

Vocal fold elasticity of the Rocky Mountain elk (*Cervus elaphus nelsoni*) – producing high fundamental frequency vocalization with a very long vocal fold

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SUMMARY

The vocal folds of male Rocky Mountain elk (*Cervus elaphus nelsoni*) are about 3 cm long. If fundamental frequency were to be predicted by a simple vibrating string formula, as is often done for the human larynx, such long vocal folds would bear enormous stress to produce the species-specific mating call with an average fundamental frequency of 1 kHz. Predictions would be closer to 50 Hz. Vocal fold histology revealed the presence of a large vocal ligament between the vocal fold epithelium and the thyroarytenoid muscle. In tensile tests, the stress–strain response of vocal fold epithelium and the vocal ligament were determined. Elasticity of both tissue structures reached quantitative values similar to human tissue. It seems unlikely that the longitudinal stress in elk vocal folds can exceed that in human vocal folds by an order of magnitude to overcome the drop in fundamental frequency due to a 3:1 increase in vocal fold length. Alternative hypotheses of how the elk produces high fundamental frequency utterances, despite its very long vocal fold, include a reduced effective vocal fold length in vibration, either due to bending properties along the vocal fold, or by actively moving the boundary point with muscle stiffening. The relationships between an individual's average fundamental frequency, vocal fold length and body size are discussed.

Key words: vocal ligament, hysteresis, stress–strain response, bioacoustics, sound production, mammal.

INTRODUCTION

A number of interconnected size-dependent factors make small mammals apt to produce (and hear) high acoustic frequencies, while large mammals generally are specialized toward lower frequencies (Fig. 1A). During the mating season, the Rocky Mountain elk (*Cervus elaphus nelsoni*) produces sounds with relatively high fundamental frequencies (Murie, 1932; Struhsaker, 1968; Feighny et al., 2006), the average of which (approx. 1 kHz) is well beyond the expected value for an animal that size (Fig. 1A).

There is an inverse relationship between vocal fold length and average fundamental frequency. The elk's average fundamental frequency is well beyond the expected value for a 3 cm-long vocal fold (Fig. 1B).

We investigate here how the elk's vocal fold can produce such high fundamental frequencies, when compared with others in the taxon. If, in a first approximation, vocal fold tissue is considered to behave like a simple string (e.g. Titze et al., 1989), then fundamental frequency depends inversely on string length:

$$F_0 = \frac{1}{2L} \sqrt{\frac{\sigma}{\rho}}, \quad (1)$$

where L is the vocal fold length, σ is the stress applied to the vocal fold (force per unit area), and ρ is the tissue density (1.02 g cm^{-3}).

Although vocal folds can be found in an enormous morphological variety across species (e.g. Negus, 1949; Schneider, 1964; Harrison, 1995), little is known about how tissue composition has evolved. We study the elk's morphological adaptation for producing high fundamental frequencies, first because we expect that the evolutionary transformation of the male vocal organ, which is under

the strong influence of sexual selection, resulted in a specialization of the vocal folds to produce very high fundamental frequencies. Vocal activity is very high in male elk during the rut, but very much reduced throughout the rest of the year. Females, however, also vocalize high-frequency calls (among other call types), but most likely not as often as males. Second, we expect that the male elk vocal fold can serve as a model for an adaptation to produce high fundamental frequencies. Many large mammals produce calls with fundamental frequencies above 1 kHz, e.g. several pig species in screaming and squeaking (Schön et al., 2001; Tembrock, 1959; Tembrock, 1996) and chimpanzees in pant hoot calling (Riede et al., 2007).

A third motivation to study elk vocalization is the severe acoustic contrast between the elk's mating calls and that of its well-studied close relative, the European red deer (*Cervus elaphus scoticus*). In contrast to the elk, the red deer produces a mating call with low fundamental frequency (200 Hz on average). The red deer's vocal system is under severe sexual selection pressure (Clutton-Brock and Albon, 1979; McComb, 1987; McComb, 1991), which has led to a sexual dimorphism in overall larynx size (Köhler, 1982) and vocal tract length (Fitch and Reby, 2001). The red deer, like the elk, live in a polygynous social system. Temporal groupings of females find attractive feeding grounds, which are defended by single stags during the rut season in the fall (Struhsaker, 1968). The reproductive success of a red deer stag depends on the successful take-over and defense of a harem (Clutton-Brock and Albon, 1979). Body size is a key factor for the outcome of physical fights between male deer (Clutton-Brock and Albon, 1979; McComb, 1991). In red deer, vocalization indicates body size, as there is a positive correlation

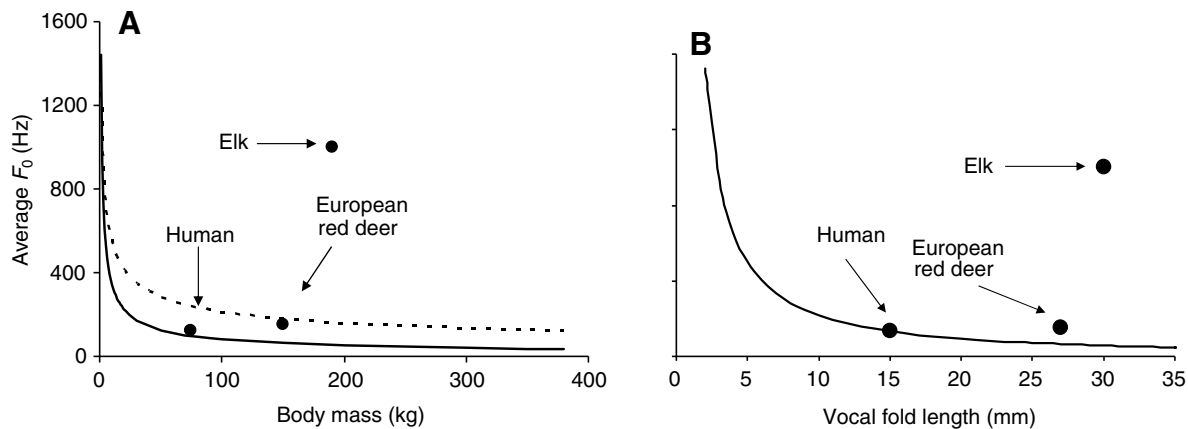


Fig. 1. (A) Relationship between body mass and average fundamental frequency (F_0) for non-human primate species [solid line (from Tembrock, 1996); 122 species considered; $y=1443x^{-0.623}$] and several mammals, except primates [dotted line (from Fletcher (Fletcher, 2004); unknown number of species considered; $y=1403x^{-0.409}$)]. (B) Relationship between vocal fold length and average fundamental frequency ($y=3537x^{-1.214}$). The regression is based on human vocal fold data and average F_0 in the speaking voice [fig. 7.7 in Titze (Titze, 2000)]. In A and B, the North American elk, European red deer and an average male human are indicated by filled circles.

between acoustic parameters (calling rate, formant frequencies) and body size (Clutton-Brock and Albon, 1979; Reby and McComb, 2003). Female red deer as well as rivaling males pay attention to those acoustic parameters in the vocalization of their potential mates or competitors (McComb 1991; Charlton et al., 2007). Whether a similar selection mechanism on the vocal system is active in the elk and the red deer is unknown. The present study is directed toward understanding the functional morphology of the elk's vocal system.

Vocal fold elasticity, morphology and fundamental frequency

Elasticity is the property of a material that causes it to be restored to its original shape after deformation. Elasticity of vocal fold tissue is a key factor in the control of fundamental frequency in vocalization (Titze, 1988; Alipour-Haghighi, Titze, 1991). An important feature of the vocal folds is their multilayered structure (Hirano, 1974). Mammalian vocal folds generally consist of at least two layers: a relatively loose 'cover' layer and a muscular 'body' layer (Hirano and Kakita, 1985). The human vocal folds also have a ligament between the cover and body layers. Some species lack this ligament (Kurita et al., 1983), although that situation is unknown for most non-human mammals. Functionally, most of the vocal fold vibrations occur in the cover layer because of its high degree of compliance and proximity to the aerodynamic driving forces. The vibrations are regarded as a result of the energy transfer from the glottal airflow to the vocal fold motion (Titze, 1988).

Each tissue layer (cover, ligament and muscle) has its specific mechanical properties. One such property is longitudinal elasticity, where a tissue fiber direction is specified and stress-strain measurements are made along this single direction. Longitudinal elasticity varies from one vocal fold layer to another. Vocal folds are variably stretched, for example to produce different fundamental frequencies (Nishizawa et al., 1988; Schuster et al., 2005). As a result, each tissue part reacts with a different tension. The tension of the cover is controlled by vocal fold length only. The same is true for the vocal ligament. The body, however, has active contractile properties (the thyroarytenoid muscle), which means that its tension is not only determined by length, but also by active stiffening of the muscle.

In the human voice, very high fundamental frequencies are reached by severe stretching of the vocal fold [reviewed in Titze

(Titze, 2000), chapter 8]. In such a situation the vocal ligament, which reaches the highest longitudinal elasticity values, was suggested to be the main stress-carrying tissue (Min et al., 1995; Titze and Hunter, 2004). In the elk we therefore expect to find tissue elements in the vocal fold that are characterized by comparatively higher elasticity. If the simple string formula holds, in order to produce a 1 kHz sound, the tissue of a 1 cm-long vocal fold needs to sustain a stress of 400 kPa (solving Eqn 1 for σ). A woman's vocal fold (approx. 1 cm long) has been demonstrated to be able to sustain such a stress *in vitro* (e.g. Min et al., 1995; Chan et al., 2007). A soprano singing a /C6/-note (1080 Hz), e.g. as Tytania in '*Be kind and courteous*' (in the English opera *A Midsummer Night's Dream*, by Benjamin Britten), is likely to produce such a stress. A three-times longer vocal fold (for example that of a male elk), would need to sustain a nine-times larger tension (i.e. 3.6 MPa; note the square root relation) to produce the same 1 kHz sound if an elk vocal fold behaves similarly to a human vocal fold, and a simple string approximation holds.

In the present study, we first use histology and different staining techniques to analyze the elk's vocal fold tissue composition. We then perform experiments to quantify the passive elasticity of the epithelium and the vocal ligament. The hypothesis tested in this study is that the stress of the elk's vocal fold can exceed that of the human vocal fold by an order of magnitude for a given strain.

MATERIALS AND METHODS

50 larynges were retrieved from hunter harvested elk *Cervus elaphus nelsoni* Nelson 1902 submitted to the Colorado Division of Wildlife's chronic wasting disease surveillance program, and one female and one male larynx were retrieved from butchered animals from a Wisconsin elk farm. Tissue was collected within 24 h after death.

Morphology

Vocal folds were dissected from two female and six male elk larynges, which were retrieved from the Colorado Division of Wildlife, as well as one female and one male from farmed elk. With a caliper (± 0.1 mm accuracy), the length of the vocal fold was measured from the insertion of the vocal fold at the thyroid cartilage to the tip of the vocal process of the arytenoid cartilage

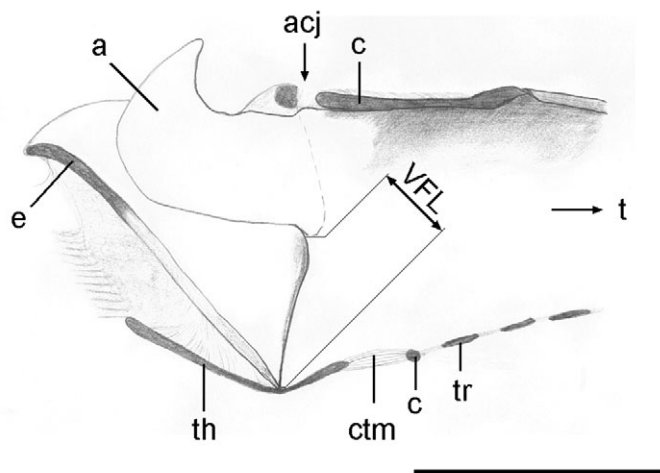


Fig. 2. Schematic of a midsagittally opened elk larynx indicating the anatomical markers to measure vocal fold length. A, arytenoid cartilage; acj, crico-arytenoid joint; c, cricoid cartilage; ctm, cricothyroid muscle; e, epiglottis; t, trachea; th, thyroid cartilage; tr, tracheal ring; VFL, vocal fold length. The dotted line indicates the contour of the arytenoid cartilage. The dorsal end of the vocal fold is determined by palpating the processus vocalis of this cartilage. Scale bar, 5 cm.

(Fig. 2). Note that the *in situ* vocal fold length is measured in the intact larynx (not cut open). Access to the vocal fold for this *in situ* measurement is possible by cutting a window (2 mm × 5 mm), mid-sagittal at the cranio-dorsal edge of the cricoid cartilage. This allows access for a small narrow caliper, paralleling the vocal fold in its complete length, without the need to stretch or bend any structure of the larynx framework. Vocal fold length was also measured in 42 additional larynges, which were retrieved from the Colorado Division of Wildlife. Specimen age was estimated by evaluation of tooth replacement and wear (Quimby and Gaab, 1957).

After the measurement, the larynges were fixed in 10% buffered formalin for 4 weeks. After fixation, larynges were cut in half in the midsagittal plane, and the vocal folds were carefully isolated. Mid-membranous coronal sections (5 µm thick) of the vocal folds were stained with Haematoxylin-Eosin, Masson's Trichrome (for collagen fiber stain) and Elastica-Van Gieson (for elastic fiber stain). Sections were scanned, viewed and measured with Imagescope software (v. 8.2.5.1263; Aperio Tech., Vista, CA, USA). Three thickness measurements (epithelium, vocal ligament, thyroarytenoid muscle) and the cross sectional area of the vocal ligament and the thyroarytenoid muscle were taken. Distance measurements were taken at the free edge of the vocal fold. In eight specimens, Masson's Trichrome and Elastica-Van Gieson stained sections were available from the same level of one vocal fold. Imagescope software allowed a positive pixel count. This algorithm looks for positive areas and shades them orange and negative areas and shades them blue. Neutral areas that are neither positive nor negative are white. The intensity of colors reflects the intensity of the staining. The percentages of positive pixels in both stains were determined and then are set into a collagen/elastin (C/E) ratio.

Stress-strain measurements

Experiments were performed on fresh specimens within 2 days after death (10 female and 10 male elk larynges from the Colorado Division of Wildlife). Tissue was kept at 4°C until the experiment.

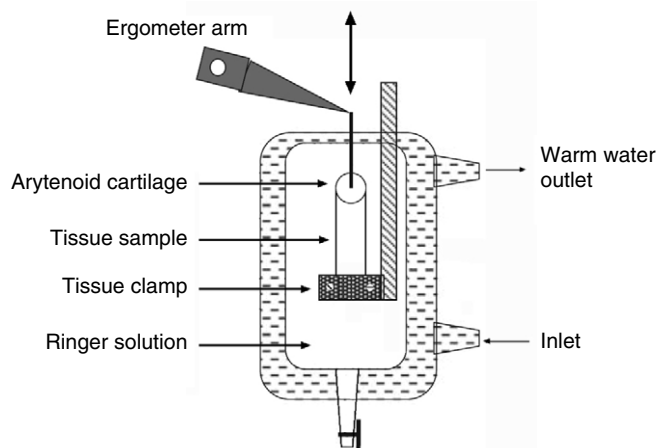


Fig. 3. Schematic of a sample mounting and the chamber.

One-dimensional stress-strain measurements were obtained: first, using a simple weight suspension system and, second, using a dual-servo force elongation ergometer system. For the weight suspension approach, stepwise stretch was applied by which the vertically positioned isolated vocal fold was stressed with increasing weights. Length changes were measured after each increase in load. For the ergometer approach, a 1 Hz sinusoidal stretch and release was applied by a dual servo motor system. The former procedure allowed a larger stress range to be applied to the complete vocal fold, while the latter procedure had a higher resolution and a faster response time, so that some dynamics of the stretch-release response could be captured. The ergometry system is most commonly used for human vocal fold tissue, but could be applied only to a sub-segment (approx. one third) of the vocal fold, because of the limited size of the ergometer arm (Fig. 3).

Stepwise stress-strain relationship

Only the vocal ligament was tested. The epithelium and the vocalis muscle were removed. The vocal ligament remained attached to the arytenoid and a piece of the thyroid cartilage. The arytenoid cartilage was fixed with a suture on a 50 cm high steel rack with the vocal ligament and the remaining small piece of thyroid cartilage freely hanging vertically. A 2 g cup was attached to the thyroid cartilage end piece. Defined weights were added to the cup. A ruler (as a known reference distance) was positioned next to the vocal fold. 30 s after placing a new weight in the cup, a picture of the vocal fold and the reference distance was taken (Sony DSC-S85 camera, 2272 × 1704 pixel resolution). Vocal fold length was measured for each increase in load with reference to the reference distance using Scion Image software (version 4.0.3.2., available at www.scioncorp.com). Some vocal folds were stressed until irreversible damage occurred. The weight of the vocal ligament and the remaining piece of thyroid cartilage were determined after the experiment. During the experiment the ligament was kept wet by covering it with wet cellulose tissue that was continuously sprayed with warm (38°C) saline solution.

1 Hz sinusoidal (cyclic) stretch and release

Larynges were midsagittally sectioned. A 2 cm long piece of vocal fold remained attached to a small portion of the arytenoid cartilage. The thyroarytenoid muscle was removed, while either the vocal ligament or epithelium remained intact. In one set of experiments

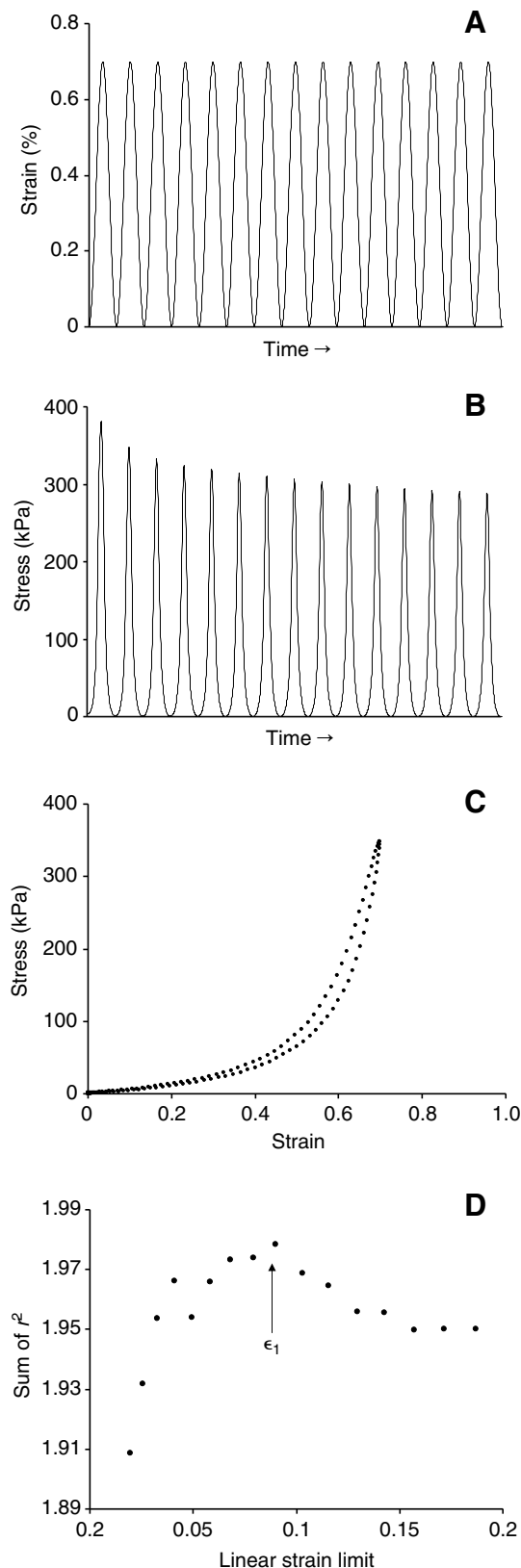


Fig. 4. (A–C) Stress–strain response in time from a 1 Hz sinusoidal elongation of vocal ligament. Note that the amplitude of strain remains constant (A) while stress (B) decreases over time. The decrease in stress is a result of tissue hysteresis, a phenomenon resulting from viscous properties of the tissue. (C) Stress–strain relationship for the same data set. The upper part of the ‘banana-shaped’ curve is the loading phase (stretching). The lower part is the unloading phase (relaxation). The difference between both curves is due to hysteresis of the tissue, i.e. lower stress in the tissue during the unloading phase. The low strain region of the loading phase was fitted with a linear regression line, while the high-strain region was modeled with an exponential function. (D) The limit of the linear region (‘Linear strain limit’) determined by maximizing the sum of the two regression coefficients (‘sum of r^2 ’). The maximum linear strain limit (ϵ_1) in this example is approx. 0.09.

ventral end of the vocal fold tissue in a fixed position. Slippage was minimized by wrapping the free end of the tissue with dry cellulose before clamping. The tissue part of interest was kept wet during the whole process of clamping. The tissue was vertically mounted in a water surrounded chamber containing saline solution (Ringer solution) maintained at 38°C (Fig. 3). The mounted tissue was close to a 1 cm length without any slack in the suture. The actual length between the cartilage and the fixation point in the clamp was measured with a caliper (± 0.1 mm accuracy).

We labeled the outermost layer dissected off from the vocal fold as epithelium. However, it is likely that it consisted of epithelium plus a thin portion of the superficial layer of the lamina propria.

The force–elongation data were obtained by 1 Hz sinusoidal stretch and release of the vocal fold by means of a dual-mode servo-control lever system (Aurora Scientific Model 305B, Aurora, ON, Canada); (resolution 1 μ m and 0.3 mN). Fractional displacement known as strain (Fig. 4A) and force (Fig. 4B) of the lever arm were recorded. Elongation was applied in a longitudinal direction (along the direction of the tissue fibers), followed by a release to the original length. The present set of experiments was conducted with a system under electronic displacement control, which was considered the input, and the elastic force response was measured as output. A controlled sinusoidal displacement was applied to the lever arm so that the vocal fold was stretched and released (loading–unloading condition) 15 times at a frequency of 1 Hz. The force and elongation signals were then transmitted *via* a 16-bit analog-to-digital acquisition board (Windaq Model DI722, DATAQ Instruments; Akron, OH, USA) at 1 kHz sampling frequency to a computer.

A pre-strain of 20% was applied to each specimen. Pre-strain is the elongation (relative to total specimen length) imposed on the specimen prior to each stretch–release test. Vocal fold length differs between *in situ* (intact larynx) and *ex situ* (vocal fold excised from the cartilage framework of the larynx) most likely due to elastic properties of the vocal fold tissue. A 20% pre-strain is compensating for length changes due to isolating the tissue and was based on vocal fold length measurements in 15 adult female and male elk larynges before and after excising the vocal fold from the cartilage framework. Vocal fold length was measured with a caliper. The distance from insertion points at the thyroid and arytenoid cartilages is $23.8 \pm 4.5\%$ shorter (mean \pm s.d., $N=15$) after isolation of the vocal fold. Differences in length between males (m) and females (f) before and after excising the vocal fold were not significant (Mann–Whitney test; $U=18$; $P=0.24$; $N_f=9$, $N_m=8$). The 20% pre-strain was applied for 10 min before experiments started. For comparison, in human vocal ligaments a 5–10% pre-strain (Hunter et al., 2004) and in arytenoid muscle a 20–41% pre-strain (Perlman et al., 1984) was described.

the vocal ligament was removed, and in a second set the epithelium was removed. Great care was taken to make sure that remaining fibers were not damaged. A suture connected the cartilage to the lever arm of the servo-control lever system, and a clamp held the

The lever system was calibrated by applying known magnitudes of displacement and force. The output signals were monitored and the measured displacement and force was adjusted to be within 1% of the operating range of the system.

All specimens were obtained within 24–48 h after death. Material was cooled at 4°C immediately after death.

Three measurement uncertainties in laryngeal tissue (tissue weight, length and density) have been identified as important for the sensitivity of elastic properties (Hunter et al., 2007). Mass measurements are uncertain due to hydration (tissue bath, see above) of the isolated tissue. For example, thyroarytenoid muscle absorbs water and mass increases by up to 13% within 5 min (Hunter et al., 2007). We tested elk vocal fold epithelium and vocal ligament (four epithelium and four vocal ligament specimens from two larynges) over a period of 10 min (1 min bath followed by a mass measurement) and found a mass increase of $11.9 \pm 4.4\%$ after 10 min and $12.3 \pm 7.5\%$ (mean \pm s.d.), respectively. We therefore corrected all mass measurements by 12%, since our mass measurements were taken after the experiment. Uncertainties in length measurements are difficult to quantify; however, they seemed to remain in an acceptable range (Hunter et al., 2007). Uncertainties in density measurements are negligible (Hunter et al., 2007). We therefore adapted density values from an earlier study (Min et al., 1995).

Data analysis

Tensile strain (ϵ) was calculated as a specimen's length change (displacement at unconstrained end) divided by its original mounting length:

$$\epsilon = \Delta l / l_0, \quad (2)$$

where l_0 is the mounting length and Δl is the change in length of the specimen during stretching. Sometimes in this paper, strain is given as percentage. The term on the right side of Eqn 2 is multiplied by 100 in those cases.

Tensile stress (σ) is defined as the ratio between the applied longitudinal force (F) and the cross-sectional area (A_0 in m^2) of the specimen. Assuming tissue incompressibility and uniform specimen cross-sectional area with roughly cylindrical geometry, the cross-sectional area is calculated as:

$$A_0 = m / \rho(l_0 + \Delta l), \quad (3)$$

where m is the specimen mass and ρ is the tissue density. This equation considers the time-varying cross-sectional area, which decreases as the specimen is elongated and increases as the specimen returns to its initial mounting length. With this time-varying cross-sectional area, the tensile stress (σ in Pa) can be calculated as:

$$\sigma = F\rho(l_0 + \Delta l) / m. \quad (4)$$

This establishes the relationship between fundamental frequency (Eqn 1) and stress (Eqn 4).

The two major fibrous protein components, collagen and elastin, contribute differently to the mechanical properties at certain strains. In the resting position, collagen fibrils are coiled in a helical fashion and become extended once the vocal fold elongates. Therefore they contribute little resistance during low strain, but they contribute more with higher strain. The elastic fibers, however, almost like a rubber band or a spring, continuously contribute to resistance, but relatively more in the low strain region. The overall stress–strain response of viscoelastic tissue has been differentiated into a linear low-strain and a nonlinear high-strain region, for example in blood vessels (e.g.

Roach and Burton, 1957; Armentano et al., 1991) or in vocal fold tissue (Fig. 4C) (Hunter and Titze, 2007; Chan et al., 2007), in order to account for the different contributions of collagen and elastin. The low-strain region is therefore modeled with a linear function (Eqn 5), while the high-strain region is best approximated with an exponential equation (Eqn 6):

$$\sigma = a\epsilon + b, \quad \epsilon \leq \epsilon_1, \quad (5)$$

where a is the slope of the curve, and b is the y -axis intercept;

$$\sigma = Ae^{B\epsilon}, \quad \epsilon \geq \epsilon_1, \quad (6)$$

where A and B are constants.

The constants in Eqn 5 and Eqn 6, as well as the upper limit of the linear low-strain region ('linear strain limit', ϵ_1), are derived by fitting a linear and an exponential regression line, respectively, to the empirical data, while maximizing the sum of both regression coefficients (Fig. 4D). The maximization process is performed by a stepwise movement of the linear strain limit, and performing a linear and exponential regression, respectively, on the two data sets (low strain and high strain region) at each step. The two resulting regression coefficients are added up at each step. The relationship between linear strain limit and the sum of both regression coefficients is exemplified in Fig. 4D.

The transition between the models is supposedly continuous, both in stress and the stress derivative (known as tangent Young's modulus). Continuity σ and $d\sigma/d\epsilon$ at $\epsilon = \epsilon_1$ requires that:

$$a = AB e^{B\epsilon_1}, \quad (7)$$

and

$$b = (1 - B\epsilon_1)A e^{B\epsilon_1}. \quad (8)$$

However, fitting of empirical data combined with optimized regression coefficients did result in some discontinuity. One explanation for this discontinuity is the sudden onset of resistance to elongation by the collagen fibers when they are fully uncoiled.

In the linear segment of the stress–strain region, the tangent Young's modulus is a constant (a). In the nonlinear segment of the stress–strain region, the tangent Young's modulus is given by the relation:

$$E = d\sigma / d\epsilon = AB e^{B\epsilon}, \quad (9)$$

which changes exponentially in proportion to the stress itself (Eqn 6). The range of tangent Young's modulus (E in kPa) is described later in tables.

Hysteresis was estimated as the difference between the area-under-the-curves of the loading and the unloading phase of the 1 Hz-sinusoidal stress–strain response (recall Fig. 4C). Hysteresis is frequency dependent because it involves the strain rate with respect to time. We provide here only one estimate for a 1 Hz loading–unloading regime. The stress–strain responses (loading phase and unloading phase separately) were fitted with an exponential curve according to Eqn 6. The area under the curve between ϵ_1 and maximum applied strain was estimated by integrating both exponential equations. The difference in the area under the curve between loading and unloading stress–strain response was considered hysteresis and expressed as a percentage. The area under the curve for the loading phase would represent 100%.

Force–elongation data were obtained for the left and right vocal fold of each larynx. The linear and exponential coefficients, linear strain limit, and hysteresis were averaged for the left and right vocal fold for each larynx, so that only one data set per larynx was considered in further analyses.

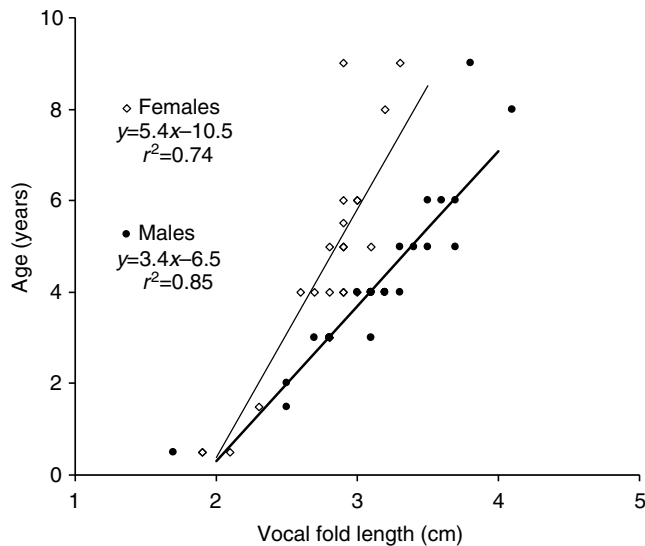


Fig. 5. Relationship between vocal fold length (cm) and estimated age (years). Females (open diamonds) and males (closed diamonds) are indicated separately. Regression function and coefficient (r^2) are shown.

Nonparametric tests were used for correlations (Spearman rank) and comparison (Mann–Whitney test) of averages of unmatched samples. Regression coefficients are used to describe the quality of the fit of the linear and the exponential model (Eqn 5 and Eqn 6).

RESULTS Morphology

Vocal folds were on average 3.0 ± 0.42 cm long (mean \pm s.d.; $N=52$), ranging between 1.7 and 4.1 cm for individuals between 0.5 and 9 years old. Correlations between age and vocal fold length were significant for both males (Spearman rank correlation, $r=0.93$; $P<0.001$; $N=30$) and for females (Spearman rank correlation, $r=0.70$; $P<0.001$; $N=22$) (Fig. 5).

Epithelium thickness ranged between 33 and $75 \mu\text{m}$. The vocal ligament ranged in cross-sectional area between 20 and 40 mm^2 . The largest diameter of the thyroarytenoid muscles ranged between 4.5 and 7 mm (Table 1).

Unlike in humans, the vocal ligament reached much deeper into the vocal fold (Fig. 6) extending almost 45% of the distance between the epithelium and the thyroid cartilage (see dotted line in Fig. 6A

between two short lines). In humans, this ligament depth would be only about 10% of the total depth. The collagen/elastin ratio was slightly larger in five males than in three females (Table 1), however not significantly.

We observed a depression (a cavity of approx. $5 \times 5 \times 5$ mm) on the caudo-lateral side of each vocal ligament (Fig. 7). A thick branch of the thyroarytenoid muscle projects into that depression. It is located about two-thirds down of the vocal fold length from the thyroid cartilage insertion. A contraction of this branch of thyroarytenoid muscle fibers would supposedly shorten the vocal fold length to a distance between the thyroid cartilage insertion point and this depression, taking out the dorsal part of the vocal fold. Such a mechanism would allow some degree of flexibility in effective vocal fold length, but only if the different portions of the thyroarytenoid muscle can be controlled separately.

Stress–strain relationship

Stepwise stress–strain relationship

Elk vocal fold ligaments can be stretched 200–300% of their original length before rupture occurs (Fig. 8). Independent of sex, older specimens reach smaller strain values before damage (Spearman rank correlation; age *versus* maximum strain before rupture; $r=-0.94$; $P<0.01$; $N=8$ larynges). Ligaments from seven larynges ruptured between 3 and 4 kg mass (30–40 N), corresponding to a stress of 2.2 ± 0.9 MPa (mean \pm s.d.; range: 0.9–4.4 MPa), but those of one older male (estimated 9 years) at 49 and 52 N (2.2 MPa), respectively.

Damage occurred not to the ligament itself but to the anchoring connection, either at the ligament–arytenoid or the ligament–thyroid connection. 14 ligaments (from eight larynges) were extended until rupture occurred. Seven ruptures occurred at the ligament–arytenoid and seven at the ligament–thyroid connection.

Because of these plastic deformations, ligaments demonstrated a pseudo-exponential stress–strain relationship (Fig. 8). Nevertheless, we determined the exponential model of the stress–strain relationship (according to Eqn 6) for a strain range from 0 to 80% (Table 2). The average exponential function for stress in four male vocal fold ligaments is:

$$\sigma = 1.1.e^{6.5\epsilon}, \quad (10)$$

and in five female vocal fold ligaments:

$$\sigma = 1.1.e^{6.1\epsilon}. \quad (11)$$

The regression model for data between 0 and 80% strain are exemplified in Fig. 8 for two individuals by overlaying those

Table 1. Raw data of macroscopic and histological measurements in 10 elk larynges

Larynx	Sex	Age (years)	VF length (cm)	Epithelium thickness (μm)	LV		TA		C/E ratio
					Area (mm^2)	Thickness (mm)	Area (mm^2)	Thickness (mm)	
1	M	4	3.2	57.0	24.9	4.9	43.5	5.7	0.82
2	M	3	2.8	46.6	39.5	5.9	53.3	5.8	0.72
3	F	4	3.0	44.0	36.2	3.1	54.3	5.5	0.75
4	F	6	2.9	33.0	19.7	3.9	59.7	4.5	0.66
5	F	>8	2.9	75.3	23.0	3.9	65.6	6.3	0.78
6	M	5	3.2	40.7	23.6	4.5	43.8	5.2	0.75
7	M	>8	3.8	71.0	38.8	5.7	72.1	7.0	0.74
8	M	0.5	1.7	88.9	14.0	2.8	39.0	5.5	–
9	M	4	3.2	68.4	33.8	5.0	76.4	6.3	0.79
10	M	2	2.9	55.0	30.0	4.9	62.1	6.0	–
Mean \pm s.d.			3.0 ± 0.5	54.5 ± 14.6	29.9 ± 7.4	4.4 ± 1.0	57.0 ± 12.5	5.8 ± 0.7	0.76 ± 0.05

VF, vocal fold; LV, vocal ligament; TA, thyroarytenoid muscle; C/E, collagen/elastin.

models onto the actual data. Beyond a strain of 0.8, data suggest some degree of yielding in the molecular bonds of the endpoint tissue. We have attempted to exclude any slippage of the sutures in

the cartilages, or stretching of the sutures themselves, and therefore assume that the shift around 100% strain is due to some internal material damage.

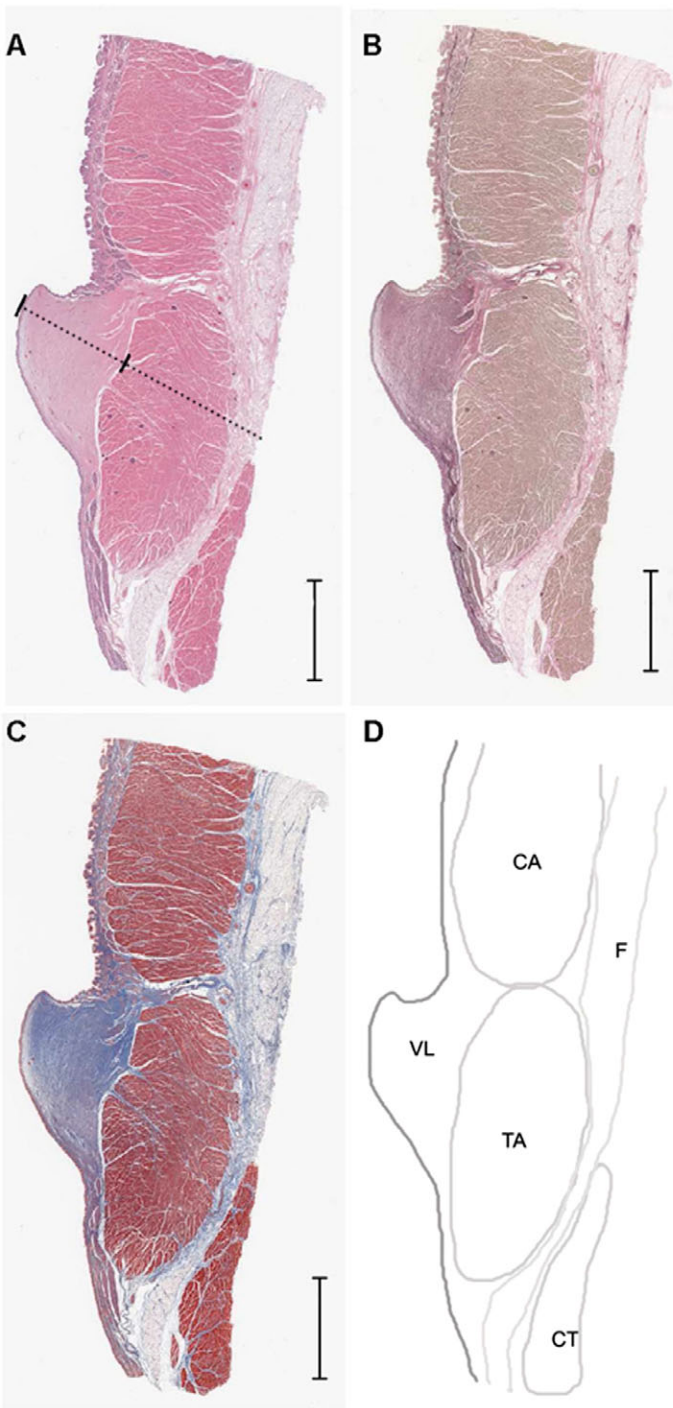


Fig. 6. Three successive histological sections of the mid-membraneous part of the vocal fold of a 4-year-old elk. (A) Haemalaun-Eosin stain, (B) Elastica-van Gieson stain indicating elastic fibers in black stain, (C) trichrome stain indicating collagen fibers in blue stain. Scale bars, 5 mm. Thickness measurements of the epithelium, the vocal ligament and the thyro-arytenoid muscle were taken along the dotted line in A. (D) Schematic of the histological sections: CA, lateral cricoarytenoid muscle; TA, thyroarytenoid muscle; CT, cricothyroid muscle; VL, vocal ligament; F, fat layer that sits on the thyroid cartilage.

1 Hz sinusoidal stretch and release

Fig. 4C shows the stress–strain response for tensile stress–release deformation of one vocal ligament specimen (male, ca. 6 years), at a loading rate of 1 Hz. During the unloading phase, energy loss relative to the loading phase (hysteresis) was observed in all specimens. All data sets were better fitted with a combination of a linear and nonlinear model than with a single model. Table 3 shows summaries of the fitting parameters for the linear portion of the loading phase.

The constants *a* and *b*, as well as the linear strain limit, ϵ_1 , are not significantly different between males and females, nor for the vocal ligament or the vocal fold epithelium (Mann–Whitney tests). The A and B constants for the exponential portion (Table 4) are not significantly different between males and females for the vocal fold mucosa. Also the A constant for the vocal ligament is not significantly different between males and females (at significance levels $P=0.05$, tested with Mann–Whitney tests). However, the exponent B was significantly different between females and males for the vocal ligament (Mann–Whitney test, $Z=-2.2$, $P<0.01$, $N_{1,2}=6$). In summary, the average exponential functions for six male vocal fold ligaments were:

$$\sigma = 3.3e^{6.5\epsilon}, \quad \text{for } \epsilon > \epsilon_1, \tag{12}$$

and for six female vocal fold ligaments:

$$\sigma = 3.1e^{5.9\epsilon}, \quad \text{for } \epsilon > \epsilon_1. \tag{13}$$

In the strain range tested, stress in the elk vocal ligament was on average smaller than in a human vocal ligament (Fig. 9).

Hysteresis was 13.6% for female vocal ligaments and 14.4% for male vocal ligaments (Mann–Whitney test, $Z=-0.16$; $P=0.87$; $N_{1,2}=6$) as shown in Table 3. Hysteresis is 13.7% for female vocal fold epithelia and 13.9% for male vocal fold epithelia, also a non-significant difference (Mann–Whitney test, $Z=-0.31$; $P=0.83$; $N_{1,2}=5$) (Table 3).

DISCUSSION

The results of our experiments provide three new findings that contribute to the understanding of the functional morphology of the elk vocal fold. We found a large vocal ligament in both males and females. We found tissue properties comparable to humans, and we found some sex differences.

Our initial hypothesis, that the elk vocal fold is able to sustain tension at least 9 times larger than that acting on a human vocal fold while producing a 1 kHz sound cannot be accepted at this stage (Fig. 9). However, the strain range above 0.8 still needs to be tested in a cyclic procedure. The stepwise stretching showed plastic deformation at very high strain ranges. It is not clear if such a deformation would occur in a cyclic procedure with much shorter

Table 2. Constants A and B for fitting the exponential stress–strain relationship (according to Eqn 6) derived in a stepwise experiment

Specimen	A	B	r^2	Young's modulus (kPa)
Elk vocal ligament				
Male	1.1±0.3	6.5±1.3	0.966±0.01	14–182
Female	1.5±0.2	6.1±0.8	0.957±0.01	17–191

Values are means ± s.d. ($N=4$ for each sex). r^2 , regression coefficient.

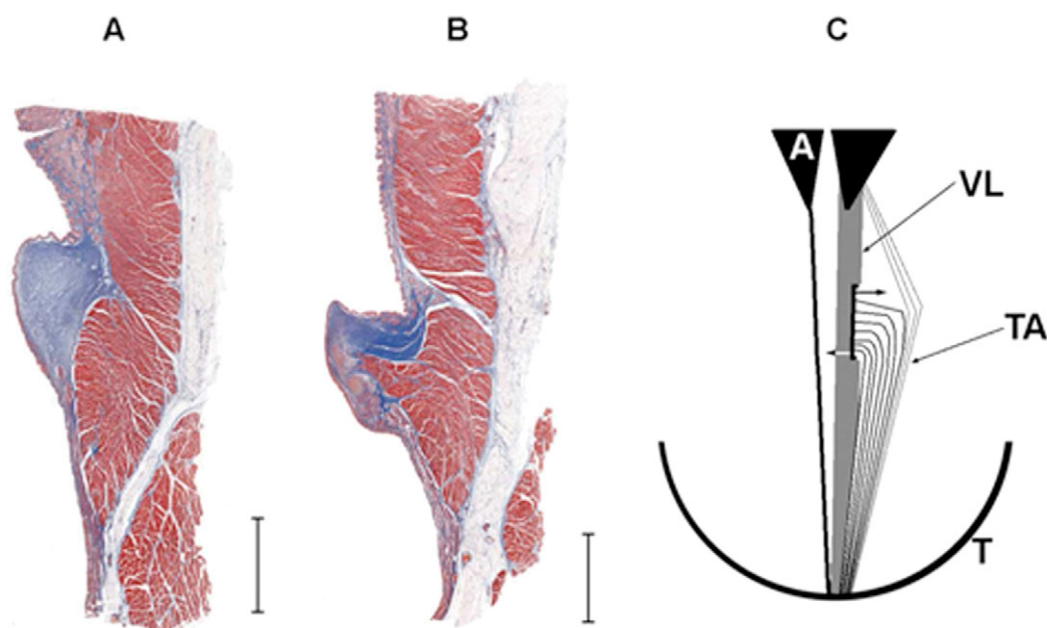


Fig. 7. Histological sections of a mid-membraneous part (A) and a dorsal-membraneous part (B) of the vocal fold of a 4-year-old elk (Masson's Trichrome stain). Scale bars, 5 mm. Note the bundle of muscle fibers leading deeply into the ligamentum vocale in dorsal section but not in the mid-membraneous one. (C) Schematic of a top view of the elk larynx. The position of a depression in the vocal ligament (VL), where a portion of the thyroarytenoid muscle (TA) inserts and could cause an effective shortening of the vocal folds, is shown. Arrows indicate likely directions of out-forces. Note that a line at the insertion point of the TA muscle at the base of the depression was drawn to indicate likely directions of forces; however, whether the tissue has different elastic properties at this point is unknown. A, arytenoid cartilage; T, thyroid cartilage.

stretching times, or *in vivo* with intact repair mechanisms and presumably also shorter stretching times. We therefore also believe it is too early to reject the hypothesis that the exponential function may be much steeper in elk than in humans, but alternative hypotheses should be considered.

It is possible that the elastic properties of the vocal fold could have changed post mortem. Post mortem changes include the release of proteolytic enzymes from the epithelial cells to the connective tissue. However, within reasonable times post mortem changes of elasticity are predictable. Chan and Titze (Chan and Titze, 2003) demonstrated that viscoelastic properties of vocal fold tissue did not change for 24 h when kept in saline solution at room temperature.

A large vocal ligament bears the stress

The tissue with the largest elastic modulus is presumably the one with the largest stress-bearing potential, which is the vocal ligament in the elk, as it is in humans (Min et al., 1995). Epithelium showed a weaker stress-strain response, but epithelium does not bend itself as well to uniaxial stress application because it is not fibrous. Also, it is difficult to dissect away all the superficial lamina propria, making the exact cross sectional area of an epithelium sample somewhat suspect (Perlman et al., 1984).

An isolated elk vocal ligament can bear stress around 0.5 MPa for strain up to 0.8. In this range, the stress-strain data increased exponentially in both sexes and stepwise results were similar to the cyclic data. With more elaborate instrumentation, we predict that in the cyclic procedure (or *in vivo*) stresses up to 4 MPa could be reached even below strains of 1.0.

It is unknown how much an elk can stretch its vocal folds *in situ*, but preliminary testing in a cadaver larynx suggest 50–70%. Length changes *in situ*

depend on the flexibility of the cartilage framework, mostly the movability of the crico-arytenoid joint and the crico-thyroid joint, as well as the size of the crico-thyroid space. In humans vocal fold lengths vary as little as 21% and as much as 111% over an entire fundamental frequency range of an individual (Nishizawa et al., 1988). The amount of vocal fold length change that occurs with the change of fundamental frequency is very individual-specific and does not necessarily follow a general pattern (Nishizawa et al., 1988).

Furthermore, there is hysteresis in the cyclic stress-strain curve that depends on the viscous properties of the tissue. It ranges between 9 and 23% in elk vocal ligaments. This is comparable, for example, to mammalian limb tendons, in which hysteresis ranges between 5% and 11% (Bennett et al., 1988; Pollock and Shadwick, 1994). Hysteresis results from continuous stress relaxation (viscous leakage) during the stress-strain cycle. In other words, the tissue is weaker during the release phase than during the stretch phase. This relaxation over time may account for the weakening of the tissue in the very high strain region. The duration of an elk call with a high fundamental frequency may therefore be limited (unless there

Table 3. Parameters of the linear model (Eqn 5) and linear strain limit (ϵ_1) stress-strain response of the vocal ligament and vocal fold epithelium, based on the 1 Hz sinusoidal stretch and release

Specimen	N	a	b	r^2	ϵ_1
Elk vocal ligament					
Male	6	47.8±18.2	0.1±0.1	0.993±0.004	0.113±0.022
Female	6	43.6±15.7	0.1±0.1	0.993±0.002	0.142±0.039
Elk mucosa					
Male	5	86.8±30.7	0.3±0.3	0.987±0.002	0.211±0.037
Female	5	96.6±28.6	-0.2±0.6	0.988±0.005	0.194±0.049

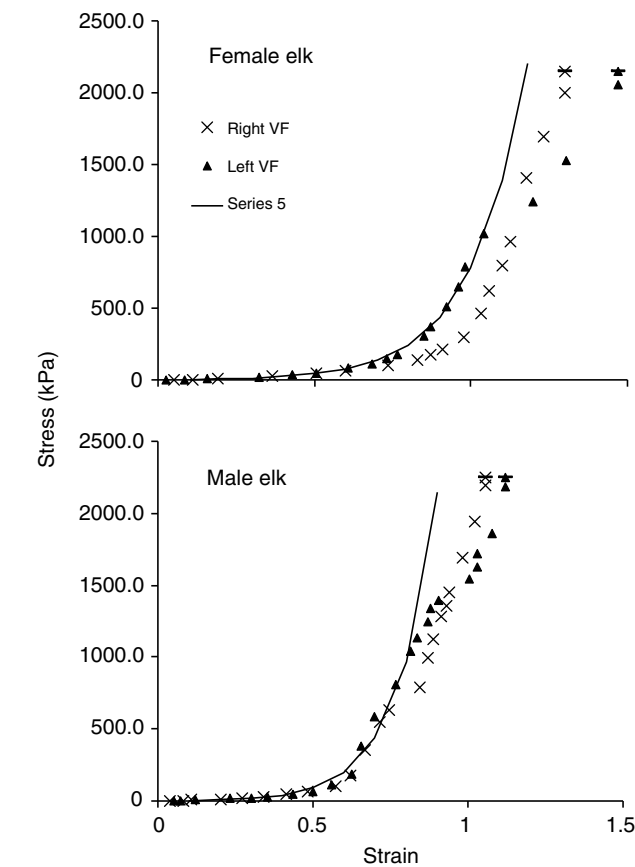


Fig. 8. Stress–strain relationship for vocal ligaments from a female (top) and male (bottom) elk vocal fold derived in a stepwise procedure. The symbols with a strike-through indicate the rupture point of the respective vocal fold. The lines represent the regression lines calculated for stress–strain data between 0 and 0.8 strain, in both cases only for the left vocal fold. Note that data points ‘slip off’ from the regression model above strain 1.0 in the female and strain 0.8 in the male. This holds true for all ligaments investigated.

is some counterbalance, for instance, by increasing muscle activity and further stretching the tissue).

Our initial hypothesis was based on the assumption that vocal folds can be approximated with an ideal string-model. However this assumption might be too simple. A reduction of the effective string length in vibration can help explain why high fundamental frequencies can be produced with a 3 cm long vocal fold. The

Table 4. Parameters of the exponential model (Eqn 6) for curve-fitting the empirical stress–strain response of the vocal ligament and vocal fold mucosa for strains larger than ϵ_1 , based on the 1 Hz sinusoidal stretch and release

Specimen	N	A	B	r^2	Young's modulus (kPa)	Hysteresis (%)
Elk vocal ligament						
Male	6	3.3±1.3	6.5±0.3	0.996±0.002	44–547	14.4±3.9
Female	6	3.1±1.0	5.9±0.4	0.995±0.002	42–346	13.6±1.9
Elk mucosa						
Male	5	9.7±4.2	3.9±0.7	0.994±0.003	86–264	13.9±3.3
Female	5	9.6±3.5	4.1±0.7	0.996±0.001	86–303	13.7±1.3

Young's modulus (from ϵ_1 to 50% strain), according to Eqn 7.
Hysteresis was calculated for a range between ϵ_1 and maximum applied stress.

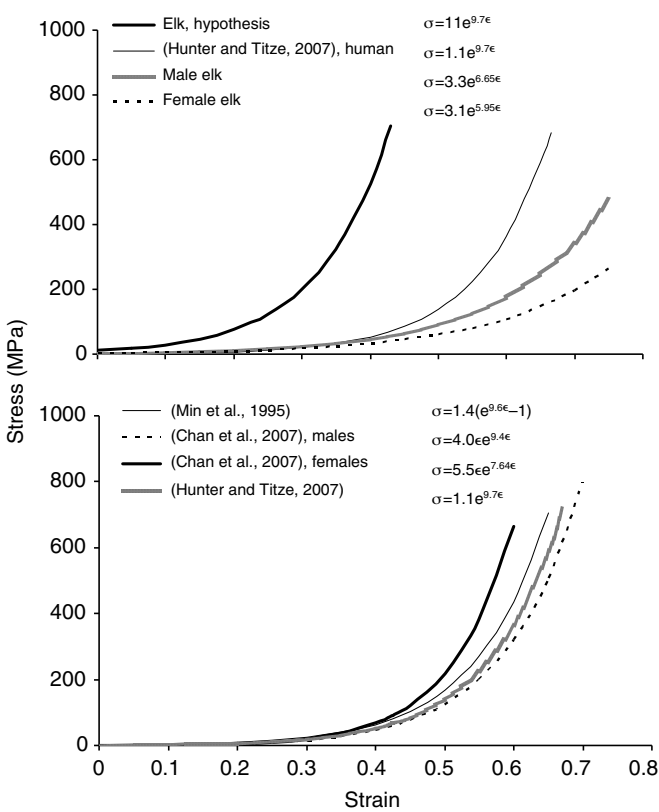


Fig. 9. Comparative stress–strain relationship according to Eqn 8 for different species and tissue types. Top, stress–strain response as predicted for the elk (hypothesis), as measured in male and female elk, and data from one study in humans. Bottom, different studies in human vocal folds.

hypothesis of the vocal fold acting like an ideal string assumes no bending stiffness at the endpoints, i.e. the tissue is vibrating at its full length in integer half-wavelengths (reviewed in Titze and Hunter, 2004). However, ligament end points are somewhat stiffer and fan out at the cartilage attachments (macula flavae) (Haji et al., 1992). Furthermore, the entire cross-section may vary as the tissue is stretched, making a broad end point even stiffer (in comparison to the center). Our data are somewhat supportive of this hypothesis. The cyclic elongation experiments delivered stronger stress–strain responses than the stepwise stretching experiments (the A constant was larger in the cyclic elongation experiments, comparing Eqn 10 and Eqn 12). In the former, only a fraction of the vocal fold (plus the insertion portion to the arytenoid cartilage) was tested. This portion is supposedly stiffer than the rest of the membranous part of the vocal fold. Quantifying the bending characteristics of the vocal fold tissue will help to evaluate this hypothesis. It has been shown that the combination of bending stiffness and *macula flavae* widening of the ligament can increase fundamental frequency by a factor of two in humans (Titze and Hunter, 2004; Hunter and Titze, 2007). Effective vocal fold length might also be affected actively in elk by moving the boundary point. We observed a depression

on the caudo-lateral side of each vocal ligament. A contraction of a small branch of thyroarytenoid muscle fibers would supposedly shorten the vocal fold length to a distance between the thyroid cartilage insertion point and this depression (Fig. 7C). Such a mechanism would allow some degree of flexibility in effective vocal fold length, but only if the different portions of the thyroarytenoid muscle can be controlled separately. A recent study of neuromuscular junctions in the intrinsic laryngeal muscles of various mammalian species demonstrated a species-specific pattern (Lima-Rodrigues et al., 2006); however, this is unknown for the elk. A longer vocal fold would allow the elk to produce powerful sounds with a low fundamental frequency, while the shorter vocal fold would allow a high fundamental frequency.

In non-humans, little is known about effective vocal fold length. Data from a small mammal, the squirrel monkey (*Saimiri boliviensis*), suggest that the vocal fold at its complete length is involved in vibration (Brown et al., 2003).

Similar material properties but different design results in stress similarity?

We found that elastic properties were similar between elk vocal fold tissues and human vocal fold tissues described before. Conservative tissue properties across species are actually not surprising and could be compensated by design differences in order to accommodate different stress constraints. For example, in mammals of different sizes, limb tendons possess similar mechanical properties (e.g. Pollock and Shadwick, 1994). Body posture is altered in those species to achieve certain mechanical functions but remain within safety limits (e.g. Biewener, 1989).

Instead of using the same design and vary its composition or properties of some parts, a new structure could serve a new function. Vocal membranes, thin extensions on top of the free edge of the vocal fold, are often found in bats or non-human primates. They seem to be an adaptive design to reach high fundamental frequencies (Mergell et al., 1999). An intra-specific polymorphism of this structure has been reported for dogs (Riede et al., 2000). However, such structures have not been observed in the histology of any of the ten elk larynges investigated in the morphology experiment.

Sex differences in the elastic properties

In cyclic tensile tests, we found sex differences. Males showed a slightly stronger stress-strain response. The stepwise stretching experiments showed a similar tendency, and the causal relationship may be simple. Sex-related differences in tissue elasticity could be related to the relative densities of collagen and elastin in different layers of the vocal fold (Hammond et al., 1998; Hammond et al., 2000). Sex steroids affect the elastin and collagen composition. In fact, an increase in testosterone increases the collagen/elastin ratio (e.g. Fischer and Swain, 1980). The male elk is under heavy testosterone influence during the rut season (e.g. Haigh et al., 1984) and all larynges were collected during (or very shortly after) peak mating season. Gender-related differences in tissue elasticity have also been found in human vocal folds (Chan et al., 2007).

Differences in staining intensities between male and female vocal ligaments were only suggestive. However, we selected larynges for histology to cover a wide range of ages, particularly in males, and age is an important factor affecting the collagen/elastin ratio in tissues (e.g. Stephens and Grande-Allen, 2007). We predict that the collagen/elastin ratio will be larger in males than in females, particularly during the rut season.

Vocal fold length, age, body size and honest signaling

We found a positive correlation between age and vocal fold length (Fig. 5). Since a positive relationship between age and body size, at least for male elk, has been demonstrated (e.g. Bender et al., 2003), it seems reasonable to assume a positive relationship between vocal fold length and body size. In humans it is known that body height and vocal fold length correlate positively [(e.g. Hirano, 1983; Filho et al., 2005); note that vocal fold length was measured during the resting position or in cadavers]. Despite this positive relationship in anatomical parameters, a relationship between body size and fundamental frequency parameters is somewhat inconsistent (e.g. Rendall et al., 2005). The relationship is roughly inverse if a large age range (adult and juvenile humans) and both sexes (male and female humans) are considered (see for example Fig. 1B this study). However, within the same sex and similar age classes, the F_0 -body size relationship is less clear. Studies of the vocal folds' biomechanical properties help to understand why it is unlikely, or at least very difficult, to find a strong relationship, despite a positive relationship based on morphology between the vibrating source and body size. Fundamental frequency is determined not only by vocal fold length, but also by mean vocal fold tissue stress and maximum stress-bearing potential. Those biomechanical parameters change with age and physical condition. Life-span changes lead to individual-specific patterns of vocal fold settings for particular F_0 production, as reviewed earlier using human data (Nishizawa et al., 1988) and studied by others (Hollien, 1960; Hollien and Moore, 1960). The choice of the 'correct' F_0 parameter to correlate with body size seems important in animal behavior. Average speaking F_0 in humans (Hirano, 1983), minimum F_0 in baboon wahoo-calling (Fischer et al., 2004), or maximum F_0 in chimpanzee pant hoot calling (Riede et al., 2007) demonstrated better results than studies using a species' overall mean fundamental frequency, in the search for honest signaling parameters of physical condition.

The question why elk mimic small animals remains open. Elk call to attract females and to repel competing males. Maybe they signal their physical condition in fundamental frequency parameters unlike the European red deer, who use vocal tract parameters to communicate physical fitness (Charlton et al., 2007). Maybe males, who drive their F_0 to a maximum or who can hold a high F_0 the longest, are most attractive? Further acoustic analyses and behavioral observations must show whether such a 'vocalizing at the edge' mechanism, as proposed for chimpanzee pant hoot calling (Riede et al., 2007), is at work.

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