Title:

Beyond dichotomy: diversity of rat's ultrasonic vocalizations

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Summary

Understanding animal emotions is of great interest to researchers in various fields, from neuroscience to animal welfare. Ultrasonic vocalizations (USVs) in rats (*Rattus norvegicus*) have been established as an ideal model for studying emotional expression because they reflect internal emotional states. Since the discovery of USVs in rats, it has gradually been revealed that two types of adult rats' USVs with distinct acoustic features (high-short and low-long) are closely linked to positive and negative affect, respectively. Thus, for many years, most studies have grouped USVs into dichotomous categories: 50-kHz (positive) USVs and 22-kHz (negative) USVs, using these numbers as labels rather than measured values. However, the diversity of rats' USVs may have been overlooked due to this 50- / 22-kHz dichotomous framework, as several recent studies claim to have found new categories in USVs outside the framework. Here, through a systematic survey of descriptions of rats' USVs in the past literature, we confirmed a non-negligible number of reports on USVs that are outside the dichotomous framework. Our results support the abundance of diverse USVs beyond the dichotomy. This extends the traditional association between acoustic features and emotional states, highlighting the importance of research on diverse vocalizations that might reflect complex affective states beyond the simple distinction between positive and negative.

Main text

Understanding animal emotions is of great interest to researchers in various fields, from neuroscience to animal welfare. Ultrasonic vocalizations (USVs) in rats (*Rattus norvegicus*) have been established as an ideal model for studying emotional expression because they reflect internal emotional states. Since the discovery of USVs in rats[1], it has gradually been revealed that two types of adult rats' USVs with distinct acoustic features (high-short and low-long) are closely linked to positive and negative affect, respectively[2]. Thus, for many years, most studies have grouped USVs into dichotomous categories: 50-kHz (positive) USVs and 22-kHz (negative) USVs, using these numbers as labels rather than measured values. However, the diversity of rats' USVs may have been overlooked due to this 50- / 22-kHz dichotomous framework, as several recent studies claim to have found new categories in USVs outside the framework[3–5]. Here, through a systematic survey of descriptions of rats' USVs in the past literature, we confirmed a non-negligible number of reports on USVs that are outside the dichotomous framework. Our results support the abundance of diverse USVs beyond the dichotomy. This extends the traditional association between acoustic features and emotional states, highlighting the importance of research on diverse vocalizations that might reflect complex affective states beyond the simple distinction between positive and negative.

Rats' USVs have attracted interest in many fields, including medical, pharmacological, and behavioral studies, as reflected in a surge of publications over the past 2–3 decades (**Figure 1A**). In parallel with the increase in publications, the 50- / 22-kHz dichotomous view has become dominant, with most papers mentioning only these two frequencies in their abstracts as labels for vocalization categories rather than actual measurements (**Figure 1B**). Despite several attempts to capture diversity in rat USVs by identifying subtypes[6,7], the dichotomous framework remains dominant (*i.e.*, these subtypes are regarded as subcategories under the two main categories), and little experimental

evidence has been accumulated on the behavioral functions of each subtype.

Several recent studies claim to have found new categories in rat USVs outside of the dichotomous framework along with evidence to infer their behavioral functions (*e.g.*, 30 kHz for responding to 50-kHz USVs[3]; 40 kHz for feeding[4]; 31 kHz for social inequality and isolation[5]). In light of these claims, revisiting past literature finds that there are indeed several reports on USVs whose acoustic features go beyond the dichotomous framework. For example, a previous study reported a possible cluster of USVs outside acoustic features of 50- or 22-kHz USVs[8]. Given these findings, we speculated that the practice of analyzing USVs by classifying them into 50- or 22-kHz USVs based on a single boundary might have undermined the possibility of detecting new vocalizations and corresponding behavioral contexts. Therefore, we systematically surveyed publications on rat USVs to provide evidence supporting the existence of vocalizations outside 50- or 22-kHz USVs. We also present a perspective for future studies on the behavioral functions of USVs beyond the dichotomy.

We found 413 articles based on an online database search and included 378 original research papers for adult rats' USVs that were available in English. We then comprehensively examined the description of USV features reported there and eventually extracted the frequency and duration of USVs from 84 articles (**Figure 1C**), excluding data obtained from physiological/genetic manipulations. As a result, we found clear clusters around the two dichotomous USVs (**Figure 1D**), while 41 cases in a total of 257 data points fall outside the dichotomous USVs, which we named "non-dichotomous" USVs. Such cases were found in 20 articles (**Table S1** in the **Supplementary Information**). Several other articles reported non-dichotomous USVs in histograms or scatter plots (**Table S2**). Our literature survey revealed that non-dichotomous USVs have been reported in a non-negligible number, which supports recent studies that claimed new categories of USVs outside the 50-/22-kHz dichotomous framework. Since various types of non-dichotomous USVs have been

repeatedly reported in both sexes and different strains (**Figure 1E**), it is safe to assume that they are not exceptional or outliers nor biased toward any particular strain or sex. Although causal manipulations of behavioral contexts are required to establish functional categories, the results indicate that there may be far more acoustic categories of USVs than previously thought.

Our results do not negate the existence of 50- and 22-kHz USVs but suggest that analyzing data without the dichotomous scheme will improve our current understanding of USVs and their communicative functions. As a guideline for such attempts, we recommend clearly describing the frequency and duration of the vocalization and the calculation process of these values. Showing spectrograms would also be helpful. To detect the 50-/22-kHz USVs without counting intermediate vocalizations, which may blur the results, it would be preferable to define their boundaries tightly in frequency and duration ranges instead of separating them with a single boundary frequency, such as 32 kHz. Here, we tentatively used the following boundaries to define the dichotomous USVs according to the results of the literature survey: 44–85 kHz, 4–250 ms for the high-short (50-kHz) USVs; 19–29 kHz, 250–3000 ms for the low-long (22-kHz) ones (see **Figure S1** for boundary estimation).

The existence of diverse USVs beyond the dichotomous framework raises the possibility that rat USVs can be mapped onto a continuum, not just a few categories. In line with this idea, recent studies revealed that laboratory mouse USVs do not show clear clusters but form a continuum[9]. Thus, it may be reasonable to assume that the continuous acoustic structure of USVs is linked to the various emotional states. In conclusion, our study supports the abundance of non-dichotomous USVs beyond the 50- / 22-kHz dichotomous framework, strongly encouraging research on diverse vocalizations. Future studies investigating the connections between vocalizations and emotions from the non-dichotomous perspective will provide deeper insights into the mechanisms of emotional

communication in animals.

Acknowledgements

This work was supported by grants of MEXT/JSPS KAKENHI (23H05428 to K.O.; 23H03243 and 23H04342 to T. I.; 22H05157 to J. M.; 24K06624 to S.O.; 23H04336 to T. I. S.; 22KJ0702 to S. H.), JST PRESTO (JPMJPR22S8 to T. I. S.), JSP FOREST Program (JPMJFR2151 to T. I.; JPMJFR2320 to J. M.), and the Smoking Research Foundation (to T. I.).

References

1. Anderson, J.W. (1954). The Production of Ultrasonic Sounds by Laboratory Rats and Other Mammals. Science 119, 808–809. https://doi.org/10.1126/science.119.3101.808.
2. Knutson, B., Burgdorf, J., and Panksepp, J. (2002). Ultrasonic vocalizations as indices of affective states in rats. Psychological Bulletin 128, 961–977. https://doi.org/10.1037/0033-2909.128.6.961.
3. Berz, A.C., Wöhr, M., and Schwarting, R.K.W. (2022). Response Calls Evoked by Playback of Natural 50-kHz Ultrasonic Vocalizations in Rats. Front. Behav. Neurosci. 15. https://doi.org/10.3389/fnbeh.2021.812142.
4. Champeil-Potokar, G., Kreichati, L., Rampin, O., Denis, I., Darcel, N., and Bombail, V. (2023). Rats chirp with their mouth full: During an experimental meal, adult male Wistar rats emitted flat ultrasonic vocalisations upon feeding. Front. Behav. Neurosci. 17. https://doi.org/10.3389/fnbeh.2023.1089631.
5. Okabe, S., Takayanagi, Y., Yoshida, M., and Onaka, T. (2023). Novel 31-kHz calls emitted by female Lewis rats during social isolation and social inequality conditions. iScience 26. https://doi.org/10.1016/j.isci.2023.106243.
6. Wright, J.M., Gourdon, J.C., and Clarke, P.B.S. (2010). Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context. Psychopharmacology 211, 1–13. https://doi.org/10.1007/s00213-010-1859-y.
7. Brudzynski, S.M., Bihari, F., Ociepa, D., and Fu, X.-W. (1993). Analysis of 22 kHz ultrasonic vocalization in laboratory rats: Long and short calls. Physiology & Behavior 54, 215–221. https://doi.org/10.1016/0031-9384(93)90102-L.
8. Takahashi, N., Kashino, M., and Hironaka, N. (2010). Structure of Rat Ultrasonic Vocalizations and Its Relevance to Behavior. PLOS ONE 5, e14115. https://doi.org/10.1371/journal.pone.0014115.
9. Goffinet, J., Brudner, S., Mooney, R., and Pearson, J. (2021). Low-dimensional learned feature

spaces quantify individual and group differences in vocal repertoires. eLife 10, e67855. https://doi.org/10.7554/eLife.67855.

Figure

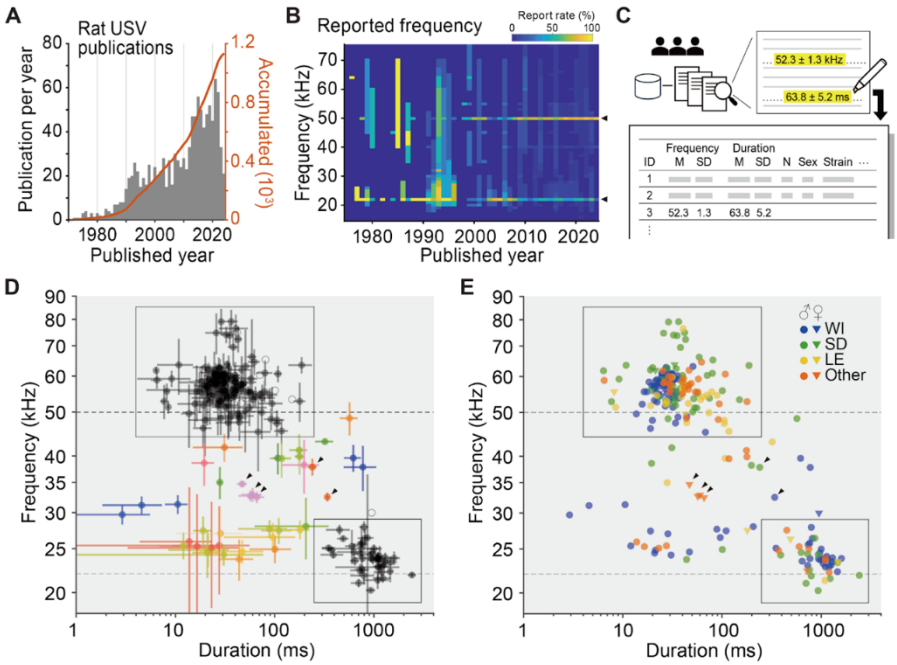

Figure 1. Rat's USVs are more diverse than previously thought.

(**A**) The number of publications per year on rat USV. (**B**) Appearance ratio of frequency values (kHz) in the abstracts of publications per year. Reports of 50- and 22-kHz USVs have been predominant (as pointed out by arrowheads). (**C**) Procedures of the paper survey and extraction of numerical values. Over 400 articles were selected from those published before 2024, and the numerical values of frequency, duration, and other information in the text were extracted. (**D**) Relationship between extracted USV frequency and duration. Squares indicate the ranges of 50-/22-kHz USVs (see **Supplementary Information**). Colors other than black correspond to the respective article reporting non-dichotomous USVs. Thick and thin error bars are SD and min-max ranges, respectively. Open circles indicate that representative values were reported without ranges. The black arrowhead indicates data from papers cited in the main text[3–5]. (**E**) Relationship between USV frequency and duration with the sex and strain labels (WI: Wistar, SD: Sprague-Dawley, LE: Long-Evans).

Supplementary Information

"Beyond dichotomy: diversity of rat's ultrasonic vocalizations"

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Supplementary methods

Yearly publication analysis

To investigate the change over time in the number of papers published that mention rat vocalization, we searched the following search key in the Pubmed paper database: "((vocalization[Title/Abstract]) OR (vocalizations[Title/Abstract])) AND (ultrasonic[Title/Abstract]) AND ((rat[Title/Abstract]) OR (rats[Title/Abstract]))". As a result, 1134 papers were found (searched on August 9, 2024). The number of published papers was counted per year and plotted (**Figure 1A**).

Frequency reporting rates in paper abstracts

We searched for USV-related papers the following search key in the Web of Science: "AB=(kHz (rat OR rats) (vocalization OR call OR vocalizations OR calls))". As a result, 567 papers were found (searched on August 13, 2024). Descriptions of frequency in kHz in abstracts of the papers were automatically detected as single point numbers or range expressions, such as "X-Y kHz", "between X and Y kHz", etc. Unclear range descriptions (i.e., shown with "above", "higher than", ">", "<", etc.) were excluded from further analysis since almost all of them were related to analysis or stimulus conditions. After these extractions, we counted the detection for every frequency number from 15 to 80 kHz in 1 kHz steps. When the same frequency was reported multiple times in a paper, we counted it only once to avoid redundancy. For the range expressions, we added one for all frequency bins within that range. We then created a two-dimensional histogram with publication year as the horizontal axis and frequency as the vertical axis. The raw histogram was divided by the number of papers that reported the frequency in their abstracts to obtain the frequency reporting rates for each publication year (**Figure 1B**).

Paper survey and selection for acoustic parameters

We searched for USV-related papers using the following search key in the Web of Science: "AB=(rat (ultrasonic OR kHz) (call OR vocalization) NOT(pup OR infant OR neonatal))", Document

type: "Article", Citation Topics Micro: "1.5.1511 Ultrasonic Vocalization". We only included papers that were classified in the following "WoS Categories": Neurosciences, Behavioral Sciences, Pharmacology Pharmacy, Psychiatry, Psychology Biological, Zoology, Multidisciplinary Sciences, Clinical Neurology, Veterinary Sciences, Psychology, Psychology Multidisciplinary, Biology, Audiology Speech Language Pathology, Developmental Biology, Endocrinology Metabolism, Substance Abuse, Medicine Research Experimental, Acoustics, Genetics Heredity, Physiology, Anesthesiology, Linguistics, Cell Biology, Ecology, Engineering Biomedical, Environmental Sciences, Evolutionary Biology, Otorhinolaryngology, Psychology Experimental. As a result, we found 398 articles (searched on April 18, 2024). We further added 15 papers that authors found by chance during the article survey, and hence the literature list size became 413. After collecting these articles, we started to read the literature and omitted articles that were reviews or book chapters but not original papers.

To extract the USV data (duration, frequency, sample size, sex, and strain), we marked the corresponding descriptions in paper PDFs by drawing highlights using the reference management software Zotero (as shown in **Figure 1C**). We carefully excluded USV data obtained from pups, electric or optogenetic stimulation, and genetically or pharmacologically manipulated animals, whereas data from wild-type and saline controls were included.

Many papers in our collection described the "mean frequency" or "peak frequency" as the frequency characteristic of targeted USV syllables. The mean frequency typically indicates the mean of the frequency trace in a syllable, whereas the peak frequency is the frequency at the time point where the amplitude showed its peak in the syllable. In rare cases, we found different descriptions, such as "representative frequency" or "dominant frequency." We labeled these characteristics differently during the data extraction. The USV characteristics were typically described as "mean ± SD" or "mean ± SE" form in the vast majority of articles that were calculated across subjected animals. We also found median values or the value range as "minimum - maximum" forms in some cases. We marked these statistics differently.

For simple visualization in the main figures (**Figure 1D, E**), we intentionally ignored the differences in the frequency characteristics (i.e., mean, peak, or other), and converted the standard error (SE) to standard deviation (SD) if we could obtain the corresponding sample size (N) by multiplying the SE value by the square root of N.

As described in the section below, we defined the boundary between the dichotomous and non-dichotomous USVs, and we further examined the non-dichotomous USVs (**Table S1**). We also report several example papers that reported histograms or scatter plots corresponding to non-dichotomous USVs (**Table S2**).

Table S1. Literature reporting non-dichotomous USVs with exact numbers of frequency and duration.

Article information	Type*	Frequency (kHz) mean ± SD	Duration (ms) mean ± SD	N	Sex	Strain
Haney M & Miczek KA (1993) Journal of Comparative Psychology [S1]	Fx	27.5 ± 2.5	180 ± 84.9	8	F	LE
Oliveira AR et al. (2006) Anesthesia & Analgesia [S2]	Fx	29.7 ± 2.9	2.91 ± 5.3	10	M	WI
	Fx	31.16 ± 2.8	4.57 ± 5.5	10	M	WI
	Fx	31.3 ± 3.0	10.56 ± 5.4	10	M	WI
Schwarting RKW et al., (2007) Behavioural Brain Research [S3]	Fm	24.83 ± 4.2	21.35 ± 25.7	8	M	WI
	Fm	27.38 ± 4.1	19.02 ± 7.9	9	M	WI
	Fm	25.39 ± 3.4	12.04 ± 5.2	8	M	WI
	Fm	24.49 ± 5.9	20.95 ± 12.3	8	M	WI
	Fm	27.63 ± 2.5	88.75 ± 91.7	11	M	WI
	Fm	27.4 ± 3.6	110.05 ± 73.2	10	M	WI
	Fm	24.24 ± 2.6	30.64 ± 91.2	9	M	WI
Takahashi N et al. (2010) PLoS ONE [S4]	Fm	41.78 ± 5.9	31.18 ± 32.4	6	M	SD
Natusch C & Schwarting RKW (2010) Pharmacology Biochemistry and Behavior [S5]	Fm	23–31**	10–47	-	M	WI
Rao RP et al. (2014) eLife [S6]	Fm	28 ± 8.6	206.5 ± 287.0	(299)***	both	WI
Willadsen M et al. (2014) Journal of Comparative Psychology [S7]	Fp	30	950	(14)	F	WI
Burke CJ et al (2017) Behavioural Processes [S8]	Fm	43.03 ± 1.0	321 ± 130.6	24	M	LE
	Fx	34.99 ± 5.7	28.05 ± 3.2	24	M	LE
Shimoju R et al. (2020) Journal of Physiological Sciences [S9]	Fp	39.5 ± 7.3	117.6 ± 56.7	6	M	WI/ST
	Fp	41.2 ± 7.1	176.6 ± 71.1	6	M	WI/ST
	Fp	39.9 ± 7.0	178.5 ± 38.4	6	M	WI/ST
Lara-Valderrábano L et al. (2022) Epilepsy Research [S10]	Fx	23.7 ± 4.9	43.9 ± 33.1	(10)	M	SD
Narihara I et al. (2022) Scientific Reports [S11]	Fm	38.6 ± 8.4	19.5 ± 9.9	17	M	SD
Berz AC et al. (2022) Frontiers in Behavioral Neuroscience [S12]	Fp	32.48 ± 1.5	340 ± 30.0	18	M	WI
	Fp	37.82 ± 3.2	240 ± 50.0	18	M	SD
Tryon SC (2023) Genes Brain and Behavior [S13]	Fp	39.58 ± 6.3	107.8 ± 24.3	8	-	LE

Olszynski KH et al. (2023) Progress in Neuro-Psychopharmacology and Biological Psychiatry [S14]	Fp	25.9 ± 16.4	13.8 ± 18.9	37	-	-
	Fp	25.1 ± 13.2	23.1 ± 39.6	16	-	-
	Fp	25.4 ± 11.2	27.6 ± 56.3	20	-	-
	Fp	25.3 ± 15.3	16.5 ± 19.2	19	-	-
Cullins MJ et al. (2023) Behavioural Brain [S15]	Fp	38.21 ± 10.4	197.97 ± 161.2	7	M	SD
Okabe S et al. (2023) iScience [S16]	Fm	32.86 ± 2.3	60 ± 27.7	12	F	Lewis
	Fm	34.7 ± 1.1	47 ± 10.4	12	F	Lewis
	Fm	32.67 ± 1.8	57 ± 27.7	12	F	Lewis
	Fm	32.45 ± 1.9	66 ± 24.2	12	F	Lewis
Bartsoen E & Wöhr M (2023) Physiology & Behavior [S17]	Fp	24.92 ± 3.4	100.82 ± 90.0	(158)	M	SD
	Fp	48.44 ± 8.1	568.46 ± 229.0	(14)	M	SD
Olszyński KH et al (2024) eLife [S18]	Fp	37.8 ± 8.7	775 ± 559.5	17	M	WI
	Fp	39.6 ± 5.4	619.6 ± 239.6	17	M	WI
Perrier L et al. (2024) Bioacoustics [S19]	Fx	26.49 ± 4.0	96 ± 130.0	4	M	WI
	Fx	24.62 ± 2.9	47 ± 59.0	4	M	WI

Abbreviations. M: male; F: female; SD: Sprague-Dawley; LE: Long-Evans; WI: Wistar

* Reported type of frequency characteristics. Fm: mean frequency; Fp: peak frequency; Fx: other or unknown.

** Range (minimum to maximum) was reported.

*** number in parentheses indicates sample size of (pooled) syllables but not animals

Table S2. Literature that reported non-dichotomous USVs in histograms or scatter plots.

Article information	Data reported in Figure1D, E	Notes
Brudzynski SM & Holland G (2005) Neuroscience & Biobehavioral Reviews [S20]	yes	The spectrogram in Fig.4 shows that the first call after air-puff presentation exhibits higher frequency than normal 22-kHz USV.
Schwarting N et al (2007) Behavioural Brain Research [S3]	yes	Histograms in Fig.5 indicate the observation of USVs different from the typical 50-kHz and 22-kHz USVs.
Wöhr M &Schwarting RKW (2009) Pharmacology Biochemistry and Behavior [S21]	-	The scatterplot in Fig.4 shows a few numbers of short USVs around 30-40 kHz obtained from adult rats.
Takahashi N et al (2010) PLoS ONE [S4]	yes	USVs around 40 kHz are shown in the scatter plots in Fig.1, which are associated with food intake.
Mahler SV (2013) Behavioural Brain Research [S22]	-	The scatterplot in Fig. 5A shows that a tone cue prior to rewards elicited a few USVs around 35 kHz in the "flat" category.
Burke CJ et al. (2017) Behavioural Processes [S23]	yes	The scatterplot in Fig. 2 shows USVs around 30 kHz as an "extended flat/trill combo" category obtained during playing behavior.
Lenell C & Johnson AM (2017) Laryngoscope [S24]	yes	The frequency distribution plotted in Fig.4 indicates two peaks located at around 40 and 55 kHz for male "flat" USVs.
Taylor JO et al. (2017) Behavioural Neuroscience [S25]	-	A few plots around 40 kHz can be found in Fig. 3A before foot shock.
Costa G et al. (2021) Brain Sciences [S26]	-	The spectrogram in Fig. 2 depicts USVs around 40 kHz as the "flat 50-kHz" category.
Karwicka W et al. (2021) Brain Sciences [S27]	-	The scatterplot in Fig. 4 exhibits relatively lower "50 kHz" USVs between 30 and 45 kHz.
Berz AD et al. (2022) Frontiers in Behavioral Neuroscience [S12]	yes	Sonogram and scatter plots provided in Fig. 4 and Fig. 5 demonstrate that the calls emitted in response to the 50-kHz playback fall into the frequency bands distinguishing 22-kHz and 50-kHz calls.
Bartsoen E & Wöhr M (2023) Physiology & Behavior [S17]	yes	Scatter plots and sonograms provided in Fig. 1 demonstrate that rats emitted various types of USVs during acoustic startle response tests.
Cullins MJ (2023) Behavioural Brain Research [S15]	yes	Frequency distribution plots in Fig. 4 show a peak around 30 kHz.

Estimation of dichotomous vs non-dichotomous boundary

The boundary between dichotomous and non-dichotomous USVs ("D-ND boundary") in our meta-analysis was tentatively determined. The distribution of duration-frequency data points appears to exhibit sufficiently different clusters for dichotomous and non-dichotomous USVs, *i.e.* non-dichotomous USVs are unlikely artifacts due to inappropriate preprocessing. However, the D-ND boundary was not consistent across several clustering methods; therefore, in this study, we propose tentative boundaries that approximately cover the clustering results. The details of this analysis are as follows.

First, we evaluated the distribution shape of the scatter plot of the duration-frequency data. By approximating the data using a Gaussian mixture model (#components: $k = 3$) on the logarithmic axis, we found that the distribution was a mixture of two components corresponding to dichotomous USVs and a component that contained other USVs (**Figure S1A**). The centers (μ) of the three components resembled the representative frequencies of dichotomous USVs and their intermediate values (μ_1: 56.95 kHz, 31.25 ms; μ_2: 23.78 kHz, 908.15 ms; μ_3: 43.49 kHz, 49.68 ms). This suggests that dichotomous USVs show well-clustered distributions and non-dichotomous data points should not be deviated data from them.

Next, we attempted to define the D-ND boundaries using four clustering methods: (1) clustering based on the Gaussian mixture approximation obtained above, (2) 'DBSCAN' [S28], [S29], the algorithm views clusters as areas of high density separated by areas of low density (with parameters: *eps*=0.4, *min_samples*=20), (3) 'OPTICS' [S30], also focusing on density in similar to DBSCAN with a relaxed limitation in its parameters (*min_samples*=20, *max_eps*=0.5), and (4) 'BIRCH' [S31], which estimates the best approximation of a tree-like hierarchical structure of subclusters (*threshold*=0.5, *branching_factor*=50, *n_clusters*=5). The frequency and time length values were standardized by dividing them by the SD after logarithmic conversion. For the three methods other than Birch, we selected the optimal parameters to make it easier to see the clusters corresponding to dichotomous USV while dividing them into three clusters. For Birch, we set the maximum number of clusters to 5. All clustering processes were performed with functions implemented in a toolbox for MATLAB (Statistics and Machine learning toolbox, R2024b) and a library for Python (scikit-learn ver. 1.5.2 with python ver. 3.12).

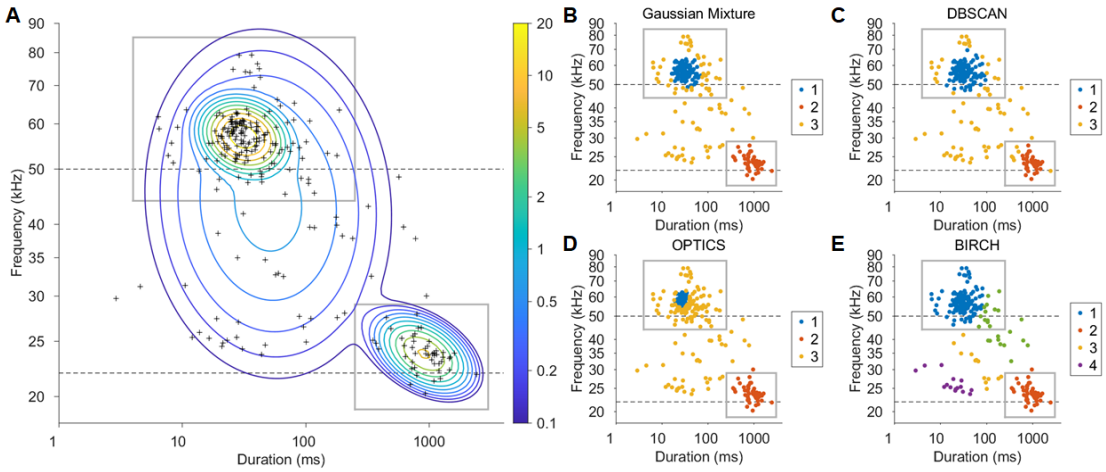

Figure S1. Distribution and clustering of data points on a duration-frequency plane. **A.** Data points and Gaussian mixture modeling. The contour line shows fitted Gaussian distributions. Gray square areas indicate the proposed boundary for dichotomous USVs. **B-E.** Clustering results were obtained using the Gaussian mixture model (B), DBSCAN (C), OPTICS (D), and BIRCH (E).

As a result, all four methods detected clusters at two core regions, which correspond to dichotomous USVs (**Figure S1B-E**). The results also showed the third additional cluster outside of the dichotomous two clusters (as outliers in DBSCAN and OPTICS, or as a composite of three clusters in BIRCH). This suggests that the non-dichotomous USV data are not artifacts that were artificially excised from the tail of the original dichotomous distribution during preprocessing. However, when we examine the marginal data points in detail, there is variation among the methods regarding which cluster they are included in. This is likely due to the small data size rather than indicating that there is no boundary.

Although the similar tendency was observed across the clustering methods, the results were inconclusive in determining the D-ND boundaries. Therefore, while considering these clustering results, we defined tentative boundaries conservatively (including as many data points as possible in the dichotomous USVs), as shown below. Rat's USV typically exhibits 'trills', intense frequency modulations with repetitive up-down movement, in the positive situation (50-kHz USV). The trill increases the syllable-average frequency by 10–30 kHz, thus, we interpreted that 70–80 kHz data points in our dataset reflected syllables containing the trill. Therefore, we set frequency boundaries to include them in the dichotomous USV. In addition, the syllable duration value could vary depending on how long gaps are allowed within the syllable during segmentation; therefore, we set a

certain extent for duration boundaries. The clusters corresponding to the 22-kHz USV were sufficiently cohesive, so we decided to set boundaries that were approximately symmetrical around the center of the distribution. Based on these considerations, the D-ND boundaries were tentatively set to the range of dichotomous USVs, as follows: high-short region (50-kHz USV) with 44–85 kHz and 4–250 ms; low-long region (22-kHz USV) with 19–29 kHz and 250–3000 ms.

Author contributions

RW, SH, and ROT mainly wrote manuscripts. ROT performed data processing, visualization, and preparation for supplementary information. RW, SH, RK, and GT originally started the USV data survey project. All authors joined in the survey. SO and KO supervised the project. All authors were involved in revising and editing the draft.

Data availability

All data and MATLAB scripts can be found in the OSF repository:
https://osf.io/78ag9/?view_only=61ce4341902b4082875215a9a7cd361c

Supplemental References

[S1] M. Haney and K. A. Miczek, "Ultrasounds during agonistic interactions between female rats (*Rattus norvegicus*)," J Comp Psychol, vol. 107, no. 4, pp. 373–379, Dec. 1993, doi: 10.1037/0735-7036.107.4.373.

[S2] A. R. Oliveira and H. M. T. Barros, "Ultrasonic Rat Vocalizations During the Formalin Test: A Measure of the Affective Dimension of Pain?:," Anesthesia & Analgesia, vol. 102, no. 3, pp. 832–839, Mar. 2006, doi: 10.1213/01.ane.0000196530.72813.d9.

[S3] R. K. W. Schwarting, N. Jegan, and M. Wöhr, "Situational factors, conditions and individual variables which can determine ultrasonic vocalizations in male adult Wistar rats," Behav Brain Res, vol. 182, no. 2, pp. 208–222, Sep. 2007, doi: 10.1016/j.bbr.2007.01.029.

[S4] N. Takahashi, M. Kashino, and N. Hironaka, "Structure of Rat Ultrasonic Vocalizations and Its Relevance to Behavior," PLoS ONE, vol. 5, no. 11, p. e14115, Nov. 2010, doi: 10.1371/journal.pone.0014115.

[S5] C. Natusch and R. K. W. Schwarting, "Using bedding in a test environment critically affects 50-kHz ultrasonic vocalizations in laboratory rats," Pharmacology Biochemistry and Behavior, vol. 96, no. 3, pp. 251–259, Sep. 2010, doi: 10.1016/j.pbb.2010.05.013.

[S6] R. P. Rao, F. Mielke, E. Bobrov, and M. Brecht, “Vocalization–whisking coordination and multisensory integration of social signals in rat auditory cortex,” eLife, vol. 3, p. e03185, Dec. 2014, doi: 10.7554/eLife.03185.

[S7] M. Willadsen, D. Seffer, R. K. W. Schwarting, and M. Wöhr, “Rodent ultrasonic communication: Male prosocial 50-kHz ultrasonic vocalizations elicit social approach behavior in female rats (*Rattus norvegicus*),” J Comp Psychol, vol. 128, no. 1, pp. 56–64, Feb. 2014, doi: 10.1037/a0034778.

[S8] C. J. Burke, T. M. Kisko, H. Swiftwolfe, S. M. Pellis, and D. R. Euston, “Specific 50-kHz vocalizations are tightly linked to particular types of behavior in juvenile rats anticipating play,” PLoS One, vol. 12, no. 5, p. e0175841, 2017, doi: 10.1371/journal.pone.0175841.

[S9] R. Shimoju, H. Shibata, M. Hori, and M. Kurosawa, “Stroking stimulation of the skin elicits 50-kHz ultrasonic vocalizations in young adult rats,” The journal of physiological sciences: JPS, vol. 70, no. 1, p. 41, Sep. 2020, doi: 10.1186/s12576-020-00770-1.

[S10] L. Lara-Valderrábano, R. Ciszek, I. Bañuelos-Cabrera, P. Andrade, and A. Pitkänen, “Ultrasonic vocalizations – Novel seizure-related manifestation in rats,” Epilepsy Research, vol. 183, p. 106927, Jul. 2022, doi: 10.1016/j.eplepsyres.2022.106927.

[S11] I. Narihara et al., “Increased self-triggered vocalizations in an epidermal growth factor-induced rat model for schizophrenia,” Sci Rep, vol. 12, no. 1, p. 12917, Jul. 2022, doi: 10.1038/s41598-022-17174-3.

[S12] A. C. Berz, M. Wöhr, and R. K. W. Schwarting, “Response Calls Evoked by Playback of Natural 50-kHz Ultrasonic Vocalizations in Rats,” Front. Behav. Neurosci., vol. 15, p. 812142, Jan. 2022, doi: 10.3389/fnbeh.2021.812142.

[S13] S. C. Tryon et al., “ChAT::Cre transgenic rats show sex-dependent altered fear behaviors, ultrasonic vocalizations and cholinergic marker expression,” Genes Brain Behav, vol. 22, no. 1, p. e12837, Feb. 2023, doi: 10.1111/gbb.12837.

[S14] K. H. Olszyński, R. Polowy, A. D. Wardak, A. W. Grymanowska, J. Zieliński, and R. K. Filipkowski, “Spontaneously hypertensive rats manifest deficits in emotional response to 22-kHz and 50-kHz ultrasonic playback,” Prog Neuropsychopharmacol Biol Psychiatry, vol. 120, p. 110615, Jan. 2023, doi: 10.1016/j.pnpbp.2022.110615.

[S15] M. J. Cullins, C. Lenell, M. R. Ciucci, and N. P. Connor, “Changes in ultrasonic vocalizations after unilateral cerebral ischemia in a rat stroke model,” Behavioural Brain Research, vol. 439, p. 114252, Feb. 2023, doi: 10.1016/j.bbr.2022.114252.

[S16] S. Okabe, Y. Takayanagi, M. Yoshida, and T. Onaka, “Novel 31-kHz calls emitted by female Lewis rats during social isolation and social inequality conditions,” iScience, vol. 26, no. 3, p. 106243, Mar. 2023, doi: 10.1016/j.isci.2023.106243.

[S17] E. Bartsoen and M. Wöhr, “Detailed spectrographic analysis of rat ultrasonic vocalizations emitted during the acoustic startle response test,” Physiology & Behavior, vol. 270, p. 114290, Oct. 2023, doi: 10.1016/j.physbeh.2023.114290.

[S18] K. H. Olszyński et al., “Rats emit aversive 44-kHz ultrasonic vocalizations during prolonged Pavlovian fear conditioning,” eLife, vol. 12, Apr. 2024, doi: 10.7554/eLife.88810.2.

[S19] L. Perrier, A. Cascarino, C. Mounichetty, F. Levréro, N. Mathevon, and F. Roche, “Sleep fragmentation disrupts vocal interactions in rats,” Bioacoustics, vol. 33, no. 1, pp. 91–101, Jan. 2024, doi: 10.1080/09524622.2024.2303691.

[S20] S. M. Brudzynski and G. Holland, “Acoustic characteristics of air puff-induced 22-kHz alarm calls in direct recordings,” Neuroscience & Biobehavioral Reviews, vol. 29, no. 8, pp. 1169–1180, 2005, doi: 10.1016/j.neubiorev.2005.04.007.

[S21] M. Wöhr and R. K. W. Schwarting, “Ultrasonic communication in rats: Effects of morphine and naloxone on vocal and behavioral responses to playback of 50-kHz vocalizations,” Pharmacology Biochemistry and Behavior, vol. 94, no. 2, pp. 285–295, Dec. 2009, doi: 10.1016/j.pbb.2009.09.008.

[S22] S. V. Mahler et al., “A rodent ‘self-report’ measure of methamphetamine craving? Rat ultrasonic vocalizations during methamphetamine self-administration, extinction, and reinstatement,” BEHAVIOURAL BRAIN RESEARCH, vol. 236. ELSEVIER, RADARWEG 29, 1043 NX AMSTERDAM, NETHERLANDS, pp. 78–89, Jan. 2013. doi: 10.1016/j.bbr.2012.08.023.

[S23] C. J. Burke, T. M. Kisko, S. M. Pellis, and D. R. Euston, “Avoiding escalation from play to aggression in adult male rats: The role of ultrasonic calls,” BEHAVIOURAL PROCESSES, vol. 144. ELSEVIER SCIENCE BV, PO BOX 211, 1000 AE AMSTERDAM, NETHERLANDS, pp. 72–81, Jan. 01, 2017. doi: 10.1016/j.beproc.2017.09.014.

[S24] C. Lenell and A. M. Johnson, “Sexual dimorphism in laryngeal muscle fibers and ultrasonic vocalizations in the adult rat,” The Laryngoscope, vol. 127, no. 8, Aug. 2017, doi: 10.1002/lary.26561.

[S25] J. O. Taylor, C. M. Urbano, and B. G. Cooper, “Differential Patterns of Constant Frequency 50 and 22 kHz USV Production Are Related to Intensity of Negative Affective State,” BEHAVIORAL NEUROSCIENCE, vol. 131, no. 1. AMER PSYCHOLOGICAL ASSOC,

750 FIRST ST NE, WASHINGTON, DC 20002-4242 USA, pp. 115–126, Feb. 2017. doi: 10.1037/bne0000184.

[S26] G. Costa, M. Serra, and N. Simola, "Association between Novel Object Recognition/Spontaneous Alternation Behavior and Emission of Ultrasonic Vocalizations in Rats: Possible Relevance to the Study of Memory," BRAIN SCIENCES, vol. 11, no. 8. MDPI, ST ALBAN-ANLAGE 66, CH-4052 BASEL, SWITZERLAND, Aug. 2021. doi: 10.3390/brainsci11081053.

[S27] W. Karwicka, M. Wiatrowska, K. Kondrakiewicz, E. Knapska, M. B. Kursa, and A. Hamed, "Relaying Aversive Ultrasonic Alarm Calls Depends on Previous Experience. Empathy, Social Buffering, or Panic?," BRAIN SCIENCES, vol. 11, no. 6. MDPI, ST ALBAN-ANLAGE 66, CH-4052 BASEL, SWITZERLAND, Jun. 2021. doi: 10.3390/brainsci11060759.

[S28] M. Ester, H.-P. Kriegel, J. Sander, and X. Xu, "A density-based algorithm for discovering clusters in large spatial databases with noise," kdd, vol. 96, no. 34, pp. 226–231, 1996.

[S29] E. Schubert, J. Sander, M. Ester, H. P. Kriegel, and X. Xu, "DBSCAN Revisited, Revisited: Why and How You Should (Still) Use DBSCAN," ACM Trans. Database Syst., vol. 42, no. 3, p. 19:1-19:21, Jul. 2017, doi: 10.1145/3068335.

[S30] M. Ankerst, M. M. Breunig, H.-P. Kriegel, and J. Sander, "OPTICS: ordering points to identify the clustering structure," SIGMOD Rec., vol. 28, no. 2, pp. 49–60, Jun. 1999, doi: 10.1145/304181.304187.

[S31] T. Zhang, R. Ramakrishnan, and M. Livny, "BIRCH: an efficient data clustering method for very large databases," in Proceedings of the 1996 ACM SIGMOD international conference on Management of data - SIGMOD '96, Montreal, Quebec, Canada: ACM Press, 1996, pp. 103–114. doi: 10.1145/233269.233324.