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The role of ultrasonic vocalizations in rat laryngological investigations

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ABSTRACT

Rat ultrasonic vocalizations (USVs) have traditionally been used in psychosocial and psychobiological studies to understand emotion, social behavior, cognition, and associative learning. However, recent studies have expanded the goal of USVs to include the study of the laryngeal system and the effects of disease processes on vocal sensorimotor control. Without the foundational understanding of the goals of this area of laryngological research, fundamental differences in study objectives between psychobehavioral and laryngological studies can easily be missed, leading to misconceptions and misinterpretations of the role USVs play in laryngology-focused studies. Standardization of terminology and methods are also needed to improve communication, enhance study replicability, and prevent ambiguity that can lead to misinterpretations of study objectives and findings in this line of research.

The primary objective is to describe the role of USVs in studies of laryngeal anatomy and physiology, with a focus on their connections to the neuromuscular and neurological aspects of the laryngeal system, particularly in relation to vocal sensorimotor control and voice disorders. It is intended for novice investigators interested in laryngology-specific USV research. Researchers experienced in USV studies within the context of the larynx and vocal sensorimotor control first outline the development and refinement of various USV elicitation methods. They provide insights into how these approaches have been tested across different studies and laboratories. Finally, they advocate for standardizing terminology and methodologies to enhance study replicability, reduce ambiguity, and foster collaboration across research groups.

1. Introduction

Rats are highly social mammals that produce vocalizations in the ultrasonic range for various biological imperatives, including mating, signaling distress, and establishing social hierarchies. These ultrasonic vocalizations (USVs) are well-established biomarkers in psychosocial and psychobiological studies to understand emotion, social behavior, and cognition. Such studies using USVs as outcome variables include observations of emotional behaviors (*e.g.*, pleasure, distress, aggression), rodent interactions (*e.g.*, play, informing food location, avoidance, mating, and mother-pup communication), learning paradigms (*e.g.*, operant conditioning and extinction), and to study the relationships among these behaviors and the brain [1–5]. USVs have also been used to study neurobiology, psychiatric and developmental disorders, and the effects of pharmacological agents on the nervous system [1,6–14].

Although most studies using USVs as outcome variables have focused on social, emotional, and cognitive factors, more recent studies (including studies from our collective labs), have focused on the anatomy and physiology of USV production. These studies have confirmed that USVs are produced by the larynx, which is comprised of skeletal muscles encapsulated within cartilaginous framework [15–17]. Activation of the intrinsic laryngeal muscles within this cartilaginous

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framework results in partial vocal fold adduction and subsequent increased laryngeal resistance essential for vocal production. This laryngeal resistance plays a critical role in USV productions by transforming exhaled air from the lungs through the larynx into aerodynamic and acoustic energy via either an alar edge whistle tone or glottal air jet impingement [18,19]. The amount and type of laryngeal muscle activation for vocal fold closure can result in differences in USV acoustic output: USV duration, fundamental frequency, frequency contour and modulation (complexity), and intensity. Our previous work has shown that increasing the amount (*i.e.,* dose) of laryngeal muscle activation over multiple weeks of vocal training not only has an influence on USV acoustics but also results in neuromuscular changes in the larynx [20–22]. As such, the acoustic features of USVs provide robust non-invasive insights into the complex nature of laryngeal function and dysfunction as it relates to peripheral and central vocal sensorimotor control.

Because the rat larynx has laryngeal musculature, cartilaginous structure, and neural innervation similar to humans [15], vocalizations generated by the larynx in rodents can be used as a proxy to study laryngeal musculoskeletal, neuromuscular, and nervous system deficits common in humans. USVs have been used in various laryngological studies, including the effects of senescence on laryngeal neuromusculature, the role of the larynx in neurodegenerative disease (*e.g.,* Parkinson Disease, Amyotrophic Lateral Sclerosis), laryngeal adaptations to increased vocal demands, recurrent laryngeal nerve injury and recovery, and laryngeal studies of sexual dimorphism and the effects of sex hormones. These studies typically elicit USVs through sexual encounters and/or food rewards. However, the goal of these studies is not to investigate learned behaviors of USV productions. Instead, these studies (1) capitalize on inherent biological behavior to study laryngeal sensorimotor vocal control in its natural state, (2) facilitate anatomical and physiological changes in the larynx, and (3) study how these physiological changes in the laryngeal sensorimotor system influence USV acoustic characteristics.

The distinction between using USVs as a behavioral readout *versus* a proxy to study the laryngeal sensorimotor system is especially important considering previous work has shown that only twenty percent of rats *learned* to increase their vocalizations via an associative learning operant conditioning task [23]. Learning, in this case, was determined by the number of USVs produced based on a predetermined target number and the speed at which the rats reached the target during each training session. However, our previous work has also demonstrated that all rats increase their USVs when presented with a biological imperative—irrespective of whether this vocal behavior is learned or not—and that this increase in USVs is critical to understanding laryngeal physiology (*e.g.*, laryngeal adaptations to increased vocal demands) and pathophysiology (*e.g.,* laryngeal injury and recovery). For example, when rats are presented with a rat of the opposite sex—one type of biological motivator—they will increase their USVs substantially (75 USV/min) compared to rats whose spontaneous vocalizations are passively recorded in their home cage (0.2 USV/min) regardless of whether vocal learning or training occurred [24]. This mating paradigm is especially relevant when investigators are interested in (1) capturing and characterizing numerous USVs over a short period to study laryngeal physiology and pathophysiology or (2) increasing vocal demands from baseline USV production levels to elicit physiological changes in the laryngeal system. Our previous work has also shown that USVs obtained during sexual encounters have increased fundamental frequencies, greater vocal intensity, are more complex, and have higher tonality compared to spontaneous vocalizations produced without biological motivators [24].

In summary, USVs provide critical insights into relationships between laryngeal anatomy, physiology, and resultant vocal production for various sensorimotor laryngeal deficits and disorders. Distinguishing intent between investigations that use USVs to study associative learning and social behavior from investigations using USVs to study laryngeal anatomy and physiology as it relates to sensorimotor vocal control is essential. Otherwise, it can lead to erroneous conclusions that experimental designs that focus on eliciting or increasing USV productions independent of vocal learning are methodologically flawed (*e.g.,* Wardak et al., 2024). Understanding these distinctions in study designs and objectives is also crucial as applications of USVs become more common in academic literature. According to PubMed, fewer than 20 academic papers per year in the 1990s utilized USVs. In contrast, the 2020s have seen an annual publication record of 110 to 150 papers, with the number of studies using USVs expected to grow exponentially in the coming years. This increase in the use of USVs for study outcomes is driven by greater accessibility to USV acquisition and analysis, facilitated by lower equipment costs and the availability of open-source software.

Because laryngological studies using USVs as outcome measures are less common than the use of USVs in psychobehavioral studies, there is a possibility flawed interpretations (*e.g.,* Wardak et al., 2024) in how USVs are used for laryngological studies are not isolated events. As such, the primary objective of this paper is to explore the use of USVs in studies on laryngeal anatomy and physiology, sensorimotor vocal control, and voice disorders. Drawing insights from laryngological research experts who utilize USVs as outcome metrics across various laboratories, this paper serves as a resource for novice investigators interested in incorporating USVs into laryngology-related experimental designs. Rather than offering a comprehensive literature review, it highlights key examples of how USVs can be applied to study the laryngeal system in vocal function and dysfunction.

We begin by summarizing previous studies that have used USVs as outcome measures to investigate the larynx and sensorimotor vocal control across various rat models of vocal function and dysfunction. Next, we review different methods for eliciting USVs, highlighting the historical development and evolution of these approaches across multiple studies and research labs focused on the laryngeal sensorimotor system. Finally, we propose recommendations for standardizing vocabulary and methodology to enhance study replicability, improve transparency, and minimize misinterpretations.

2. Summary of previous work on the laryngeal sensorimotor system

2.1. The use of USVs to study the effects of vocal exercise dose and senescence on laryngeal muscle

Our previous work in rat models of typical and aging rats has demonstrated that USV elicitation can be used to facilitate increases in vocal demands that yield neuromuscular plasticity within the larynx. In one of our studies, using sexual pairings and food rewards to increase USV production across multiple weeks reduced or eliminated age-related changes in the larynx and impacted corollary USV acoustics [25]. Specifically, rats who were enrolled in the vocal exercise group (*i.e.*, elicited increases in USV rate over multiple weeks) produced USVs with increased duration, intensity, and fundamental frequencies after 1–2 months of vocal exercise training, compared to their pre-vocal training time points and compared to rats who received no USV elicitation motivators.

To assess changes in USV acoustics resulting from the vocal training, pre- and post-vocal training USV recordings were elicited through sexual pairing and withdrawal without rewards in the rat's homecage with no biological motivators. The observed acoustic changes in USVs from pre- to post-training without presentation of food rewards or sexual pairings could suggest that differences between groups had just as much to do with physiological changes in the laryngeal system as learned associative vocal behaviors (*e.g.,* operant conditioning, see Wardak et al. 2024). These results were further supported by differences in laryngeal neuromuscular junction morphology in rats who were trained to vocalize, compared to those who were not. These findings demonstrate the important role USVs play in laryngeal neuromuscular plasticity.

Findings from this work are relevant to people with presbyphonia (age-related voice issues) who commonly report concerns with vocal projection and changes in their vocal pitch. Results are promising in the context of understanding the role vocal exercise training plays in helping to improve laryngeal physiology and preserve voice function in advanced age.

A second study we conducted examined the role of vocal exercise dose (increased USV emission rate and complexity over 4- and 8-weeks) on laryngeal neuromusculature in young adult male rats using sexual pairing and food reward [21]. Reduced dispersion of the neuromuscular junction endplates in the thyroarytenoid muscle of the larynx was present after eight weeks of increased USV production but not after four weeks. These findings suggest increasing rate and complexity of USVs over several months result in adaptive laryngeal neuromuscular plasticity, likely as means to accommodate these chronic changes in vocal demands. The neuromuscular adaptation patterns observed in the laryngeal muscle resemble those previously identified in limb muscles undergoing low-intensity endurance training with gradually increases in exercise dose over several months. These findings highlight the crucial role of vocal exercise in promoting neuroplastic adaptations in the laryngeal muscle, emphasizing its importance in the design of vocal training programs.

In a third study, we examined the effects of increased vocal demands on neuromuscular and musculoskeletal mechanisms in the larynx of young and old rats [22]. Similar to the previous study, rats who increased their USVs had reduced dispersions in neuromuscular junction motor endplates in the lateral thyroarytenoid muscle of the larynx after multiple weeks of vocal training. However, increasing the rate of USVs over multiple weeks had no effect on laryngeal muscle fiber size or type. These findings suggest that increasing vocal demands improves neuromuscular efficiency (*e.g.,* coordination, endurance) in the larynx but does not enhance muscle strength (*i.e.*, hypertrophy). The results of this work provide key information on how the larynx adapts to increased vocal demands in younger and older adults and is critical for understanding the role the larynx plays in the context of vocal exercise in vocal function and dysfunction irrespective of learned behaviors.

Together, these three studies demonstrate the utility of using vocal training and USVs to study the effects of increased vocal demands on laryngeal neuromusculature, regardless of whether associative vocal behavior was learned or not.

2.2. USVs to study the effects of sex hormones on laryngeal muscle

Rat USVs are an important tool to study the effects of sex hormones on laryngeal anatomy and physiology and play a vital role in understanding how sex hormones influence voice production and voice disorders. Overall, sex hormones are critical for maturation and maintenance of the laryngeal structures [26]. Chronic changes to sex hormones lead to remodeling of the vocal folds, which has been observed in both gonadectomy models and hormone replacement models [27–30]. For example, the loss of ovarian hormones, a common animal model of menopause, has been shown to result in increased vertical thickness of the vocal folds, decreased collagen I and III, decreased elastin, and decreased hyaluronic acid [30]. Several studies have corroborated the hormone-dependent effects on the cover of the vocal fold in both humans and rats [27–29,31–33]. It is also well established that the vocal fold cover thickens after menopause [28,29,33,34], which is attributed to the drop in pitch in post-menopausal women [35,36].

Hormones including androgen receptors and estrogen receptors (ERβ1, ERB2, and G protein-coupled estrogen receptor [GPER]) are located in the rat vocal fold [30,37] and GPER has been hypothesized to mediate rapid estrogenic effects of the vocal fold mucosa. A recent review summarized the effects of sex hormones on the vocal folds, suggesting that changes in estrogen and progesterone hormone production and expression in the ovaries are primarily responsible for remodeling the mucosal layer of the vocal fold influencing vocal fold stiffness and viscosity [38]. Furthermore, increased androgen (*e.g.,* testosterone) expression is also thought to result in increased vocal fold muscle mass [38]. These findings highlight the duality of the effects of sex hormones on the structure and function of the vocal folds.

Similar to human larynges, male rat vocal folds are also larger (*e.g.,* have greater mass from increased androgen exposure) and have greater muscle fiber size in the lateral thyroarytenoid muscle of the larynx important for vocal production [39]. These anatomical differences result in some USV subtypes produced with lower frequencies in male rats compared to female rats. Female rats also differ from male rats in the number of USV emissions that can vary throughout their estrous cycle [39,40]. In addition to differences in USVs, female rats also have greater fragmentation of acetylcholine receptors in the neuromuscular junctions of the thyroarytenoid muscle of the larynx [41].

Of note, the laryngeal structure and function differences observed between the sexes do not correlate with body weight, and body weight does not correlate with USV frequency or vocal fold size [39]. These findings suggest differences in body weight between the sexes cannot be directly attributed to sexual dimorphism in relation to USVs and the larynx. As such, because of these sexual dimorphisms in laryngeal structure and function, USVs are a useful biomarker for understanding the role of hormones and sex on the larynx. However, only 58 % of studies using a rat model have used both male and female rats, requiring more rigor in studies across the sexes, especially in the context of laryngeal physiology [42].

The application of USVs in these hormone-related studies is different from studies using USVs to increase vocal demands, described in the previous section. USVs in this case are used to monitor vocal changes across the estrous cycle or compare hormone differences between groups, not elicit changes in vocal demands or behaviors. Spontaneous USVs can be recorded over multiple hours. However, increasing USV rate via biological motivators like sexual pairings and food rewards facilitates more USV productions over a shorter time. This reduces time allocated to manual labor (*e.g.,* the need for intermittent recording equipment and software checks to make sure USVs are recorded over multiple hours) and reduces acoustic file size.

2.3. USVs to study the effects of parkinson disease on vocalization

Parkinsonian studies utilizing rat models are extensive and have provided significant insights into the neural mechanisms underlying Parkinson's disease (PD). These models have been widely used to investigate motor deficits, neurochemical changes, and therapeutic interventions, making them a critical tool in PD research. However, a comprehensive review of the vast body of literature on rat models of PD is beyond the scope of this paper. Instead, we will focus on key applications of these models in studying the effects of PD on vocal motor control. By highlighting specific ways in which rat models contribute to understanding vocal deficits associated with PD, we aim to underscore their relevance in investigating the neural and biomechanical underpinnings of PD-related communication impairments.

The pathology of PD is complex, widespread, and involves multiple mechanisms (*e.g.,* systemic inflammation, pathologic misfolding of α-synuclein, formulation of Lewy bodies, and death of dopaminergic neurons). While research has shown that PD affects both the central and peripheral nervous systems, its impact on vocal motor control and the voice issues common in PD patients remains poorly understood. Furthermore, treatments aimed at modulating dopamine to improve motor function in the limbs do not reliably improve motor function in the vocal sensorimotor system. To address gaps in knowledge, rat USV studies using the *Pink 1 -/-* strain have been instrumental in improving our understanding of the effects of PD on the vocal sensorimotor system.

Specifically, we have systematically modulated dopamine pharmacologically and with lesion studies in the *Pink 1 -/-* strain and found that antagonism of D_1 and D_2 receptors showed that USV acoustic

degradations were most prominent when D_1 and D_2 receptor subtypes were antagonized simultaneously. However, the D_1 receptor subtype may contribute largely to certain acoustic parameters, such as increased peak acoustic frequency [43]. We also found lesions to nigrostriatal dopamine pathways with the neurotoxin 6-OHDA causes the intensity and complexity of USVs to diminish and that levodopa does little to improve these deficits in these USV acoustic parameters [44]. These findings are highly translational, as humans with PD often struggle with the intensity of their voices and the mainstay of behavioral voice therapy focuses on increasing vocal loudness [45,46]

We also found that the administration of levodopa and apomorphine (a dopamine agonist) increases the rate of USVs [47]. These findings indicate that mechanisms beyond simple nigrostriatal dopamine depletion may play a role in vocalization. Recent research has highlighted that even before dopamine loss becomes evident, there is significant degeneration of noradrenergic (norepinephrine, NE) neurons in the locus coeruleus [48], confirming other mechanisms may be at play in PD vocal deficits. Another series of studies examined the modulation of noradrenergic receptors using amphetamine and found that various acoustic characteristics of USVs changed, including USV rate. These studies became the basis of assessing the effects of various NE adrenoceptor agonists (*e.g.*, Cirazoline, Clonidine) and antagonists (*e.g.,* Prazosin, Atipamezole, Propranolol) on USV parameters. We found that NE signaling alterations significantly modified various USV acoustic parameters, including USV intensity, bandwidth, duration, and peak frequency. Specifically, agents that increased NE neurotransmission, such as Atipamezole, or those that activated alpha-1 receptors, such as Cirazoline, generally led to increased USV intensity and duration. Conversely, agents that reduced NE signaling, like Clonidine, or blocked alpha-1 receptors, such as Prazosin, resulted in decreased USV rate, intensity, and bandwidth. Notably, the beta-receptor antagonist Propranolol was associated with increases in USV call rate, duration, and intensity [49].

In summary, rat models of PD (especially the *Pink 1 -/-* strain) present valuable opportunities for addressing research questions specific to the vocal system that were previously difficult to explore. These bodies of literature have shown that while there is some degree of USV changes with dopamine depletion, there is also significant effects to USV when other regions of the brain are involved and with non-dopamine pharmacological treatment. The use of USVs to study the role of the vocal sensorimotor system in PD has expanded our understanding of PD's multifaceted impact on vocalization and vocal motor control.

2.4. USVs to study the effects of early-stage alzheimer's disease on laryngeal muscle

Similar to Parkinson disease, Alzheimer's disease (AD) is a whole-body disease, affecting both peripheral and central mechanisms, even in its prodromal stages [50–55]. Previous research has indicated that vocal impairments and disrupted communication due to nervous system dysfunction can occur before the onset of cognitive and memory deficits [56]. Despite the importance of studying these early-stage deficits, rat models capable of such investigations have only become available in recent years. Preclinical laryngological studies focused on vocalization deficits are crucial for enhancing our understanding of AD-related vocal dysfunction and overall pathophysiology, which can aid in early detection and intervention strategies.

Various investigations have been conducted to address methodological shortcomings that have made studying the effects of AD on the vocal motor control system accessible. For example, in a study conducted by our group, we analyzed USVs from transgenic TgF344-AD rats, which express human APP with the Swedish mutation (APP K670_M671delinsNL-Swedish) and human PSEN1 with the Δ exon 9 mutation [57]. Other studies have revealed that TgF344-AD rats exhibit hallmark AD characteristics, including age-related amyloid beta accumulation, tau pathology, gliosis, and neuronal apoptosis [58–60]. These rats also demonstrated neuropathological and behavioral dysfunction, as well as prodromal neuroinflammation similar to that seen in human AD. Additionally, we validated a model specifically for laryngological investigations through demonstrations of vocalization deficits, AD-related pathology in the thyroarytenoid muscle of the vocal folds, and AD-specific inflammatory cytokines in the thyroarytenoid muscle. Specifically, in these studies we found dysregulated inflammatory and AD-related gene expression in the thyroarytenoid muscle of the vocal folds and confirmed early and progressive vocalization deficits. USVs in these rats showed that simple and frequency-modulated USVs had reduced mean power (dB/Hz)—a measure of vocal intensity—and increased high-frequency components (kHz) over time compared to wildtype rats. Together, these findings underscore the importance of USVs for the study of vocal communication impacts in the TgF344-AD rat model. These studies have also validated the use of USVs as a valuable biomarker for studying the onset and progression of laryngeal system deficits in AD.

2.5. USVs to study the effects of vocal fold movement impairments

Our recent work has demonstrated that injury to the recurrent laryngeal nerve that directly innervates the vocal fold muscles results in USV acoustic characteristic changes and correlates well with vocal fold movement impairments [24]. After laryngeal nerve transection, USVs of rats elicited with sexual encounters and during spontaneous USV recordings had significantly lower fundamental frequencies (kHz), vocal intensity (dB), and tonality (signal-to-noise ratio), compared to their pre-injury time points. USV duration and complexity were also decreased after nerve injury, but only in the rats in the sexual encounter group and not in the spontaneous vocalization cohort. These findings highlight the importance of using biological motivators to yield acoustic changes in USVs to better understand physiological capabilities in the laryngeal system.

Fig. 1 is a visual representation of how USVs have been used across various investigations and across multiple labs to study various aspects of the laryngeal system and vocal sensorimotor control in vocal function and dysfunction.

3. USV elicitation, training, reward, and outcome metrics

Although USVs have been used to study psychology, biology, pharmacology, and behavior since the 1970s, in the 2010s, investigators at the University of Wisconsin-Madison (*e.g.,* Ciucci, Johnson) first began experimenting and optimizing protocols to elicit, reward, and quantify USV productions to study the laryngeal system and sensorimotor vocal control. This section covers the evolution of these protocols, their continued refinement, and their current applications across various labs.

3.1. USV elicitation and training approaches

Over the years, we tested multiple elicitation methods, including tickling, sexual pairings, familiar/unfamiliar rat pairings, odor presentations, and audio playback. Early odor-based setups, such as piping scents into cages [61], proved inconsistent, leading to the adoption of sexual pairings as the gold standard. Sexual encounters, especially with experienced, receptive female rats in estrus, reliably produced 50-kHz USVs while minimizing 22-kHz alarm calls. As training progressed, sexual pairings were phased out in favor of food and water rewards.

There is a vast body of psychological and behavioral literature on food and water motivation that is beyond the scope of this paper (see [62–64] for examples). The focus of this section is instead on how these standard motivative behaviors can be capitalized to study laryngeal anatomy, physiology, and vocal motor control. Rats produce more USVs when they are hungry or thirsty and anticipate the availability of food or water. Studies employing food deprivation protocols, typically maintaining rats at 80–90 % of their baseline body weight, combined with

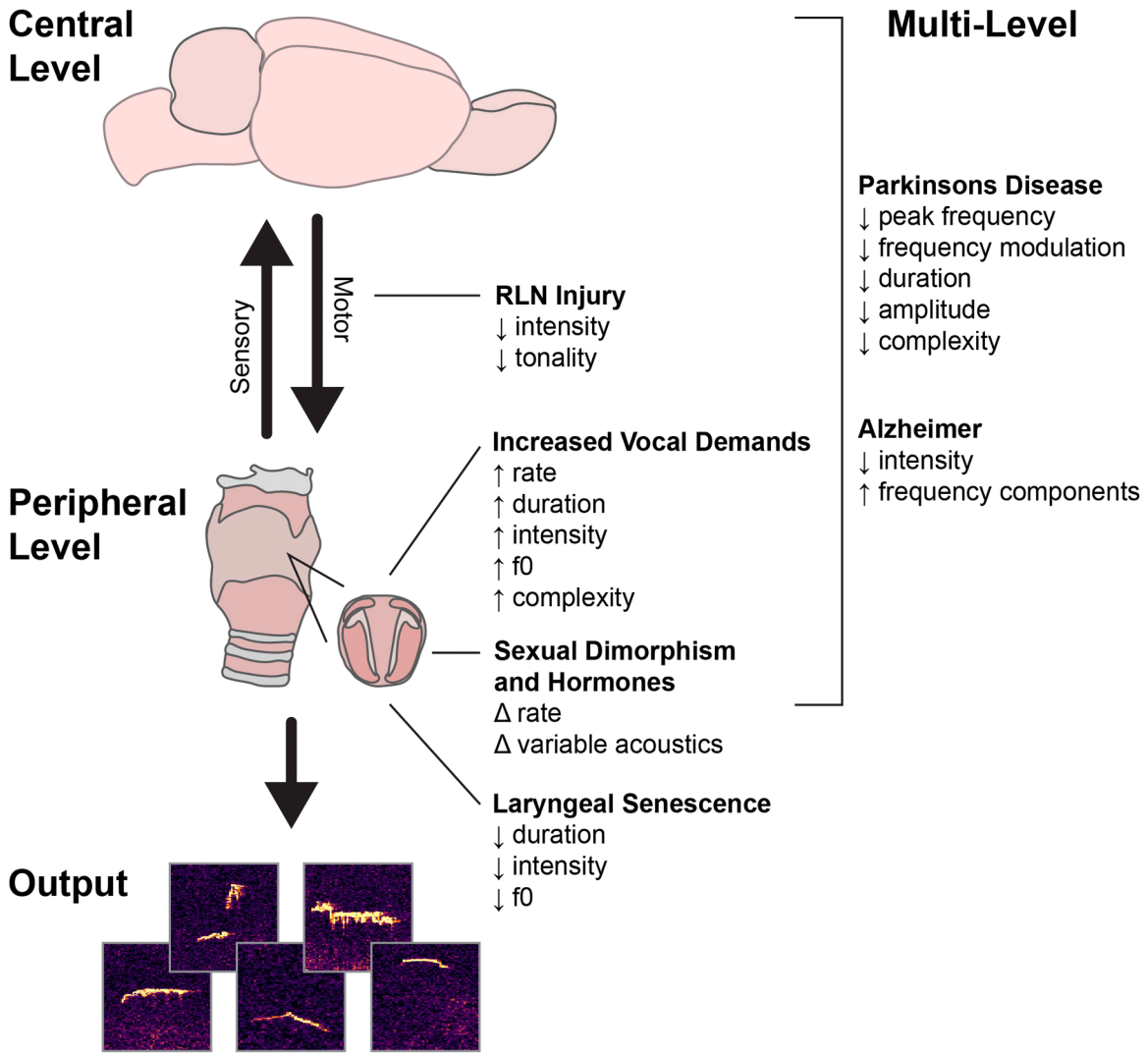

Fig. 1. Use of Ultrasonic Vocalizations Across Laryngological Studies. Ultrasonic Vocalizations (USVs) serve as robust outcome measures for studying various vocal conditions, including recurrent laryngeal nerve injury, increased vocal demands, sexual dimorphism and hormonal influences, laryngeal muscle senescence (aging), Parkinsons disease, and Alzheimer disease. These conditions affect vocalization through central, peripheral, systemic, and multi-level mechanisms. The acoustic characteristics of USVs vary depending on the rat model and the specific condition being studied. *f0 = fundamental frequency; RLN = recurrent laryngeal nerve; ↑ = increase in acoustic USV characteristics; ↓ = decrease in acoustic USV characteristics; Δ = change in acoustic USV characteristics.*

high-value food rewards with nutritive value, consistently demonstrate increased USV production over multiple weeks. Various food rewards include nutritional pellets with sucrose, sugary cereals (*e.g.*, Fruit Loops, Frosted Flakes), and peanut butter.

We found that when rats were given 100 % sucrose pellets, they quickly ceased vocalizing within minutes and rarely met their vocal training targets. However, when the food motivator was replaced with nutritionally balanced pellets containing proteins, fats, carbohydrates, vitamins, and minerals as well as sucrose, the rats produced more USVs over a longer period and performed better during USV training. This suggests that motivation declines when rats consume only pure sucrose, as their physiological needs for a well-rounded diet remains unmet. Previous research has also shown that rats rewarded with nutritionally complete pellets with sucrose exhibit more frequent and sustained performance in motor tasks over time [22,65].

One possible explanation is that nutrient-rich and nutrient-balanced rewards more effectively influence brain chemistry than pure sucrose, leading to stronger and more sustained motivation [65]. Additionally, the presence of essential nutrients may better facilitate satiety signals and overall health, contributing to a greater willingness to perform tasks for such rewards. The lack of available water when consuming sugar pellets may further reduce appetitive behaviors. This could explain why a study on vocal learning found that only 20 % of rats increased their USV production when pure sucrose pellets were used as the primary motivator, no food deprivation protocol was implemented, and no water was provided during training sessions [23].

An alternative to food deprivation is water deprivation, with training cages equipped with water bottles to encourage USV production. Although 30 % sucrose solution in water is commonly used in behavioral studies, we found that it presents several practical challenges. Mold rapidly develops in the bottles, and rats frequently refuse to drink the solution, leading to decreased USV production. Water with sucrose also causes liquid dippers to stick and malfunction. Furthermore, based on our experience, water deprivation must last at least 21 h for water rewards to serve as a significant motivator.

Food and water rewards paired with additional auditory or visual cues, further enhance USV training. In previous experiments, we observed that rats retrieved food rewards 50 % of the time without additional sensory stimuli. However, when the food reward was paired with an auditory cue, such as a pen click, retrieval rates increased significantly. This effect is likely due to the rats' rapid orientation toward the reward following the auditory signal. Since USVs are often

produced during locomotion, auditory cues may help the rats locate rewards more efficiently. Furthermore, incorporating a variable reward schedule, once rats recognize food or water as a positive reinforcer, has been shown to further improve USV production rates.

4. *Additional considerations for usv elicitation and training*

4.1. Strain Differences

Rodent strain is an important consideration when developing experimental designs with USVs as outcome measures. This is due to the variability in USV production and acoustics previously observed across strains. In our experience, Long Evans rats are easiest to train with high USV rates, while other strains (*e.g.*, F344BN, Wistar, Sprague Dawley) show lower USV production or require additional coaxing. Strain differences in laryngeal muscle composition may contribute to these variations. For example, while the F344BN strain has type IIL fibers in the thyroarytenoid muscle that range between 0 and 40 % [21,22,66–68], Sprague-Dawley and Wistar strains have the same fiber type that range between 20 and 30 % [69–72].

4.2. Training Timing

We also found that the timing of USV training is important to consider in study designs. Female USV production varies with estrous cycle, with increased alarm calls 1–2 days post-estrus. USVs are also more frequent during the dark cycle, necessitating reverse light cycles in experimental setups.

4.1. USV acoustic parameters

Studying both the acoustic characteristics of USVs and the rate of their production provides valuable insight into how USVs are generated via the larynx, vocal sensorimotor function, and the impact of disease states on vocal production. The specific acoustic features of interest depend largely on the rat disease model, experimental design, and should always be guided by the research hypothesis. For example, in the rat model of laryngeal senescence, USV intensity and duration may be affected due to age-related loss of muscle bulk or impaired neuromuscular coordination in the laryngeal muscles (*e.g.,* lower intensity and shorter duration) [20,22,25]. Our studies using a rat model of Alzheimer's disease have identified significant differences in USV intensity, peak frequency, and the highest frequency ranges [57]. Similarly, we have found that USV complexity—specifically sinuosity and frequency bandwidth—is a strong marker in rat models of Parkinson's disease [73–75]. In a rat model of laryngeal nerve injury, where laryngeal muscle function and tonicity are reduced, we identified frequency, intensity, and tonality as the most robust acoustic USV markers [24].

4.2. USV classification methods

Various methods for classifying USVs have been proposed, utilizing different acoustic features. A common approach is classification based on USV frequency (kHz), which is often used to study emotion and affect (*e.g.*, 50-kHz, 40-kHz, and 22-kHz USVs). Within this framework, 22-kHz USVs are typically associated with negative affective states, while 50-kHz USVs correspond to positive states. USV classification has identified six subtypes of 22-kHz and 14 subtypes of 50-kHz USVs based on their shape or duration [76,77]. More simplified classification systems for 50-kHz USVs also exist, such as distinguishing between flat and frequency-modulated calls [78] or categorizing them as flat, frequency-modulated, and harmonic USVs [25].

Beyond frequency-based categorization, other classification methods, such as machine-learning clustering models, rely on frequency and tonality contours to perform supervised or unsupervised clustering. Supervised clustering allows researchers to manually define classification categories, whereas unsupervised clustering uses an elbow-optimized k-means algorithm to determine the optimal number of USV categories based on frequency, shape, and duration parameters. The choice of classification method depends on the research question and experimental design. For example, frequency-based shape and contour classifications may be most appropriate for studying behavior related to emotion or affect, as these studies often focus on how vocal behaviors emerge, recur, or change over time. Examining categorical shifts in USV patterns is particularly useful when studying positive emotional states, which are often associated with frequent high-pitched vocalizations and distinct prosodic features (*e.g.*, trills in the 50-kHz range). In contrast, studies on laryngeal function and vocal physiology benefit more from analyzing acoustic characteristics such as intensity, duration, and tonality, as these features directly reflect the biomechanics of USV production.

While manual classification is the most commonly used method, it is subject to investigator bias, as it relies on visually identifying USV shapes in a spectrogram, which can be subjective. Automated methods, such as unsupervised clustering, reduce user error but require researchers to still weigh frequency, duration, and shape parameters to define USV categories. Using a 14-subtype manual classification system may help identify altered USV patterns in specific vocalization subtypes, but rats rarely produce all 14 USV subtypes, which can impact statistical analyses. Therefore, researchers must carefully consider their dataset, research question, and experimental conditions when selecting a classification method. In laryngological research, the primary focus is on characterizing USV acoustics in relation to laryngeal sensorimotor control, regardless of whether associative behavioral learning has occurred. However, depending on the experimental design, researchers may need to account for the effects of behavior on USV production, particularly in studies involving locomotion, sniffing, or grooming. This consideration is especially relevant in certain rat models, such as those used to study Alzheimer's or Parkinson's disease.

4.3. USV analysis software

The two most commonly used software programs for analyzing rodent ultrasonic vocalizations (USVs) in our collective labs are Avisoft-SASLab, developed by Avisoft Bioacoustics (Glienicke/Nordbahn, Germany), and DeepSqueak, an open-source program that runs in MATLAB [79]. Other available programs also include Sonotrack and UltraVox but the focus will be on the former two USV analysis software programs, each of which have their distinct advantages and disadvantages.

Avisoft-SASLab is widely used for studying USVs in rodents and bats and has several advantages. First, the software generates customizable spectrograms that enhance data visualization, facilitate noise removal, and support detailed USV characterization. Second, it includes detection software capable of classifying USVs, with customizable settings tailored to specific experimental needs (*e.g.*, frequency range adjustments, detection thresholds). Third, the program supports multiple audio formats, ensuring compatibility with various recording equipment and software. The ability to process multiple files simultaneously improves its efficiency, particularly in large datasets. Finally, technological support to assist users with Avisoft-SASLab technical issues is exceptional. However, Avisoft-SASLab has several drawbacks. The first is a steep learning curve with the extensive customization options that require significant time to learn and master. The second is the substantial computing power and memory needed for optimal performance.

The second commonly used USV data analysis program in our labs, DeepSqueak, is designed for automated detection, analysis, and classification of USVs using deep learning and machine learning algorithms in MATLAB [79]. Its advantages include reduced reliance on manual detection, enabling rapid processing of large datasets. The deep learning algorithms are trained for consistent and precise USV classification. The automated analysis minimizes variability in data interpretation. The program can be user friendly in that the graphical interface is accessible and requires minimal coding knowledge as long as no modifications to the MATLAB code are needed. Users can adjust settings to fit specific experimental designs. Because it is open source, it can be more cost

effective than proprietary alternatives (although it does require a MATLAB license).

Despite these strengths, there are also several limitations with DeepSqueak. The first is the learning curve. Although it is user-friendly, it still requires time to understand its full functionality. Second, incorrect parameter settings may lead to excessive noise detection or missed USVs, compromising data accuracy. Third, a powerful computer is necessary for processing large datasets of complex models. Fourth, it is dependent on a MATLAB license, which can add a financial barrier to some users. Fifth, accuracy depends on the quality of the data used to train the algorithms and poorly trained models may misclassify subsequent USV detection and analysis. Like any machine-learning tool, DeepSqueak may perform well on trained datasets but poorly on new ones, requiring additional validation and MATLAB coding expertise. Finally, as an open-source tool, it relies on community contributions for updates and compatibility improvements.

5. Standardization of usv terminology

Standardization of terminology is important for several reasons. First, clear and precise definitions provide common ground, ensure consistent communication, and reduce ambiguity and misunderstandings. Second, the use of a common language makes training, education, and collaborations easier across labs conducting investigations with USVs. Third, common terminology makes it easier to compare and contrast findings across studies and between different labs. In the following section, we have provided several examples of terminology discrepancies across studies as well as possible solutions to improve communication and reduce ambiguity.

5.1. Vocal training, vocal learning, and operant conditioning

Vocal training is the preferred term when describing increased USV rates or alterations of selected USV acoustic properties. While vocal training has traditionally been considered in the context of operant conditioning—*i.e.,* elicit target behavior, reward target behavior, increase subsequent target behaviors—associative learning is not a desired outcome of vocal training to study the laryngeal system. The primary goal of vocal training is to increase laryngeal muscle activation or to study USVs in the context of vocal sensorimotor control regardless of whether the behavior increased because of associative learning. In fact, rats often engage in secondary learned behaviors associated with increased USV rates (*e.g.,* spinning) which often increase with vocal training because they are inadvertently paired with USV motivators. These behaviors are typically ignored because they are not direct targets in laryngeal studies. In other cases, certain learned behaviors require extinguishment. For example, in one of our studies using an automated pellet dispenser to elicit USVs, rats quickly learned that scratching the metal food trough under the pellet dispenser also triggered food pellet rewards, requiring modifications to the training environment to extinguish the undesired behavior.

5.2. Ultrasonic vocalizations, calls, songs, and squeaks

The term ultrasonic vocalization should replace the terms call or song in the murine vocalization literature. This terminology originates from previous bird literature, where songs and calls are differentiated based on whether vocalizations in birds were learned from other birds (songs) or inherent to their biology (calls). Applying these principles to murine studies, post-ejaculatory USVs in the male rat have been characterized as a 'song' consisting of 22-kHz vocalizations that corresponded with the refractory period of copulation [80,81]. However, defining what constitutes a 'song' *versus* other types of USVs is challenging in a murine model without knowing the rat's communicative intent and without knowing if the USV was an inherent biological behavior (call) or based on an associative learned behavior (song). The term 'squeaks' should also not be used to describe USVs as these are vocal productions in the sonic range that are typically used to signal distress or fear when the rat feels threatened or cornered, is handled roughly, or is injured, and is reserved to deter predators who have sonic hearing (*e.g.,* felines, humans). Frequent elicitations of audible 'squeaks' in the lab setting can result in higher 22-kHz alarm calls in rodent colonies [82].

5.3. Estrous versus estrus

The terms estrous and estrus have been used interchangeably across various studies [83–86]. However, *estrus* is the specific stage of the *estrous cycle*. The term *estrus* refers to the specific timeframe when the female rat is fertile, accepts copulation, and is often associated with mating behaviors such as darting, spinning, ear wiggling, lordosis, and increased rates of 50-kHz vocalizations.

5.4. Gender versus sex

Similar to the above, the terms gender and sex have been used interchangeably in the USV literature [87–89]. However, gender is a social construct of humans, and in rat research, the term "gender" should be replaced with "sex". Interchanging these terms contributes to confusion both within and outside scientific communities. Confusing the two terms or using them interchangeably can also play a role in perpetuating harmful stigmas placed on transgender communities whose sex assigned-at-birth do not align with their gender. Using correct terminology can help alleviate confusion and stigma.

6. Standardization of usv methods

Standardization of methods to elicit, record, and analyze USVs is important for several reasons. First, having consistency across studies, investigators, and labs ensures data collected can be directly compared, contrasted, and replicated. Second, standardizing methods can minimize variability and reduce recording or analysis errors, which are all essential for scientific integrity and for drawing meaningful conclusions. Third, having established protocols via standardization can make it easier to integrate study findings into larger bodies of knowledge, which is essential to understanding the biological and behavioral significance of USVs. Fourth, from a training perspective, standardization of methods can provide clear and concise protocols for training new researchers and students and ensure that they acquire the necessary skillsets and knowledge to conduct high-quality research. Finally, a common set of standards can ensure that researchers from different institutions and disciplines can more effectively collaborate, which leads to more interdisciplinary insights and innovative USV approaches. There are several standardization factors to consider, including rat acclimation periods, USV elicitation approaches, optimization of recording environments, rat characteristics, and best practices for USV characterization and classification. We have provided a checklist for investigators to consider prior to implementing study protocols (Table 1).

6.1. Rat acclimation

Standardization of the acclimation period is important; otherwise, it can influence results between rats, across studies, and across labs, making it challenging to generalize study findings. For example, rat handling comfort levels, previous history of sexual encounters, and familiarity with food rewards can all affect the rate of USV productions. These variables should all be explicitly reported in the USV literature. Ensuring that rats are responding consistently, especially with sexual encounters or variable ratios of food rewards, is critical before moving on to the next steps in the study protocol. A week's worth of consistent handling by study team members can reduce anxiety and help maximize rats' comfort levels with handlers, which will increase the likelihood of

Table 1
Checklist of USV study considerations to standardize methods across investigations.

Study Methods	Checklist
Animal Acclimation	☐ Daily animal handling to increase comfort levels with study team (~1–2 weeks) ☐ Minimize strong scents (*e.g.*, perfumes, lotions) ☐ Keep animals on reverse light cycle ☐ Confirm minimal alarm calls with pre-study USV recordings
Animal Characteristic Considerations	☐ Scientific justification of: ○ Strain ○ Sex ○ Age
Elicitation Considerations	☐ Ensure consistency with motivators and reward system (*e.g.*, sexual encounters, nutritional sucrose pellets) ☐ Ensure type, duration, and stimulus of stimulus exposure is held constant ☐ Automate training whenever possible to reduce variability that can occur with human contact and manual training
Recording Considerations	☐ Test recording environment for extemporaneous ultrasonic noise (*e.g.*, background, cage) ☐ Isolate animals in sound-attenuating environment ☐ Standardize sampling rate, microphone placement, and recording duration
USV Analysis Considerations	☐ Match USV analysis methods to research question ☐ Consider acoustic characteristic outcomes over USV frequency contours to characterize group or condition differences

maximizing or reaching USV production targets. Keeping strong scents like lotions and perfumes to a minimum and consistent throughout the protocol can reduce anxiety because it prevents rats from feeling like there is a foreign intruder in their environment, which can also help ensure optimal USV productions, especially for USVs in the 50-kHz positive communicative range.

The light cycle can also affect the rate of USV production. Because rats are most active during their dark cycle, reversing the light cycle (*i.e.,* training rats in their dark cycle) can maximize the probability of sufficient USV production rates. Pre-study recordings in the rats' new environments can capture alarm calls, which means rats may not yet feel comfortable in their new environment, which can influence results. Confirming rats are producing minimal alarm calls via USV recordings ensures the rats' comfort levels with their environment and their level of readiness for study protocols.

6.2. Rat characteristics

The strain, age, and sex of the rats should be held constant within and between studies. The Long-Evans strain is known for their abundant vocalizations; they are also docile and easy to vocally train. USVs can vary significantly with age, with older rats producing less frequent USVs than younger rats [22,25]. Females typically vocalize more than males, although their USV rate can depend on their estrous cycle [42].

6.3. Elicitation practices

The type, duration, and timing of stimulus exposure used to elicit USVs should be held constant. The behavioral context in which vocalizations are recorded should also be consistent. For example, social interactions should follow a standardized protocol to minimize variability across animals. USV data should be complemented with behavioral observations wherever possible to provide context and ensure consistency and replicability within and across studies. Automating the elicitation and training process as much as possible is also advantageous. Not only is it timesaving, but it can also improve consistency as it removes human variability that comes with manual training. Automated training can yield more controlled responses to elicitation (*e.g.*, rewards) and minimizes human encounters, which can reduce stress. Finally, automated training allows for higher throughput of experiments because multiple rats can train simultaneously with one investigator observing these sessions [61].

6.4. Recording environments

To ensure standardization of USV recordings across studies and investigators, various recording factors should be considered. First, recording environments should be tested prior to initiating formal study protocols to ensure they are free of confounding ultrasonic noise. We have found that common sources of ultrasonic noise include motion detectors on room lights, computer monitors, and cell phones. Various cage noises can also result in ultrasonic recording confounds. We found that toys, bedding, metal food hoppers, water bottles, and investigator keys can all be sources of noise that get picked up on ultrasonic recordings and may be erroneously labeled as USVs, especially when using automated USV detection programs. Microphone qualities should be suitable for capturing vocalizations in the ultrasonic frequencies and recording settings, including sampling rate, microphone placement, and recording duration should be held constant. Finally, rats should be isolated during recordings and their recording environments should consist of sound-attenuating material embedded between cages to reduce the possibility of microphone bleed, especially when multiple rats vocalize simultaneously.

6.5. USV characterization and classification

Despite various attempts across the years to characterize and standardize USVs into distinct categories, there are currently no universal classification systems. Part of the issue is that USV classifications will ultimately depend on the research questions being asked, rat models used, elicitation and training methods being implemented, and the types of outcome variables being measured, making it challenging to encapsulate these different factors into one or two overarching USV categorization methods. Furthermore, vocalizations consist of highly variable and skilled sensorimotor vocal behaviors. As such, the types of USVs produced will depend on rat affect and vocal production abilities. For example, studies that investigate positive affect or are interested in the physiological role of the larynx in the context of communicative intent will typically attempt to elicit as many USVs around 50-kHz as possible. In contrast, investigators conducting studies on anxiety, depression, or arousal may instead be interested in eliciting 22-kHz USVs. Whereas USVs around 50-kHz are typically shorter in duration, more complex in their frequency modulations, and more varied across productions, USVs around 22-kHz are typically much longer in duration, are centered on a single frequency with minimal frequency modulation, and are consistent in frequency across productions.

7. Future directions

We recognize that the studies described in this review and experiences provided are from one cohort of investigators and from an laryngological perspective. As such, we encourage the mobilization of cross-discipline consortiums to enrich our approaches to investigations involving USVs across different disciplines. Various disciplines bring unique perspectives and methodologies necessary to study USVs more comprehensively. More collaborative involvement across disciplines via conferences or focus groups (1) can help further improve standardization of methods, (2) broaden knowledge bases and skillsets, (3) better identify subtle patterns and correlations across studies, and (4) foster innovation and speed up scientific discoveries. Combining insights from various fields ensures that study interpretations are thorough, considered from all possible angles, unbiased, and reduces the potential for misinterpretations of experimental designs. Cross-disciplinary

involvement also allows for validation and replication of findings through different methodologies, strengthening the reliability of results.

CRediT authorship contribution statement

Adrianna C. Shembel: Writing – review & editing, Writing – original draft, Project administration, Investigation, Funding acquisition, Conceptualization. **Aaron M. Johnson:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Michelle R. Ciucci:** Writing – review & editing, Writing – original draft, Conceptualization. **Charlie Lenell Lunaris:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Robert A. Morrison:** Writing – review & editing, Writing – original draft, Conceptualization. **Denis Michael Rudisch:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

None

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