Ultrasonic output from the excised rat larynx

Aaron M. Johnson,^{a)} Michelle R. Ciucci, and John A. Russell

*Department of Surgery and Department of Communicative Disorders, Clinical Science Center,
University of Wisconsin-Madison, 600 Highland Avenue, Madison, Wisconsin 53792
johnson@surgey.wisc.edu, ciucci@surgey.wisc.edu, russell@surgey.wisc.edu*

Michael J. Hammer

*Department of Surgery, University of Wisconsin-Madison, Madison, Wisconsin 53792
hammer@surgey.wisc.edu*

Nadine P. Connor

*Department of Surgery and Department of Communicative Disorders,
University of Wisconsin-Madison, Madison, Wisconsin 53792
connor@surgey.wisc.edu*

Abstract: The source of ultrasonic vocalizations (USVs) produced by rats is thought to be within the larynx. The purpose of this investigation was to determine if the rat larynx is capable of producing ultrasounds with the full range of frequencies reported *in vivo*. Acoustic output of excised rat larynges with and without vocal fold constriction was measured. At biologically-reasonable airflow rates and pressures, only larynges with a constriction produced the full range of ultrasounds reported *in vivo*, providing support for the hypothesis that a constriction within the larynx is likely the source of rat USVs.

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1. Introduction

Rats produce both audible and ultrasonic vocalizations.^{1,2} These vocalizations differ in both their communicative intent and acoustic properties; audible vocalizations are typically in response to pain and fear from a perceived external threat and occur between 2 to 4 kHz,² while ultrasonic vocalizations (USVs) are used for intra-species communication, such as predator warning, mother-pup locating, and mating.³ Ultrasonic vocalizations in rats are often classified based on the fundamental frequency that occurs in two distinct bandwidths, centering on either 22 kHz or 50 kHz, with some variability within each of these classifications.³

Audible vocalizations and USVs are likely produced via different mechanisms in lower mammals, and the mechanisms appear to vary across species. While audible vocalizations from rats are produced with vocal fold vibration,^{2,4} USVs are likely produced by a whistle mechanism with a constriction somewhere in the upper airway, possibly at the larynx.^{5,6} In contrast, USVs produced by echolocating bats are accomplished by vibration of very thin, specialized vocal membranes projecting upward from the main body of the vocal folds.⁷ Therefore, generalizations regarding vocalization mechanisms within and across lower mammals and types of vocalizations are not possible and particular vocalization types and animal models must be studied directly.

Evidence for the larynx as the source of rat USVs comes primarily from laryngeal denervation studies of young rats, in which unilateral or bilateral resection of either the recurrent or the superior laryngeal nerve disrupted and/or eliminated USVs.^{6,8} However, it has also been reported that unilateral laryngeal denervation has no effect on the intensity, duration, or frequency of USV of adult rats.⁹ These conflicting reports may be due to differences in the

^{a)} Author to whom correspondence should be addressed.

Fig. 1. Schematic of the excised larynx equipment (not to scale).

strains or ages of the rats studied, or methodological differences, such as the way in which USVs were elicited. Therefore, it is unclear if USVs are created by a constriction at the larynx or some other location in the upper airway.

While results of prior laryngeal denervation studies implicate the larynx as the site of this constriction,^{6,8} direct observation of the larynx during USV production would provide more compelling evidence for the larynx as the source of USVs. Although endoscopic procedures for *in vivo* observation of the rat larynx have been developed,¹⁰ these methods cannot be used to visualize laryngeal movement in the awake rat. Laryngeal constriction observed via endoscopy in anesthetized rats has been reported during USVs elicited by stimulation of the periaqueductal gray area.⁵ However, these USVs were in the 20–46 kHz range and there are no systematic studies of the rat larynx that show it is capable of producing the full range of USVs (50 kHz and above). Therefore, this study used an excised larynx model with different degrees of vocal fold closure to directly test if the rat larynx is capable of producing the full range of ultrasounds reported *in vivo*.

2. Methods

Larynges and proximal 1 cm of trachea from 6 nine-month-old Fischer 344/Brown Norway male rats were excised immediately after animals were euthanized. To simulate different degrees of vocal fold adduction, three experimental conditions were used; (1) open glottis with no adduction, (2) anterior glottal adduction created with a single suture adducting the anterior third of the vocal folds, and (3) both anterior and posterior glottal adduction created with an anterior suture adducting the anterior third of the vocal folds and a posterior suture adducting the vocal processes of the arytenoid cartilages.

Larynges were mounted on a plastic tube and secured onto an existing excised larynx apparatus (Fig. 1).¹¹ Humidified air was delivered in 1 L/min airflow increments from 1 to 6 L/min, the reported peak expiratory flow of the rat.¹² Phosphate buffered saline was applied to the larynx throughout the experiment to approximate *in vivo* moisture conditions. Subglottal air pressure directly below the mounting tube was monitored with a U-tube manometer (Dwyer Instruments, Michigan City, IN) to ensure it remained within known physiologic limits.¹³

A bat detector system (Anabat II with ZCAIM, AnaBat, East Brisbane, Australia) was mounted 10 cm away from the larynx out of the airstream to detect ultrasonic output. The AnaBat II with ZCAIM (zero crossings analysis interface module) is a frequency division bat detector that uses zero crossings analysis to record fundamental frequency over time; amplitude and harmonics of the acoustic signal are not preserved. This analysis method was chosen because our research question focused on measurement of acoustic output from the larynx alone and did not involve characterizing the formants or other aspects of the acoustic signal. The bat detector output was monitored and recorded using a handheld computer (iPAQ hx2495, Hewlett-Packard, Houston, TX).

Fig. 2. Box and whisker plots of the ultrasonic output from the adduction conditions. The box contains the inter-quartile range (IQR) of the data with the median data point indicated by a black dot. The whiskers extend to the last observation within 1.5 times the IQR. An asterisk indicates a significant difference from the open glottis condition ($p < 0.05$).

For each combination of the three adduction conditions and six airflow levels, acoustic output (kHz) was measured and recorded for 5 s. Additionally, to ensure that any ultrasonic acoustic output was a result of the larynx and not an artifact of the equipment, the acoustic output of the mounting tube without a larynx was recorded at each airflow rate. The modal frequency, defined as the frequency that occurred most often within each 5 s sample, was extracted during offline acoustic analysis.¹⁴

The modal frequencies of the three adduction conditions at each airflow rate were compared using repeated measures analysis of variance (ANOVA). Post hoc pair-wise comparisons were made between adduction conditions using Fisher's protected least significant difference tests (LSD). All analyses were performed using SAS statistical software (SAS Institute Inc., Cary, NC). The critical value for obtaining statistical significance was set at $\alpha = .05$ level.

3. Results

The mounting tube alone without a larynx did not produce any ultrasonic acoustic output at any airflow rate. The maximum modal frequency of the equipment alone was 19.39 kHz at the highest airflow rate of 6 L/min. This finding verified that the study apparatus was not responsible for generating any ultrasounds, and that any ultrasounds recorded in the adduction conditions came from the larynx.

The adduction conditions produced ultrasounds at airflow rates of 4, 5 and 6 L/min. Mean subglottal pressure during ultrasonic output was 37.41 cm H₂O with a standard deviation of 17.76 cm H₂O. While there were upper outliers in these data that were excessive, the mean subglottal pressures observed were biologically realistic.¹³

A significant main effect for adduction condition was found at airflow rates of 3 L/min ($F_{[2,4]} = 14.88$, $p = 0.01$), 4 L/min ($F_{[2,6]} = 54.75$, $p = 0.0001$), and 6 L/min ($F_{[2,7]} = 11.69$, $p < 0.01$). Post-hoc paired comparisons revealed that modal frequency was significantly greater in both the anterior and the anterior/posterior adduction conditions at airflow rates of 3, 4, and 6 L/min compared with the open glottis condition ($p < 0.05$). These data are summarized in Fig. 2. Additionally, the open glottis configuration did not produce ultrasounds above 42 kHz, indicating that some degree of laryngeal adduction was necessary to produce the full frequency range of rat USVs observed *in vivo*.

4. Discussion

This study shows that the rat larynx is capable of producing ultrasounds, as evidenced by the ultrasonic output in all three laryngeal adduction conditions. Furthermore, the results indicate a

laryngeal constriction is required to produce the full range of ultrasounds reported *in vivo* (up to 50 kHz). This was demonstrated by the 50 kHz and higher output of the anterior and anterior/posterior adduction conditions, in contrast to the maximum 42 kHz output of the open glottis adduction condition. In addition to a laryngeal constriction, an airflow rate of at least 4 L/min and subglottal pressure of 27 cm H₂O was necessary to achieve ultrasounds at 50 kHz and above. Although direct study of subglottal pressure during USV production is lacking, one study of rats reported subglottal pressures of 30 cm H₂O during vocal fold closure.¹³ Thus, it is likely that variations in laryngeal adduction, airflow, and subglottal pressure are used by the rat to achieve a wide frequency range of USVs *in vivo*.

An excised larynx has obvious differences from *in vivo* physiology and has inherent limitations. First of all, vocal fold adduction in the excised larynx was created surgically and not via muscle contraction. As such, the simulated configuration of the glottis in the excised larynxes may have differed from configurations typically used during USV productions in nature. Further, the properties of excised tissue may undergo biomechanical changes such as stiffening and/or relaxing. Changes in biomechanical and viscoelastic properties were not quantified over the course of the experiment. However, the results obtained from the simulated adduction conditions provide evidence that adduction at the level of the larynx is necessary for the production of 50-kHz USVs and likely has a role in USV production *in vivo*.

This study measured steady-state acoustic output produced by applying a constant airflow to a static laryngeal constriction. *In vivo*, however, USVs are often characterized by rapid frequency modulations and transitions from one type of call to another. These rapid modulations may be achieved through the mechanisms of laryngeal adduction and/or varying airflow and subglottal pressure, or by altering other structures in the upper airway, such as the tongue. Further *in vivo* studies are needed to determine the precise source of USV modulation.

Excised larynxes from other species have been used to study aspects of vocal fold vibration,¹⁵ but behavioral animal models for the study of voice production and voice disorders are scarce.^{16–18} Although it is unlikely that USVs are created by vocal fold vibration,⁴ they are similar to human vocalizations in that they require control of airflow, subglottal pressure, and glottal adduction and share some common neural pathways.^{19,20} Therefore, rat USVs may be a useful model to study the underlying peripheral and central neural mechanisms of vocal control, as well as the effects of interventions and disease.¹⁶

Our results indicate that biologically relevant ultrasounds can be generated by the rat larynx at airflow rates and pressures that approximate physiologic conditions and are thus consistent with our hypothesis that a laryngeal constriction is a source of rat USVs. Further *in vivo* experimentation is needed to show that the larynx is a necessary and not just sufficient component of USV production, and to further understand how USVs are rapidly modulated. Because the larynx is the likely source of USVs, these vocalizations can serve as a model of laryngeal control to study the mechanisms underlying the disorders/diseases of human laryngeal control.

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