stiffness in the left nerve compared to the right and greater stiffness in the piglet compared to the adolescent pig (at low strains).

The findings of Campbell et al. were, however, different for the percentage of collagen that comprises the epineurium and the percent perineurium. The authors found that the epineurium of the

left RLN exhibited a greater percentage of collagen than the right and also that piglet samples had a greater percentage of collagen found in the epineurium than adolescent pigs. There was also a significant difference found between the proximal and distal segments of the adolescent pig, with the proximal segments exhibiting more collagen than the distal segments.

When taking a look at percent perineurium, the authors of the histological study found a larger percentage of perineurium for the left RLN compared with the right. In addition, the average percentage of perineurium was higher in the piglet RLN than in the adolescent pig.

The mechanical testing done in this study was purely tensile. It has been reported that perineurium may be the primary contributor to tensile strength of peripheral nerves [25,31–33]. If this is true then the results of the mechanical data are what is expected given the results of the histological study [35]. More perineurium in the right RLN and the piglet RLN may lead to greater values of tensile strength in these specimens. While epineurium is a contributing factor to tensile strength of a nerve, it is the collagen fibers of the epineurium that provide the primary resistance to stretch [25,31–33,35]. This may mean that the larger percentage of epineurial collagen in the left RLN, the piglet RLN, and the proximal RLN of the adolescent pig is a contributing factor to the higher levels of stiffness we found in these segment groups.

To the author's knowledge the only other study on the mechanical behavior of the RLN is the study we ourselves previously published in Alexander et al. [36]. However there have been studies on the mechanical properties of other peripheral nerves that may allow us to make some useful comparisons.

Other research groups have performed studies on the mechanical behavior and/or the microstructure of other peripheral nerves including but not limited to the ulnar nerve, tibial nerve, median nerve, and sciatic nerve [32,42–49]. Ma et al. published a study of adult human cadaver ulnar nerves stretched at a rate of 0.05 mm/s up to 10% strain found an average maximum value of tensile stress to be 0.18 MPa which is lower on average than the stresses we have calculated in the porcine RLN [43]. In another study of the sciatic nerve of female guinea pigs, Young's Modulus was reported at 5%, 10%, 15%, and 20% strain, being 32 MPa (± 13) at 15% which is the maximum stretch we achieved in our study [46]. This is higher than the values we reported which may be due to differences in species and/or differences in the amount of force imposed on the sciatic nerve than the RLN.

Table 1 Summary of key main effects identified for age, location, and side for all independent variables (TM, W , α , β , FWHM, mean mode)

		Age (piglet versus adolescent pig)	Side (left versus right)	Location (proximal versus distal)
TM (MPa)	Mean difference	1.25	1.50 ^a	1.19
	Standard error	1.39	1.14	1.14
	P	0.516	0.002	0.191
W (MPa)	Mean difference	1.33 ^a	1.19 ^a	1.06
	Standard error	1.13	1.05	1.05
	P	0.038	0.000	0.196
α (MPa)	Mean difference	2.05 ^a	1.28	1.12
	Standard error	1.26	1.16	1.16
	p	0.006	0.093	0.480
β (MPa)	Mean difference	3.25 ^a	2.08	1.29
	Standard error	1.6	1.61	1.73
	p	0.014	0.116	0.610
FWHM (deg)	Mean difference	1.01	1.00	1.00
	Standard error	1.01	1.00	1.00
	p	0.292	0.349	0.304
Mean mode (deg)	Mean difference	2.48	4.56	8.08 ^a
	Standard error	5.65	4.27	4.27
	p	0.826	0.265	0.004

^aThe mean difference is significant at the 0.05 level.

Table 2 Summary of all pairwise comparisons made for all significant interactions

Tangential modulus (TM) (MPa)				
Side $\times$ location		Mean difference	Standard error	<i>p</i>
Left	Proximal/distal	1.81 ^a	1.20	0.001
Right	Proximal/distal	−1.31	1.20	0.139
Distal	Left/right	−1.04	1.21	0.847
Proximal	Left/right	2.24 ^a	1.21	0.000
Strain energy density (W) (MPa)				
Age $\times$ location		Mean difference	Standard error	<i>p</i>
Distal	Piglet/adolescent pig	1.48 ^a	1.14	0.008
Proximal	Piglet/adolescent pig	1.19	1.14	0.200
Adolescent pig	Proximal/distal	1.19 ^a	1.06	0.007
Piglet	Proximal/distal	1.05	1.07	0.504
Side $\times$ location		Mean difference	Standard error	<i>p</i>
Left	Proximal/distal	1.23 ^a	1.07	0.002
Right	Proximal/distal	−1.09	1.07	0.215
Distal	Left/right	1.03	1.07	0.708
Proximal	Left/right	1.37 ^a	1.07	0.000
α (MPa)				
Side $\times$ location		Mean difference	Standard error	<i>p</i>
Left	Proximal/distal	1.58	1.27	0.056
Right	Proximal/distal	−1.26	1.27	0.339
Distal	Left/right	−1.06	1.27	0.801
Proximal	Left/right	1.86 ^a	1.27	0.011
FWHM (deg)				
Side $\times$ location		Mean difference	Standard error	<i>p</i>
Left	Proximal/distal	1.01 ^a	1.01	0.004
Right	Proximal/distal	−1.02 ^a	1.01	0.000
Distal	Left/right	1.02 ^a	1.01	0.004
Proximal	Left/right	−1.02 ^a	1.01	0.000

^aThe mean difference is significant at the 0.05 level.

Future research in our laboratory will include a finite element analysis of the left RLN and its interaction with the aortic arch which it loops around. The material properties determined from these tensile experiments will be implemented in a parametric analysis to simulate the effects of stretch on nondiseased RLN tissue. Additionally we would like to observe the effects of compression on the RLN. The custom built apparatus designed for the tensile experiments has also been adapted to allow compressive loading of the nerves while simultaneously imaging them as we have done here. The final goal is to directly test whether individuals with idiopathic onset of left-sided UVP exhibit aortic aneurysm or have an increased aortic compliance compared to age-matched controls. Prior clinical research has identified that a small proportion of those previously identified with idiopathic left-sided UVP had a thoracic aneurysm [16,17]. Thus, compliance changes will be studied to investigate the possibility that compliance change alone may be linked to onset of left-sided UVP. Related to this goal is to determine the range of deformations the RLN undergoes throughout a cardiac cycle as it passes around the aortic arch and in close proximity to the pulmonary artery.

This study was not without limitations. The most significant limitation was the time required to acquire multiphoton images. Each z-stack of images takes approximately 10 min to complete. This prevented us from measuring fiber data as a continuous function of stretch. The multiphoton microscope also restricts us to less than 300 μ m of depth through the nerve specimens that equates to less than 25% through the largest diameter specimens and just over 50% through the smaller specimens. The left RLN in

particular is subject to a complex mechanical environment. While it is tethered to the aortic arch by way of the ligamentum arteriosum and the pulmonary artery, we do not expect sliding between the nerve and the aorta; however, we do expect it to be subjected to pulsatile tensile and compressive forces caused by the internal pressure of the arteries and also aortic root motion. This study measures the ability of the RLN to withstand stretch but not compression, which is a focus of our future research in RLN biomechanics. Finally, the cost and availability of pigs also restricted the age of the tissue that was available to us. It would be interesting to also observe mechanical and microstructural behavior of much older specimens to further look at changes with aging.

In conclusion, the mechanical properties and microstructure of the left RLN are heterogeneous with the proximal region, which loops around the aortic arch, exhibiting greater stiffness than the distal or neck region. The higher stiffness and greater degree of collagen fiber splay may provide this region of the RLN the necessary structure to counter forces imposed by the aortic arch, pulmonary artery, and any neighboring tissues that contact it. There were not any significant differences found in the microstructure between the adolescent pigs and the piglets, however W and α were significantly greater in piglets while β was significantly greater in adolescent pigs. This may mean that the piglet RLN tissues are more resistant to stretch until greater levels of strain ($>10\%$) are reached at which point the adolescent pig RLN tissues become more stiff. This study applies an innovative biomechanical and imaging approach that allows simultaneous imposition of nerve stretch while directly observing and measuring connective tissue changes within the epineurium. Ongoing work in our laboratory will focus on exploring other methods of nerve deformation, including compression.

Acknowledgment

The authors would like to thank Mark Borgstrom (Office of Student Computing Resources, University of Arizona) for his statistical support.

This research was funded by the NIH, Grant No. NIDCD-1R01DC011311-01A1. MJW was also partially supported by the CMMBS Training Grant—NIH Grant No. GM084905. Imaging was performed on an NIH sponsored shared device NIH/NCRRS10RR023737.

References

- [1] Heitmiller, R. F., Tseng, E., and Jones, B., 2007, "Prevalence of Aspiration and Laryngeal Penetration in Patients With Unilateral Vocal Fold Motion Impairment," *Dysphagia*, **15**(4), pp. 184–187.
- [2] Dedo, H. H., 1970, "The Paralyzed Larynx: An Electromyographic Study in Dogs and Humans," *Laryngoscope*, **80**(10), pp. 1455–1517.
- [3] Duncan, I. D., Griffiths, I. R., McQueen, A., and Baker, G. O., 1974, "The Pathology of Equine Laryngeal Hemiplegia," *Acta Neuropathol.*, **27**(4), pp. 337–348.
- [4] Hillstrom, R. P., Cohn, A. M., and McCarroll, K. A., 1990, "Vocal Cord Paralysis Resulting From Neck Injections in the Intravenous Drug Use Population," *Laryngoscope*, **100**(5), pp. 503–506.
- [5] Rosin, D. F., Handler, S. D., Potsic, W. P., Wetmore, R. F., and Tom, L. W. C., 1990, "Vocal Cord Paralysis in Children," *Laryngoscope*, **100**(11), pp. 1174–1179.
- [6] Ward, P. H., and Berci, G., 1982, "Observations on So-Called Idiopathic Vocal Cord Paralysis," *Ann. Otol., Rhinol., Laryngol.*, **91**(6 Pt 1), pp. 558–563.
- [7] Haller, J. M., Iwanik, M., and Shen, F. H., 2012, "Clinically Relevant Anatomy of Recurrent Laryngeal Nerve," *Spine*, **37**(2), pp. 97–100.
- [8] Kelchner, L. N., Stemple, J. C., Gerdeman, E., Le Borgne, W., and Adam, S., 1999, "Etiology, Pathophysiology, Treatment Choices, and Voice Results for Unilateral Adductor Vocal Fold Paralysis: A 3-Year Retrospective," *J. Voice*, **13**(4), pp. 592–601.
- [9] Mu, L., and Yang, S., 1991, "An Experimental Study on the Laryngeal Electromyography and Visual Observations in Varying Types of Surgical Injuries to the Unilateral Recurrent Laryngeal Nerve in the Neck," *Laryngoscope*, **101**(7 Pt 1), pp. 699–708.
- [10] Quiney, R. E., and Michaels, L., 1990, "Histopathology of Vocal Cord Palsy From Recurrent Laryngeal Nerve Damage," *J. Otolaryngol.*, **19**(4), pp. 237–241.

- [11] Sannella, N. A., Tober, R. L., Cipro, R. P., Pedicino, J. F., Donovan, E., and Gabriel, N., 1990, "Vocal Cord Paralysis Following Carotid Endarterectomy: The Paradox of Return of Function," *Ann. Vasc. Surg.*, **4**(1), pp. 42–45.
- [12] Teixido, M. T., and Leonetti, J. P., 1990, "Recurrent Laryngeal Nerve Paralysis Associated With Thoracic Aortic Aneurysm," *Otolaryngol., Head Neck Surg.*, **102**(2), pp. 140–144.
- [13] Cuning, D. S., 1955, "Unilateral Vocal Cord Paralysis," *Ann. Otol., Rhinol., Laryngol.*, **64**(2), pp. 487–493.
- [14] Huppler, E. G., Schmidt, H. W., Devine, D., and Gage, R. P., 1955, "Causes of Vocal-Cord Paralysis," *Proc. Staff Meet. Mayo Clin.*, **30**(22), pp. 518–521.
- [15] Sulica, L., 2008, "The Natural History of Idiopathic Unilateral Vocal Fold Paralysis: Evidence and Problems," *Laryngoscope*, **118**(7), pp. 1303–1307.
- [16] Bando, H., Nishio, T., Bamba, H., Uno, T., and Hisa, Y., 2006, "Vocal Fold Paralysis as a Sign of Chest Diseases: A 15-Year Retrospective Study," *World J. Surg.*, **30**(3), pp. 293–298.
- [17] Coscaron Blanco, E., Blanco Garcia, J. L., and Gomez Gonzalez, J. L., 2006, "An Uncommon Cause for Unilateral Vocal Fold Paralysis: Aortic Aneurysms. Case Report and Literature Review," *Otorrinolaringol. Ibero. Am.*, **33**(5), pp. 481–487.
- [18] Escribano, J. F., Carnés, J., Crespo, M. A., and Antón, R. F., 2006, "Ortner's Syndrome and Endoluminal Treatment of a Thoracic Aortic Aneurysm: A Case Report," *Vasc. Endovasc. Surg.*, **40**(1), pp. 75–78.
- [19] Wunderlich, C., Wunderlich, O., Tausche, A. K., Fuhrmann, J., Boscheri, A., and Strasser, R. H., 2007, "Ortner's Syndrome or Cardiovascular Hoarseness," *Int. Med. J.*, **37**(6), pp. 418–419.
- [20] Lundborg, G., 1998, "Intraneural Microcirculation," *Orthop. Clin. North Am.*, **19**(1), pp. 1–12.
- [21] Peters, A., Palay, S. L., and Webster, H. D., 1991, *The Fine Structure of the Nervous System: Neurons and Their Supporting Cells*, 3rd ed., Oxford University Press, NY, p. 494.
- [22] Sunderland, S., 1965 "The Connective Tissues of Peripheral Nerves," *Brain*, **88**(4), pp. 841–854.
- [23] Sunderland, S., 1970, "Anatomical Features of Nerve Trunks in Relation to Nerve Injury and Nerve Repair," *Clin. Neurosurg.*, **17**, pp. 38–62.
- [24] Sunderland, S., 1978, *Nerves and Nerve Injuries*, 2nd ed., Churchill Livingstone; Longman, Edinburgh, p. 1046.
- [25] Sunderland, S., 1990, "The Anatomy and Physiology of Nerve Injury," *Muscle Nerve*, **13**(9), pp. 771–784.
- [26] Sunderland, S., and Bradley, K. C., 1961, "Stress-Strain Phenomena in Denerated Peripheral Nerve Trunks," *Brain*, **84**, pp. 125–127.
- [27] Sunderland, S., and Bradley, K. C., 1961, "Stress-Strain Phenomena in Human Peripheral Nerve Trunks," *Brain*, **84**, pp. 102–119.
- [28] Sunderland, S., and Bradley, K. C., 1961, "Stress-Strain Phenomena in Human Spinal Nerve Roots," *Brain*, **84**, pp. 120–124.
- [29] Thomas, P. K., 1963, "The Connective Tissue of Peripheral Nerve: An Electron Microscope Study," *J. Anat.*, **97**, pp. 35–44.
- [30] Ushiki, T., and Ide, C., 1990, "Three-Dimensional Organization of the Collagen Fibrils in the Rat Sciatic Nerve as Revealed by Transmission- and Scanning Electron Microscopy," *Cell Tissue Res.*, **260**(1), pp. 175–184.
- [31] Haftek, J., 1970, "Stretch Injury of Peripheral Nerve. Acute Effects of Stretching on Rabbit Nerve," *J. Bone Jt. Surg. Br.*, **52**(2), pp. 354–365.
- [32] Rydevik, B. L., Kwan, M. K., Myers, R. R., Brown, R. A., Triggs, K. J., Woo, S. L., and Garfin, S. R., 1990, "An In Vitro Mechanical and Histological Study of Acute Stretching on Rabbit Tibial Nerve," *J. Orthop. Res.*, **8**(5), pp. 694–701.
- [33] Nicholson, K. J., and Winkelstein, B. A., 2011, "Nerve and Nerve Root Biomechanics," *Neural Tissue Biomech.*, **3**, pp. 203–229.
- [34] Sunderland, S., and Bedbrook, G. M., 1949, "The Cross-Sectional Area of Peripheral Nerve Trunks Devoted to Nerve Fibres," *Brain*, **72**, pp. 428–449.
- [35] Campbell, E. O., Samlan, R. A., McMullen, N. T., Cook, S., Smiley-Jewell, S., and Barkmeier-Kraemer, J., 2013, "Developmental Changes in the Connective Tissues of the Porcine Recurrent Laryngeal Nerve," *J. Anat.*, **222**(6), pp. 625–633.
- [36] Alexander, M. J., Barkmeier-Kraemer, J. M., and Vande Geest, J. P., 2010, "Biomechanical Properties of Recurrent Laryngeal Nerve in the Piglet," *Ann. Biomed. Eng.*, **38**(8), pp. 2553–2562.
- [37] Sunderland, S., 1945, "The Adipose Tissue of Peripheral Nerves," *Brain*, **68**, pp. 118–122.
- [38] Sunderland, S., 1951, "A Classification of Peripheral Nerve Injuries Producing Loss of Function," *Brain*, **74**(4), pp. 491–516.
- [39] Peti-Peterdi, J., and Bell, P. D., 2003, "Confocal and Two-Photon Microscopy," *Methods Mol. Med.*, **86**, pp. 129–138.
- [40] Kirkpatrick, N. D., Hoying, J. B., Botting, S. K., Weiss, J. A., and Utzinger, U., 2006, "In Vitro Model for Endogenous Optical Signatures of Collagen," *J. Biomed. Opt.*, **11**(5), p. 054021.
- [41] Raghavan, M. L., and Vorp, D. A., 2000, "Toward a Biomechanical Tool to Evaluate Rupture Potential of Abdominal Aortic Aneurysm: Identification of a Finite Strain Constitutive Model and Evaluation of Its Applicability," *J. Biomech.*, **33**(4), pp. 475–482.
- [42] Borschel, G. H., Kia, K. F., Kuzon, W. M., Jr., and Dennis, R. G., 2003, "Mechanical Properties of Acellular Peripheral Nerve," *J. Surg. Res.*, **114**(2), pp. 133–139.
- [43] Ma, Z., Hu, S., Tan, J. S., Myer, C., Njus, N. M., and Xia, Z., 2013, "In Vitro and in Vivo Mechanical Properties of Human Ulnar and Median Nerves," *J. Biomed. Mater. Res. A*, **101**(9), pp. 2718–2725.
- [44] Millesi, H., Zoch, G., and Reihnsner, R., 1995, "Mechanical Properties of Peripheral Nerves," *Clin. Orthop. Relat. Res.*, **1995**(314), pp. 76–83.
- [45] Phillips, J. B., Smit, X., De Zoysa, N., Afoke, A., and Brown, R. A., 2004, "Peripheral Nerves in the Rat Exhibit Localized Heterogeneity of Tensile Properties During Limb Movement," *J. Physiol.*, **557**(Pt 3), pp. 879–887.
- [46] Rickett, T., Connell, S., Bastijanic, J., Hegde, S., and Shi, R., 2011, "Functional and Mechanical Evaluation of Nerve Stretch Injury," *J. Med. Syst.*, **35**(5), pp. 787–793.
- [47] Topp, K. S., and Boyd, B. S., 2006, "Structure and Biomechanics of Peripheral Nerves: Nerve Responses to Physical Stresses and Implications for Physical Therapist Practice," *Phys. Ther.*, **86**(1), pp. 92–109.
- [48] Wall, E. J., Kwan, M. K., Rydevik, B. L., Woo, S. L., and Garfin, S. R., 1991, "Stress Relaxation of a Peripheral Nerve," *J. Hand Surg. Am.*, **16**(5), pp. 859–863.
- [49] Wall, E. J., Massie, J. B., Kwan, M. K., Rydevik, B. L., Myers, R. R., and Garfin, S. R., 1992, "Experimental Stretch Neuropathy. Changes in Nerve Conduction Under Tension," *J. Bone Jt. Surg. Br.*, **74**(1), pp. 126–129.
- [50] Deslauriers, J., 2007, "Anatomy of the Neck and Cervicothoracic Junction," *Thoracic Clin.*, **17**, pp. 529–547.