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Sound Production in the Isolated Mouse Larynx

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Sound Production in the Isolated Mouse Larynx

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Sean William Berquist

2013

ABSTRACT OF THE THESIS

Sound Production in the Isolated Mouse Larynx

by

Sean William Berquist

Master of Science in Physiological Science

University of California, Los Angeles, 2013

Professor Walter Metzner, Chair

Recent evidence indicates that laboratory mice use elaborate ultrasonic songs, and this behavior possibly may be learned. In the present study, air pressure was manipulated while producing sound in isolated larynges from wild type male laboratory mice *post mortem*. The present study indicates that, consistent with findings in other mammals, changes in subglottal pressure induce linear changes in the fundamental frequency of periodic sound, sudden non-linear frequency jumps, biphonation, and sudden changes from periodic to aperiodic sound depending on the absolute value of air pressure applied. The linear and non-linear changes in phonations by the isolated mouse larynx cover virtually all aspects of natural vocalizations produced by adult male mice. Data from simulated contractions of the main laryngeal muscle for frequency control, the cricothyroid, suggest that adduction of the thyroid plays only a minor (if any) role for frequency control in the mouse larynx, especially when compared with the larynx of bats. Therefore, most spectral aspects of mouse vocalizations appear to be based upon changing the tracheal air flow rather than contracting the cricothyroid muscle.

The thesis of Sean William Berquist is approved.

Peter M. Narins

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Walter Metzner, Committee Chair

University of California, Los Angeles

2013

DEDICATION

For My Family

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Chapter 1

ACKNOWLEDGEMENTS

"Whatever you do, don't panic. It only makes things worse." Dr. Z, instructor at the UC Davis Veterinary School had intended these words for future surgeons who may encounter serious procedural complications, however, the phrase applies as a general life lesson that will stay with me throughout my career.

In addition to the above adage, the following people will remain in my mind as I pursue my personal ambitions: Dr. Walter Metzner, my research mentor, for teaching me how to be an independent investigator and pushing me to put forth my best effort; Drs. Peter Narins and Stephanie White, who form the rest of my thesis committee and have inspired me through their teachings and guidance as my professors during my undergraduate and graduate years; Drs. Rachelle Crosbie and Jim Tidball for their support with providing tissue for the project; Scott Arno, a graduate student at UCLA for helping with additional support related to the live mouse recordings; the Metzner Lab, including Khota Kobayasi, Steffen Hage, Tinglei, Sen, and Jennifer Ho, who I owe a great debt for raising me to be a creative and curious scientist; my family, William, Korinne, Eric, Becca, Ethan, Kari, Angie, and Eve, for all their support and love; my friends, especially Valerie Wang, Daniel Tupy, Priscilla Soule, Spencer Naar, Scott Dobroski, Michael Kasian, Alex Nguyen, Nate Ricketts, and Becky B for all their much needed stress relief; and last but not least, Dr. Garfinkel, for his wonderful instruction in bootstrapping statistics that became such an integral part of my research projects.

INTRODUCTION

A wide variety of animal models, such as bats, frogs, birds, nonhuman primates, and various other mammals have long been vital in improving our understanding of basic neurobiological and evolutionary aspects in vertebrate vocalization. However, one model, the house mouse, *Mus musculus*, has only recently been discovered as another tool in communication biology that benefits from genetic tractability. Of particular interest in mouse vocal communication is the adult ultrasonic vocalization (USV); adult laboratory mice have long been known to vocalize in the ultrasonic range (Anderson, 1954; Nyby and Whitney, 1978; Whitney and Nyby, 1983; Sales and Pye, 1974), and behavioral contexts of these vocalizations have been elucidated (Blumberg, 1992; Nyby, 2001; Portfors, 2007) to suggest a communicative function. Indeed, mouse USVs have been proposed as a powerful tool for behavioral assays in studying communicative deficits of many neurodevelopmental disorders (Scattoni et al., 2008).

Mice, unlike other rodents, usually only emit ultrasonic vocalizations during non-aggressive social situations such as mating, with a frequency range between 30-110kHz and exhibiting frequency modulations, sudden frequency jumps and harmonic stacks (Wysocki et al., 1982; Liu et al., 2003; Holy and Guo, 2005; Gourbal et al 2004). In mating situations, males produce nearly all the ultrasonic vocalizations while females tend to be quiet (Warburton et al., 1989; White et al., 1980). Males will vocalize in the presence of a female or to female urine (Nygby, 1979) while females tend to vocalize when kept with other females (Maggio and Whitney, 1985; Moles and D'Amato, 2000) or alone, particularly when her litter is removed (Ehret 2005). Although females do not normally emit USVs with quite the same complexity as males, recent evidence suggests that they possess the underlying neural and physical capability (Kimchi et al., 2007), meaning that the production of complex USVs seen mainly in males are

most likely innate across both genders and not learned. Although there appear to be significant changes in complexity of USVs over the course of development (Grimsley et al., 2011), these changes are not known definitively to be the result of natural organ development or from vocal learning. However, mouse vocalizations may prove useful in studying the evolution of vocal communication and possibly correlates to human communicative disorders and diseases such as Autism Spectrum Disorders and orofacial dyspraxia, the latter of which has been shown to be caused by a mutation in one regulatory protein, FOXP2 (White et al., 2006; White, 2010; Fischer and Hammerschmidt, 2011; Crawley, 2007; Jamain et al., 2008; Scattoni et al., 2008; Silverman et al., 2010).

Spectral complexity and nonlinear phenomena, such as those found in mouse USVs, have been found in several several vertebrate species as being important to acoustic communication (Suthers et al., 2006; Wilden et al., 1998; Fee et al., 2002; Lavenex et al., 1999; Fitch et al., 2002). One study in mice showed that experienced males produced complex USVs more frequently when presented with unfamiliar female urine versus familiar male urine (Musolf et al., 2010), possibly implicating complex USVs being emitted in a mating context to indicate recognition of a potential partner. Additionally, it has been previously thought that to give rise to increased complexity in vocalizations, there generally is a concomitant increase in neural circuitry (Gaunt, 1983; Simpson & Vicario, 1990), despite evidence to the contrary that such nonlinearities may be produced by the vocal organ (Suthers et al., 2006). Either a highly organized neural vocal motor program orchestrates the production of complex USVs and is highly active during appropriate behavioral contexts, or USVs are based on a more simple process involving increased vocal motor drive and innate nonlinear properties of the larynx.

Isolated larynx models have been used to uncover the linear and nonlinear innate properties of the larynx in relation to natural vocalizations in a variety of species, including frogs (Suthers et al., 2006), dogs (Finnegan et al., 2009), birds (Fee et al., 1998), monkeys (Brown et al., 2003), bats (Kobayasi et al., 2012), cows, sheep, and pigs (Alipour and Jaiswal, 2008; Alipour and Jaiswal, 2009). Assuming the phonations are produced by vibrating vocal folds, these nonlinear phenomena arise from complex interactions between the intrinsic biomechanical properties of the folds and aerodynamics of subglottal (Wilden et al., 1998; Mergel et al., 1999; Fitch et al., 2002; Suthers et al., 2006) and possibly supraglottal forces (Titze, 2008). In mice, however, it is still uncertain as to which nonlinear characteristics of phonation, such as chaotic "noise" and biphonations, result from a complex neural motor program or from the biomechanical properties of the larynx itself.

The mechanism of USVs has been unconfirmed for over fifty years. Much like in other mammals, it is thought that mice produce vocalizations with the larynx (Nyby and Whitney, 1978; Roberts 1975). However, before embarking on the task of correlating effects in mouse models to those in humans, some basic questions regarding the basis of USVs still remain. Although studies have shown various types of contextual, hormonal, and neural influence on mouse USVs, the mechanism of sound production has not been clearly demonstrated. Early evidence suggests that USVs are produced in the larynx and emitted through the oral cavity (Roberts, 1975), and the rodent larynx has been shown to have anatomical similarity to other mammals and act as a sound source in a similar fashion (Roberts, 1975; Thomas et al., 2009). In particular, the cricothyroid muscle, an easily accessible muscle that is known to increase tension in vocal folds, is well developed (Roberts, 1975) and has an action that can be mimicked in an excised larynx model (Kobayasi et al., 2012). In one denervation study, transecting either the

superior or inferior laryngeal nerve led to a loss in USV production (Nunez et al., 1985). Studies looking into the mechanism of laryngeal USV production have been inconclusive, with one study using the effects of helium on voiced sound to support a whistle mechanism (Roberts, 1975) while another suggesting the idea of two mechanisms (Holy and Guo, 2005). Uncovering the mechanism of USV production in the mouse larynx will have several implications, including how the mouse model could be genetically altered to mimic human speech pathologies.

The epilaryngeal tube, a small structure that serves as part of the sound filter in vocalization production, has been studied in a linear acoustic interaction model with the voice source (Titze & Story, 1997; Chan & Titze, 1998) as well as recently in a nonlinear model (Titze, 2008). Increased inertance - the pressure gradient required to induce a change in air flow - and impedance, as a result of the dimensions of the structure, have been proposed to make several contributions to vocalized sound: 1) lower phonation pressure, 2) increase vocal efficiency (reduction of vocalization onset pressure), 3) produce the output formant, and 4) affect the mode of vibration in vocal folds (Titze, 1988; Rothenberg, 1988; Titze, 2001; Titze & Laukkanen, 2007; reviewed in Titze, 2008). Additionally, it has been proposed that such a structure has an effect on nonlinear attributes such as hysteresis and bifurcations. The role of the epilaryngeal tube, or epilarynx, however, has to date not been studied thoroughly using excised larynx models but only using theoretical models and simulations. Additionally, understanding how the epilarynx may influence nonlinear aspects of vocalization may more specifically identify which structures give rise to each particular nonlinear acoustic attribute.

Although much is known about the type, structure, and, to a lesser degree, behavioral function of mouse USVs, several basic questions about the production of mouse vocalizations remain. First, the degree to which the larynx itself can produce the full range of linear frequency

changes as well as nonlinear phenomena seen in vocalizations has yet to be determined. By proxy, the characteristics of mouse vocalization may not be produced by mere subglottal pressure changes, i.e. neural control over inspiratory and expiratory muscles, but involve more complex types of neuromuscular control via the vocal motor pathways. Second, although first studied over 35 years ago (Roberts, 1975), it remains to be determined if there is only one or maybe multiple mechanisms that could be responsible for different types of vocalizations, e.g., one for audible vocalizations and a separate mechanism for USVs. Third, the role of supra- and infra-glottal structures in affecting spectral features and vocal economy in mice have yet to be investigated. While previously studied extensively in laboratory larynx models and simulations (Zhang et al., 2006; Chan and Titze, 1998; Story et al., 2001; Titze, 2004; Chan & Titze, 2006; Titze and Laukkanen, 2007), the role of extra-glottal structures has yet to be tested in a mouse model and may further provide insight into the sound production mechanism.

In the current study, we attempt to further the current understanding of mouse USV production through a series of experiments using isolated mouse larynges in an *in vitro* model of vocalization. Firstl, we looked at linear nonlinear characteristics of the larynx by applying short ramp increases of air pressure to assess the biomechanical properties of the isolated mouse larynx to the range of natural vocalizations. Second, we mimicked the action of the cricothyroid muscle, a highly developed and visible muscle in the mouse (Roberts, 1975), to increase tension in the vocal folds as a possible mechanism for increasing the linear range of USV (Kobayasi et al., 2012). Third, helium was applied in place of atmospheric air through a set of larynges to assess whether the USV mechanism was by vocal fold vibration or by whistle, extending a similar study done with live rodents (Roberts, 1975) and frogs (Rand and Dudley, 1993) by

applying physics of helium's effect on vocalization (Fant & Lindquist, 1968). Last, all tissue above the laryngeal cartilages was removed to assess the role of the epilarynx.

Our findings show that, consistent with findings in other mammals, changes in subglottal pressure induce linear changes in the fundamental frequency of periodic sound, sudden non-linear frequency jumps, biphonation, and sudden changes from periodic to aperiodic sound depending on the absolute value of air pressure applied. The linear and non-linear changes in phonations by the isolated mouse larynx cover virtually all aspects of natural vocalizations produced by adult male C57BL/6J mice. We also simulated contractions of the main laryngeal muscle for frequency control, the cricothyroid, by adducting the thyroid cartilage towards the crocoid. The data suggest that adduction of the thyroid plays only a minor (if any) role for frequency control in the mouse larynx, especially when compared with the larynx of bats. Therefore, most spectral aspects of mouse vocalizations appear to be based upon changing the tracheal air flow rather than contracting the cricothyroid muscle.

MATERIALS AND METHODS

Animals

We recorded from 3 male wild-type (WT) mice (C57BL/6J) prior to them being sacrificed as part of a different experiment (Dr. I. Fried, UCLA). 2 female WT mice from the same lab were used in the behavioral assay to obtain natural vocalization recordings. Additionally, 10 female and 20 male WT were obtained *post mortem* from the laboratories of Drs. R. Crosbie-Watson and J. Tidball and, as done with the first 3 mice mentioned, larynges were harvested shortly after the lab sacrificed the mice through a tissue sharing protocol of the Institutional Animal Care and Use Committee (IACUC) at UCLA. All mice were adults with an age range of 11-13 months. Mice were originally purchased from the Jackson Laboratories (Bar Harbor, ME) for breeding and experimental use under IACUC guidelines before transfer of remaining tissue. Tissue used in these experiments would have otherwise been discarded.

Natural vocalization assay and recordings

Recordings of USVs were obtained (Figure 1) following a modification of a previously described protocol (Holy and Guo, 2005) used in training naive male mice to vocalize in the presence of female mouse urine. When the mice showed their highest activity levels during the day-night cycle, a naive male WT was placed in a naive female WT's cage in a dark, sound attenuated container. Water and food had been removed from the cage and the bedding had not been changed for 2 days. After waiting for a 2-minute period to allow the male to become accustomed, we recorded his vocalizations for a 5-minute period. Recordings were made using a condenser ultrasonic microphone (CM16, Avisoft-Bioacoustics, Berlin, Germany; flat ±4dB frequency response between 10 and 150 kHz), mobile ultrasonic recording interface (UltraSoundGate 116, Avisoft Bioacoustics, Berlin, Germany; maximum sample rate: 750 kHz,

8-bit resolution), and commercial recording software (Avisoft-RECORDER; Avisoft Bioacoustics), before being analyzed using analysis software (Avisoft-SASLab Pro, version 4.3; Avisoft Bioacoustics). The microphone was secured to a metal wire rack and directed downward towards the center of the cage such that its tip was approximately 8-15 centimeters from the mouse. To assess whether the male was vocalizing, a video recording during the 5-minute period was taken with a night vision camera (Model QS2814C, Q-See, Anaheim CA, USA) and analyzed for physiological indicators (chest and abdomen movement) of vocalization.

***In vitro* tissue preparation and setup**

Mouse tissue was promptly frozen, within 30 minutes *post mortem*, for storage at -5º C. On the day of experimentation the tissue was quickly thawed by being sealed inside of a plastic bag before exposure to water of ~ 25º C. Tissue was then prepared following an approach previously described for the bat larynx (Kobayasi et al., 2012). The chest cavity was exposed and the entire respiratory tract was removed including the lung, trachea, larynx, and lingua, initially cutting just dorsal to the soft palate. The lungs were completely removed along with as much of the lingua and excess connective tissue as possible to relieve the larynx of any forces that may increase onset pressure. The remaining tissue was transported and temporarily placed in an isotonic saline solution (0.9% NaCl) until the larynx could be used in the experimental apparatus (Figure 2). An opening was made in the trachea just superior to the primary bronchioles before inserting a small piece of polyethylene tubing (2 cm long, 1.14 mm interior diameter, 1.57 mm outer diameter; Clay Adams Inc. New York, NY, USA) through the opening and securing the trachea in place with a cotton string. The tubing was connected to polyvinyl chloride tubing (4.7 mm internal diameter, 7.9 mm outer diameter; Fisher Scientific, CA, USA) through a 29 gauge needle (21 gauge Precision Glide, Becton Dickinson & Co, Franklin Lakes NJ, USA). The

tubing provided warmed (35-40º C) humid (>90%) air as depicted in Figure 2 using a Büchner flask. Air was first supplied from a pressurized outlet into the flask top opening, which was sealed with a rubber stopper, and sent through a highly porous aquarium air diffuser, or "air stone", that was submerged in warm water. This humidified air then exited through the hose barb near the neck of the flask and out into the polyvinyl chloride tubing. Subglottal air pressure was altered manually and measured relative to atmospheric air pressure using an electric pressure sensor (MPX5050DP; Freescale Semiconductor, Austin, TX, USA) and a 0.45 correction factor from the smaller diameter tracheal tubing compared to the diameter of the sensor membrane. The voltage output of the sensor was converted to pressure as kPa. To prevent the tissue from drying, which has been known to increase tissue viscosity and thereby increase onset pressure as well as decrease phonation frequency (Zhang et al 2009, Jiang et al 1999; Jiang et al 2000; Sivasankar and Leydon 2010; Witt et al 2009), isotonic saline solution was applied to the larynx every 5 minutes. To control for sound produced by tube vibration, recordings were conducted of air applied through the apparatus without larynges.

Experiment 1. In vitro sound production in isolated larynges

In order to explore how changes in air pressure affect phonations from the larynx, subglottal air pressure was manually altered within a range of 0 to 3.5 kPa, with air flow being altered up to 2 L/min, in a series of linear increases and decreases. Each linear change lasted between 1 to 20 s and was repeated at least 100 times per larynx preparation. In many cases, pressure was ramped up until the onset of phonation, after which the pressure was continually ramped up further and subsequently reduced to determine the relationship of subglottal air pressure and the dominant frequency of phonations. This experiment was performed on all larynges.

Experiment 2. Simulation of cricothyroid (CT) muscle contraction

The effect of cricothyroid muscle contraction was assessed by adducting the thyroid towards the cricoid cartilage. This increases tension on the vocal folds and thus increases their resonant frequency as a function of stiffness/mass. A pair of forceps (Dumont #5, Fine Science Tools, Foster City, CA; width of tips: approximately 50 μm) was used to manually simulate cricothyroid muscle contractions by placing one tip superior to the ventromedial edge of the cricoid and the other just inferior to the ventromedial protrusion of the thyroid. To quantify the degree of adduction, we attached a slide potentiometer (linear motion, Type V448 MONO, CTS Electronic Components, Kaohsiung, Taiwan), positioned between the shanks of forceps (approximately 3 cm from the tip ends), using glue and cotton string. Squeezing the forceps to adduct the cartilage would alter the DC voltage output from the device and allow for measurement of adduction within an accuracy of 5 μm. These experiments were performed on five male larynges.

Experiment 3. Effects of helium on larynx phonations

Following a previous investigation of ultrasonic vocalization mechanisms (Roberts, 1975) we set out to use a helium medium instead of atmospheric air. 100% pure helium (Worthington Cylinders, Columbus, OH) was applied shortly after producing an ultrasonic phonation using atmospheric air. If the mechanism of phonation is that of a whistle, the sound frequency should increase as a function of sound velocity. This follows the idea that in a whistle mechanism, sound is produced by turbulent vibrations of air particles and is subjected to equation 1:

(1) $$V_S = f \times \lambda$$

where V_s equals sound velocity, *f* is the frequency of vibrations, and λ is the wavelength. If the sound is produced in a less dense medium such as helium, the speed of sound will increase and, hence, the frequency. The wavelength is assumed to remain constant in a whistle since it is determined by the physical properties of the resonating chamber. This relationship does not exist in a system where sound is produced by a string-based mechanism, such as vocal cord vibration, in which the sound frequency is determined primarily by the vibration rate of the vocal cords. Knowing the relative molecular weights of atmospheric air (78% N_2, 21% O_2, 0.04% CO_2) and 100% He_2 we could calculate the expected increase in sound velocity and the increase in sound frequency using equation 2:

(2) $$V_S = \sqrt{\frac{\gamma RT}{M}}$$

where γ is the adiabatic constant, R is the universal gas constant (8.314 J/mol K), T is the absolute temperature, and M is the molecular mass of the gas. Assuming a constant temperature and a direct relationship between sound velocity and frequency, we have the following relationship:

(3) $$f \propto \sqrt{\frac{\gamma}{M}}$$

We can then calculate the expected changes in sound velocity easily, assuming the use of dry air instead of humid air. Since the use of humid air would add the effect of introducing more complexities in calculating the precise adiabatic constant and average molecular weight, we decided to only use dry air for this experiment in order to more accurate predictions. When using dry air, the following values apply: $\gamma_{air} = 1.4$, the adiabatic constant for dry air; $\gamma_{He} = 5/3$, adiabatic constant for helium; $M_{air} = 0.02895$ kg/mol, the average molecular mass for dry air;

M_{He} = 0.004 kg/mol, the molecular mass for helium. The expected change in sound velocity is described by the following:

$$(4) \qquad {f_{He}}/{f_{air}} = \sqrt{\frac{\gamma_{He}}{M_{He}}} \Bigg/ \sqrt{\frac{\gamma_{air}}{M_{air}}} = \sqrt{\frac{1.66}{0.004\ kg/mol}} \Bigg/ \sqrt{\frac{1.4}{0.02895\ kg/mol}} = 2.93$$

This gives an increase in sound frequency of 2.93 times in helium when compared to dry air. This experiment thus provides an indirect method of deducing the mechanism of ultrasound phonations by comparing frequency ranges in both mediums, and confirms a decades old hypothesis of rodent vocalizations.

Experiment 4. Effects of supraglottal structures on phonation.

To assess the role of supraglottal structures that were not removed in the initial tissue preparation, in approximately 12 larynges we subsequently removed the epiglottis, pharyngeal structures, and what has been identified as the "epilaryngeal tube", which has been implicated in increasing vocal economy as well as effecting nonlinear attributes of larynx phonation (Titze 2008; Titze 2009; Titze and Laukkanen 2007; Titze and Story 1997; Story et al 2001; Chan and Titze 1998; Dollinger et al 2006) from several larynges. Tissue was removed superior to the larynx and down to the superior border of the cricoid and thyroid cartilages to ensure that as much of the epilaryngeal area was removed as possible.

Recording procedure, data analysis and statistical analysis

For larynx phonations, a quarter-inch ultrasonic microphone (model 4939 with preamplifier 2633, Brüel & Kjær, Nærum, Denmark) was placed 15 cm away from and pointed directly towards the rostral end of the larynx for recording. The recording system had a flat (± 3dB) frequency response between 10 kHz and 100 kHz. Sound, air pressure, and cricothyroid

adduction data were analyzed first through an A/D signal processor (Micro1401-3, 500 kHz maximum sample rate, 32-bit ARM7 processor; Cambridge Electronic Design, Cambridge, England) and fed into a data acquisition system software (Spike2, Version 7; Cambridge Electronic Design, Cambridge, England). FFT length used for sound spectrograms was 1024 points with 94% overlap (spectral resolution of 195 Hz). Phonations were visually analyzed and isolated for data extraction in order to identify artifacts and audible sound, such as ambient noise (dominant frequency below 25 kHz). Commercial sound analysis software (Avisoft-SASLab Pro; Avisoft Bioacoustics, Berlin, Germany) was used to extract frequency, amplitude and bandwidth (frequency minimum and maximum at 20 dB below peak frequency) of sound files. Computing software (MATLAB, version 7.1; Natick, MA) extracted pressure data, graphically displayed combined data and was used in the statistical analysis.

Statistical Methods

To avoid the requirement of a Gaussian normal distribution and the effect of outliers, no statistical methods requiring the use of means or squaring were used. Medians and median absolute deviation (MAD) were calculated to determine the Bootstrap resampling and Monte Carlo using medians to test for correlations was used for statistical analysis. Resampling does not assume data are of a particular distribution, such as a normal distribution, and thus is a useful method of statistical analysis that uses the distribution of the data itself. The Pearson's Coefficient was calculated using 10,000 resamples and statistical significance was determined if a probability of error of <5% was achieved over 95% of all resampled coefficient values. Median and median absolute deviation (MAD) were chosen as descriptors that were not dependent on the existence of a normal distribution. Bootstrap was also employed to test for

differences among groups using 10,000 resamples (statistically significant difference if overlap of <5% of the resampled test statistic).

RESULTS

Natural mouse vocalization recordings

We recorded from two naive male C57BL/6J mice which emitted several bouts of USVs over approximately 4 sessions, each lasting 5 minutes. Two representative samples from each male are shown in detail over an approximately 2 s period (Figure 1). Sounds were grouped spectrally into two frequency "bands", a lower band that ranged from 40 kHz to 75 kHz and a higher band that ranged from approximately 60 - 85kHz. The amplitude of the emitted sounds were always larger in the lower band with amplitudes up to 95 dB SPL (as measured using Avisoft automated analysis). Vocalizations generally consisted of three types of syllables: a tonal, frequency modulated sound emitted within the lower frequency band (Figure 1 - "A"); a frequency modulated sound that quickly sweeps upward within the higher frequency band before suddenly jumping down to the lower frequency band ("B"); an upward frequency modulation within the higher band followed by a jump down to the lower band and finally a jump back up to the higher band ("C"). Of the three types, the third was the rarest and is only shown once in Figure 1. The vocalizations in our WT mice were very similar to those found in other mouse lines such as JA that have been characterized in detail (Holy & Guo, 2005). The sound frequencies between the higher and lower bands (such as indicated by B and C in Fig. 1) are non-harmonically related, indicating a fast transition from one dominant vibratory mode to another. Additionally, any two frequency modulations before and after a jump contained virtually no period of overlap. The amplitude across a frequency jump always increased, indicating that there may be a consistent use of increased vocal power to drive the vocal system into producing such a nonlinearity. No other nonlinearities, such as biphonations or chaos, were found in our recordings of natural USVs.

Experiment I. In vitro sound production in isolated larynges

Recordings from isolated mouse larynges were successful in 20 larynges (out of a total of 30 harvested) to produce phonations over a maximum absolute pressure range of 2 kPa, consistent with findings in similar work on bat larynges (Kobayasi et al., 2012). Representative phonations are depicted in Figure 3. Figure 3A and C depict male larynx phonations while B and D were female larynx phonations. The absolute air pressure used to generate these phonations is indicated in the lower traces. Matching the behavioral observations in natural mouse USVs, larynx phonations occupy two discrete frequency bands, a higher band and lower band that had an inverse relationship to subglottal pressure; low pressures generally produced frequencies within the higher band while higher pressures produced frequencies within the lower band. All four examples shown exhibit frequency jumps, with Figure 3A showing a consistent pressure at which the jump occurs across three cycles of pressure increases and decreases. Subharmonics occurred at higher pressures (Figures 3A and D). Broadband, "noisy" phonations, present in 10 preparations, can be seen in Figure 3C. Biphonations (not depicted) were present but only in 4 preparations. Noise and biphonation characteristics, shown in Table 1, were fairly consistent across subjects. Noise bandwidth was roughly 6.05 ±1.37 kHz with an onset pressure of 2.27±0.34 kPa and center frequency of 45.49±2.52 kHz. Biphonations contained a lower frequency sound at 8.50±3.50 kHz and a higher frequency sound at 55.58±8.58 kHz at an onset pressure of 2.71 ±0.34 kPa.

Isolated larynx phonations were analyzed further in detail for sound emitted within air pressure changes of 0.05 kPa (0.05 kPa bin widths) yielding the frequencies, amplitudes, and bandwidths for one male and one female larynx phonation for different air pressures (Figure 4). These examples represent several cycles of pressure changes pooled together. For the male

larynx (top two rows), a single cycle is highlighted in red and represents frequencies generated when the pressure was decreasing, whereas the blue symbols indicate frequencies generated when pressure increased. Figure 4 B1-3 shows a statistical representation of the cycles, showing that frequency jump occurred consistently at the same pressures for both upward and downward jumps: The downward jump occurred within a 0.1 kPa window, while the upward jump occurred within a 0.15 kPa window (windows correspond to the number of bins required to shift the median frequency between the high and low bands). The frequency jumps always coincided with sudden changes in amplitude (Figure 4 A2 and B2): downward frequency shifts were accompanied by sudden upward shifts in amplitude and *vice versa*. They also coincided with a temporary increase in bandwidth (Figure 4 A3 and B3) that is most likely an artifact from measuring frequency both before and after the jump. As pressure continued to climb, bandwidth began to increase again as the larynx began to enter a state that produced large bandwidth "noise". The example of the female larynx (third row) produced somewhat more variable data: The pressures at which the frequency jumps occurred was broader and the concurrent jumps in amplitude and bandwidth were missing. However, the transition to a "noisy" state at the highest pressures was present. We have preliminary evidence that these differences were due to variation in tissue integrity after thawing.

Despite individual differences in males and females, there were no overall spectral features that separated the sexes (Figure 5). In both sexes, amplitudes remained within a range between 70 and up to 100 dB SPL (Figure 4). This value is consistent with findings observed elsewhere (Portfors, 2007). There were no major differences in frequency measured at phonation onset and offset or before and after jumps. There was a difference observed at peak pressure, however, although this value was based on a variable measure of pressure that changed from

cycle to cycle; the peak pressure was different depending on the maximum pressure manually set by the human operator. Additionally, no statistical differences were found between frequency ranges emitted at low or high pressure.

Following a previous analysis (Holy and Guo, 2005; Figure 6A), frequency jumps and linear ranges of isolated larynx phonations were visualized using a technique that compared frequency at time point t with frequency at time point t + 5 milliseconds (Figure 6B). Approximately 65% of the range in linear changes of natural vocalizations recorded in our mouse strain could be explained by subglottal pressure in the isolated larynx. However, this range is shifted down, producing USV frequencies not seen in our limited sample of natural USVs recordings. Taking into account the shift, isolated larynges produce only approximately half the amount of frequencies than could be recorded behaviorally. Three types of frequency jumps appeared as data clusters, with two down jumps and one up jump; this unique pattern introduced a new second type of downward jump not seen in the natural mouse recordings of our study, and could possibly be due to a novel characteristic only seen in larynges that have experienced a loss of tensile forces and tissue integrity.

Further preliminary analysis compared sounds produced from three groups: male mating mice, larynges from mating mice, and all other male larynges (Figure 7). In Figure 7A, natural mouse vocalizations (filled green circles) had an phonation onset frequency approximately twice that produced by larynges isolated from the same animals and tested *post mortem* (filled red circle at Fmin of the high frequency band). However this 2:1 ratio decreased when comparing frequencies of sounds emitted before and after jumps, as well as for frequencies at phonation offset. Only two statistical differences existed when comparing phonations from larynges of mice used in the behavioral assay (i.e., in which natural USVs were recorded) and those of other

mice, in which natural USVs were not recorded: the frequency of sound at phonation onset (filled red circle and filled red square at Fmin in the high frequency band) and the frequency of sound after a downward frequency jump (open blue circle and open blue square under Fmax in the high frequency band). However, these differences had a small effect size and the small sample size of larynges from mice that were behaviorally recorded (circles, n=2) indicated that more data needs to be obtained to establish a more robust relationship. A comparison of frequency ranges for the three groups (Figure 7B) showed a striking similarity between mice recorded from during the behavioral assay (linear range approximately 20-30 kHz) and phonations obtained from their own larynges, with similar ranges reported for the higher frequency band, although the similarity was absent in the lower frequency band, where the frequency ranges of isolated larynges more closely matched those of larynges from mice not used in behavioral assays (linear range approximately between 5 and 15 kHz). Linear ranges of larynges from mice not used behaviorally were consistently within an approximation of 5-15 kHz.

A preliminary comparison of frequency jumps observed across groups of larynges and mice recorded in the behavioral assay is shown in Figure 7C and D. No differences were found between either isolated larynx group. Vocalization jumps consistently occurred at frequencies just under a 2:1 ratio with phonations from isolated larynges (Figure 7C). However, the relationship between vocalizations and phonations was closer to a 2:1 ratio with respect to jump size (Figure 7D).

Experiment 2. Simulation of cricothyroid (CT) muscle contractions

Following experiments that showed laryngeal denervation led to a loss of USV production (Nunez et al., 1985), we mimicked the action of the cricothyroid (CT) muscle, which

is innervated by the superior laryngeal nerve and shown to be well developed in the mouse (Roberts, 1975). CT contractions were simulated by adducting the thyroid towards the cricoid cartilage during larynx phonations caused by air passing through the vocal folds at a constant pressure (Figure 8A and B). Out of 10 preparations in which this method was attempted, only 3 produced viable results (Figure 8C). As the CT distance decreased, the frequency of laryngeal phonations changed only within a range of up to 1-2 kHz over approximately 0.55 mm in distance between the caudal tip of the anterior portion of the cricoid cartilage and the rostral tip of the anterior portion of the thyroid cartilage. Regression lines from three larynges were used to form a general regression (black line, Figure 8C, R^2 0.2255), indicating that there is no clear correlation between simulated CT contraction and emitted frequency.

Experiment 3. Effect of helium on larynx phonations

In six preparations, atmospheric air was replaced by helium (Figure 9) to obtain preliminary data on the underlying mechanism of sound production. Sound traveling in a helium medium, which has a lower density that oxygen, has a higher velocity. If phonations in the mouse larynx are based on a whistle-like mechanism, phonation frequencies should increase (Roberts, 1975). In contrast, if sound production involves a vibration-based mechanism, sound frequencies should not depend on sound velocity. We found that at similar pressures, the larynx phonated at a similar fundamental frequency (Fig. 9A,B). Frequencies at phonation onset and offset, at the maximum value for the low pressure band, at the minimum value for the high pressure band, and at the maximum pressure were similar between helium and atmospheric air (Figure 10A). Median frequencies were approximately 40 kHz at phonation onset, 35-40 kHz at phonation offset, 45 kHz at the maximum value for a low pressure band phonation, 40 kHz at the minimum value for a high pressure band phonation, and 45-50 kHz at maximum pressure.

Pressure values at each of these frequencies were similar (Figure 10C). The ranges of linear frequency change at low pressures were also not statistically different in the two media (Figure 10B). However, the range of linear frequency change at high pressures was at least twice as large in air compared with helium(5 kHz vs. 1-2 kHz). At first glance, the change in medium appeared to have no dramatic effects on laryngeal phonation.

However, measuring additional vocal parameters did indicate some differences between atmospheric air and helium: Vocal efficiency, defined as the change in frequency per unit pressure change (dF/dP), differed (Figure 10D). At low pressures, larynges had a consistently greater vocal efficiency in atmospheric air (8 and 10 kHz/kPa) than in helium (5 and 7 kHz/kPa). This difference was not observed at higher pressures. Another difference became obvious when considering sudden frequency jumps. Although generally the frequency differences before and after the jumps were small (Figure 10E), they were nevertheless slightly larger for helium than for air (approximately 8 kHz vs. 5.5 kHz) and the pressures at which jumps occurred were higher (approximately 0.25 kPa difference for downward jumps and over 0.5 kPa for upward jumps; Figure 10F and G). These results are consistent with the finding that vocal efficiency is greater at lower pressures for atmospheric air. Jumps would therefore also occur at lower pressures in atmospheric air.

Experiment 4. Effect of supraglottal structures on larynx phonations.

Removing supraglottal structures and impacting the structural integrity of the epilaryngeal tube that resides just superior to the vocal folds produced few effects on the spectral aspects of laryngeal phonation. Frequency measures at phonation onset and offset, at the maximum value for the low pressure band and at the minimum value for the high pressure band, and at the maximum subglottal pressure were not significantly different ($p>0.05$, 95% confidence

interval overlap) in larynges with supraglottal structures intact or absent (Figure 11A). Similarly, there were no significant differences in the range of linear frequency changes between the two groups, although there was a slight trend for the median linear frequency ranges to be greater in larynges with supraglottal structures present (Figure 11B). Greater differences emerged when examining the pressure values at spectral landmarks and vocal economy (Figure 11C and D). Median pressures were lower by 0.5 kPa lower at phonation onset, by 0.3 kPa at phonation offset, and consistently lower at all spectral landmarks (Figure 11C) when supraglottal structures are intact. Additionally, vocal economy at lower pressures in these larynges was improved by 2 kHz/kPa (Figure 11D).

Spectral qualities of frequency jumps were similar between groups. Frequencies at which jumps occurred did not differ between the two groups, with the exception of a slight decrease in the frequency at the end of an upward jump in larynges lacking a supraglottis (Figure 11E). Additionally, no differences were observed in the size of the frequency jumps (Figure 11F). However, the pressures at which jumps occurred differed significantly, with jumps occurring at lower pressure values in larynges having an intact set of supraglottal structures (Figure 11F; difference in median pressures: 0.4 kPa to 0.6 kPa). The effect was especially pronounced in upward jumps.

DISCUSSION

Our current study aimed at determining which details of natural mouse USVs corresponded to phonations from an isolated mouse larynx, i.e. were based on biomechanical properties of the larynx, and which may require direct neuromuscular control. We found that the isolated mouse larynx, both from male and female mice, produced most features seen in natural USVs during mating behavior, such as linear upward and downward frequency modulations within the two frequency bands, a higher and a lower one, also occupied by natural USVs, as well as non-linear sudden jumps between these bands. We even observed broadband "noise" and biphonations, which we did not see in our natural USVs but which is a common feature in most vertebrate vocalizations (Suthers et al., 2006; Wilden et al., 1998; Fee et al., 2002; Lavenex et al., 1999; Fitch et al., 2002; Kobayasi et al., 2012). As evidenced from one investigation in birds (Elemans et al., 2010), the absence of nonlinear phenomena, such as dense spectra, in natural vocalizations that are observed in the isolated vocal organ may be a result of precise operation of the vocal motor system from neural control. Nevertheless, the replication of features from natural USVs in larynx phonations suggests that most features of natural mouse USVs were mainly based upon biomechanical properties of the larynx and did not require direct neuromuscular control, similar to what was observed in other vertebrates, such as birds, frogs and bats (Fee et al., 2002; Suthers et al., 2006; Kobayasi et al., 2012). The entire range of frequencies produced by isolated mouse larynges, however, was shifted down significantly from that produced in live mice (Figures 6, 7A and C). We did not observe such a shift in our previous work on bat larynges (Kobayasi et al., 2012) and attribute this effect to a loss in tissue tension in the mouse larynges which is much more delicate than that in bats.

Male and female laboratory mice produce spectrally similar USVs (Moles et al., 2007; Portfors, 2007; Ehret, 2005; Warburton, 1989). It is therefore not surprising that male and female mouse larynges also produced spectrally similar phonations (Figure 5), with similar onset and offset frequencies and frequency bands. Preliminary evidence suggests that the larynges in male and female mice are also morphologically similar (Roberts, 1975; Thomas et al., 2009), although additional histological and biomechanical studies are needed to verify that. Female mouse vocalizations, therefore, appear to differ from those of males mainly in the behavioral context in which they are produced: females will either vocalize with other females or when alone and especially after litter removal (Moles and D'Amato, 2000; Ehret, 2005), while males tend to vocalize only in a mating context (Sales, 1972; Holy and Guo, 2005; Guo and Holy, 2007).

Understanding the biomechanics of the mouse larynx depends on determining the relative contribution of three major possible mechanisms of sound production: tissue vibration, vocal fold vibration, and whistling. Only two studies to date addressed this problem; the first used helium in various rodent species, none of which were *Mus musculus*, and concluded that mice do not use vocal fold vibration (Roberts, 1975). The second study showed that denervation of the superior or inferior laryngeal abolished USVs, suggesting that neuromuscular control, and thus contraction of laryngeal muscles were part of the sound production mechanism in mice (Nunez et al., 1985). In our present study, we present preliminary results using helium instead of ambient air, which suggest instead that vocal fold vibrations may indeed play a role in laryngeal phonation (Figures 7 and 8): Whether in a medium of atmospheric air or helium, laryngeal phonation frequency remained unchanged, which is counter indicative of a whistle mechanism (which does not involve vocal fold vibrations). As detailed in the Methods section, whistle

based sound frequencies will change depending on the density of the gas medium, unlike in tissue vibrations or vocal fold vibrations (Fant and Lindquist, 1968). Recording natural USVs in live mice breathing an oxygen-helium mixture (instead of oxygen-nitrogen) and, ideally, visualizing vibrations of the vocal folds are needed to test our preliminary results and determine if vocal fold vibrations are involved in generating natural USVs.

We further explored the role of neuromuscular control for laryngeal phonations especially by the cricothyroid (CT) muscle (Roberts, 1975). Contractions of the CT muscle in mammals in general contribute significantly to altering vocal frequency (Perlman and Alipour-Haghighi, 1988; Kitajima et al., 1979). Following a similar protocol used in previous work (Kobayasi et al., 2012), we adducted the thyroid towards the crocoid cartilage and tested the contribution of this simulated contraction of the CT muscle to frequency changes in larynx phonations. Surprisingly, we found no relationship between the amount of adduction and phonation frequency (Figure 8). Since USVs have been shown to be abolished by laryngeal nerve transaction (Nunez et al., 1985), the possibility remains that other muscles may influence spectral features of phonations. In addition, removal of accessory, supporting tissue around the delicate mouse larynx may have lowered the compliance of the larynx below its natural level and thus altered the effects of thyroid adduction in our preparation. Eliciting phonations in an *in-situ* preparation while electrically stimulating the CT muscle or its motor nerve (the superior laryngeal nerve) may allow one to verify our preliminary results.

Supraglottal structures, in particular the epilaryngeal tube, have been studied extensively in theoretical studies examining nonlinear coupling in source-filter interactions (Titze, 2007; Titze, 2004). The epilargyneal tube area appears to have a direct relationship with phonation threshold pressure, glottal air flow, and vocal fold dynamics (Titze and Story, 1997; Dollinger et

al., 2006; Chan and Titze, 2006). Evidence also points to the vocal tract and possibly supraglottal structures as a player in producing nonlinear phenomena such as broadband "noise" (chaos), frequency jumps, and bifurcations (Hatzikirou et al., 2006). Our data comparing phonations from isolated larynges with and without supraglottal structures support the idea that a narrow supraglottal housing facilitates phonation, vocal efficiency, and increasing the range of frequencies produced by the isolated larynx (Figure 11). However, nonlinear phenomena such as broadband "noise" (chaos) and rapid frequency jumps were still present and similar in larynges with a supraglottis present or lacking it. It has been suggested (Hatzikirou et al., 2006) that the presence of a tube alone surrounding vibrating the vocal folds could also produce nonlinear phenomena in laryngeal phonation. Thus, the presence of the trachea, subglottal structures, or some remnant supraglottal structures that maintain a tube structure associated with the vocal folds may have aided in producing nonlinear phenomena in the isolated larynges.

In summary, it is still unclear how mouse USVs are generated by the larynx and the brain. To date there has not been conclusive evidence on a single mechanism or the specific roles that various features of the larynx may play in producing the full range of USVs. The present study furthers our understanding in that simple pressure changes can elicit a significant range of ultrasonic frequencies as well as prominent nonlinear features of vocalization. The long held hypothesis of a whistle mechanism for mouse USV production has been called into question, similar to the role of extrinsic laryngeal muscle contraction. Further studies are required to fully understand what certainly is a complex process of producing mouse vocalizations that may ultimately provide important cues in understanding human communicative disorders and speech pathologies.

FIGURES

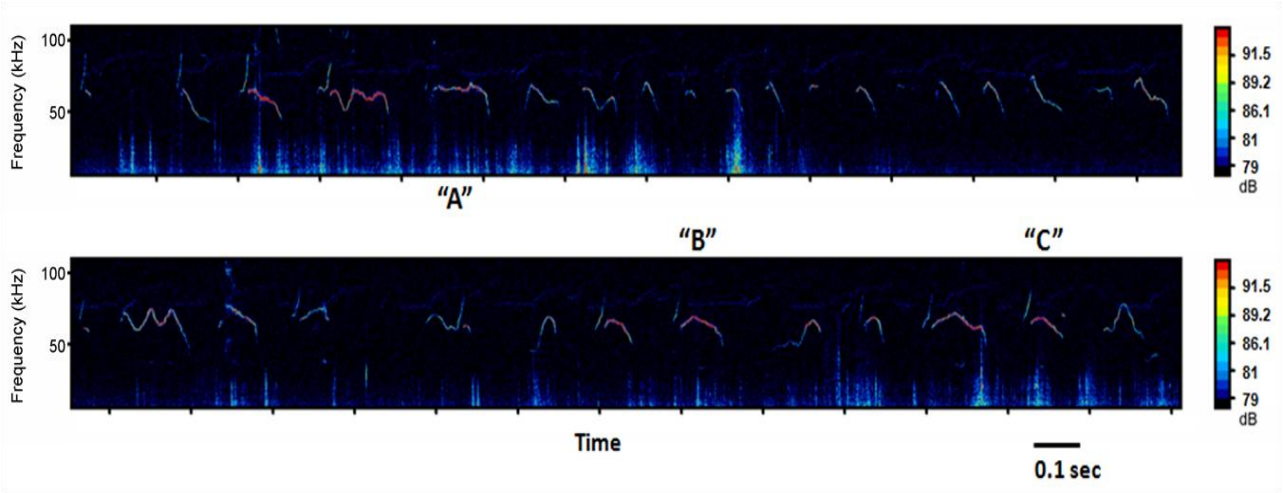

Figure 1. Natural ultrasonic male mouse vocalizations in response to the presence of a female. Representative samples of two recordings from 2 different naive males (upper vs. lower trace) while in the presence of a naive female. Both males produced both linear, tonal frequency modulations within a distinct frequency band between 40 and 75 kHz, such as the calls indicated by "A". In addition, both male mice emitted sudden, non-linear jumps (see "B") from an initial upward sweep within a higher frequency band (60 – 85 kHz) to the lower band between 40 and 75 kHz. In one male, an upward jump followed a downward jump (see "C"). For more details, see text.

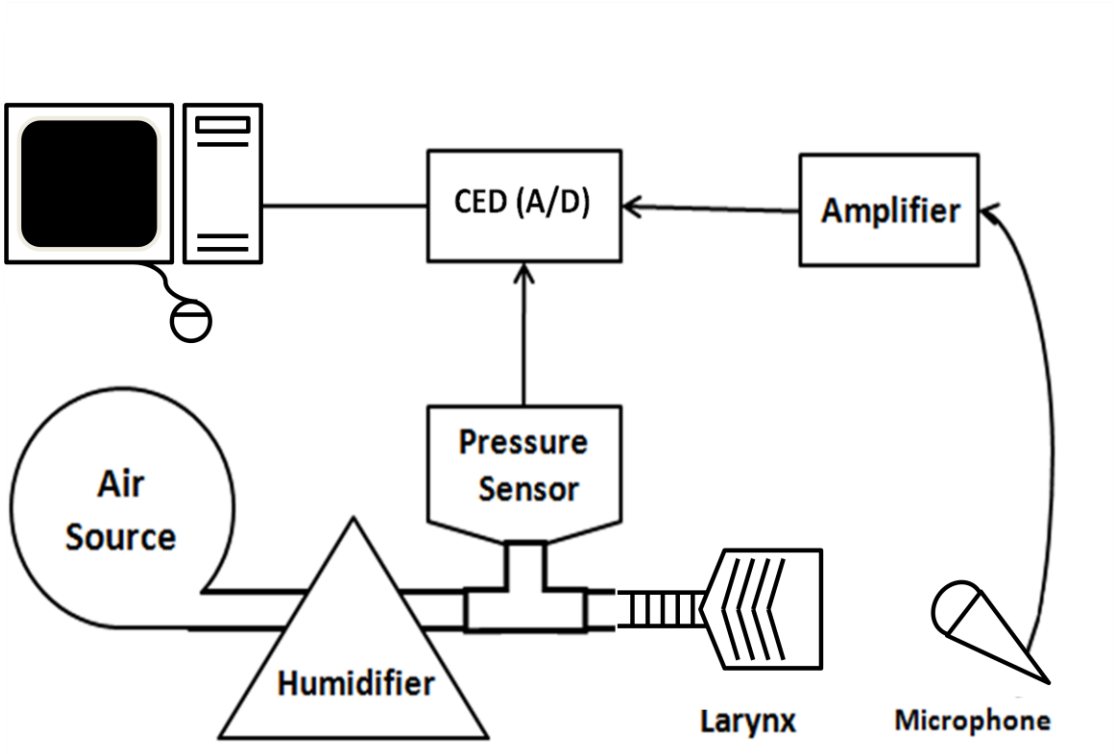

Figure 2. Basic scheme of the general experimental setup. CED (A/D) – consolidated electronic disc anologue/digital converter. Humidifier composed of Büchner flask. For further details, see text.

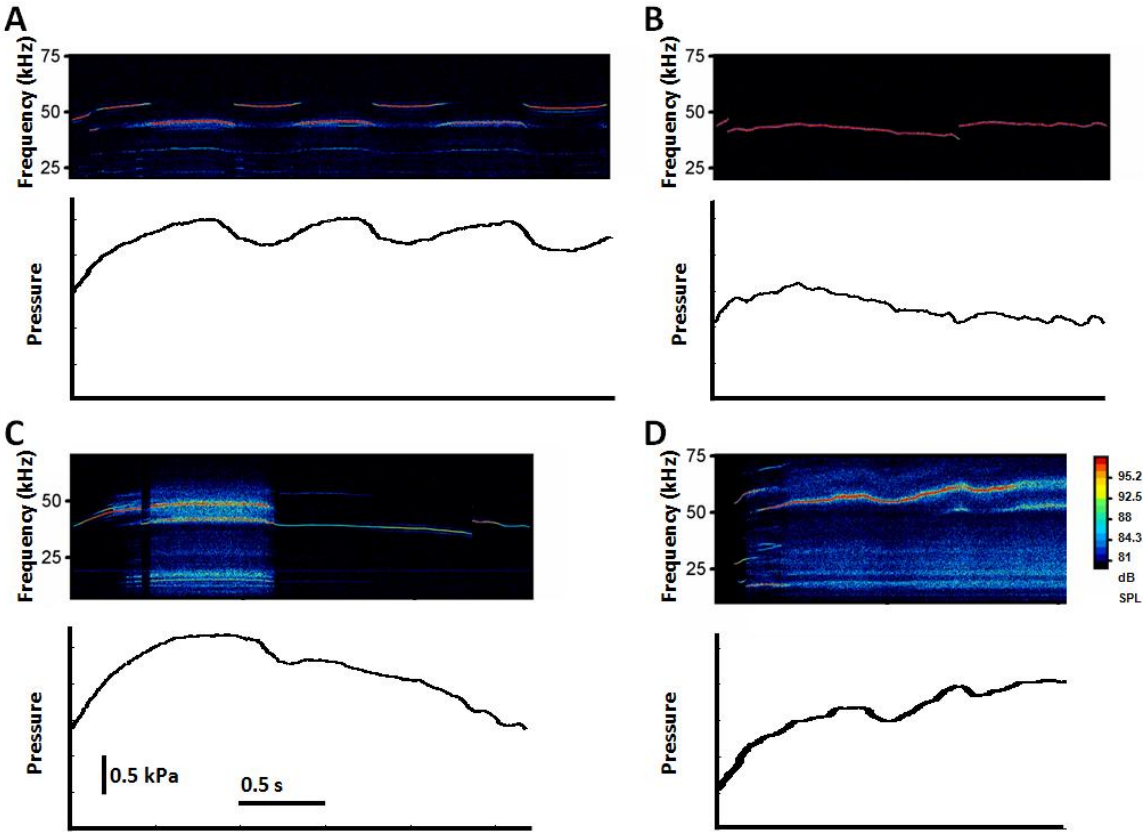

Figure 3. Representative phonations of male and female larynges (spectrograms in upper traces) resulting from changes in air pressure (lower traces). Phonations of 2 different male larynges in **A** and **C**, and of 2 female larynges in **B** and **D**. Initial increases in subglottal air pressure lead to the onset of phonation, typically with a dominant frequency between 30 and 50 kHz. The dominant frequency increased linearly with further increases in subglottal air pressure until a sudden jump to lower frequencies occured. In some cases, (e.g. **C**), a further increase in subglottal pressure lead to a transition into a "noisy" broadband sound. All frequency changes reversed with decreasing air pressures.

	Noise (n = 10)			Biphonations (n = 4)			
	Bandwidth		Center Frequency (kHz)	CF_1 (kHz)	CF_2 (kHz)	Δ Frequency (kHz)	Onset Pressure (kPa)
	Δ Frequency (kHz)	Onset Pressure (kPa)					
Median	6.05	2.27	45.49	55.58	8.50	40.00	2.71
MAD	1.37	0.34	2.52	8.58	3.50	6.50	0.34

Table 1 - Nonlinear Characteristics of Larynx Phonations. In addition to nonlinear frequency jumps, noise and biphonations were present in a number of different preparations.

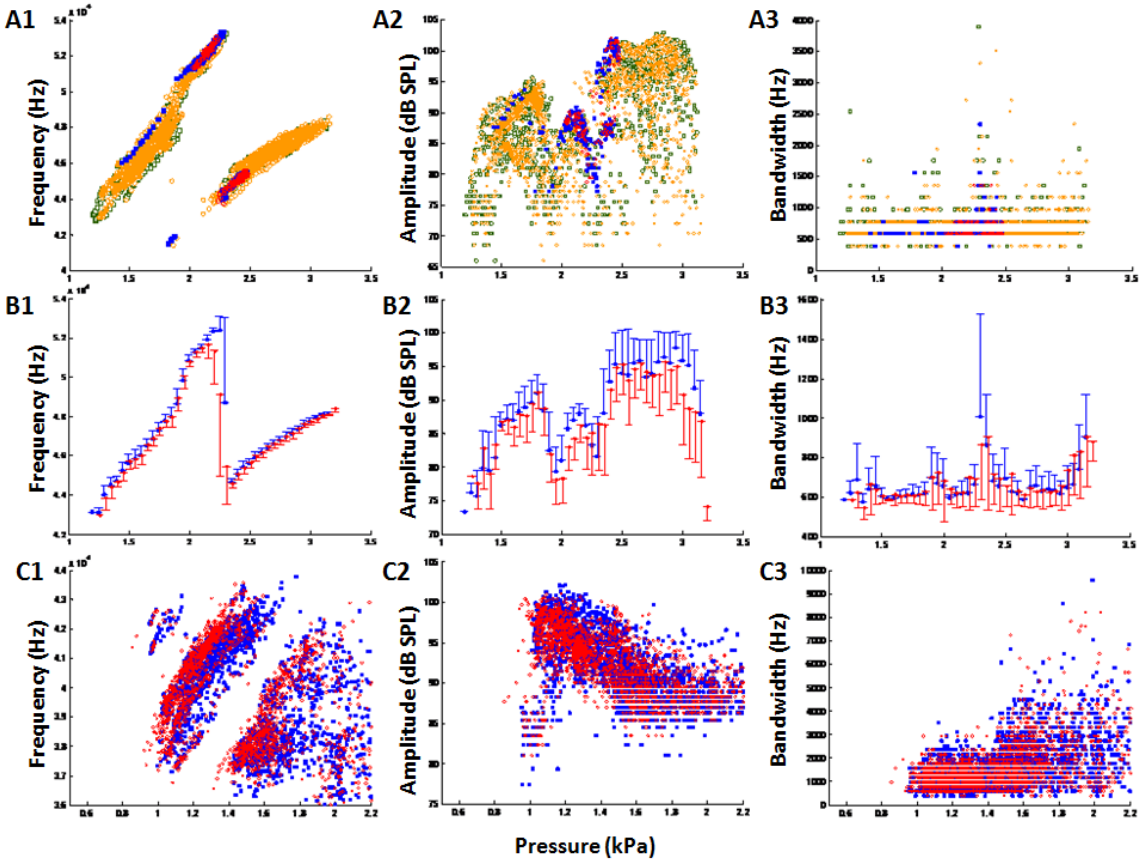

Figure 4. Examples (pooled and averaged) of phonations from 2 larynges. Pooled data from multiple trials for one male mouse larynx (first 2 rows) and one female larynx (bottom row). Each trial consisted of one continuous phonation. Trials were manually selected on the basis of detecting a dominant frequency in the ultrasonic range. Frequency, amplitude, and bandwidth were then calculated over a range of subglottal air pressures for each larynx. Columns relate air pressure to three different variables: **Column 1**: frequency; **Column 2:** amplitude; and **Column 3**: bandwidth. Rows represent the following: **Row A -** data from one male larynx with many averaged trials indicated in yellow/green while one trial is highlighted in red and blue; yellow and red indicate increasing pressures, respectively, green and blue indicate that pressure was decreasing; **Row B** - medians and 95% confidence intervals in 0.05 kPa windows of data from Row A, with red indicating pressure decreases and blue indicating pressure increases; and **Row C -** several trials of a female larynx with red representing pressure increases and blue representing pressure decreases.

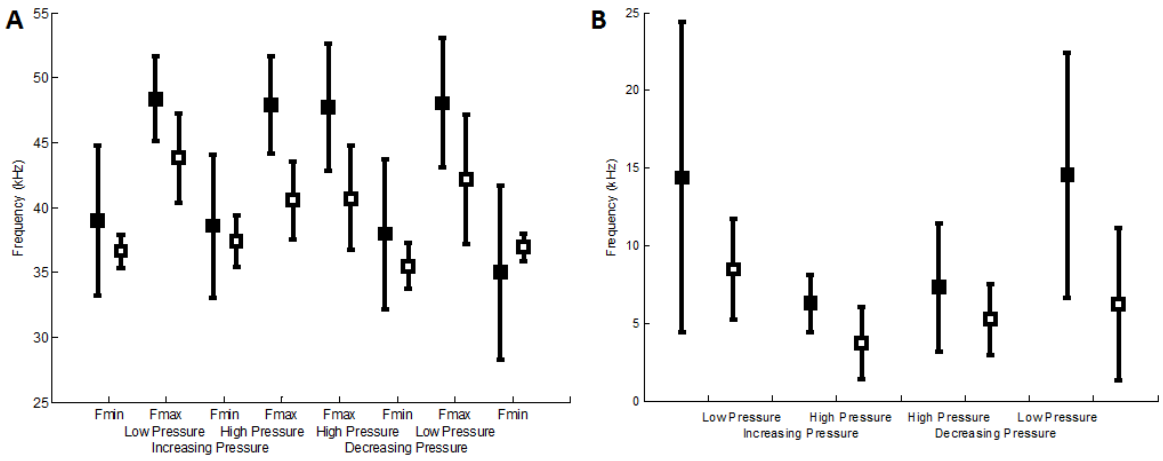

Figure 5. Sex comparison for phonation frequencies produced by isolated larynges. Median values of different frequency characteristics of phonations produced by male (filled squares) and female (open squares) larynges for pressure increases and decreases. Whiskers indicate median absolute deviation. Significant differences were determined based on 95% confidence intervals of each median. **(A)** Average male and female phonation frequencies for: low pressure phonation onset (Fmin, Low Pressure, Increasing Pressure), low pressure phonation frequency maximum during pressure increases (Fmax, Low Pressure, Increasing Pressure), high pressure phonation onset (Fmin, High Pressure, Increasing Pressure), high pressure phonation frequency maximum during pressure increases (Fmax, High Pressure, Increasing Pressure), high pressure phonation frequency maximum during pressure decreases (Fmax, High Pressure, Decreasing Pressure), high pressure phonation offset (Fmin, High Pressure, Decreasing Pressure), low pressure phonation frequency maximum during pressure decreases (Fmax, Low Pressure, Decreasing Pressure), and low pressure phonation offset (Fmin, Low Pressure, Decreasing Pressure). **(B)** Frequency ranges in male and female larynges in different stages of pressure increases and decreases, separated for ranges of low and high pressure, respectively.

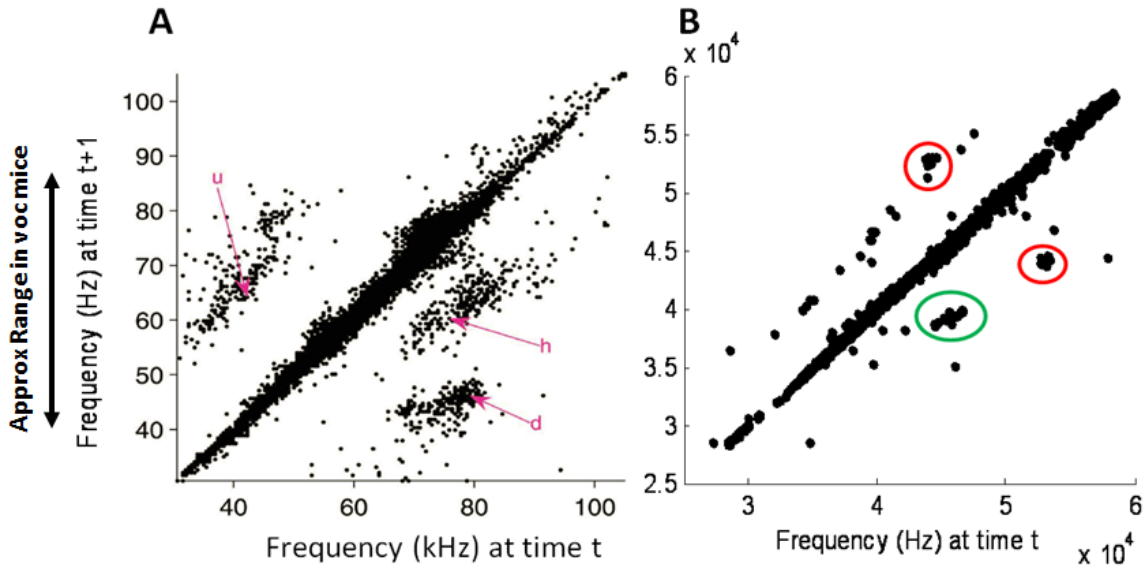

Figure 6. Frequency plot of vocalizations (A) and phonations (B). Frequency was plotted on the abscissa against frequency at a small time step (dt = 5 ms) on the ordinate. **(A)** Figure reproduced from Holy and Guo, 2005 and showing vocalizations from experienced male mice of the B6D2F1 strain (a cross between C57Bl/6 and DBA2/J) to illustrate various types of jumps as follows: 'u' was a 20 – 30 kHz jump up; 'h' was a 15 kHz jump down; 'd' was a 30 kHz jump down. The linear range of vocalizations recorded in this study is indicated on the left. **(B)** Same plot as in A using our data with the C57BL/6J strain. Frequency time step plot using phonation data to illustrate the linear range of frequency produced along with frequency jump clusters. Red circles indicate up and down jumps of ~9.7 kHz while the green circle indicates a jump down of 6.5 kHz.

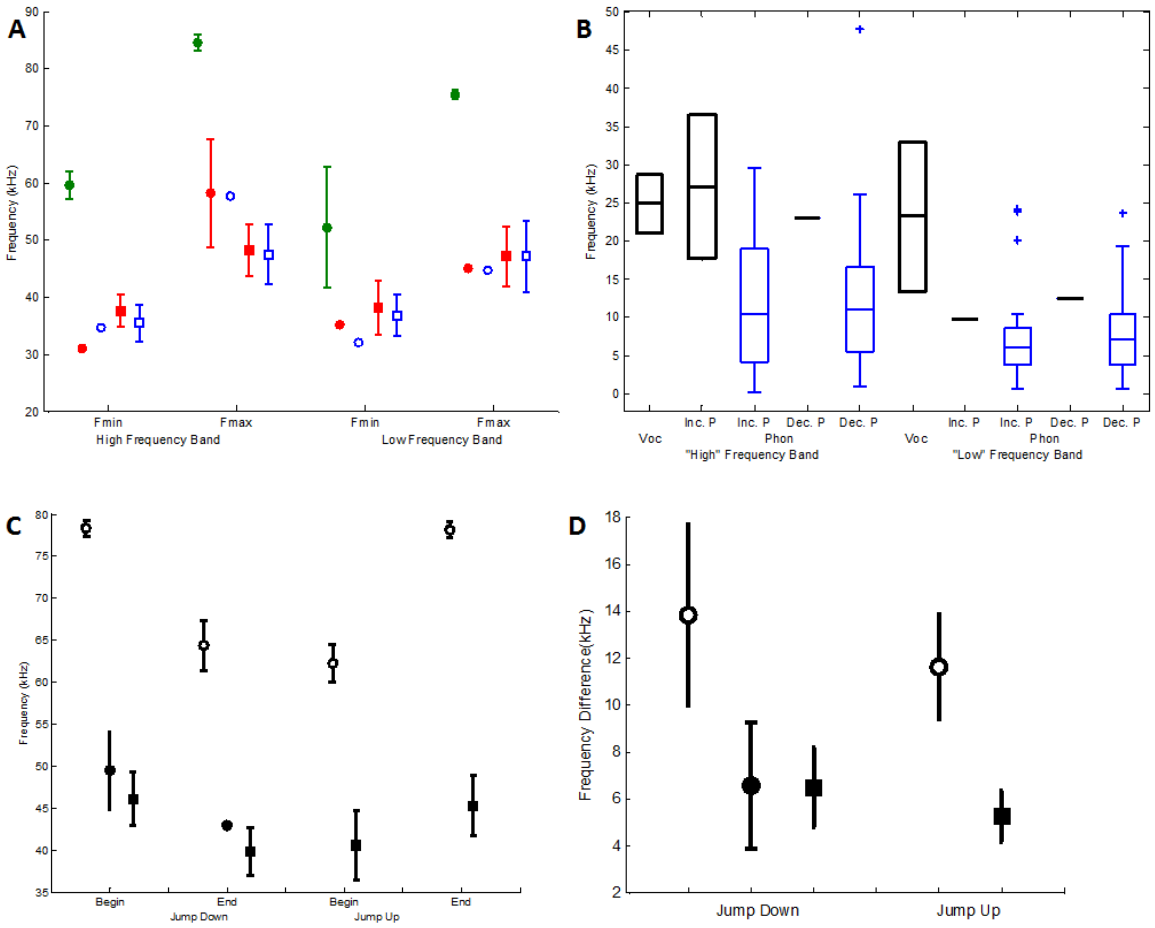

Figure 7. Comparison of sounds from mating male mice, their harvested larynges, and all other larynges. Whiskers indicate median absolute deviation in all but **B**. Significant differences were determined based on 95% confidence intervals of each median. **(A)** Ranges of natural vocalizations (green filled circles) and Phonations (red and blue circles are from larynxes of mice used to obtain natural vocalizations; red is increasing pressure and blue is decreasing pressure). **(B)** Boxplot indicating median and quartiles of frequency ranges emitted in natural vocalizations (Voc) and Larynx Phonations (Phon); Thick black lines indicate mice used for both natural vocalizations and phonations, blue lines indicate mice with only phonation data. **(C)** Frequency jumps in male natural mouse vocalizations (open circles), larynx phonations of male mice used for natural vocalizations (closed circles, not enough data for jump up), and larynx phonations. **(D)** Frequency jump size in male natural mouse vocalizations (open circles), larynx phonations of male mice used for natural vocalizations (closed circles, not enough data for jump up), and larynx phonations.

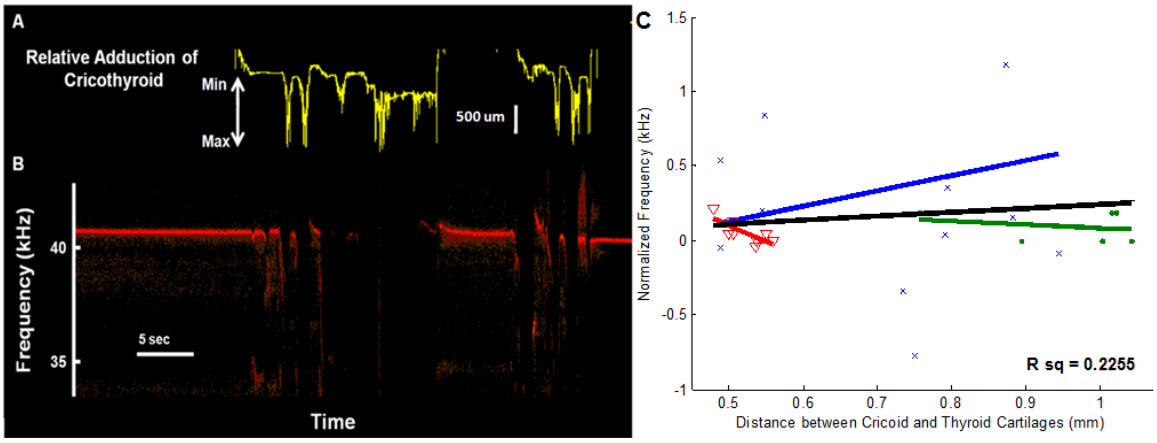

Figure 8. Effect of simulated cricothyroid contraction by adducting the thyroid towards the cricoid cartilage. (A) Relative adduction of thyroid, with 'Max' indicating maximum adduction and thus the smallest distance between cricoid and thyroid. Adduction less than 'Min' was due to the distance between forceps arms increasing past the distance between the caudal tip of the cricoid and rostral tip of the thyroid. **(B)** Spectrogram of larynx phonations during simulated contractions of the CT. Manipulation did not occur until a constant phonation frequency was achieved (see first 10 seconds of spectrogram). **(C)** Lines of best fit for three different larynges (indicated by different colors) and a combined line of best fit (black) for phonation frequencies as a function of amount of thyroid adduction normalized to the constant frequency achieved just prior to cricothyroid manipulation.

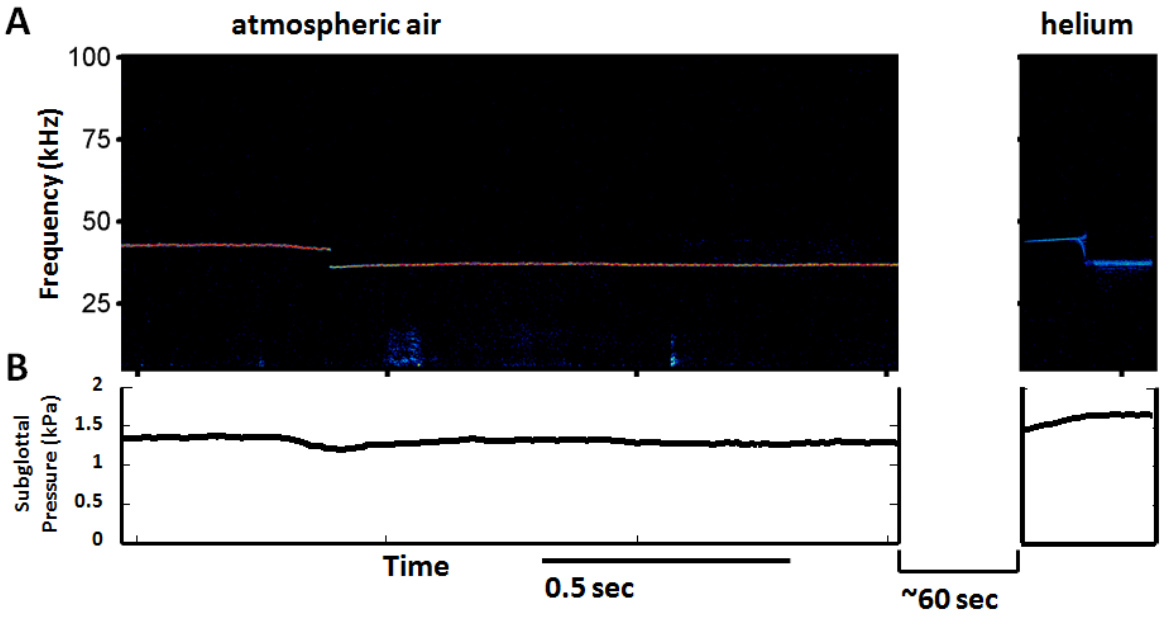

Figure 9. Representative example comparing larynx phonations based on flow of atmospheric air (left) *vs.* helium. (A) Spectrogram of larynx phonations in an atmospheric air medium (left) followed by phonations in helium medium (right). **(B)** Corresponding absolute subglottal pressures. A 60 s interim allowed for manual switching of atmospheric air source to a helium source.

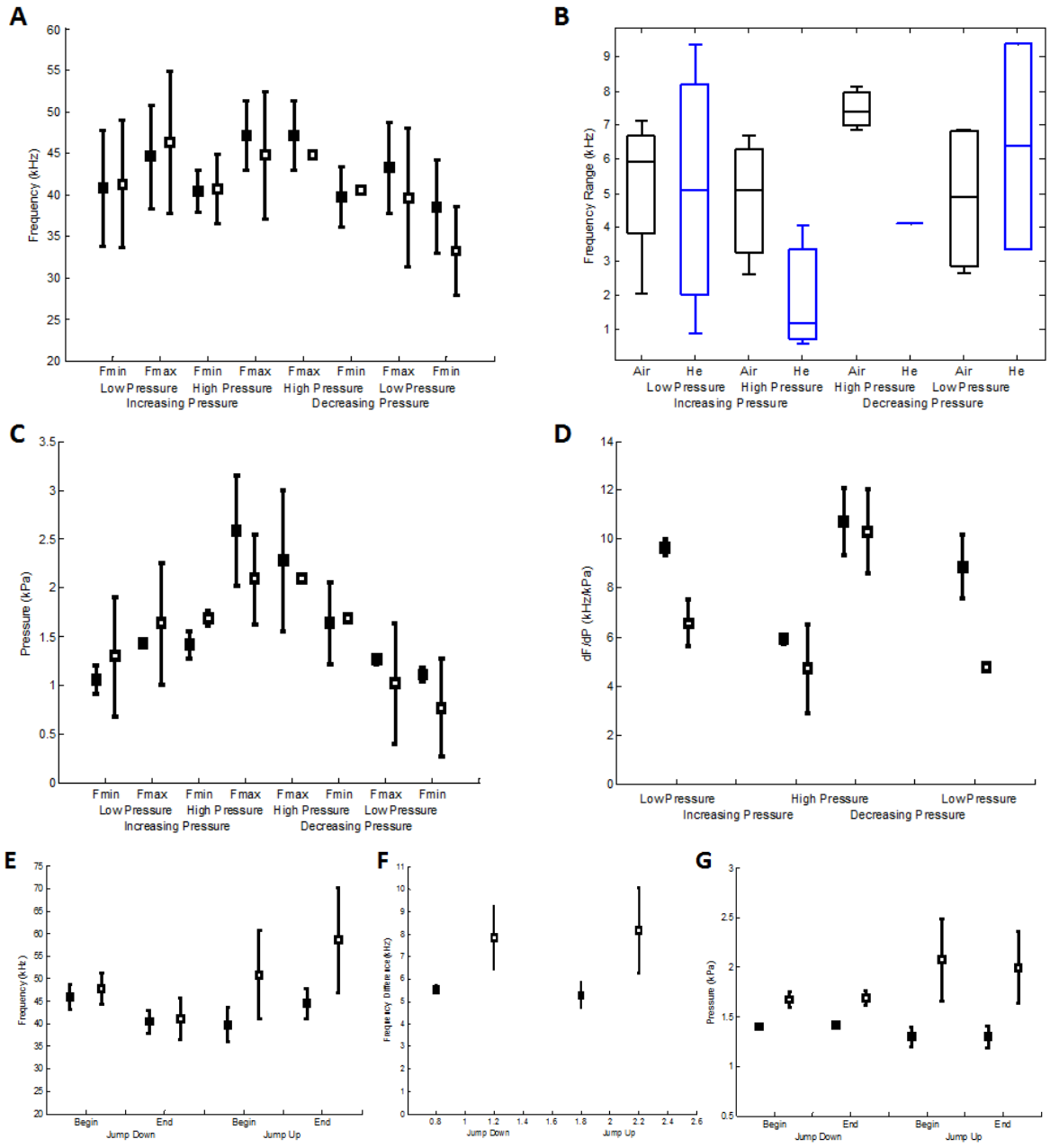

Figure 10. Comparison of larynx phonations in atmospheric air (closed squares) and helium (open squares). Whiskers in all but **B** indicate median absolute deviation. Significant differences were determined based on 95% confidence intervals of each median. **(A)** Phonation frequency at various spectral landmarks (see Figure 5 legend for definitions). **(B)** Boxplot indicating median and quartile values of frequency ranges at different stages of pressure increases and decreasesfor low pressure and high pressure flows. **(C)** Pressure values at various spectral landmarks (same landmarks as in **A**). **(D)** Change in frequency per unit pressure change at different stages of pressure increases and decreases (similar to **B**). **(E)** Frequency values immediately before and after jumps. **(F)** Jump size of upward and downward jumps. **(G)** Pressure values at the point of upward and downward frequency jumps.

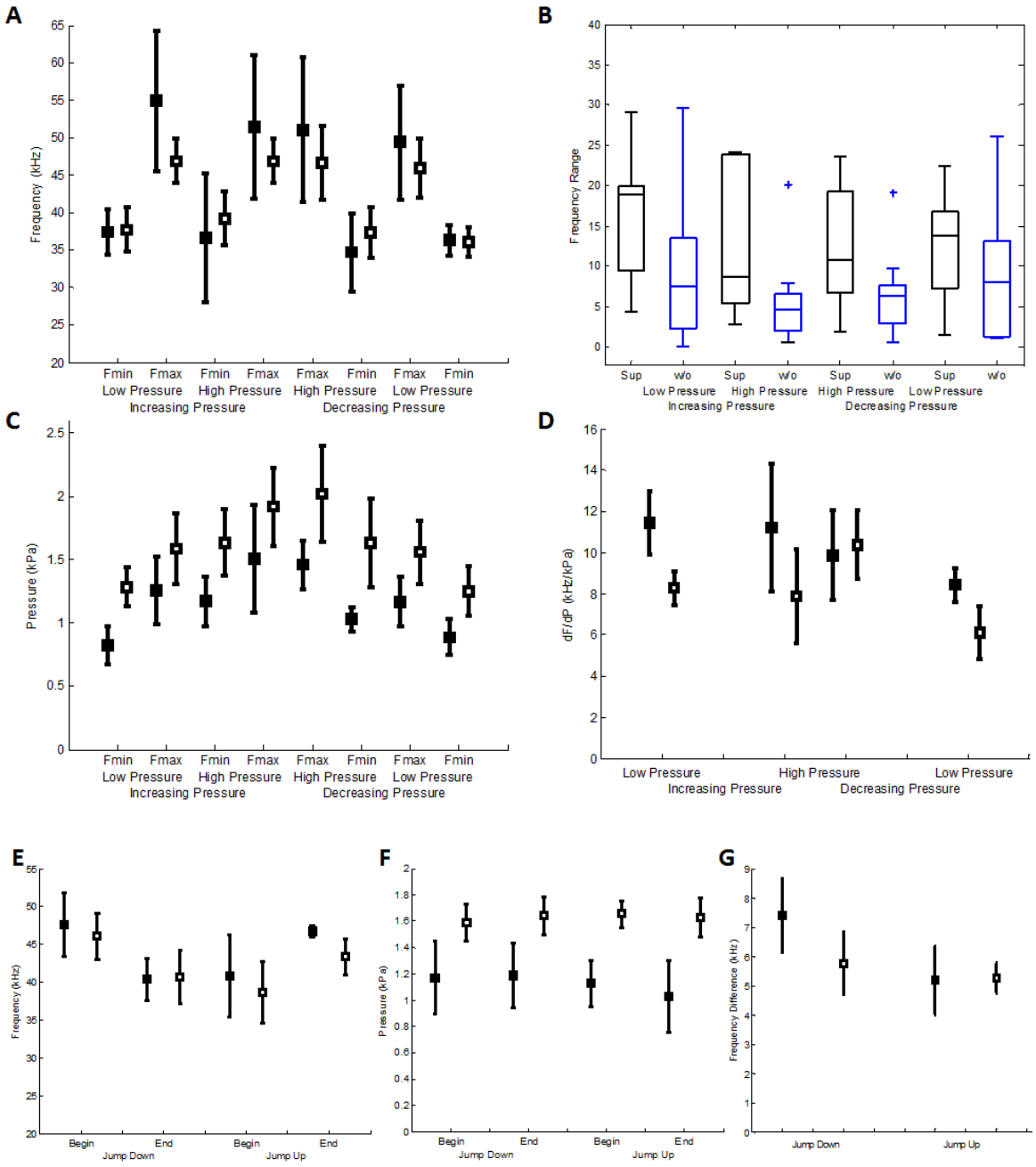

Figure 11. Comparison of larynges with supraglottal structures present (closed squares) or removed (open squares). Whiskers indicate median absolute deviation in all but **B**. Significant differences were determined based on 95% confidence intervals of each median. **(A)** Phonation frequencies at various spectral landmarks (see Figure 5 legend for definitions). **(B)** Box plot indicating median and quartile values of frequency ranges at different stages of pressure increases and decreases for ranges of low and high pressures, respectively. **(C)** Pressure values at various spectral landmarks (same landmarks as in **A**). **(D)** Change in frequencies per unit

pressure change at different stages of pressure increases and decreases (similar as in **B**). **(E)** Frequency values immediately before and after jumps. **(F)** Size of upward and downward jumps. **(G)** Pressure values at the point of upward and downward frequency jumps.

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