J Neurophysiol 123: 966–979, 2020.
First published January 22, 2020; doi:10.1152/jn.00590.2019.

RESEARCH ARTICLE | *Control of Movement*

Role of deep breaths in ultrasonic vocal production of Sprague-Dawley rats

Tobias Riede, Charles Schaefer, and Amy Stein
Department of Physiology, College of Graduate Studies, Midwestern University, Glendale, Arizona

Submitted 15 September 2019; accepted in fina form 21 January 2020

Riede T, Schaefer C, Stein A. Role of deep breaths in ultrasonic vocal production of Sprague-Dawley rats. *J Neurophysiol* 123: 966–979, 2020. First published January 22, 2020; doi:10.1152/jn.00590.2019.—Deep breaths are one of three breathing patterns in rodents characterized by an increased tidal volume. While humans incorporate deep breaths into vocal behavior, it was unknown whether nonhuman mammals use deep breaths for vocal production. We have utilized subglottal pressure recordings in awake, spontaneously behaving male Sprague-Dawley rats in fiv contexts: sleep, rest, noxious stimulation, exposure to a female in estrus, and exposure to an unknown male. Deep breaths were produced at rates ranging between 17.5 and 90.3 deep breaths per hour. While overall breathing and vocal rates were higher in social and noxious contexts, the rate of deep breaths was only increased during the male's interaction with a female. Results also inform our understanding of vocal-respiratory integration in rats. The rate of deep breaths that were associated with a vocalization during the exhalation phase increased with vocal activity. The proportion of deep breaths that were associated with a vocalization (on average 22%) was similar to the proportion of sniffin or eupnea breaths that contain a vocalization. Therefore, vocal motor patterns appear to be entrained to the prevailing breathing rhythm, i.e., vocalization uses the available breathing pattern rather than recruiting a specifi breathing pattern. Furthermore, the pattern of a deep breath was different when it was associated with a vocalization, suggesting that motor planning occurs. Finally, deep breaths are a source for acoustic variation; for example, call duration and fundamental frequency modulation were both larger in 22-kHz calls produced following a deep inhalation.

NEW & NOTEWORTHY The emission of a long, deep, audible breath can express various emotions. The investigation of deep breaths, also known as sighing, in a nonhuman mammal demonstrated the occasional use of deep breaths for vocal production. Similar to the human equivalent, acoustic features of a deep breath vocalization are characteristic.

augmented breath; breathing; sigh; subglottal pressure; ultrasonic vocalization; vocal production

INTRODUCTION

Breathing is one of three movements besides laryngeal valving and upper vocal tract posturing that must be coordinated to produce acoustic signals for vocal communication in mammals (Titze 2000). Dysfunctional breathing or difficultie to integrate with the other two movements can negatively affect sound output in humans (e.g., Rubin et al. 2014) and in nonhuman mammals (e.g., rats and mice; Hülsmann et al. 2019; Riede et al. 2015). Although our understanding of normal movement control in vocal production of nonhuman mammals is expanding (e.g., Bennett et al. 2019; Jürgens 2009; Okobi et al. 2019; Riede 2018; Takahashi et al. 2015; Tschida et al. 2019), normal vocal breathing is maybe the least understood behavior of those three (Del Negro et al. 2018). This study was conducted to explore the role of one particular breathing type, the deep breath, in vocal production in the Sprague-Dawley rat. The deep breath, also known as "sigh" or "augmented breath," is a breathing variant that occurs only a few times per hour. Its regular occurrence is critical for maintaining functionality of lung alveoli (Bartlett 1971; Haldane et al. 1919; Thet et al. 1979). In humans it is associated with the expression of emotional states (e.g., Vlemincx et al. 2011). The incorporation of deep breaths in vocal communication of rodents has been suggested (Hernandez et al. 2017; Riede 2014; Soltysik and Jelen 2005) but not investigated.

Many muroid rodents rely on ultrasonic vocalizations (USVs) for acoustic communication (Brudzynski 2018; Dent et al. 2018). Rodent USVs are aerodynamic whistles produced during the exhalation phase of the breathing cycle and require the control of both breathing and laryngeal movements (Riede 2018). Breathing movements during ultrasonic whistle production must be controlled to maintain subglottal pressure within narrow boundaries (Riede 2013). Subglottal air pressure is a primary factor in controlling voice onset, offset, and intensity. It also contributes to variations in fundamental frequency (Riede 2011, 2013). A positive subglottal pressure and an open glottis create an outward going airflo (Riede 2011). The airflo passes through the glottis and is then directed over the opening of a small intralaryngeal air sac, the "ventral pouch." An ultrasonic whistling sound is generated when an airflo fluctuatio is generated due to an interaction between the airflo and the ventral pouch cavity (Riede et al. 2017). Two critical variables, geometry of glottis and ventral pouch, are controlled by intrinsic laryngeal musculature (Riede 2013).

Rat USVs have been categorized into groups labeled as 22-kHz and 50-kHz calls. Both call categories have behavioral and neurophysiological correlates (Brudzynski 2013). USVs in the 28- to 90-kHz range ("50-kHz calls") are produced by males and females in mating and other positive interactions or in response to the expectation of reward. USVs in the 19- to 28-kHz range ("22-kHz calls") are produced in aversive settings such as anticipation of pain and danger. Both categories can also be concatenated into a single breath appearing as a

Address for reprint requests and other correspondence: T. Riede, Dept. of Physiology, College of Graduate Studies, Midwestern University, 19555 N. 59th Ave., Glendale, AZ 85308 (e-mail: triede@midwestern.edu).

single call, potentially reflectin an ambiguous state of the sender (Burke et al. 2017; Hernandez et al. 2017).

Various breathing patterns have been described in rodents: eupnea (1–4 Hz), sniffin (5–10 Hz), and deep breaths (15–40 breaths/h) (e.g., Bartlett 1971; Carnevali et al. 2014; Kabir et al. 2010; Welker 1964). Sirotin et al. (2014) suggested that vocal activity is mostly associated with periods of olfactory exploration during which rodents show a breathing pattern referred to as sniffing Therefore, USVs are frequently associated with active sniffing However, anecdotal observations suggest that the concatenation of multiple calls is sometimes preceded by a deep breath (Hernandez et al. 2017; Riede 2014), but overall it was unknown whether slower breathing patterns are also involved in vocal production. Deep breaths are relevant for the expression of emotions in human vocal communication (e.g., Poggi et al. 2018; Teigen 2008; Vlemincx et al. 2011), and rat USVs are discussed in the context of changing behavioral and neurophysiological states (Brudzynski 2018). We therefore tested four hypotheses. 1) The rate of deep breaths changes with different behavioral contexts. *2*) Deep breaths are associated with USVs in rats. *3*) If deep breath are used for vocal production, the associated breathing pattern will change. *4*) A vocalization produced with a deep breath will also demonstrate characteristic acoustic changes.

METHODS

Animals. The data presented were obtained from a total of 10 male Sprague-Dawley rats, 6–12 mo old and weighing between 250 and 450 g. The animals were housed one per cage and maintained on a reversed 12:12-h light-dark cycle so that experiments were performed in the rat's dark phase. Temperature was controlled (20°C). Rats had free access to food (standard pellets) and water. All procedures performed in studies involving animals were approved by the Institutional Animal Care and Use Committee at Midwestern University (Glendale, AZ).

Subglottal pressure and sound monitoring. Subglottal pressure, i.e., the pressure inside the trachea below the glottis, is a good proxy for breathing movements; simultaneously, it is one key variable in vocal production (Titze 1989). Subglottal pressure is determined by the force with which respiratory muscles contract and thereby expand (inhalation) or compress (exhalation) the thoracic cavity. Subglottal pressure also depends on the size of the glottal valve. Pressure increases with increasing lung pressure und with vocal fold adduction.

Subglottal pressure was measured through a small stainless steel tube (1-mm outer diameter, 0.8-mm inner diameter) implanted between the fift and seventh tracheal rings. The tube opening was implanted flus with the tracheal wall so that it did not constrict the airway. The other end of the tube pointed caudally. The tube was connected to a pressure transducer (model FHM-02PGR-02; Fujikura) by a silastic tube. The transducer was housed in a backpack mounted onto a rodent jacket. A tether was fixe to the rat's backpack and connected by a swivel to a horizontal bar 15 cm above the cage, which allowed the animal to move freely in the cage.

Surgery was performed under general anesthesia by administering ketamine-xylazine (80 and 8 mg/kg). Anesthesia depth was monitored by watching the rat respiration rate, testing pinch reflex and monitoring whisker micromovements. None of the rats required a second dose. Following surgery, the rat received fluid (Ringer lactate), nonsteroidal anti-inflammator drugs (Rimadyl), and antibiotics (Baytril). The rat was placed in the home cage, tethered, and allowed to recover overnight. During the recovery period breathing rate was monitored using the subglottal pressure recording.

Ultrasonic vocalizations produced by the rats were recorded using an ultrasonic microphone (CM16/CMPA-5V; Avisoft-Bioacoustics) placed 5 cm over the center of the male cage. The voltage output from the pressure transducer was amplifie (500 to 1,000 times; model 440; Brownlee, San Jose, CA). The subglottal pressure signal was calibrated at the end of the experiment (handheld pressure meter model HHP-90; Omega, Stamford, CT). Pressure signal and sound signal were recorded simultaneously and acquired through a NiDAQ 6212 acquisition device, sampled at 200 kHz, and saved as uncompressed file using Avisoft Recorder software (version 3.4.2; Avisoft-Bioacoustics, Berlin, Germany).

For simplicity, we will use "deep breath" and "sigh" interchangeably throughout the remainder of the text referring to a subglottal pressure signal of characteristic shape. Sighs were visually identifie and counted in the time domain signal of the subglottal pressure signal. Sighs were characterized by a biphasic inhalation followed by a longer exhalation (Ramirez 2014). The firs inhalation phase resembled a normal eupneic inhalation; the second phase showed a large amplitude inhalation. The subsequent exhalation was always associated with a positive subglottal pressure inside the trachea (Fig. 1).

Experimental situations. A standard rat cage was divided by an acrylic plate into two equal-sized compartments. The experimental male was placed in compartment 1 immediately after surgery.

Subglottal pressure was recorded during anesthetic sleep for 20 min (1- to 2-h recovery period after surgery; animal was not responsive to a tail pinch). The animal was allowed to recover for 24 h. Subglottal pressure and sound above *compartment 1* were recorded during a 10-min habituation period immediately preceding one of three types of sessions when the following stimuli were presented: *1*) noxious stimulus, *2*) female near estrus (estimated through behavioral observation; Erskine 1989), and *3*) unknown male. For a noxious stimulus, the experimenter blew fiv short air puffs through a long, narrow tube to the facial region of the animal. The air puff functions as an aversive stimulus (Knapp and Pohorecky 1995) and can trigger the animal to start vocalizing within a few seconds from the start of the air puffs. For female and male exposure, the second animal was placed into *compartment 2*. Animals in *compartments 1* and *2* were able to have physical contact through six 10-mm holes in the acrylic plate divider. The "female" and the "male" exposure began at the end of a 10-min habituation period ("rest") by adding bedding from the respective female or male to *compartment 1*. *Compartment 2* was still empty. After 3 min, the female or male was placed into *compartment 2*. Time between two sessions without recording was at least 60 min.

Analysis. Overall breathing rate, sigh rate, and vocalization rate were determined in each context (sleep, rest, noxious stimulus, male, female). The fiv contexts were not available in all 10 rats for different reasons (see Supplemental Table S1 at https://doi.org/10.5281/zenodo.3618631 for total observation times and available contexts for each rat). Four animals recovered faster than expected after surgery and no sleeping context was available. Furthermore, the pressure tubes had variable lifetimes between 12 and 65 h after surgery depending on how soon the animals began to manipulate the rodent jacket and the tubing, which caused eventual detachment of the tube from the pressure transducer. At this point the experiment was ended, and the animal was euthanized with CO_2.

Vocalizations were counted using a pitch-tracking tool in the software PRAAT (version 5.3.80, retrieved January 2014 from http://www.praat.org/). Visual inspection of each call confirme an associated pressure fluctuation Visual confirmatio ensured that only vocalizations of the experimental male were counted but not vocalizations from the female or the second male.

Breathing and sigh rate were estimated from the subglottal pressure signal. Breathing rate (breaths/min, bpm) was calculated by dividing the number of zero-crossings by 2 and by the duration of the recording session. Sigh rate (sighs/h, sph) was estimated by dividing the number of sighs by the duration of the recording session. The comparison of breathing and sigh rates between different contexts and vocal sigh rates among contexts were analyzed using linear mixed-effects regression, with individual rat included as a random effect. A linear

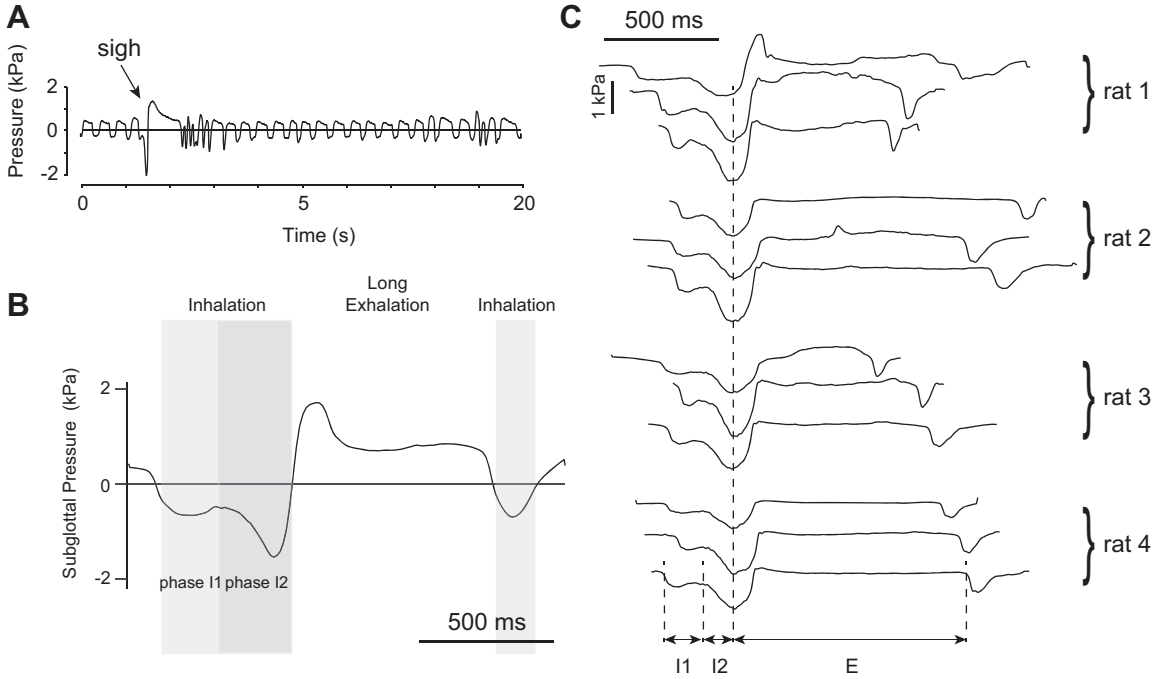

Fig. 1. All rats produce sighs, and sighs can be recognized by a typical subglottal pressure patterns. Sighs are embedded in between normal breaths during eupnea when the animal is at rest (*A*) and in between faster breathing, for example, during vocal activity when interacting with a female (*B*). Three example sighs from each of 4 rats are shown (*C*). Note the two-phase inhalation (*phases I1* and *I2*, indicated in the last sigh example; *bottom*) followed by a long exhalation (E). The two-phase inhalation is characterized by a shallow firs phase followed by a large-amplitude second phase. The subsequent long exhalation is always associated with a positive subglottal pressure.

mixed-effects model is a type of hierarchical linear regression model that includes both fixe and random effects, and was fi using the nlme package (Pinheiro et al. 2019). Pairwise contrasts were performed and *P* values adjusted for multiple comparisons with the Tukey method.

The relationship between breathing rate, sighing rate, and vocal rate, respectively, was tested by repeated measure correlations (rmcorr), a statistical technique for determining within-individual association for paired measures assessed on two or more occasions for multiple individuals (Bakdash and Marusich 2017). Rmcorr evaluates the overall relationship of common intraindividual association between two measures. The three variables (breathing rate, sighing rate, and vocal rate) have been determined in multiple experiments in each individual. Instead of averaging the repeated measures for each rat before the correlation is performed, rmcorr accounts for nonindependence among observations by using analysis of covariance to adjust for interindividual variability. This removes measured variance between rats, and rmcorr provides the best linear fi for each participant by using parallel regression lines (same slope) with varying intercepts. The rmcorr coefficien (r_{rm}) is bounded by −1 to 1 and represents the strength of the linear association between two variables. Null hypothesis is that there is no effect or relationship, and the alternative hypothesis is that there is a relationship.

We also investigated whether the occurrence of a vocalization in the exhalation phase of a sigh is associated with changes in breathing pattern or in acoustic features of the vocal behavior. Subglottal pressure shape was compared between sighs that contain no vocalization ("nonvocal sighs") and those containing a vocalization ("vocal sighs"). Subglottal pressure shape was quantifie through six variables: area under the curve for the inhalation and the exhalation phase (AUC_I and AUC_E), peak subglottal pressure during the inspiratory and expiratory phase (P_I and P_E), and duration of both phases (Dur_I and Dur_E). Comparisons were made between sighs with and without vocalizations using a one-way repeated measures analysis of variance (RM ANOVA).

One acoustic analysis focused on two features (average fundamental frequency and call duration) in various 50-kHz call types. A sigh vocal sample consisted of up to 10 calls per call type and rat, but usually less. The non-sigh vocal sample of 10 calls of the same type was randomly selected. Comparisons were made between vocalizations produced during a sigh and during another breathing pattern using a one-way RM ANOVA. In a second analysis, 22-kHz calls produced in bouts of fiv or more calls were analyzed. Five acoustic features (call duration, fundamental frequency at the beginning and end of a call, fundamental frequency modulation, call intensity) were compared between the sigh vocalization and the bout average. We included call intensity in the analysis because the animal was not moving, and therefore the call intensity measurement was more reliable as microphone-head distance and orientation were constant. Call duration was measured manually in the time waveform. Fundamental frequency and call intensity were measured using the pitch-tracking and intensity-tracking tools in PRAAT. We tested whether the 22-kHz call produced after a deep inhalation was different from the bout average of 22-kHz calls associated with normal breaths and produced in the same bout by using a linear mixed-effects model with bout and individual as crossed random effects and sigh/non-sigh included as the fixed-effec variable of interest.

Variables were transformed as appropriate to address issues with heteroscedasticity and skewness based on the residual plots. Vocal types within rats were analyzed with a nonparametric signed rank test. All statistical analysis was done in R (version 3.5.1; R Core Team 2014), and statistical significanc was assessed at the 0.05 level.

RESULTS

A total of 1,916 deep breaths from 10 male Sprague-Dawley rats were analyzed (Table 1). Overall, sigh rates ranged between 17.5 and 90.3 sph (Fig. 2*A*). Average sigh rate during sleep was 42.0 ± 7.5 sph (mean ± SE; $n = 6$ rats), at rest it

Table 1. *Overall number of sighs, sigh rate, number and proportion of sighs associated with a vocalization, vocal sigh rate, vocal rate, and proportion of vocalizations produced during a sigh*

Rat	No. of Sighs	Sigh Rate, sighs/h	No. and Proportion (%) of Sighs Associated with a Vocalization	Vocal Sigh Rate, vocal sighs/h	Vocal Rate, calls/min	Proportion of Vocalizations Produced During a Sigh, %
110	62	31.8 ± 6.3	14 (22.6%)	8.7 ± 4.4	19.3 ± 10.5	0.82 ± 0.79
111	361	56.3 ± 2.9	44 (12.2%)	11.2 ± 4.4	12.8 ± 8.2	1.31 ± 0.49
136	15	21.1 ± 7.5	5 (33.3%)	7.0 ± 3.8	76.7 ± 37.5	0.11 ± 0.10
137	214	70 ± 6.8	30 (14.0%)	9.0 ± 3.5	32.4 ± 15.1	1.31 ± 1.57
140	242	34.9 ± 2.5	39 (16.1%)	11.9 ± 3.6	29.9 ± 32.5	5.26 ± 9.49
141	165	38.5 ± 1.6	18 (10.9%)	2.9 ± 1.1	30.4 ± 33.9	0.35 ± 0.38
143	262	51.3 ± 8.7	7 (2.7%)	4.3 ± 2.1	11.1 ± 11.1	0.62 ± 0.76
144	69	26.7 ± 2.9	24 (34.8%)	10.9 ± 2.7	45.8 ± 39.9	0.48 ± 0.29
145	51	37.0 ± 2.6	27 (52.9%)	18.2 ± 6.3	35.6 ± 30.2	1.89 ± 1.4
151	475	49.2 ± 1.74	77 (16.2%)	19.6 ± 5.0	33.5 ± 24.8	1.51 ± 1.00

All values are means or means ± SD of variables recorded from 10 male Sprague-Dawley rats.

was 35.9 ± 4.2 sph (n = 10 rats), during female exposure it was 46.7 ± 5.9 sph (n = 10 rats), during male exposure it was 49.6 ± 7.2 sph (n = 7 rats), and during exposure to a noxious stimulus it was 34.6 ± 3.3 sph (n = 9 rats).

As expected, breathing and vocal rates were higher in the social and noxious contexts (Fig. 2*A*). We investigated whether sigh rate changed with context using a linear mixed model where the individual rat effect is included as a random effect. Table 2 gives the estimated marginal means for each context for sighing and breathing, and Table 3 shows all pairwise comparisons. Sigh rate did not change significantl in four contexts but was slightly higher during the female exposure

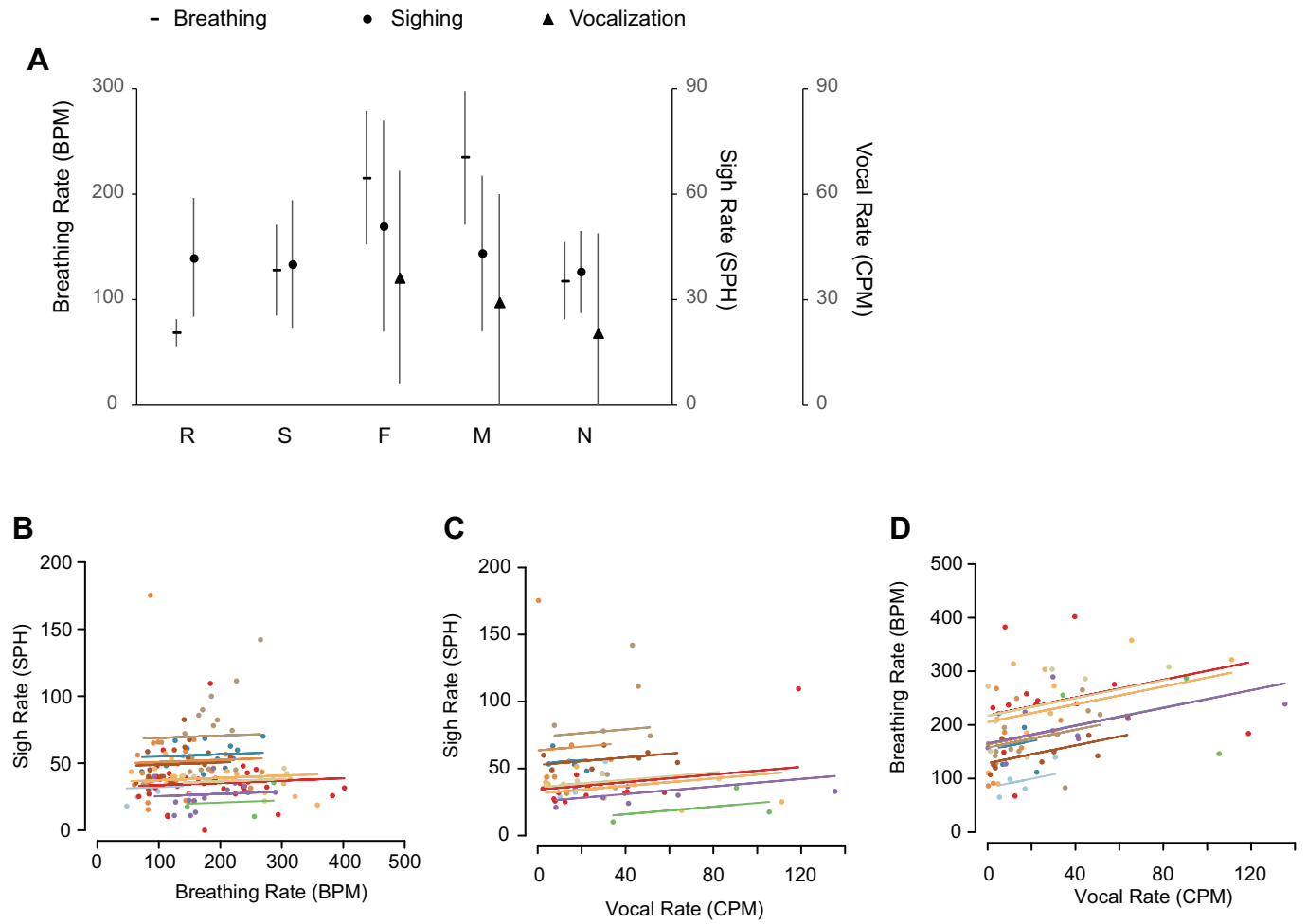

Fig. 2. Relationship between breathing, sigh, and vocal rates. *A*: the 3 variables were estimated in 5 different contexts: during sleep (S), rest (R), female exposure (F), male exposure (M), and exposure to a noxious stimulus (N). Shown are overall means and SD. *B* and *C*: sigh rate (sighs per hour, SPH) did not significantl change with increasing breathing (breaths per minute, BPM) or vocal rates (calls per minute, CPM). *D*: breathing and vocal rate, however, were related. Each data point in *B*–*D* represents a measurement from one experimental session lumping 3 contexts (exposure to a female, male, and noxious stimulus). Different colors designate different male rats.

Table 2. *Raw and estimated marginal means and 95% CI of sighing and breathing rates in different contexts*

Group	Context	Raw Mean	Estimated Marginal Mean	95% CI
Sighing	F	50.9 ± 13.4	49.0 ± 5.3	(37.63, 60.31)
	M	43.1 ± 9.9	45.7 ± 5.9	(33.38, 58.12)
	N	37.9 ± 5.1	38.7 ± 6.2	(26.02, 51.46)
	R	40.2 ± 8.1	38.7 ± 5.1	(27.55, 49.84)
	S	42.0 ± 7.5	33.1 ± 9.5	(14.28, 51.95)
Breathing	F	215.3 ± 28.3	221.6 ± 13.7	(191.83, 251.31)
	M	234.2 ± 28.3	228.1 ± 15.2	(196.18, 260.05)
	N	118.0 ± 16.3	109.1 ± 15.7	(76.41, 141.74)
	R	128.5 ± 19.2	131.0 ± 13.3	(101.68, 160.32)
	S	68.5 ± 5.8	89.7 ± 23.3	(43.27, 136.08)

Values are raw and estimated marginal means ± SE and 95% confidenc intervals (CI) of breathing (breaths/min) and sighing rates (sighs/h) in 5 different contexts, adjusted for individual rats: F, exposure to female in estrus; M, exposure to unknown male; N, noxious stimulus; R, rest; S, sleep.

than during the preceding resting period. Sigh rate changes were not associated with changes in breathing or vocal rate. While breathing and vocal rate were positively related [$r_{rm(68)} = 0.34$, 95% confidenc interval (CI) (0.11, 0.54), $P = 0.0038$; Fig. 2*B*], sighing rate appears to change with neither breathing rate [$r_{rm(153)} = 0.06$, 95% CI (−0.1, 0.22), $P = 0.45$; Fig. 2*C*] nor vocal rate [$r_{rm(56)} = 0.168$, 95% CI (−0.1, 0.41), $P = 0.21$; Fig. 2*D*].

Deep breaths and vocal activity. Rats produced vocalizations during the exhalation phase of a deep breath (Fig. 3). Vocalizations were produced in 285 of 1,916 deep breaths. The average proportion of sighs associated with a vocalization for 10 rats was 21.6 ± 14.8% (mean ± SD, $n = 10$ rats; Fig. 4*A* and Table 1). None of the three contexts had either more or fewer sighs associated with a vocalization (results for a linear mixed model where the individual rat effect is included as a random effect are presented in Tables 4 and 5). However, while we did fin that more sighs were associated with a vocalization during episodes of increased vocal activity [$r_{rm(55)} = 0.6288$, 95% CI (0.44, 0.77), $P < 0.0001$; Fig. 4*B*], calls produced during a sigh were rare events. Average call rates were as high as 76 calls/min (Table 1), but the average proportion of vocalizations that were produced within sighs was only 1.37 ± 0.5% (mean ± SD, $n = 10$ rats; Table 1).

Call type distribution into sighs and into non-sigh breathing patterns was only different for 3 of the 14 different call types (upward ramps, splits, and step downs; Fig. 5; paired nonparametric signed-rank test with associated *P* values are listed in Table 6).

Vocal production during deep breaths. We refer to sighs that are associated with a vocalization as "vocal sighs," as opposed to "nonvocal sighs" when a deep breath is generated without a vocal gesture in the exhalation phase. The production of ultrasonic whistles depends on the precise coordination of laryngeal and breathing movements to maintain subglottal pressure within narrow boundaries. It is therefore possible that a vocal sigh is produced differently than a nonvocal sigh. It is also possible that a call that is placed into the exhalation of a deep breath shows different acoustic features than the same call type placed into a normal breath. To address both questions, we studied subglottal pressure pattern and acoustic properties in more detail.

Averages for area under the curve of the subglottal pressure signal (AUC_I and AUC_E), peak subglottal pressure (P_I and P_E), and duration (Dur_I and Dur_E) are provided in Table 7. The 22-kHz vocal sigh demonstrates a greater expiratory area under the curve (AUC_E: $T_{369} = 11.69$, $P < 0.001$), a higher expiratory peak subglottal pressure (P_E: $T_{369} = 9.47$, $P < 0.001$), and a longer expiratory duration (Dur_E: $T_{369} = 0.27$, $P < 0.001$). The inspiratory area under the curve (AUC_I: $T_{355} = 1.96$, $P = 0.125$) and inspiratory peak subglottal pressure (P_I: $T_{355} = -1.09$, $P = 0.523$) were not different, but inspiratory duration (Dur_I) was smaller in vocal sighs ($T_{369} = -3.43$, $P < 0.01$).

The 50-kHz vocal sigh demonstrates a greater AUC_E ($T_{369} = 3.13$, $P < 0.01$) and a higher P_E ($T_{369} = 8.17$, $P < 0.001$) but no difference in Dur_E ($T_{369} = -2.04$, $P = 0.106$). AUC_I was greater ($T_{355} = 4.79$; $P < 0.001$) and P_I was more negative ($T_{355} = -6.55$, $P < 0.001$), but Dur_I ($T_{369} = -5.84$, $P < 0.001$) was smaller in vocal sighs. While differences in the exhalation phase are less surprising since breathing movement must be integrated with laryngeal movements to produce sound, most interesting is the observation that some of the inhalation phase variables were different. This suggests that the vocal motor program that follows during the exhalation had already affected the preceding inhalation.

If the inhalation pattern is adjusted to prepare a vocalization that is produced during the subsequent exhalation, it is expected that inhalation and exhalation are associated. We tested whether differences in AUC of the subglottic pressure signal, peak subglottal pressure, and duration of the inhalation were predictive for the exhalation (Fig. 6). The AUC of the inhalation and exhalation phase were not related in the nonvocal sigh [$r_{rm(157)} = 0.14$, 95% CI (−0.02, 0.29), $P = 0.077$], but they were negatively related in the vocal sigh [$r_{rm(189)} = -0.15$, 95% CI (−0.28, −0.04), $P = 0.043$; Fig. 6, *A* and *D*). The peak subglottal pressure of the inhalation and exhalation phase were negatively related in the nonvocal sigh [$r_{rm(157)} = -0.44$, 95% CI (−0.56, −0.31), $P < 0.001$], and they were not related in the vocal sigh [$r_{rm(189)} = 0.11$, 95% CI (−0.04, −0.25), $P = 0.13$; Fig. 6, *B* and *E*). The durations of the inhalation and exhalation phase were positively related in the nonvocal sigh [$r_{rm(166)} = 0.33$, 95% CI (0.18, 0.46), $P < 0.001$] and the vocal

Table 3. *Pairwise comparisons of breathing and sigh rates between different contexts*

	Sighing		Breathing	
Context Contrast	Sigh rate, sighs/h	*P* value	Breathing rate, breaths/min	*P* value
R – S	5.6 ± 8.7	0.9687	41.3 ± 20.9	0.2830
R – F	10.3 ± 3.6	**0.0388**	90.6 ± 8.6	**<0.0001**
R – M	7.1 ± 4.3	0.4789	97.1 ± 10.3	**<0.0001**
R – N	0.1 ± 4.9	1.00	−21.9 ± 11.6	0.3523
F – S	15.9 ± 8.8	0.3812	131.9 ± 21.1	**<0.0001**
F – N	10.2 ± 5.2	0.2792	112.5 ± 12.3	**<0.0001**
F – M	3.2 ± 4.8	0.9616	−6.5 ± 11.4	0.9875
M – S	12.6 ± 9.4	0.6618	138.4 ± 22.4	**<0.0001**
M – N	7.0 ± 5.6	0.7261	119.0 ± 13.5	**<0.0001**
N – S	5.6 ± 9.5	0.9760	19.4 ± 22.7	0.9128

Values are means ± SE of pairwise comparisons showing the difference in breathing and sigh rates between 5 different contexts: F, exposure to female in estrus; M, exposure to unknown male; N, noxious stimulus; R, rest; S, sleep. *P* values were adjusted for multiple comparisons with the Tukey method; bold type indicates significan difference ($P < 0.05$).

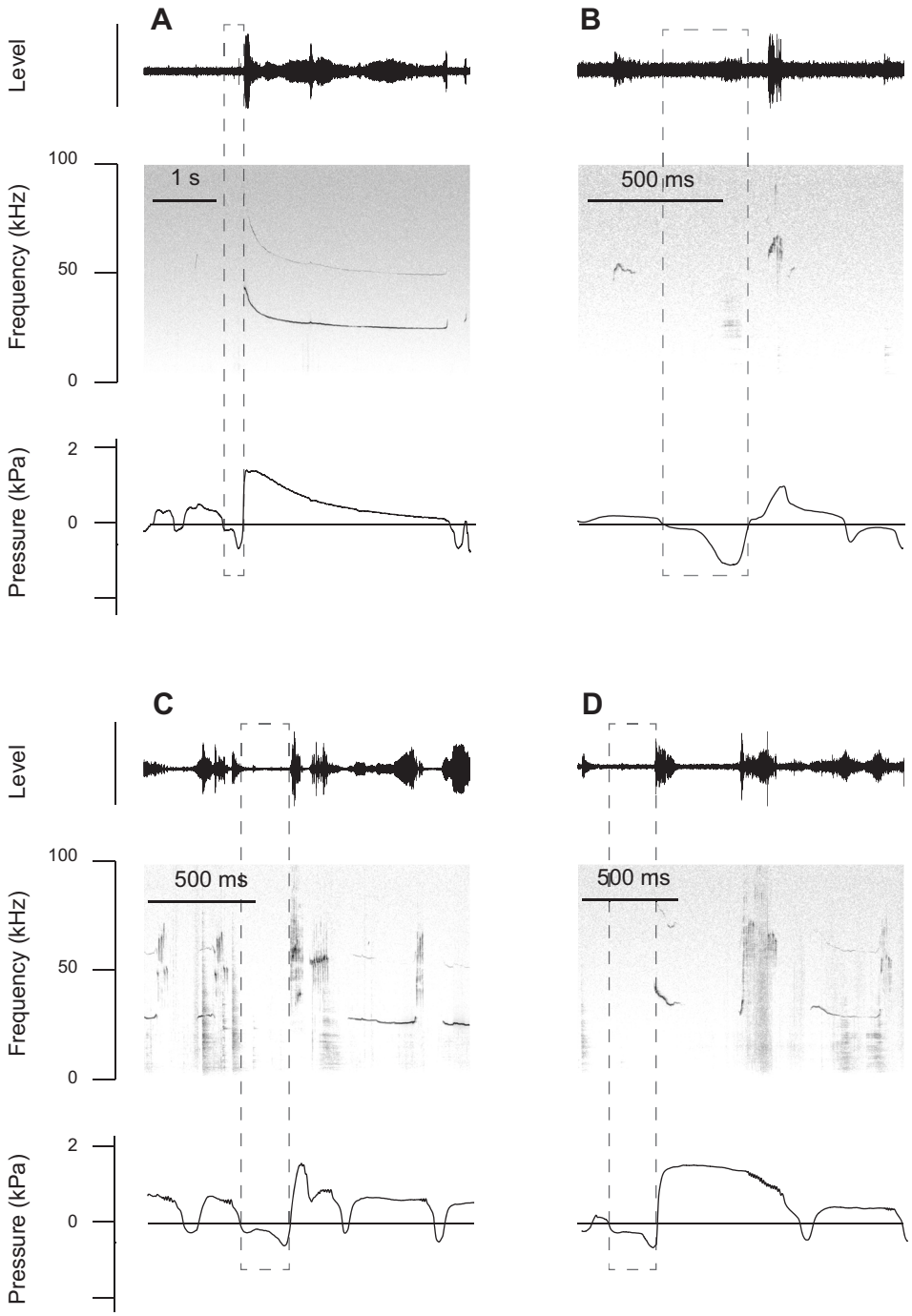

Fig. 3. Different call types are produced during deep breaths. *A*: a very long 22-kHz call. *B*: a single 50-kHz complex call. *C* and *D*: two 50-kHz calls are separated by a short silence but still produced during the same long exhalation following a deep breath. Sound is depicted as a time waveform (*top*; level indicates relative change in output voltage of microphone signal) and spectrographically (*middle*). For subglottal pressure (*bottom*), horizontal line indicates ambient pressure.

sigh [$r_{rm(193)} = 0.23$, 95% CI (0.09, 0.36), $P < 0.01$; Fig. 6, *C and F*).

Next, we investigated whether acoustic features would change if a call is associated with a deep breath. We compared two acoustic variables (call duration and average fundamental frequency) between a set of various 50-kHz call types that were produced during a sigh and a randomly selected set of same 50-kHz call types that were produced during normal breathing or sniffing Mean values for individual rats are presented in Table 8. Average fundamental frequency ($F_{1,251} = 0.08$, $P = 0.77$) and call duration ($F_{1,251} = 2.96$, $P = 0.09$) were not different between sigh and non-sigh vocalization.

We also investigated acoustic features of 22-kHz calls, which were produced in bouts of fiv or more calls. One call within the bout was produced in a sigh. Figure 7 shows four examples. We tested whether the one sigh vocalization was different from the remaining calls of the same bout. Five acoustic variables (call duration, fundamental frequency at the beginning and end of the call, fundamental frequency modulation, and call intensity) were compared between the call produced in a sigh and the bout average. Mean values for individual rats are presented in Table 9. Call duration was longer if produced in a deep breath ($T_{59} = 8.88$, $P < 0.001$). Fundamental frequency at the beginning of a call ($T_{59} = 6.84$, $P < 0.001$), but not at the end of the call ($T_{59} = -0.25$, $P =$

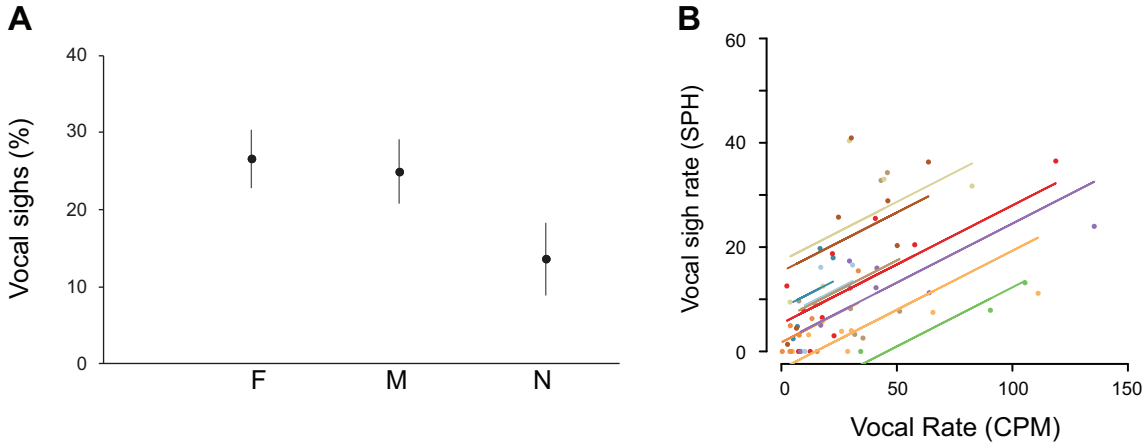

Fig. 4. During increased vocal activity, ultrasonic vocalizations (USVs) are more often associated with a deep breath. *A*: in 3 different contexts (F, female; M, male; N, noxious stimulus) associated with high rates of vocal behavior, USVs were found, on average, in 21% of deep breaths ("vocal sighs"). *B*: the proportion of deep breaths that include a vocalization (vocal sigh rate; sighs per hour that are associated with a vocalization, SPH) increases with vocal activity (vocal rate; calls per minute, CPM). Each data point in *B* represents a measurement from one experimental session lumping 3 contexts together (exposure to a female, male, and noxious stimulus). Different colors designate different male rats.

0.803), was significantl different. Fundamental frequency modulation was larger in calls associated with a deep breath (T_{59}= 6.2, $P < 0.001$). Call intensity was not different ($T_{59} = 1.61$, $P = 0.1$) between sigh and non-sigh vocalization.

Finally, we qualitatively describe two other features, which were observed in vocal sighs. First, rats are able to produce more than one call during a single exhalation phase (Riede 2014). The calls are separated by a pause during which laryngeal motor activity is interrupted. The deep breath generates a greater available lung volume, which could allow to produce more calls or longer vocalizations during the subsequent exhalation. While all 10 rats produced many examples of exhalations with multiple calls, we found eight vocal sighs in 3 rats with more than one call during the exhalation. All seven remaining rats also produced multiple calls in single breaths, but during sniffin or eupnea breathing patterns. Second, we found 22-kHz calls that were interrupted (Fig. 8). Spectrographically, the sound appears as two subsequent calls, but judging the underlying breathing pattern demonstrates that the sound is produced during a single exhalation. A total of 363 interrupted 22-kHz calls were found in nine rats. While the majority were produced in normal breaths, six rats produced 21 of them during the exhalation of a deep breath.

DISCUSSION

The results presented in this report provide new finding that contribute to an understanding of vocal-respiratory integration in rats. The subsequent discussion will focus on four main results: *1*) In some social contexts, sighing rates increase in the awake and spontaneously behaving male rat. *2*) The exhalation following a deep inhalation is used for the production of different call types; however, overall, vocal sighs are rare events. The proportion of deep breaths that contain a vocalization resembles the proportion of sniffin or eupnea breaths that contain vocalizations. This suggests that vocal production can be associated with any breathing pattern available and no specifi breathing pattern is recruited. *3*) Results suggest that the pattern of a deep breath is different when it is associated with a vocalization. *4*) Finally, some of the vocalizations produced with a deep breath demonstrated characteristic acoustic changes. In other words, sighing can be a source for acoustic variation not only in humans but also in nonhuman mammals.

Sigh rates and social interactions. The frequency of respiratory movements depends predominantly on the level of blood gases, but the breathing pattern (eupnea, sniffing deep breaths) is determined by additional factors. Hypoxic challenges lead to an overall increase in breathing movements (both eupnea rates and deep breaths) (rats: Bartlett 1971; Bell and Haouzi 2010; cats: Cherniack et al. 1981; Glogowska et al. 1972; brain slice preparations: Hill et al. 2011). From human studies we also know that anxiety, negative emotions, and sustained attention tasks can increase eupneic breathing rates and, simultaneously, the rate of deep breaths, but the overall breathing rate and the rate of deep breaths change independently (Feldman et al. 2003; Ramirez 2014; Shea 1996; Vlemincx et al. 2009, 2011;

Table 4. *Proportion of sighs associated with a vocalization in different contexts*

Context	Raw Mean	Estimated Marginal Mean	95% CI
F	32.1 ± 7.1	31.1 ± 5.4	(19.64, 45.59)
M	29.6 ± 10.3	26.8 ± 6.3	(13.80, 39.85)
N	19.6 ± 8.8	18.5 ± 7.1	(4.15, 32.78)

Values are raw and estimated marginal means ± SE and 95% confidenc intervals (CI) of the proportion of sighs that were associated with a vocalization (%vocal sighs) in 3 contexts, adjusted for individual rats: F, exposure to female in estrus; M, exposure to unknown male; N, noxious stimulus.

Table 5. *Pairwise comparisons of vocal sigh rates between different contexts*

Context Contrast	Vocal Sigh Rate, sighs/h	*P* Value
F – M	4.3 ± 5.7	0.7321
F – N	12.7 ± 6.8	0.1569
M – N	8.3 ± 7.2	0.4761

Values are means ± SE of pairwise comparisons showing the difference in vocal sigh rate between 3 different contexts: F, exposure to female in estrus; M, exposure to unknown male; N, noxious stimulus. *P* values were adjusted for multiple comparisons with the Tukey method.

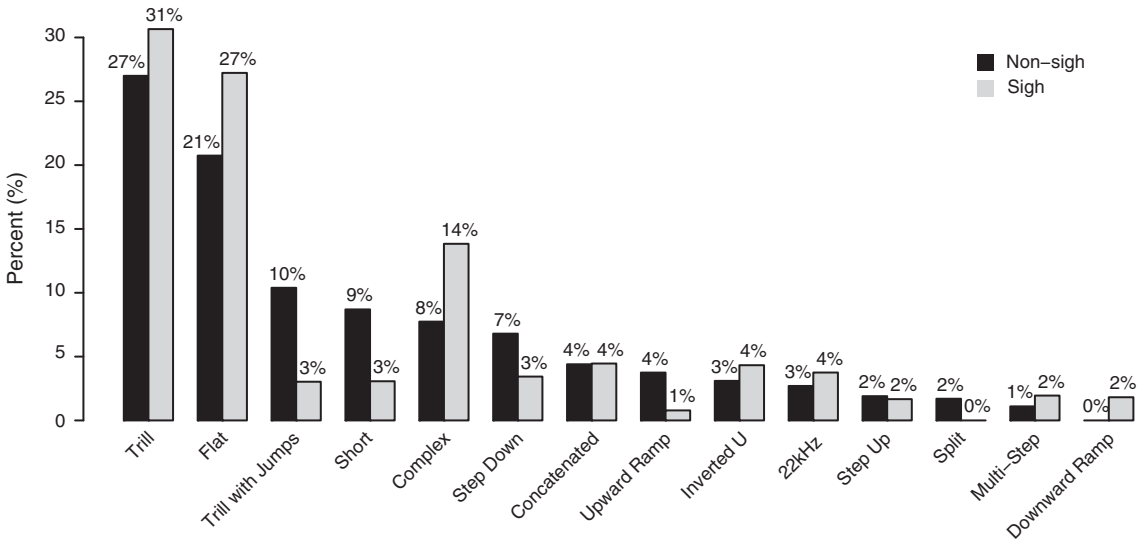

Fig. 5. For most call types, the same proportion was associated with deep breaths and with normal breathing/sniffin (differences were not significan). Three call types were produced significantl more often during eupneas: upward ramps, splits, and step downs.

Wilhelm et al. 2001). Compared with the hypoxic challenge, the association between the two patterns is less tight.

The increased breathing rates during female interactions, which we observed in this study, are a reflectio of the frequent occurrence of sniffin episodes. Sniffin is a behavioral response typical for rodents (Clarke, Trowill 1971; Grant 1963; Kepecs et al. 2007; Welker 1964) that is expressed frequently during social (Wesson 2013) or anxiety-related behaviors (e.g., Carnevali et al. 2014; Kabir et al. 2010). When male rats were exposed to a female, their sigh rates also increased. Exposure to a male or noxious stimulus did not trigger a similar increase in sigh rate. This observation supports the hypothesis that the rate of deep breaths in rats is state dependent (Soltysik and Jelen 2005).

However, Soltysik and Jelen (2005) had observed an up to a sevenfold increase in sigh rates during tail-shock experiments. We observed the rate increase was on the order of 1.5- to 2-fold during the female exposure. The smaller increase could reflec strain differences. Rat strains differ in their anxiety-like behavior and their sighing behavior differs. Carr and Lucki (2010) found that Wistar rats display greater amounts of anxiety-like behavior than Sprague-Dawley rats, and Carnevali et al. (2013) found anxiety related differences in two strains of Wistar rats. The strain demonstrating higher anxiety levels, measured by behavior in the elevated T-maze, showed significantl higher rates of deep breaths (Carnevali et al. 2013).

An important difference between our experimental approach and that of Soltysik and Jelen (2005) is stimulus type. The facial air blow represents a less intense but potentially more complex aversive stimulus than the electric tail shock (Nakagawa et al. 1981). The exposure to a potential male or female social partner also represents a more complex stimulus (e.g., Cardinal et al. 2002). Finally, our experimental design did not include any conditioning component, but it was only designed to increase the probability for high rates of vocalizations and different types of breathing rhythms.

Vocal sighs are rare events. Vocal activity in spontaneously behaving male rats can be 60–80 calls per minute and higher (e.g., Brudzynski, Holland 2005; Wöhr et al. 2008). In contrast, we found vocal sigh rates ranging between 1 and 42 vocal sighs per hour. Overall, vocalizations after deep breaths are rare events.

A deep breath was produced approximately every 90 s in awake and behaving rats with an increase when an estrous female was presented. On average, 23% of deep breaths contained vocalizations during episodes of high vocal activity. Vocal sigh rate was a function of vocal activity. The rate is comparable to vocalizations produced during sniffin episodes. Sirotin et al. (2014) noted that a considerable number of the sniffin episodes are associated with vocal production. They found that vocal utterances are placed into the exhalation phase of breaths in up to 15% of the sniffin episodes in contexts of high vocal activity (Sirotin et al. 2014). It appears that a similar proportion of sighs and sniffin episodes are associated with vocalization; however, sniffin episodes are much more common. This suggests that no particular breathing pattern is recruited in preference over another.

The pattern of the deep breath is different when associated with a vocalization. The subglottal signal during the exhalation following a deep inhalation changed when a call was produced during the exhalation. This is not surprising because ultrasonic calls are associated with stereotypic laryngeal muscle activity

Table 6. *Pairwise comparison of call types in different breath types*

Trill	Flat	Trill with Jump	Short	Complex	Step Down	Concatenated	Upward Ramp	Inverted U	22 kHz	Step Up	Split	Multistep	Downward Ramp
0.16	0.008	0.18	1	0.08	0.013	0.08	0.006	0.94	0.69	0.131	0.477	0.845	0.937

Data are *P* values for pairwise comparisons of 14 call types investigated for their appearance in a deep breath or a normal breath/sniffing Results of a nonparametric signed-rank test suggest that only 3 call types were produced more frequently in one or the other breath type.

Table 7. *Average values for measurements taken from the subglottal pressure signal for both inspiratory and expiratory phases*

	AUC_I			AUC_E		
Rat	None	50 kHz	22 kHz	None	50 kHz	22 kHz
110	0.013 ± 0.005	0.017 ± 0.003	0.023 ± 0.005	0.111 ± 0.19	0.033 ± 0.01	0.470 ± 0.25
111	0.008 ± 0.002	0.008 ± 0.002	0.008 ± 0.001	0.013 ± 0.04	0.017 ± 0.01	0.013 ± 0.03
136				0.046 ± 0.02	0.070 ± 0.03	0.023 ± 0.03
137	0.046 ± 0.02	0.066 ± 0.02	0.057 ± 0.02	0.050 ± 0.05	0.054 ± 0.02	0.154 ± 0.09
140	0.033 ± 0.01	0.038 ± 0.008	0.051 ± 0.005	0.032 ± 0.02	0.052 ± 0.04	0.343 ± 0.13
141	0.033 ± 0.01	0.030 ± 0.01	0.033 ± 0.01	0.064 ± 0.05	0.041 ± 0.02	0.520 ± 0.21
143	0.043 ± 0.001	0.098 ± 0.001	0.098 ± 0.001	0.024 ± 0.02	0.036 ± 0.01	0.111 ± 0.10
144	0.030 ± 0.01	0.037 ± 0.007	0.031 ± 0.01	0.076 ± 0.04	0.093 ± 0.07	0.279 ± 0.12
145	0.039 ± 0.01	0.038 ± 0.006	0.036 ± 0.009	0.043 ± 0.02	0.069 ± 0.04	0.300 ± 0.12
151	0.080 ± 0.04	0.093 ± 0.03	0.047 ± 0.02	0.101 ± 0.07	0.241 ± 0.09	0.379 ± 0.22
	P_I			P_E		
Rat	None	50 kHz	22 kHz	None	50 kHz	22 kHz
110	−1.02 ± 0.3	−1.44 ± 0.6	−1.56 ± 0.7	1.17 ± 0.9	2.48 ± 1.2	2.84 ± 0.5
111	−1.01 ± 0.3	−1.20 ± 0.2	−0.90 ± 0.2	0.38 ± 0.2	0.64 ± 0.2	0.95 ± 0.4
136				1.37 ± 0.8	2.26 ± 0.1	0.46 ± 0.4
137	−1.14 ± 0.6	−1.60 ± 0.6	−1.49 ± 0.4	0.55 ± 0.2	0.88 ± 0.3	0.81 ± 0.3
140	−1.03 ± 0.3	−1.26 ± 0.2	−1.19 ± 0.1	0.47 ± 0.3	0.97 ± 0.7	1.54 ± 0.7
141	−1.03 ± 0.2	−1.17 ± 0.2	−0.87 ± 0.5	0.49 ± 0.2	0.97 ± 0.6	1.36 ± 0.3
143	−0.44 ± 0.2	−1.65 ± 0.5	−1.24 ± 0.3	0.32 ± 0.2	2.52 ± 0.3	2.15 ± 1.2
144	−0.86 ± 0.3	−1.13 ± 0.2	−1.08 ± 0.2	0.58 ± 0.4	1.73 ± 0.6	1.97 ± 0.4
145	−1.11 ± 0.2	−1.21 ± 0.2	−1.01 ± 0.1	0.58 ± 0.4	1.11 ± 0.6	1.41 ± 0.6
151	−2.57 ± 1.2	−3.18 ± 0.7	−1.60 ± 0.6	1.37 ± 1.2	0.88 ± 0.4	1.80 ± 0.9
	Dur_I			Dur_E		
Rat	None	50 kHz	22 kHz	None	50 kHz	22 kHz
110	0.33 ± 0.08	0.33 ± 0.04	0.39 ± 0.11	0.53 ± 0.33	0.43 ± 0.06	2.11 ± 0.89
111	0.36 ± 0.07	0.31 ± 0.04	0.36 ± 0.04	0.81 ± 0.56	0.77 ± 0.47	2.23 ± 0.88
136	0.31 ± 0.08	0.25 ± 0.01	0.26 ± 0.16	0.38 ± 0.21	0.32 ± 0.07	0.28 ± 0.27
137	0.39 ± 0.06	0.38 ± 0.04	0.35 ± 0.04	0.62 ± 0.44	0.51 ± 0.18	1.14 ± 0.60
140	0.31 ± 0.05	0.30 ± 0.04	0.39 ± 0.02	0.60 ± 0.28	0.66 ± 0.42	2.58 ± 0.36
141	0.39 ± 0.08	0.30 ± 0.04	0.30 ± 0.12	0.96 ± 0.43	0.47 ± 0.17	3.15 ± 1.31
143	0.49 ± 0.09	0.26 ± 0.07	0.33 ± 0.05	1.67 ± 0.75	0.65 ± 0.35	1.65 ± 1.14
144	0.41 ± 0.07	0.35 ± 0.08	0.30 ± 0.07	1.19 ± 0.60	0.74 ± 0.43	1.40 ± 0.85
145	0.35 ± 0.07	0.31 ± 0.03	0.32 ± 0.03	0.81 ± 0.40	0.81 ± 0.53	1.95 ± 0.17
151	0.41 ± 0.07	0.36 ± 0.08	0.36 ± 0.07	0.77 ± 0.43	0.83 ± 0.39	2.27 ± 1.02

Values are means ± SD for 3 measurements taken from subglottal pressure signals (50 kHz, 22 kHz, and no signal) for both the inspiratory (I) and expiratory (E) phases: area under the curve (AUC; in arbitrary units), peak subglottal pressure (P; in kPa), and phase duration (Dur; in s).

(Riede 2013), changing laryngeal valving and thereby subglottal pressure during the exhalation. Puzzling is the observation that the inhalation phase of a deep breath was altered when a call was produced during the subsequent exhalation. This suggests that a certain degree of motor planning occurs that alters the subglottal pressure pattern. The respiratory pattern generator in the brain stem could be adjusted before a vocalization. Equally, it may be that the glottal aperture is adjusted during inspiration in anticipation of the call. Motor planning plays key roles in motor control. Movements that are preceded by periods of motor planning are faster and more accurate than in the absence of planning (Shadmehr and Wise 2005). The role of motor planning in vocal control deserves further investigation.

Deep breaths alter acoustic features of ultrasonic vocalizations. Acoustic features of calls associated with deep breaths were different from the same vocal type produced during a normal breath or a sniff. The rat's sigh vocalization illustrates how underlying breathing patterns can be an important source of acoustic variation. In rats and humans, the tidal volume of a deep breath is at least two times larger than the mean tidal volume in normal breaths (Golder et al. 2005; Wilhelm et al. 2001). An increased inhalation allows longer vocal utterances. Elevated lung volume levels, for example, at the beginning of a sentence, are associated with increased vocal intensity and/or longer utterance duration in humans (e.g., Dromey and Ramig 1998; Hixon 1973; Solomon et al. 2000). Our investigation is the firs for nonhuman mammals, which demonstrates that a modifie inhalation volume is associated with subsequent increase in call duration and fundamental frequency modulation.

Other acoustic features, which were sometimes unusual, included the call amplitude, the occurrence of interrupted calls, and multiple calls produced in a single breath. Previously, we speculated that multiple calls on a single breath is a consequence of the deep breath simply because more air would be available (Hernandez et al. 2017; Riede 2014). That seems not to be the case since the phenomenon (multiple calls on a single breath) occurs more often with normal breaths or sniffing

The rat's vocal sigh, which is longer and more frequency modulated, shows similarities to a human sigh. The human sigh sound can communicate emotional information such as relief or pain. Like other nonverbal types of acoustic communication

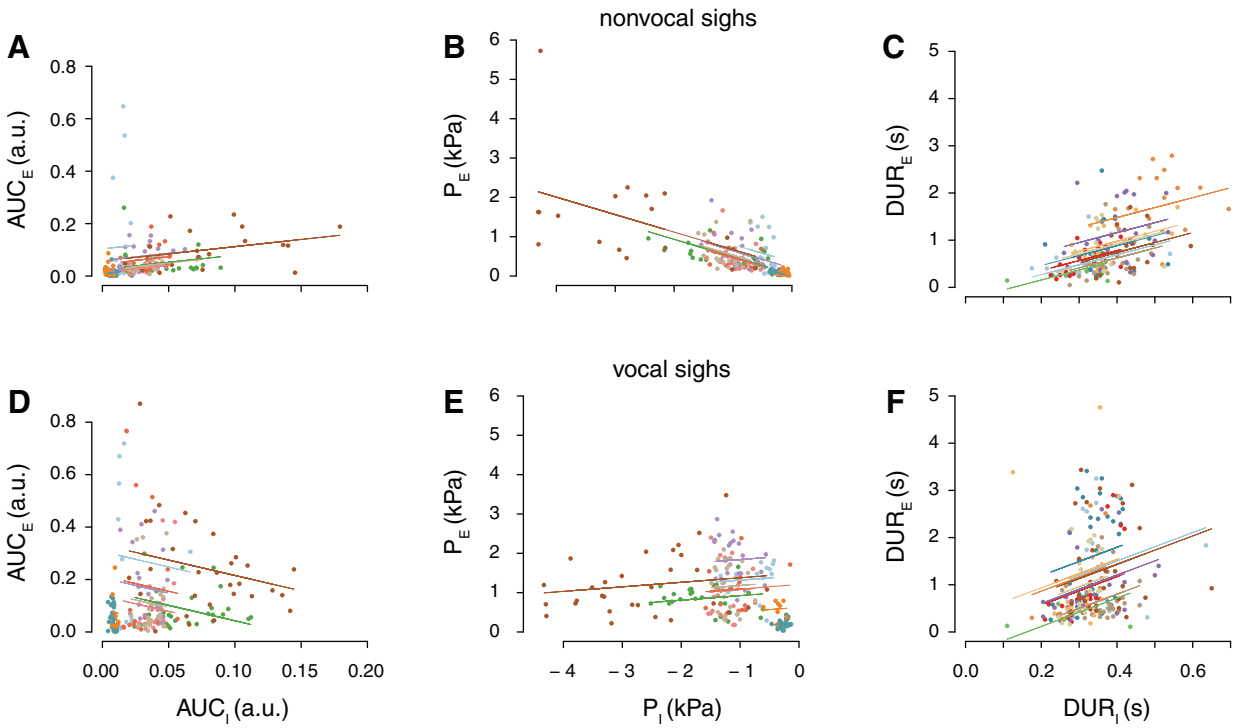

Fig. 6. Relationship between the inspiratory and expiratory phases of vocal and nonvocal sighs. Both phases were quantifie through 3 variables measured in the subglottal pressure signal. *A* and *D*: expiratory and inspiratory area under the curve (AUC_E and AUC_I). *B* and *E*: expiratory and inspiratory subglottal pressure (P_E and P_I). *C* and *F*: expiratory and inspiratory duration (Dur_E and Dur_I). Each data point represents a measurement from one experimental session lumping 3 contexts (exposure to a female, male, and noxious stimulus). Different colors designate different male rats.

signals, such as laughter (Owren and Bachorowski 2003) or crying (Miceli and Castelfranchi 2003), the human sigh is a basic cross-cultural nonverbal vocalization characterized by specifi acoustic features such as a breathy and elongated exhalation sound with or without voiced phonation (Sauter et al. 2010).

Vocal-respiratory integration. The larynx produces call type-specifi motor patterns to produce USV (Riede 2013). In what manner different breathing patterns are associated and integrated with laryngeal movements was not clear.

The positive correlation between the rate of deep breaths and vocal sigh rate (Fig. 4*B*), as well as similar results for sniffin and eupnea in experiments presented by Sirotin et al. (2014), suggests that all three breathing patterns (sniffing eupnea, and deep breaths) are used for the production of vocal types. They are associated at almost similar ratios in an "as needed" manner for vocal production. In other words, the sequence of breathing patterns is determined by multiple factors including changing blood gases, the presence of a social partner, a noxious stimulus, pain, and somatosensory feedback from the periphery.

Table 8. *Average values for measurements in different 50-kHz call types*

Rat	Call Type	*N*	Duration, ms		*F*0, kHz	
			Sigh	Non-sigh	Sigh	Non-sigh
111	Complex	7	58.6 ± 46.0	33.3 ± 9.9	55.7 ± 6.9	56.7 ± 3.5
137	Trill	6	134.5 ± 134.3	44.1 ± 26.6	65.6 ± 3.3	61.4 ± 4.1
137	Trill w/jump	8	143.8 ± 124.3	53.7 ± 18.9	62.1 ± 5.0	60.3 ± 4.7
140	Inverted U	3	27.7 ± 15.4	31.5 ± 12.2	53.4 ± 1.2	57.9 ± 6.5
140	Step down	3	71.0 ± 66.6	144.7 ± 136.7	45.7 ± 1.4	41.7 ± 5.8
140	Trill	5	35.2 ± 8.3	63.1 ± 60.9	57.1 ± 4.5	62.5 ± 5.8
141	Flat	3	112.7 ± 126.8	36.8 ± 16.1	53.7 ± 4.7	54.9 ± 7.9
141	Trill	6	35.7 ± 14.7	57.4 ± 22.9	63.3 ± 4.8	61.5 ± 4.5
143	Trill	4	69.3 ± 36.8	64.5 ± 26.4	73.0 ± 15.1	66.5 ± 10.3
144	Complex	5	51.8 ± 24.4	32.6 ± 9.1	54.8 ± 6.7	57.9 ± 6.3
144	Flat	9	46.4 ± 32.2	33.7 ± 10.1	43.8 ± 9.9	47.6 ± 7.9
144	Trill	9	69.8 ± 48.7	101.4 ± 189.7	62.6 ± 11.9	56.6 ± 6.3
145	Flat	10	36.3 ± 18.1	39.7 ± 20.0	41.5 ± 5.3	44.9 ± 9.5
151	Complex	9	59.6 ± 45.9	42.1 ± 13.1	54.5 ± 4.0	51.1 ± 8.3
151	Trill	10	88.6 ± 54.5	49.0 ± 15.6	50.4 ± 4.5	54.1 ± 3.8
151	Trill w/jump	4	46.8 ± 7.4	50.8 ± 14.8	56.8 ± 6.9	53.2 ± 4.0

Values are means ± SD for measurements of call duration and mean fundamental frequency (*F*0) taken in different 50-kHz call types. A variable number of calls associated with a sigh (*N*) were collected and compared with a sample of 10 calls of the same type and associated with a eupnea or a sniffing

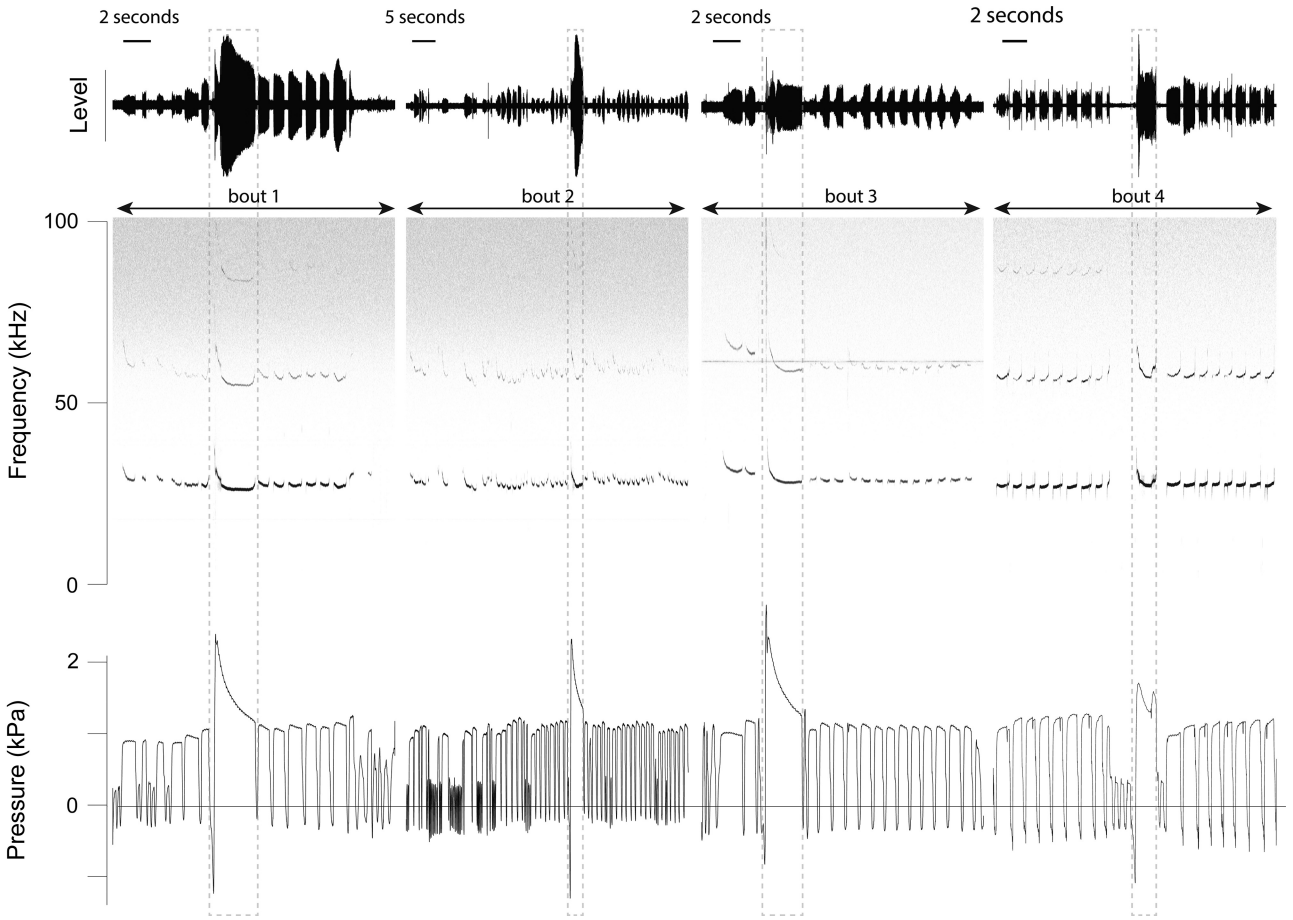

Fig. 7. Four bouts of 22-kHz calls. Each bout was produced by a different rat. In each bout there is one deep breath associated with vocal production. All other calls are produced on normal breaths. In all four cases, the sigh vocalization is louder, longer, and more frequency modulated. Sound is depicted as a time waveform (*top*; level indicates relative change in output voltage of microphone signal) and spectrographically (*middle*). For subglottal pressure (*bottom*), horizontal line indicates ambient pressure.

Laryngeal vocal motor patterns are then subsequently entrained to the breathing rhythm.

Current models of a mammalian vocal control circuitry rely on the precise entrainment of the laryngeal motor pattern onto the postinspiratory phase of the breathing cycle (Dutschmann et al. 2014; Tschida et al. 2019). Forebrain structures are responsible for initiating vocal activity (Arriaga et al. 2012; Bennett et al. 2019; Okobi et al. 2019). Those forebrain structures project through the periaqueductal gray (PAG) downstream to premotor regions in the caudal brain stem, which acts as vocal-respiratory pattern-generating circuitry (Jürgens 2002, 2009; Tschida et al. 2019). PAG stimulation studies showed that neither the precise onset nor the exact duration of a vocal utterance can be precisely timed with the

Table 9. *Average values for acoustic features measured in 22-kHz calls*

		Duration, s		*F*0 Start, kHz		*F*0 End, kHz		*F*0 Diff, kHz		Intensity, dB	
Rat	*N*	Sigh	Non-sigh	Sigh	Non-sigh	Sigh	Non-sigh	Sigh	Non-sigh	Sigh	Non-sigh
110	7	1.3 ± 0.6	0.6 ± 0.1	31.5 ± 4.9	29.2 ± 12.8	29.3 ± 1.7	31.1 ± 2.3	3.9 ± 2.0	2.4 ± 0.9	70.0 ± 6.3	66.1 ± 5.2
111	17	2.0 ± 0.8	0.7 ± 0.1	36.3 ± 3.7	28.3 ± 2.5	30.4 ± 2.6	30.4 ± 1.8	5.9 ± 2.5	2.5 ± 1.7	69.7 ± 10.1	68.4 ± 8.2
136	2	0.2 ± 0.1	0.2 ± 0.1	31.0 ± 6.6	28.9 ± 2.5	34.4 ± 6.3	30.4 ± 1.8	3.4 ± 0.4	3.2 ± 1.8	56.9 ± 8.4	60.0 ± 9.1
137	7	0.9 ± 0.9	0.5 ± 0.3	30.4 ± 3.5	28.0 ± 2.2	29.8 ± 3.0	29.0 ± 2.7	4.1 ± 2.5	2.3 ± 1.3	66.0 ± 3.6	68.2 ± 2.4
140	3	2.6 ± 0.9	0.9 ± 0.3	41.5 ± 1.7	27.6 ± 1.6	26.1 ± 2.2	25.6 ± 1.3	15.4 ± 0.5	2.0 ± 1.9	66.0 ± 5.8	64.7 ± 7.2
141	4	2.1 ± 0.8	1.3 ± 0.2	29.8 ± 4.6	25.6 ± 1.0	26.9 ± 2.0	27.8 ± 0.5	3.1 ± 2.2	2.2 ± 1.2	63.8 ± 6.3	65.9 ± 6.2
143	2	2.2 ± 0.3	0.7 ± 0.05	41.1 ± 0.2	30.8 ± 0.2	30.1 ± 0.0	30.7 ± 0.2	10.9 ± 0.2	0.3 ± 0.2	56.2 ± 1.4	54.7 ± 1.0
144	6	0.8 ± 0.5	0.4 ± 0.2	28.3 ± 5.9	28.7 ± 4.4	29.0 ± 3.2	29.0 ± 5.3	4.8 ± 1.1	2.8 ± 1.6	65.9 ± 3.3	57.5 ± 4.2
145	4	0.9 ± 0.9	0.5 ± 0.2	28.7 ± 6.9	29.1 ± 4.8	28.4 ± 6.9	27.6 ± 2.5	5.7 ± 4.3	1.9 ± 2.2	71.8 ± 3.4	69.8 ± 7.5
151	8	1.8 ± 0.9	0.8 ± 0.2	30.5 ± 3.7	27.5 ± 2.8	27.3 ± 2.0	27.5 ± 2.4	4.0 ± 2.7	0.7 ± 0.3	49.2 ± 4.8	49.9 ± 5.5

Values are means ± SD for 5 acoustic features measured in 22-kHz calls: call duration, fundamental frequency at the beginning of a call (*F*0 Start), fundamental frequency at the end of a call (*F*0 End), absolute difference between F0 Start and F0 End (F0 Diff), and average call intensity. Twenty-two-kHz calls were produced in bouts of 5 or more calls. For 10 rats, between 2 and 17 bouts (*N*) were analyzed. Each bout contained 1 sigh vocalization and 4 or more eupnea vocalizations.

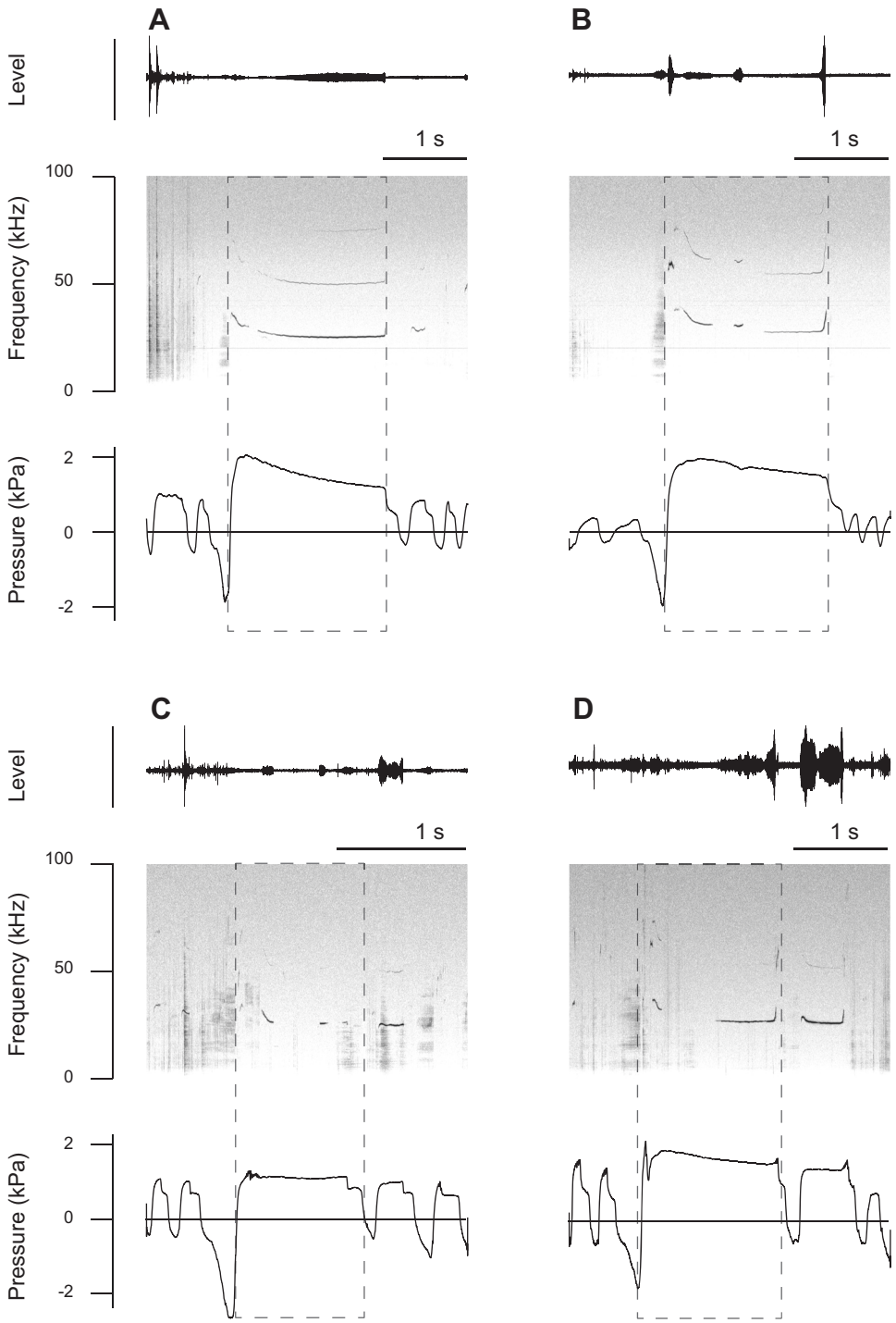

Fig. 8. Interrupted 22-kHz calls are produced during deep breaths. Two examples from each of 2 rats are shown (*A*, *B* and *C*, *D*). Sound is depicted as a time waveform (*top*; level indicates relative change in output voltage of microphone signal) and spectrographically (*middle*). For subglottal pressure (*bottom*), horizontal line indicates ambient pressure.

stimulation onset or duration (Tschida et al. 2019). Instead, results suggest that the respiratory pattern is the dominant rhythm onto which the laryngeal motor pattern becomes entrained (Tschida et al. 2019). It is hypothesized that premotor circuitry involved in generating the respiratory rhythm somehow gates the responsiveness of the laryngeal premotor circuitry, which subsequently activates laryngeal motor neurons in the nucleus ambiguus (Tschida et al. 2019). The gating ensures that laryngeal vocal motor patterns coincide with the postinspiratory or expiratory phase of the breathing cycle.

Data presented in this report and in previous studies (Hernandez et al. 2017; Riede 2014) demonstrate that rats can start a new call type at any point during the exhalation phase. When multiple calls are generated during a single exhalation, the second and subsequent calls are produced during the active expiration phase, suggesting that vocalization onset can occur during any point after the end of the inspiration phase. Our data also support the hypothesis that the respiratory pattern is the dominant rhythm onto which the laryngeal motor pattern becomes entrained, because eupneas, sniffing and deep breaths are associated at similar rates with vocal production.

Physical constraints of sound production require subglottal pressure and laryngeal motor activity to be within narrow ranges (Riede 2018). Control changes in one or both systems (lung pressure and glottal valve) causes either changes in acoustic properties or the absence of sound. While dedicated premotor circuits for breathing patterns have been identifie (e.g., Cui et al. 2016; Li et al. 2016; Tan et al. 2008; Toporikova et al. 2015), neither similar premotor circuitry for laryngeal patterns nor the circuitry responsible for respiratory entrainment have been identified The rat is a robust model system that allows comprehensive recording of breathing and vocal movements while producing naturalistic behavior (Laplagne 2018; Riede 2018). Its social behavior shows complexity that rivals that of canids and primates (e.g., Ben-Ami Bartal et al. 2011; Reinhold et al. 2019), and vocal communication is an important component. The investigation of vocal motor circuitry and of peripheral motor integration in awake and spontaneously behaving rats could therefore prove to add important information for our understanding of the evolution of vocal production.

GRANTS

T. Riede was supported by National Science Foundation Grant 1754332.

DISCLOSURES

No conflict of interest, financia or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.R. conceived and designed research; T.R. and C.S. performed experiments; T.R., C.S., and A.S. analyzed data; T.R., C.S., and A.S. interpreted results of experiments; T.R. prepared figures T.R. drafted manuscript; T.R., C.S., and A.S. edited and revised manuscript; T.R., C.S., and A.S. approved fina version of manuscript.

REFERENCES

Arriaga G, Zhou EP, Jarvis ED. Of mice, birds, and men: the mouse ultrasonic song system has some features similar to humans and song-learning birds. *PLoS One* 7: e46610, 2012. doi:10.1371/journal.pone.0046610.

Bakdash JZ, Marusich LR. Repeated measures correlation. *Front Psychol* 8: 456, 2017. [Erratum in *Front Psychol* 10: 1201, 2019.] doi:10.3389/fpsyg.2017.00456.

Bartlett D Jr. Origin and regulation of spontaneous deep breaths. *Respir Physiol* 12: 230–238, 1971. doi:10.1016/0034-5687(71)90055-7.

Bell HJ, Haouzi P. The hypoxia-induced facilitation of augmented breaths is suppressed by the common effect of carbonic anhydrase inhibition. *Respir Physiol Neurobiol* 171: 201–211, 2010. doi:10.1016/j.resp.2010.04.002.

Ben-Ami Bartal I, Decety J, Mason P. Empathy and pro-social behavior in rats. *Science* 334: 1427–1430, 2011. doi:10.1126/science.1210789.

Bennett PJ, Maier E, Brecht M. Involvement of rat posterior prelimbic and cingulate area 2 in vocalization control. *Eur J Neurosci* 50: 3164–3180, 2019. doi:10.1111/ejn.14477.

Brudzynski SM. Ethotransmission: communication of emotional states through ultrasonic vocalization in rats. *Curr Opin Neurobiol* 23: 310–317, 2013. doi:10.1016/j.conb.2013.01.014.

Brudzynski SM (Editor). *Handbook of Ultrasonic Vocalization: A Window into the Emotional Brain***.** London: Academic, 2018. Handbook of Behavioral Neuroscience 25.

Brudzynski SM, Holland G. Acoustic characteristics of air puff-induced 22-kHz alarm calls in direct recordings. *Neurosci Biobehav Rev* 29: 1169–1180, 2005. doi:10.1016/j.neubiorev.2005.04.007.

Burke CJ, Kisko TM, Pellis SM, Euston DR. Avoiding escalation from play to aggression in adult male rats: The role of ultrasonic calls. *Behav Processes* 144: 72–81, 2017. doi:10.1016/j.beproc.2017.09.014.

Cardinal RN, Parkinson JA, Hall J, Everitt BJ. Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci Biobehav Rev* 26: 321–352, 2002. doi:10.1016/S0149-7634(02)00007-6.

Carnevali L, Nalivaiko E, Sgoifo A. Respiratory patterns reflec different levels of aggressiveness and emotionality in wild-type Groningen rats. *Respir Physiol Neurobiol* 204: 28–35, 2014. doi:10.1016/j.resp.2014.07.003.

Carnevali L, Sgoifo A, Trombini M, Landgraf R, Neumann ID, Nalivaiko E. Different patterns of respiration in rat lines selectively bred for high or low anxiety. *PLoS One* 8: e64519, 2013. doi:10.1371/journal.pone.0064519.

Carr GV, Lucki I. Comparison of the kappa-opioid receptor antagonist DIPPA in tests of anxiety-like behavior between Wistar Kyoto and Sprague Dawley rats. *Psychopharmacology (Berl)* 210: 295–302, 2010. doi:10.1007/s00213-010-1832-9.

Cherniack NS, von Euler C, Głogowska M, Homma I. Characteristics and rate of occurrence of spontaneous and provoked augmented breaths. *Acta Physiol Scand* 111: 349–360, 1981. doi:10.1111/j.1748-1716.1981.tb06747.x.

Clarke S, Trowill JA. Sniffin and motivated behavior in the rat. *Physiol Behav* 6: 49–52, 1971. doi:10.1016/0031-9384(71)90013-8.

Cui Y, Kam K, Sherman D, Janczewski WA, Zheng Y, Feldman JL. Definin preBötzinger complex rhythm- and pattern-generating neural microcircuits in vivo. *Neuron* 91: 602–614, 2016. doi:10.1016/j.neuron.2016.07.003.

Del Negro CA, Funk GD, Feldman JL. Breathing matters. *Nat Rev Neurosci* 19: 351–367, 2018. doi:10.1038/s41583-018-0003-6.

Dent ML, Fay RR, Popper AN (Editors). *Rodent Bioacoustics***.** New York: Springer International, 2018. Springer Handbook of Auditory Research 67.

Dromey C, Ramig LO. The effect of lung volume on selected phonatory and articulatory variables. *J Speech Lang Hear Res* 41: 491–502, 1998. doi:10.1044/jslhr.4103.491.

Dutschmann M, Jones SE, Subramanian HH, Stanic D, Bautista TG. The physiological significanc of postinspiration in respiratory control. *Prog Brain Res* 112: 113–130, 2014. doi:10.1016/B978-0-444-63488-7.00007-0.

Erskine MS. Solicitation behavior in the estrous female rat: a review. *Hormones Behav* 23: 473–502, 1989. doi:10.1016/0018-506x(89)90037-8.

Feldman JL, Mitchell GS, Nattie EE. Breathing: rhythmicity, plasticity, chemosensitivity. *Annu Rev Neurosci* 26: 239–266, 2003. doi:10.1146/annurev.neuro.26.041002.131103.

Glogowska M, Richardson PS, Widdicombe JG, Winning AJ. The role of the vagus nerves, peripheral chemoreceptors and other afferent pathways in the genesis of augmented breaths in cats and rabbits. *Respir Physiol* 16: 179–196, 1972. doi:10.1016/0034-5687(72)90050-3.

Golder FJ, Davenport PW, Johnson RD, Reier PJ, Bolser DC. Augmented breath phase volume and timing relationships in the anesthetized rat. *Neurosci Lett* 373: 89–93, 2005. doi:10.1016/j.neulet.2004.09.063.

Grant EC. An analysis of the social behaviour of the male laboratory rat. *Behaviour* 21: 260–281, 1963. doi:10.1163/156853963X00194.

Haldane JS, Meakins JC, Priestley JG. The effects of shallow breathing. *J Physiol* 52: 433–453, 1919. doi:10.1113/jphysiol.1919.sp001842.

Hernandez C, Sabin M, Riede T. Rats concatenate 22 kHz and 50 kHz calls into a single utterance. *J Exp Biol* 220: 814–821, 2017. doi:10.1242/jeb.151720.

Hill AA, Garcia AJ 3rd, Zanella S, Upadhyaya R, Ramirez JM. Graded reductions in oxygenation evoke graded reconfiguratio of the isolated respiratory network. *J Neurophysiol* 105: 625–639, 2011. doi:10.1152/jn.00237.2010.

Hixon TJ. Respiratory function in speech. In: *Normal Aspects of Speech, Hearing and Language*, edited by Minifi F, Hixon T, Williams F. Englewood Cliffs, NJ: Prentice-Hall, 1973, p. 73–125.

Hülsmann S, Oke Y, Mesuret G, Latal AT, Fortuna MG, Niebert M, Hirrlinger J, Fischer J, Hammerschmidt K. The postnatal development of ultrasonic vocalization-associated breathing is altered in glycine transporter 2-deficien mice. *J Physiol* 597: 173–191, 2019. doi:10.1113/JP276976.

Jürgens U. Neural pathways underlying vocal control. *Neurosci Biobehav Rev* 26: 235–258, 2002. doi:10.1016/s0149-7634(01)00068-9.

Jürgens U. The neural control of vocalization in mammals: a review. *J Voice* 23: 1–10, 2009. doi:10.1016/j.jvoice.2007.07.005.

Kabir MM, Beig MI, Baumert M, Trombini M, Mastorci F, Sgoifo A, Walker FR, Day TA, Nalivaiko E. Respiratory pattern in awake rats: effects of motor activity and of alerting stimuli. *Physiol Behav* 101: 22–31, 2010. doi:10.1016/j.physbeh.2010.04.004.

Kepecs A, Uchida N, Mainen ZF. Rapid and precise control of sniffin during olfactory discrimination in rats. *J Neurophysiol* 98: 205–213, 2007. doi:10.1152/jn.00071.2007.

Knapp DJ, Pohorecky LA. An air-puff stimulus method for elicitation of ultrasonic vocalizations in rats. *J Neurosci Methods* 62: 1–5, 1995. doi:10.1016/0165-0270(95)00044-5.

Laplagne DA. Interplay between mammalian ultrasonic vocalizations and respiration. In: *Handbook of Ultrasonic Vocalization: A Window into the Emotional Brain*, edited by Brudzynski SM. London: Academic, 2018, p. 61–70. Handbook of Behavioral Neuroscience 25.

Li P, Janczewski WA, Yackle K, Kam K, Pagliardini S, Krasnow MA, Feldman JL. The peptidergic control circuit for sighing. *Nature* 530: 293–297, 2016. doi:10.1038/nature16964.

Miceli M, Castelfranchi C. Crying: discussing its basic reasons and uses. *New Ideas Psychol* 21: 247–273, 2003. doi:10.1016/j.newideapsych.2003.09.001.

Nakagawa R, Tanaka M, Kohno Y, Noda Y, Nagasaki N. Regional responses of rat brain noradrenergic neurones to acute intense stress. *Pharmacol Biochem Behav* 14: 729–732, 1981. doi:10.1016/0091-3057(81)90139-8.

Okobi DE Jr, Banerjee A, Matheson AM, Phelps SM, Long MA. Motor cortical control of vocal interaction in neotropical singing mice. *Science* 363: 983–988, 2019. doi:10.1126/science.aau9480.

Owren MJ, Bachorowski JA. Reconsidering the evolution of nonlinguistic communication: the case of laughter. *J Nonverbal Behav* 27: 183–200, 2003. doi:10.1023/A:1025394015198.

Pinheiro J, Bates D, DebRoy S, Sarkar D; R Core Team. *nlme: Linear and Nonlinear Mixed Effects Models*. R package version 3.1–139, 2019. https://CRAN.R-project.org/package=nlme.

Poggi I, Ansani A, Cecconi C. Sighs in everyday and political communication. In: *Proceedings of Laughter Workshop 2018*, edited by Ginzburg J, Pelachaud L. Paris: Sorbonne Université, 2018, p. 50–53.

R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2017. https://www.R-project.org/.

Ramirez JM. The integrative role of the sigh in psychology, physiology, pathology, and neurobiology. *Prog Brain Res* 209: 91–129, 2014. doi:10.1016/B978-0-444-63274-6.00006-0.

Reinhold AS, Sanguinetti-Scheck JI, Hartmann K, Brecht M. Behavioral and neural correlates of hide-and-seek in rats. *Science* 365: 1180–1183, 2019. doi:10.1126/science.aax4705.

Riede T. Subglottal pressure, tracheal airflow and intrinsic laryngeal muscle activity during rat ultrasound vocalization. *J Neurophysiol* 106: 2580–2592, 2011. doi:10.1152/jn.00478.2011.

Riede T. Stereotypic laryngeal and respiratory motor patterns generate different call types in rat ultrasound vocalization. *J Exp Zool A Ecol Genet Physiol* 319: 213–224, 2013. doi:10.1002/jez.1785.

Riede T. Rat ultrasonic vocalization shows features of a modular behavior. *J Neurosci* 34: 6874–6878, 2014. doi:10.1523/JNEUROSCI.0262-14.2014.

Riede T. Peripheral vocal motor dynamics and combinatory call complexity of ultrasonic vocal production in rats. In: *Handbook of Ultrasonic Vocalization*, edited by Brudzynski SM. New York: Academic, 2018, p. 45–60. Handbook of Behavioral Neuroscience 25.

Riede T, Borgard HL, Pasch B. Laryngeal airway reconstruction indicates that rodent ultrasonic vocalizations are produced by an edge-tone mechanism. *R Soc Open Sci* 4: 170976, 2017. doi:10.1098/rsos.170976.

Riede T, Zhao Y, LeDoux MS. Vocal development in dystonic rats. *Physiol Rep* 3: e12350, 2015. doi:10.14814/phy2.12350.

Rubin JS, Sataloff RT, Korovin GS. *Diagnosis and Treatment of Voice Disorders* (4th ed.). San Diego, CA: Plural Publishing, 2014.

Sauter DA, Eisner F, Ekman P, Scott SK. Cross-cultural recognition of basic emotions through nonverbal emotional vocalizations. *Proc Natl Acad Sci USA* 107: 2408–2412, 2010. [Erratum in *Proc Natl Acad Sci USA* 112: E3086, 2015.] doi:10.1073/pnas.0908239106.

Shadmehr R, Wise SP. *Computational Neurobiology of Reaching and Pointing: a Foundation for Motor Learning*. Cambridge, MA: MIT Press, 2005.

Shea SA. Behavioural and arousal-related influence on breathing in humans. *Exp Physiol* 81: 1–26, 1996. doi:10.1113/expphysiol.1996.sp003911.

Sirotin YB, Costa ME, Laplagne DA. Rodent ultrasonic vocalizations are bound to active sniffin behavior. *Front Behav Neurosci* 8: 399, 2014. doi:10.3389/fnbeh.2014.00399.

Solomon NP, Garlitz SJ, Milbrath RL. Respiratory and laryngeal contributions to maximum phonation duration. *J Voice* 14: 331–340, 2000. doi:10.1016/S0892-1997(00)80079-X.

Soltysik S, Jelen P. In rats, sighs correlate with relief. *Physiol Behav* 85: 598–602, 2005. doi:10.1016/j.physbeh.2005.06.008.

Takahashi DY, Fenley AR, Teramoto Y, Narayanan DZ, Borjon JI, Holmes P, Ghazanfar AA. The developmental dynamics of marmoset monkey vocal production. *Science* 349: 734–738, 2015. doi:10.1126/science.aab1058.

Tan W, Janczewski WA, Yang P, Shao XM, Callaway EM, Feldman JL. Silencing preBötzinger complex somatostatin-expressing neurons induces persistent apnea in awake rat. *Nat Neurosci* 11: 538–540, 2008. doi:10.1038/nn.2104.

Teigen KH. Is a sigh "just a sigh"? Sighs as emotional signals and responses to a difficul task. *Scand J Psychol* 49: 49–57, 2008. doi:10.1111/j.1467-9450.2007.00599.x.

Thet LA, Clerch L, Massaro GD, Massaro D. Changes in sedimentation of surfactant in ventilated excised rat lungs. Physical alterations in surfactant associated with the development and reversal of atelectasis. *J Clin Invest* 64: 600–608, 1979. doi:10.1172/JCI109499.

Titze IR. On the relation between subglottal pressure and fundamental frequency in phonation. *J Acoust Soc Am* 85: 901–906, 1989. doi:10.1121/1.397562.

Titze IR. *Principles of Voice Production*. Denver, CO: National Center for Voice and Speech, 2000.

Toporikova N, Chevalier M, Thoby-Brisson M. Sigh and eupnea rhythmogenesis involve distinct interconnected subpopulations: a combined computational and experimental study. *eNeuro* 2: ENEURO.0074-14.2015, 2015. doi:10.1523/ENEURO.0074-14.2015.

Tschida K, Michael V, Takatoh J, Han BX, Zhao S, Sakurai K, Mooney R, Wang F. A specialized neural circuit gates social vocalizations in the mouse. *Neuron* 103: 459–472.e4, 2019. doi:10.1016/j.neuron.2019.05.025.

Vlemincx E, Taelman J, De Peuter S, Van Diest I, Van den Bergh O. Sigh rate and respiratory variability during mental load and sustained attention. *Psychophysiology* 48: 117–120, 2011. doi:10.1111/j.1469-8986.2010.01043.x.

Vlemincx E, Van Diest I, De Peuter S, Bresseleers J, Bogaerts K, Fannes S, Li W, Van Den Bergh O. Why do you sigh? Sigh rate during induced stress and relief. *Psychophysiology* 46: 1005–1013, 2009. doi:10.1111/j.1469-8986.2009.00842.x.

Welker WI. Analysis of sniffin in the albino rat. *Behaviour* 22: 223–244, 1964. doi:10.1163/156853964X00030.

Wesson DW. Sniffin behavior communicates social hierarchy. *Curr Biol* 23: 575–580, 2013. doi:10.1016/j.cub.2013.02.012.

Wilhelm FH, Trabert W, Roth WT. Characteristics of sighing in panic disorder. *Biol Psychiatry* 49: 606–614, 2001. doi:10.1016/S0006-3223(00)01014-3.

Wöhr M, Houx B, Schwarting RK, Spruijt B. Effects of experience and context on 50-kHz vocalizations in rats. *Physiol Behav* 93: 766–776, 2008. doi:10.1016/j.physbeh.2007.11.031.