

Reviewed Preprint

v1 • April 9, 2026

Not revised

✉ For correspondence:

ursula.koch@fu-berlin.dethorsten.becker@fu-berlin.de

Competing interests: No competing interests declared

Funding: See page 27

Reviewing editor: Moriel Zelikowsky, University of Utah, United States

© 2026, Breslav et al. This article is distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use and redistribution provided that the original author and source are credited.

Separation slang – Laboratory mice use low-frequency call repertoire during physical separation

Daniel Breslav, Michal Wojcik, Ursula Koch ✉, Thorsten Becker ✉

Institute of Biology, BCP, Freie Universität Berlin, Berlin, Germany

eLife Assessment

This study represents a **useful** finding on the social modulation of the complex repertoire of vocalizations made across a variety of strains of lab mice. The evidence supporting the claims is, at present, **incomplete**, as numerous concerns regarding the appropriate categorization of vocalizations, the averaging of data points with disparate levels of occurrence, the interpretation of the function of noisy calls, and a general lack of adequate analyses of experimental data were raised. With these issues addressed, the work will be of importance to scientists studying rodent vocal communication.

<https://doi.org/10.7554/eLife.109605.1.sa3>

Abstract

The discovery of a diverse repertoire of ultrasonic vocalizations (USVs) sparked interest in understanding their role in mouse social behavior. Social communication in mice is not just vocal, but multimodal and occurs mostly in close proximity. Aiming to unravel the impact direct physical interaction has on the vocal communication of same-sex mouse dyads, we separated mice through a divider preventing direct physical interaction, but allowing visual, olfactory and some tactile interaction through holes. Separated dyads emitted a distinct call repertoire consisting mainly of calls in or just above the human audible range (but not squeaks) as well as Noisy calls, and only to a lesser degree of USVs. Increasing the possibility for direct interaction through larger holes in the divider led to an adaption of the call repertoire. The separation-induced call repertoire was neither affected by sex, nor was it mouse strain specific, even though differences in spectro-temporal parameters and call class proportion occurred. Lastly, buspirone treatment showed no observable effect, suggesting anxiety to not be the main driver underlying the separation-induced call repertoire. We show that separated same-sex mouse dyads predominantly emit a call repertoire that until now has only been observed in isolation or during aversive stimulation.

Introduction

Social situations pose challenges for the individuals involved, as messages need to be composed appropriately and receivers need to be competent in deciphering said messages to avoid misunderstandings. Interestingly, in humans non-verbal signals outweigh verbal communication when conveying emotions (Mehrabian, 1971 [↗](#)). Thus, multimodal communication is essential in social interactions. During courtship (Hanson and Hurley, 2012 [↗](#); Ronald et al., 2020 [↗](#)), territorial same-sex encounters (Gourbal et al., 2004 [↗](#)), or in the discrimination of stranger from familiar mice (de la Zerda et al., 2022 [↗](#)), mice use a mix of olfactory, acoustic, and tactile signals to communicate with one another. The resurgence of interest in mouse vocalizations during the last years has culminated in a detailed description of the neural circuit underlying mouse vocalizations (Chen et al., 2021 [↗](#); MacDonald et al., 2024 [↗](#); J. Park et al., 2024 [↗](#); Tschida et al., 2019 [↗](#); Veerakumar et al., 2023 [↗](#); Ziobro et al., 2024 [↗](#)). Mice possess a large repertoire of ultrasonic vocalizations (USVs), which has been divided into different categories ranging from eight (Holy and Guo, 2005 [↗](#)) up to 22 (Sangiromo et al., 2020 [↗](#)). Certain call categories were found

to be emitted more frequently in certain social contexts. USVs containing frequency jumps and those featuring harmonic elements occur particularly during courtship and are emitted by male mice (Chabout et al., 2015 [↗](#); Klaus et al., 2025 [↗](#)), while females emit audible squeaks concomitantly with defensive behaviors (Finton et al., 2017 [↗](#); Hood et al., 2023 [↗](#); Lupanova and Egorova, 2015 [↗](#)). Furthermore, a call with decreasing pitch (i.e. downward spectrogram slope) precede dominant social behaviors (Sangiomo et al., 2020 [↗](#)). Altogether, adult mice vocalize usually in very close proximity to one another (Neunuebel et al., 2015 [↗](#); Oliveira-Stahl et al., 2023 [↗](#); Sangiomo et al., 2020 [↗](#)), and mice that were socially deprived show increased USV emission and eagerness to interact with conspecifics (Burke et al., 2018 [↗](#); Chabout et al., 2012 [↗](#)). In most studies, where USVs were emitted, mice were in direct contact allowing for tactile and close-range olfactory cues. In contrast, mice housed in isolation conditions produced a novel broadband vocalization with noisy features and emitted only few USVs (Grimsley et al., 2016 [↗](#)), indicating that environmental conditions strongly shape the vocal repertoire. This also suggests that mice may adapt their vocalization pattern in more complex environments where non-vocal communication is restricted or severely limited.

This study investigates how the prevention of direct physical interaction (e.g. social whisking) shapes the vocal communication of same-sex mouse dyads, aiming to improve our understanding of the information transferred by mouse calls. We show a distinct, separation-induced call repertoire consisting mainly of calls in the human audible range (but not squeaks) as well as noisy calls, and only to a lesser degree of USVs. Increasing the possibility for direct interaction leads to an adaption of the call repertoire. The separation-induced call repertoire is not affected by sex and is not mouse strain specific even though differences in spectro-temporal parameters and call class proportion occur, respectively. Lastly, buspirone treatment being without any observable effect suggests anxiety to not be the main driver underlying the separation-induced call repertoire.

Results

Same-sex mouse dyads produce calls of lower frequency when separated by a divider featuring holes

Mice exhibit a remarkable diversity of vocalizations that play key roles in social interactions, with their characteristics and types varying depending on contexts such as mating, territorial disputes (Arriaga and Jarvis, 2013 [↗](#); Gourbal et al., 2004 [↗](#); Neunuebel et al., 2015 [↗](#)), and affective states (Grimsley et al., 2011 [↗](#)). While most of the produced vocalizations occur in close physical proximity, where tactile stimuli strongly influence the vocal output (Lahvis et al., 2011 [↗](#); Moles et al., 2007 [↗](#); Oliveira-Stahl et al., 2023 [↗](#)), it remains unclear how vocal communication is affected by the absence of direct physical contact.

To explore this question, we conducted experiments using 11 female pairs and 11 male pairs of adult FVB stranger mice in a controlled setting, designed to record vocalizations during physical separation. Each same-sex pair was placed in a sound-isolated environment with a transparent divider having holes in the bottom section, allowing for visual, olfactory and vocal interaction while preventing physical contact (Fig. 1A [↗](#)). Under these conditions, we observed that typical ultrasonic vocalizations (USVs) were infrequent. After 15 minutes of physical separation, the divider was removed, and recording continued for another 5 minutes as the dyads were united and able to physically interact (Fig. 1B [↗](#)). This served as a control to determine whether the removal of the divider would cause the mice to revert to their typical repertoire of USVs. We observed one exception, a female dyad emitted solely USVs with a high call rate (18.3 calls/min) regardless of being separated or being able to interact directly with one another. Hence, this dyad was excluded from the analysis. Given the low rate of detectable USVs during separation, we reanalyzed the sound recordings without any high-pass filter. This adjustment uncovered previously undetected vocalizations at lower frequencies. These vocalizations were unambiguously distinct from established USVs and occurred at markedly higher call rates across experiments.

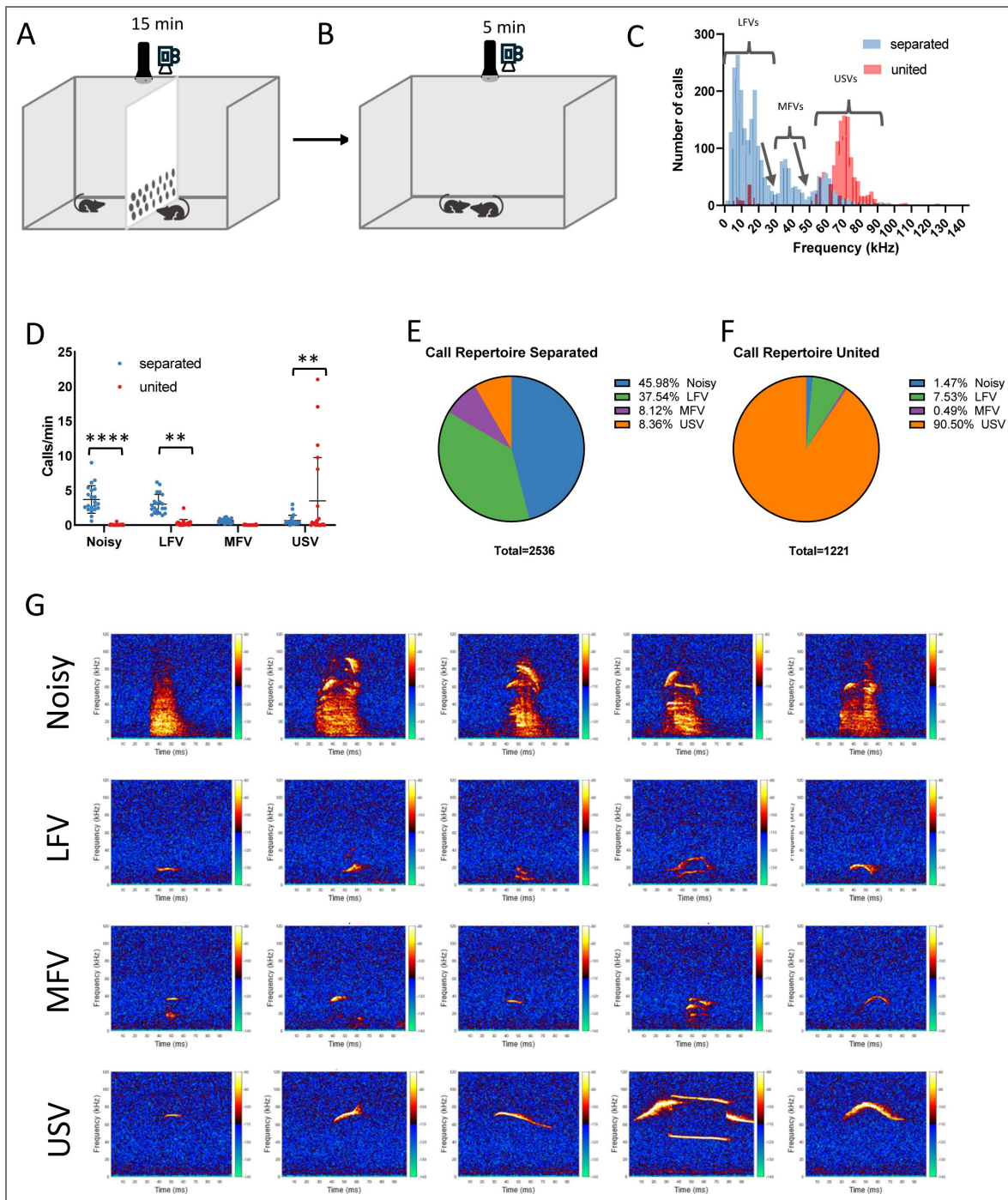


Figure 1. Same-sex mouse dyads produce calls of lower frequency when separated by a perforated divider.

(A) Schematic of experimental setup during separation phase. The arena surface area measured 930 cm² divided into two equally sized compartments. The walls were 30 cm high. Hole diameters in divider measured 0.5 cm. (B) Schematic of experimental setup during unification phase when the perforated divider was removed. (C) Histogram of peak frequencies during separation (blue) and unification (red) phases. Black arrows highlight troughs used to differentiate frequency bands. LFs = low frequency vocalizations, MFVs = middle frequency vocalizations, USVs = ultrasonic vocalizations. (D) Quantification of call rates for each call class during separation (blue) and unification (red). Each point represents one same-sex mouse dyad (n = 21 dyads). Black lines represent group mean and error bars represent SD. For statistical analysis a two-way RM ANOVA with Sidak’s multiple comparisons was employed. Call class x context interaction $F_{(3, 80)} = 14.80$, $P < 0.0001$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$. (E) Pie charts displaying total call repertoire during separation, and during unification (F). Call classes are color coded: Noisy = blue, LFV = green, MFV = purple, USV = orange. The total number of recorded calls is displayed at the bottom of each pie chart. (G) Spectrograms of exemplary vocalizations during separation phase; first row: Noisy calls, second row: lowfrequency vocalizations (LFV < 32 kHz), third row: middle-frequency vocalizations (MFV 32 – 50 kHz), fourth row: ultrasonic vocalizations (USV > 50 kHz).

Analysis of peak frequency distribution of all vocalizations during both phases revealed a trimodal distribution for calls recorded during separation with two prominent troughs at 32 kHz and 50 kHz (Fig. 1C [↗](#), blue histogram), resulting in the formation of three classes: Low-Frequency Vocalizations (LFVs; peak frequency <32 kHz), Middle-Frequency Vocalizations (MFVs; peak frequency 32–50 kHz), and USVs (peak frequency >50 kHz). Calls recorded during direct interaction showed peak frequencies nearly exclusively in the USV range, with only a few in the LFV range (Fig. 1C [↗](#), red histogram). Additionally, a fourth class termed “Noisy” was identified. Noisy calls are characterized by a broad frequency spectrum (Fig. 1G [↗](#), example spectrograms) and a distinctly warbled, noisy spectral appearance, and was distinguished based on their spectral morphology and large bandwidth (avg. 57.4 ± 6.74 kHz; Fig. 2D [↗](#)), rather than peak frequency. These vocalizations strongly resemble calls emitted during isolation as published by Grimsley and colleagues (2016) [↗](#).

Separating two mice by means of a divider affected their call rate in a class-dependent manner (call class \times separation: $F_{(3,80)} = 14.80$, $P < 0.0001$; Fig. 1D [↗](#)). While being separated, dyads emitted predominantly Noisy calls (3.7 ± 1.98 calls/min) and LFVs (3.0 ± 1.43 calls/min), however, when interacting freely call rates dropped to 0.2 ± 0.35 calls/min ($P < 0.0001$; Fig. 1D [↗](#)) and 0.9 ± 1.57 calls/min ($P = 0.0019$; Fig. 1D [↗](#)), respectively. The opposite was observed for USVs, call rate was low during separation and increased when they were united again (0.7 ± 0.32 calls/min vs. 10.5 ± 18.69 calls/min, $P = 0.0012$; Fig. 1D [↗](#)). MFVs were emitted at low call rates regardless of the context (0.7 ± 0.32 calls/min vs 0.1 ± 0.13 calls/min, $P = 0.8689$; Fig. 1D [↗](#)).

In total we recorded 2,536 vocalizations during separation, dyads emitted predominantly Noisy calls (45.98%) and LFVs (37.54%). MFVs (8.12%) and USVs (8.36%) accounted for the remaining calls (Fig. 1E [↗](#)). In contrast, of the 1,221 calls recorded during unification, over 90% were USVs and only 10% belonged to either Noisy calls (1.47%), LFVs (7.53%), or MFVs (0.49%) (Fig. 1F [↗](#)). Thus, same-sex dyads used a distinct call repertoire during physical separation compared to unification. Despite this striking difference between these call repertoires, all four call classes were present in both phases. Interestingly, like the widely known mouse USVs (Fig. 1G [↗](#), USV), both LFVs and MFVs displayed diverse spectral morphologies (Fig. 1G, LFV, MFV [↗](#)). Surprisingly, even the spectrograms of Noisy calls displayed a large variety, with often different tonal components in both mid and high frequencies being visible (Fig. 1G [↗](#), Noisy).

Impact of separation on spectro-temporal call features

These findings prompted further investigation into which temporal and spectral properties of these calls, besides their peak frequency, could be used for further characterization. For instance, categories for mouse USVs have been formed based on their spectral morphology (Holy and Guo, 2005 [↗](#); Scattoni et al., 2009 [↗](#), 2008 [↗](#)), raising the possibility for more refined categories of both LFV and MFV. Thus, we employed the convolutional neural network (CNN) VGG16 to perform call feature extraction based on the call spectrogram images resulting in 4096 different features. Dimensionality reduction to two dimensions by UMAP revealed two distinct clusters: one corresponding to mainly USVs (Fig. 2A [↗](#), yellow dots) and a second cluster encompassing LFVs, MFVs, USVs and Noisy calls. Within this latter cluster, a finer substructure was observed, with Noisy calls (Fig. 2A [↗](#), blue dots) segregating toward the north-eastern region, LFVs (Fig. 2A [↗](#), green dots) in the south-western region, and MFVs (Fig. 2A [↗](#), purple dots) in the eastern region while USVs were present throughout the cluster. Furthermore, the USV cluster consisted predominantly of USVs that were emitted during unification (Fig. 2A [↗](#) yellow dots on dark red shade), which segregated from the other call types, even USVs emitted during separation (Fig. 2A [↗](#) yellow dots on blue shade).

In conclusion, firstly, unsupervised, spectrum-based mouse call segmentation using a VGG16 CNN revealed a large part of USVs emitted during unification were distinctly different from USVs emitted when separated. Secondly, LFVs, MFVs, and Noisy calls were not further divided into distinct subclusters. This has previously been shown for USVs (Goffnet et al., 2021 [↗](#); Sainburg et al., 2020 [↗](#)). Therefore, we decided to refrain from further categorizing these classes for now.

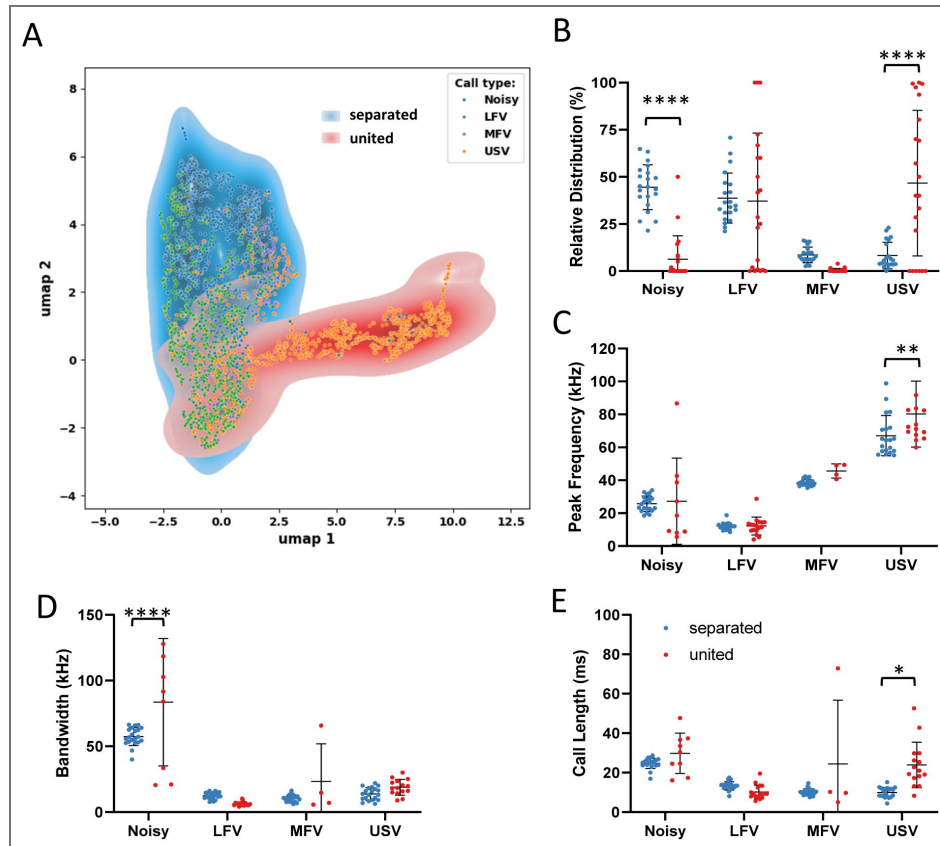


Figure 2. Spectro-temporal properties of call classes differ significantly between call repertoires used by separated and united same-sex dyad.

(A) UMAP of call classes recorded during both physical separation (blue shading) and unification (red shading) (total calls $n = 3757$). Each dot represents one call of either Noisy (blue), LFV (green), MFV (purple), and USV (orange) class. (B – E) Quantification of relative call type distribution (B), average peak frequencies (C), average bandwidths (D), and call lengths (E) during separation (blue) and unification (red). Each dot represents the average of one same-sex mouse dyad. Black lines represent group mean and error bars represent SD. Data was analyzed using two-way RM ANOVA or mixed effects model with Sidak’s multiple comparisons was employed. Relative distribution: call class \times context: $F_{(3,80)} = 25.29$, $P < 0.0001$, peak frequency: call class: $F_{(1,747, 34,95)} = 183.6$, $P < 0.0001$; context: $F_{(1,000, 20,00)} = 5.994$, $P = 0.0237$, bandwidth: call class \times context interaction: $F_{(3, 20)} = 7.600$, $P = 0.0014$, call length: $F_{(3, 120)} = 9.635$, $P < 0.0001$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$. LFV = low frequency vocalizations, MFV = middle frequency vocalizations, and USV = ultrasonic vocalizations. A total of 21 same-sex mouse dyads was used.

While all same-sex dyads emitted calls during separation, the number of calls emitted varied considerably between individual dyads (range: 42 to 269 calls, avg. 121 calls). Also, when united, 19 out of 21 dyads emitted calls, ranging from a total of 2 calls up to 317 calls (avg. 64 calls). This high variance prompted us to investigate the relative distribution of each call class per dyad and separation condition. Similar to the call rates, the relative distribution of call classes, particularly, of Noisy and USV calls was affected by the separation (call class \times separation: $F_{(3,80)} = 25.29$, $P < 0.0001$; Fig. 2B). While Noisy calls made up $44.5 \pm 11.92\%$ of calls during separation, once united they only accounted for $6.3 \pm 12.44\%$ of the dyads' call repertoire ($P < 0.0001$; Fig. 2B). The opposite was observed for USVs, during separation dyads emitted USVs rarely ($8.3 \pm 7.06\%$), however, once the animals were able to interact directly USVs made up a substantial number of calls ($46.7 \pm 38.60\%$, $P < 0.0001$; Fig. 2B). While the absolute call rate of LFVs was significantly higher during separation compared to unification (Fig. 1D), the relative call type distribution did not significantly differ between events but displayed a huge disparity between subjects ($38.7 \pm 13.28\%$ vs $37.1 \pm 36.09\%$, $P = 0.9984$; Fig. 2B). Regarding MFV distribution, same-sex dyads emitted more MFVs during separation ($8.6 \pm 4.19\%$) than during unification ($0.4 \pm 0.98\%$), however, this difference was not statistically significant (Fig. 2B). Beyond the effect separation had on the call repertoire used by same-sex dyads, we observed a lower peak frequency of 67.1 ± 12.25 kHz in USVs emitted while mice were separated compared to 80.2 ± 20.0 kHz during unification ($P = 0.0037$; Fig. 2C). Also, the average duration of USVs was substantially longer when two mice interacted freely with one another (23.9 ± 11.54 ms vs 10.0 ± 2.59 ms, $P = 0.0244$; Fig. 2E). Nevertheless, physical separation did not affect the peak frequencies or call lengths of the other call classes. Spectral properties peak frequency might not be suitable to describe Noisy calls. Instead, bandwidth seemed to be a much more fitting parameter. Indeed, we found the bandwidth of Noisy calls to be quite consistently between dyads separated by the divider (57.4 ± 6.74 kHz, Fig. 2D), while the bandwidth of Noisy calls emitted during free interaction varied more substantially (83.6 ± 48.43 kHz, $P < 0.0001$; Fig. 2D). It is noteworthy that not all dyads emitted Noisy calls when the two mice could interact freely.

Together, these results demonstrate that physical separation through a divider evoked a call repertoire containing predominantly Noisy calls and LFVs and only to a minor part MFVs and USVs, while freely interacting mice emitted mainly USVs. Furthermore, the few USVs emitted during separation were shorter and of lower peak frequency than those produced during unification. During physical separation Noisy calls exhibited a lower bandwidth than during direct physical interaction.

Temporal organization of separation-induced calls

We observed a substantial number of calls from all four classes occurring in groups or bouts. These bouts could contain solely calls from one class, i.e. Noisy, LFV, USV (Fig. 3A-C), but could also contain a mix of different call classes (Fig. 3D). Analysis of the inter-call interval (ICI) distributions of all four call classes revealed a peak at an ICI of about 90 ms during both separation (Fig. 3E-H, blue histograms) and unification (Fig. 3E-H, red histograms). All calls with an ICI of 140.6 ms or lower (the trough following the 90 ms peak) were considered to be emitted in bouts. When separated, dyads emitted Noisy calls predominantly in bouts (77.2%), while every second LFV was also embedded into a bout (54.3 %). Even though fewer MFVs and USVs were produced than both Noisy and LFV calls during separation, 44.1% of MFVs and 43.1% of USVs were emitted in bouts. Once mice were allowed to interact freely, the majority of calls recorded were USVs (87.1% in bouts), while 43.1% of LFVs were emitted in bouts. Of the few MFVs and Noisy calls emitted 50% and 17.7% were part of a bout, respectively.

Same-sex mouse dyads' call repertoire displayed a condition-dependent temporal organization. While separated, about 80% of Noisy calls were emitted in bouts. This was the case also for about 40 to 50% of LFVs, MFVs, and USVs. In contrast, in freely interacting mice about 90% of USVs emitted were structured in bouts. Our findings suggest that vocal communication in mice is highly condition-dependent, with different vocalization strategies emerging in response to physical separation.

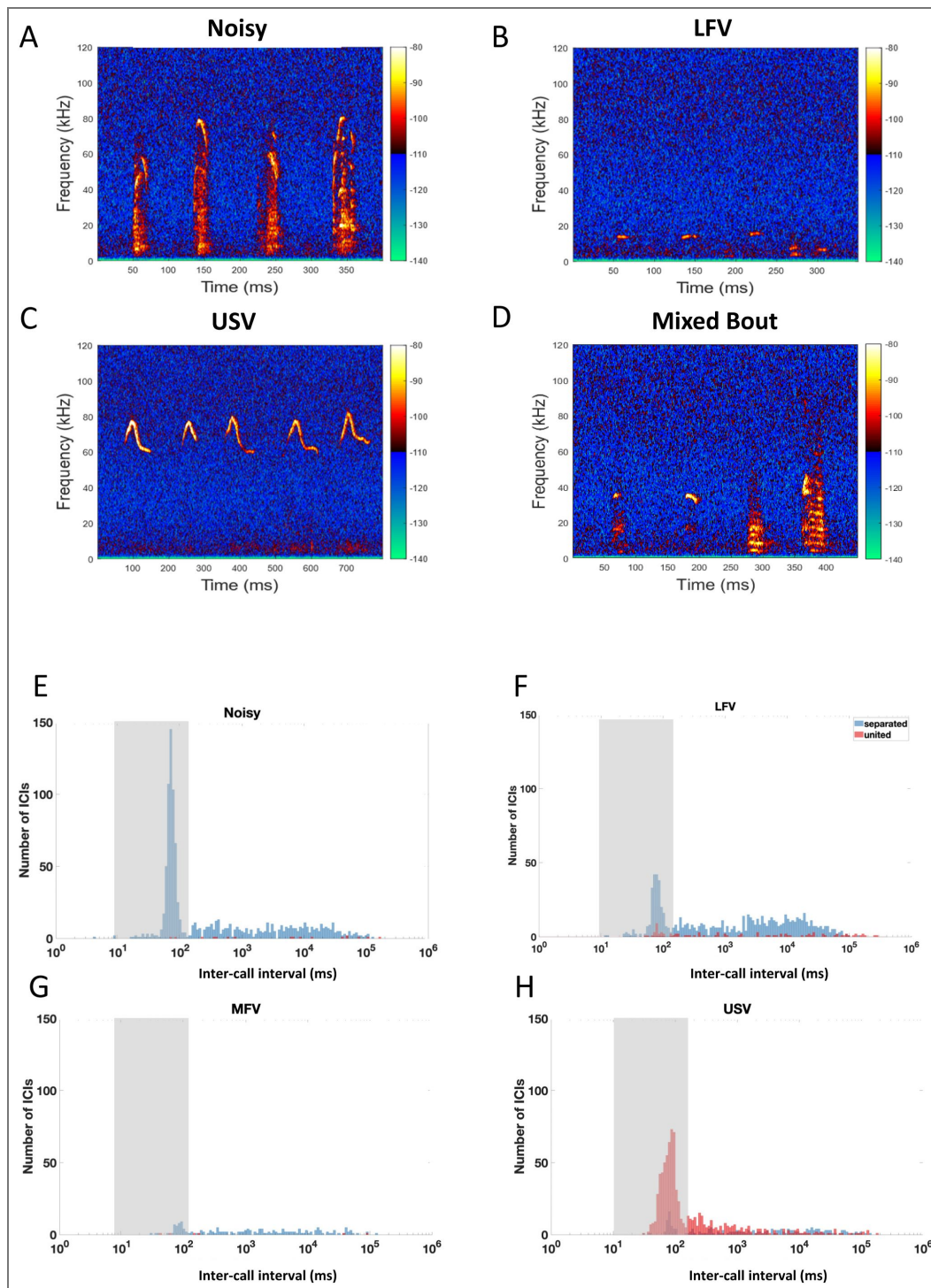


Figure 3. Temporal organization of separation-induced calls.

(A–D) Exemplary spectrograms of Noisy (A), LFV (B), USV (C), and mixed (D) calls occurring in groups (bouts) with an inter-call interval (ICI) < 140.6 ms (i.e. within one bout). (E–H) Histograms display the distribution of ICI of Noisy (E), LFV (F), MFV (G), and USV (H) calls during both separation (blue) and unification (red). The grey bar in the histograms marks ICIs shorter than 140.6 ms and thus, the number of calls occurring in bouts.

Male and female same-sex dyads emitted similar separation-induced call repertoire, but with different spectro-temporal features

Building on these findings, we next sought to investigate whether the observed differences were influenced by the sex of the mice. The call repertoire used by male same-sex dyads did not differ from that of female same-sex dyads (Fig. 4A-D). However, separated female dyads tended to emit calls at a higher rate (9.4 ± 3.79 calls/min) compared to separated male dyads (6.8 ± 2.33 calls/min, $t_{(19)} = 1.907$, $P = 0.0718$; Fig. 4E). Furthermore, female dyad calls exhibited a higher bandwidth (36.3 ± 4.80 kHz) and were longer (19.3 ± 2.01 ms) compared to male dyad calls (28.8 ± 6.66 kHz, $t_{(19)} = 2.931$, $P = 0.0086$; Fig. 4G) (16.7 ± 2.64 ms; $t_{(19)} = 2.548$, $P = 0.0197$; Fig. 4H). To verify whether these observations were context-dependent, we examined the vocalization repertoires in opposite sex pairs separated by the perforated divider. Mice are known to emit a large amount of USVs during courtship (Hanson and Hurley, 2012; Wang et al., 2008), even when separated by a divider male-female dyads emitted a substantial amount of USVs (Hood et al., 2023; Hood and Hurley, 2023). Similarly, in our separation condition mixed-sex dyads emitted predominantly USVs (80.0%; Fig. 4 - Suppl. 1). This call repertoire exhibited a close resemblance to the repertoire observed during unification (Fig. 1F), though not identical, as separated mixed-sex dyads produced a larger proportion of both Noisy calls (mixed-sex: 6.60 % vs same-sex: 1.47%) and MFVs (mixed-sex: 4.58% vs same-sex: 0.49%) compared to separated same-sex dyads. It contrasted starkly with the call repertoire of same-sex dyads that consisted mainly of Noisy calls and LFVs (Fig. 1E).

Summing up, the call repertoire and call features did not show any major differences between male and female same-sex dyads. Mixed-sex dyads emitted predominantly USVs even when separated.

Separated same-sex dyads were most likely to vocalize in close proximity while facing one another

To investigate the relationship between vocal activity and the relative position of mice to each other, we tracked mouse body parts using DeepLabcut. When separated calls were detected at nearly all snout-to-snout distances throughout the experiment in both male and female dyads. However, despite the obstruction of direct physical contact by the divider, mice emitted vocalizations particularly often in very close proximity, i.e. a snout-to-snout distance closer than one mouse body length (length: 200 px \triangleq 6 cm, Fig. 5C1, C2, D1, D2 grey bar). Separated male dyads emitted predominantly Noisy calls in very close proximity, while separated female dyads displayed a more varied call repertoire in close proximity consisting mainly of Noisy calls and LFVs, but to a lower extent also of MFVs and USVs (Fig. 5C1, D1 colored lines in grey bar). After allowing direct physical interaction by removing the divider, nearly all vocalizations recorded were produced within a distance of two body lengths (i.e. 0 to 400 px) in both male and female dyads. When united female dyads produced nearly exclusively USVs, whereas male dyads also emitted LFVs in close proximity (Fig. 5C2, D2). Incorporating the snout-to-snout angle (Fig. 5A) into the analysis revealed a positive correlation between snout-to-snout distance and angle (table 1). When separated dyads were facing one another when emitting calls in close proximity (at 0 to 100 px distance, 0 to 60° snout-to-snout angles, Fig. 5E1, F1), while they were less likely to face one another the further they were apart. After the divider was removed, same-sex dyads displayed normal social behaviors, such as anogenital sniffing and following/pursuit behavior, which was reflected by the larger number of calls emitted at greater snout-to-snout angles (Fig. 5E2, F2, 50° and upward). To investigate this further, we calculated the snout-to-tail base distance and angle between the two mice (Fig. 5B). Anogenital sniffing is represented by small snout-to-tail base distances (200 px or less), while the large range of snout-to-tail base angles represents the body position relative to one another. 0° signifies the two mice being in-line with one mouse's snout 'facing' the other mouse's tail base, while at a snout-to-tail base angle of 175° the two mice were facing in opposite direction (Fig. 5E2, F2 inset scatter plots). Calls emitted during

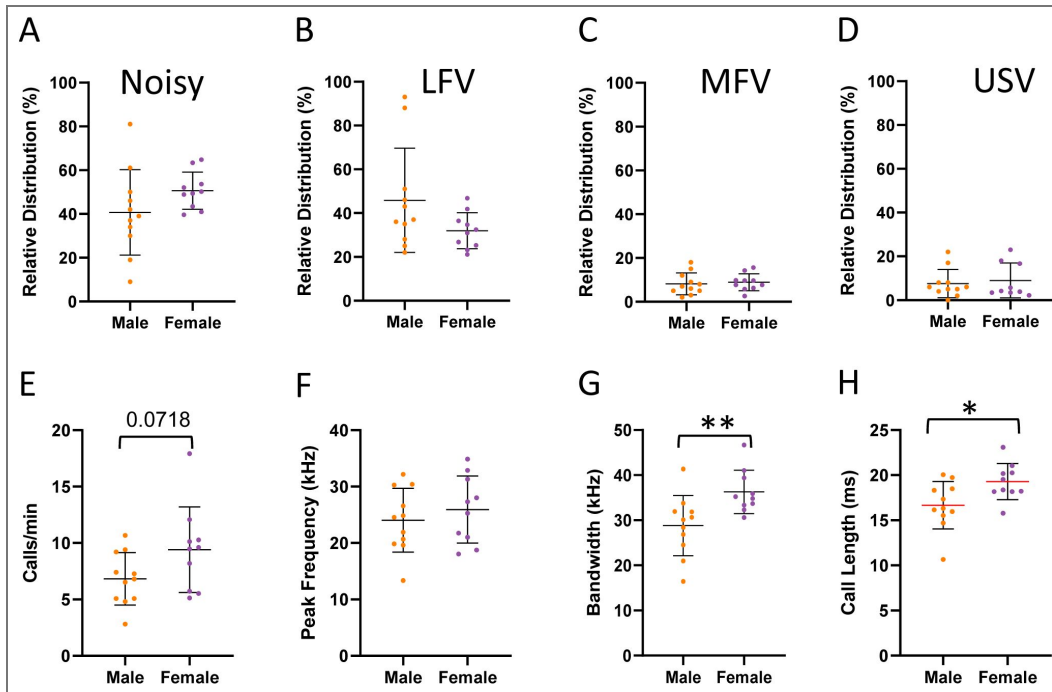


Figure 4. Female same-sex dyads produce more calls with greater bandwidth and longer call length compared to male same-sex dyads.

(A-D) Quantification of the relative distribution of Noisy calls (A), low frequency vocalizations (LFV, B), middle frequency vocalizations (MFV, C) and ultrasonic vocalizations (USV, D) between male (orange) and female (purple) same-sex dyads. Each dot corresponds to one same-sex dyad. (E - H) Quantification of spectrotemporal call parameters: call rate (E), peak frequency (F), bandwidth (G), and call length (H) between male and female same-sex dyads. Black lines represent group mean and error bars represent SD. Each dot represents the average of the respective parameter of one same-sex dyad. Differences between male and female same-sex dyads were analyzed using unpaired t-test. Call rate: $t_{(19)} = 1.907$, $P = 0.0718$, bandwidth: $t_{(19)} = 2.931$, $P = 0.0086$, call length: $t_{(19)} = 2.548$, $P = 0.0197$. * $p < 0.05$; ** $p < 0.01$. Male same-sex dyads: $n = 11$, female same-sex dyads: $n = 10$.

following/pursuit behavior were reflected by larger snout-to-tail base distances (200 px and up) and smaller snout-to-tail base angles (0 – 50°, Fig. 5E2, F2 inset scatter plots). It has to be noted that both snout-to-tail base distance and angle alone are not sufficient regarding the discrimination of whether a behavior constitutes in-line anogenital sniffing or the beginning of following/pursuit behavior, as this was not the goal of this analysis.

In summary, while direct physical interaction was obstructed by the divider, the dyad was most likely to emit calls in close proximity while facing one another at the divider. During unobstructed direct physical interaction both male and female same-sex dyads emitted mostly USVs. The emission of these USVs occurred mainly during anogenital sniffing and following/pursuit behavior. These observations suggest that two same-sex mice resort to a form of directed, vocal communication, while being prevented from direct physical interaction.

Distinct separation-induced call repertoires and spectro-temporal call properties in different mouse strains

To investigate whether these newly identified calls are caused by a specific genetic background, we analyzed separation-induced calls in two additional mouse strains with different genetic backgrounds (Fig. 6A). CBA mice were selected for their suitability in auditory research, as they maintain relatively stable hearing thresholds over age (Wu et al., 2019, Ohlemiller et al. 2010). In contrast, C57BL/6J (B6J) mice are widely used in biomedical research including vocalization behavior studies, despite their early onset, progressive hearing loss (Henry & Lepkowski, 1978). All tested strains produced the full repertoire of call classes (Fig. 6B–D), however, strain-specific differences emerged.

With a ratio of $25.5 \pm 22.7\%$, separated same-sex CBA dyads emitted fewer Noisy calls than both FVB ($44.5 \pm 11.9\%$) and B6J mice ($64.7 \pm 10.5\%$) (FVB vs. CBA $P = 0.0268$, FVB vs B6J $P = 0.0172$, CBA vs. B6J $P = 0.0003$; Fig. 6E). Conversely, CBA mice produced the highest proportion of USVs ($21.9 \pm 23.1\%$), significantly exceeding both B6J ($1.9 \pm 1.0\%$) and FVB ($8.3 \pm 7.1\%$) mice (FVB vs. CBA $P = 0.0384$; CBA vs. B6J $P = 0.0153$; Fig. 6H).

B6J mice vocalized at a significantly higher rate (33 ± 7.4 calls/min), producing more calls per minute than both CBA (4 ± 2.4 calls/min) and FVB (8 ± 3.3 calls/min) mice (FVB vs. B6J $P < 0.0001$; CBA vs. B6J $P < 0.0001$; Fig. 6I). Despite their higher call rates, B6J mice did not differ significantly from FVB mice in bandwidth or call duration (Fig. 6K, L). In contrast, CBA mice displayed a markedly narrower bandwidth (18.5 ± 8.6 kHz) compared to FVB (32.4 ± 6.9 kHz) and B6J mice (37.9 ± 6.5 kHz; Fig. 4K, FVB vs. CBA $P = 0.0014$; CBA vs. B6J $P = 0.0005$; Fig. 6K). CBA mice also displayed significantly shorter call durations (12.9 ± 2.9 ms) compared to both FVB (17.9 ± 2.7 ms) and B6J (19.6 ± 1.3 ms) mice (FVB vs. CBA $P = 0.0013$, CBA vs. B6J $P = 0.0007$, Fig. 6L).

These findings demonstrate that genetic background profoundly shapes the vocal communication repertoire in mice, influencing not only the proportional distribution of call classes but also the production rates and spectral properties of calls.

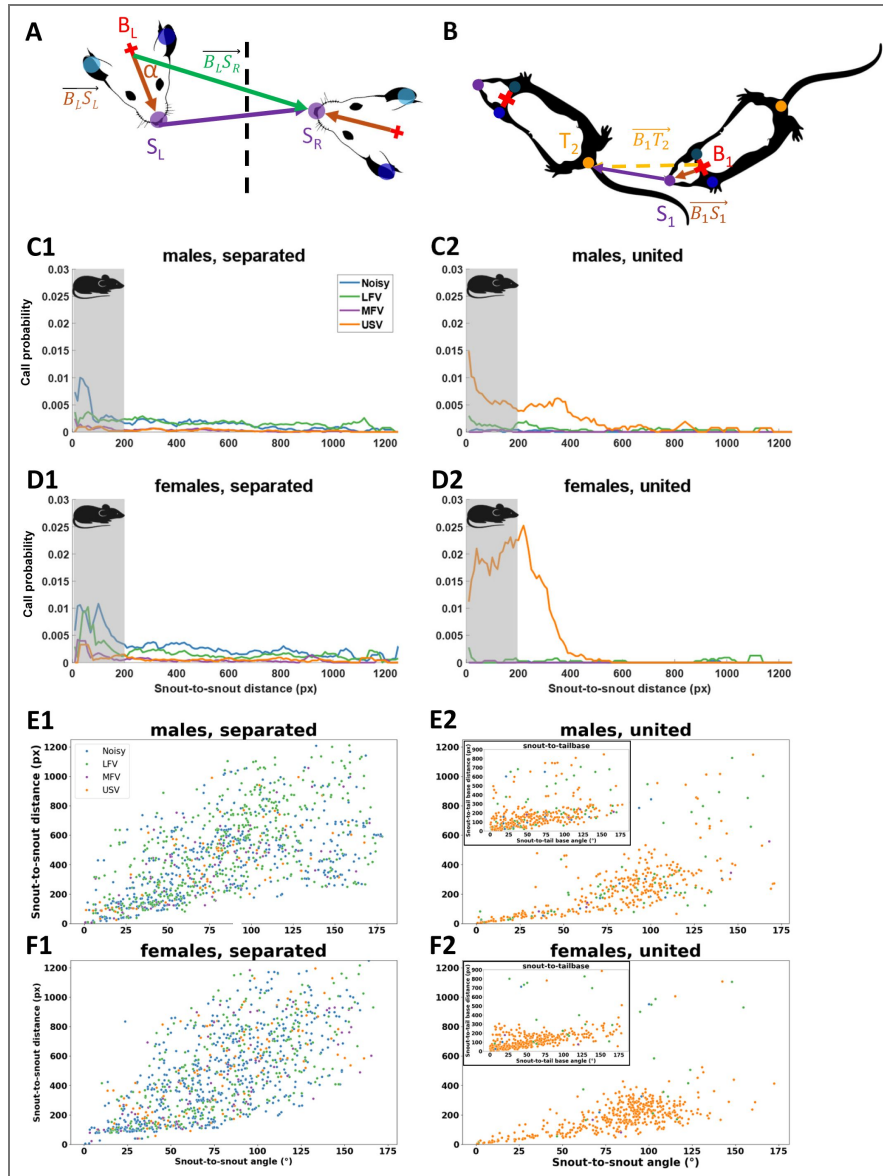


Figure 5. Physically separated same-sex mouse dyads face each other when vocalizing in close proximity.

(A) Schematic depiction of the calculation of both snout-to-snout distance and angle. Dashed black line between mouse heads represents the divider. Purple dots represent snout of left (S_L) and right (S_R) mouse. Blue and teal dots represent the left and right ear of each mouse, respectively. The middle of the distance between left and right ear is represented as a red cross and named between-ears point for both the left (B_L) and the right (B_R) mouse. The snout-to-snout distance is calculated as $|S_L S_R|$ (purple arrow). The snout-to-snout angle (α) is calculated between the vectors $\vec{B_L S_L}$ (brown arrow) and $\vec{B_L S_R}$ (green arrow). (B) Schematic depiction of the calculation of both snout-to-tail base distance and angle. Snout-to-tail base distance and angle (α) are calculated analogously to snout-to-snout distance and angle, instead of two snouts, one is replaced by the tail base, i.e. snout-to-tail base distance is calculated of the vector between the snout of the right mouse (S_R , purple dot) and the tail base of the left mouse (A_L , orange dot), $\vec{S_R A_L}$ (purple arrow). The angle is calculated between $\vec{B_R S_R}$ and $\vec{B_R A_L}$. (C1–D2) Line plots show the probabilities of Noisy calls (blue), low frequency vocalizations (LFV, green), middle frequency vocalizations (MFV, purple), and ultrasonic vocalizations (USV, orange) for all mouse-to-mouse distances found throughout the experiments for male dyads during both separation (C1) and unification (C2) and female dyads during both separation (D1) and unification (D2). The grey bar represents one mouse body length in pixels (px). (E1–F2) Scatter plots show the relationship between snout-to-snout distance and snout-to-snout angle during separation in males (E1) and females (F1), as well as during unification in males (E2) and females (F2). Inset graphs in E2 and F2 show the relationship between snout-to-tail base distance and snout-to-tail base angle. Call types are color coded: Noisy = blue, LFV = green, MFV = purple, USV = yellow. Male same-sex dyads: $n = 11$, female same-sex dyads: $n = 10$.

Sex	Call class	r_S	P
Male	Noisy	0.640	< 0.001
	LFV	0.647	< 0.001
	MFV	0.703	< 0.001
	USV	0.656	< 0.001
Female	Noisy	0.693	< 0.001
	LFV	0.680	< 0.001
	MFV	0.783	< 0.001
	USV	0.705	< 0.001

Table 1. Spearman correlation between snout-to-snout distance (px) and snout-to-snout angle (°) for separated same-sex mouse dyads.

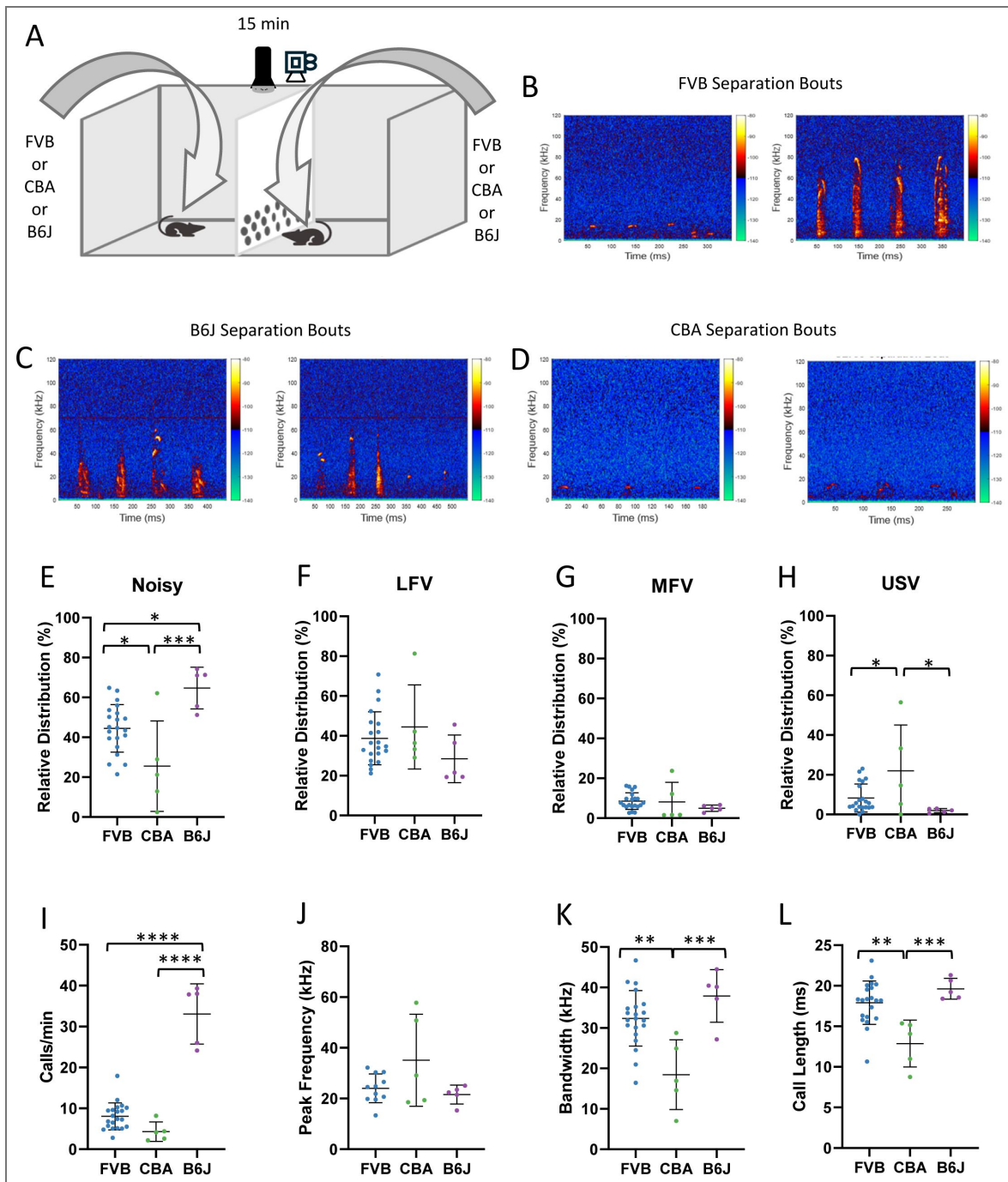


Figure 6. Genetic background influences call rates, call repertoire and acoustic call properties.

(A) Schematic depiction of the experimental design for the separation context. (B–D) Exemplary spectrograms of call bouts of FVB (B), B6J (C), and CBA (D) mice. (E–H) Quantification of the relative distribution of Noisy (E), LFV (F), MFV (G), and USV (H) calls during separation of FVB (blue), CBA (green) and B6J (purple) same-sex mouse dyads. Each dot represents one same-sex dyad. (I–L) Quantification of spectro-temporal call properties: call rate (I), peak frequency (J), bandwidth (K), and call length (L) of FVB, CBA and B6J same-sex mouse dyads. Each dot represents the average parameter for one same-sex dyad. Black lines represent group mean and error bars represent SD. FVB: n = 21, CBA: n = 5, B6J: n = 5. Data was analyzed using one-way ANOVA with Tukey’s multiple comparisons or Kruskal-Wallis test with Dunn’s multiple comparisons. Noisy distribution: $F_{(2, 28)} = 10.06$, $P = 0.0005$, USV distribution: $H_{(2)} = 7.523$, $P = 0.0233$, call rate: $F_{(2, 28)} = 86.74$, $P < 0.0001$, bandwidth: $F_{(2, 28)} = 10.56$, $P = 0.0004$, call length: $F_{(2, 28)} = 10.23$, $P = 0.0005$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$.

Varying sensory interaction during separation affects call repertoire and call features

We examined how different divider hole sizes (small, large or none) influence the call repertoire due to varying degrees of visual, olfactory, and putatively tactile interaction. Larger diameter (1 cm) of the holes resulted in a significant change of the vocal repertoire compared to that observed with the small holes (0.5 cm) or a solid divider (Fig. 7B, C, D [↗](#)). Large holes led to a markedly lower proportion of Noisy calls ($22.2 \pm 10.6\%$), compared to small ($44.5 \pm 11.9\%$) and none ($44.5 \pm 16.0\%$) ($F_{(2,34)} = 9.758$, $P = 0.0004$; Fig. 7E [↗](#)). In turn, large holes were associated with a significantly higher proportion of LFVs ($75.6 \pm 11.1\%$) compared to small ($38.7 \pm 13.3\%$) and none ($42.1 \pm 12.4\%$) ($F_{(2,34)} = 25.39$, $P < 0.0001$; Fig. 7F [↗](#)). Both MFVs and USVs accounted for about 1% of the calls when mice were separated by a divider with large holes, while both MFVs and USVs made up about 9% and 7% when the hole diameter was small or the divider was solid, respectively (MFVs: $F_{(2,34)} = 12.53$, $P < 0.0001$; Fig. 7G [↗](#), USVs: $H_{(2)} = 14.92$, $P = 0.0006$; Fig. 7H [↗](#)).

Also, the spectral and temporal call properties varied significantly with hole diameter. The hole diameter affected the call rate in separated dyads ($\chi^2(3) = 6.785$, $P = 0.0336$; Fig. 7I [↗](#)). When the divider featured large holes, mice emitted about 13 calls/min compared to both 8 calls/min with small holes and 9 calls/min with a solid divider (large vs. small $P = 0.0504$; large vs. none $P = 0.0707$, Fig. 7I [↗](#)). Peak frequency was also impacted by the hole diameter ($F_{(2,34)} = 25.86$, $P < 0.0001$; Fig. 7J [↗](#)). The lowest average peak frequency of about 9 kHz was observed with large holes, followed by an average peak frequency of about 19 kHz when the divider was not featuring any holes. Lastly, the highest average peak frequency of about 25 kHz was observed with small holes (small vs. large $P < 0.0001$, small vs. none $P = 0.0199$, large vs. none $P = 0.0031$; Fig. 7J [↗](#)). Similarly, hole diameter impacted the average call bandwidth ($F_{(2,34)} = 25.92$, $P < 0.0001$; Fig. 7K [↗](#)), with the narrowest (~ 15 kHz) occurring with large holes and similar average bandwidths with small (~ 32 kHz) or no holes (~ 29 kHz; small vs. large, $P < 0.0001$, large vs. none, $P = 0.0001$). Furthermore, the presence of holes in the divider impacted the average call length ($F_{(2,34)} = 54.62$, $P < 0.0001$; Fig. 7L [↗](#)), with shorter call length occurring when the divider featured either small (~ 18 ms) or large holes (~ 16 ms) compared to an average call length of about 28 ms when the divider was solid (small vs. none $P < 0.0001$, large vs. none $P < 0.0001$).

These findings highlight the impact of varying sensory access on both call repertoire, production rate and acoustic features of mouse vocalizations. Larger holes that allowed more direct somatosensory, visual, and olfactory interaction between the two mice resulted in more calls with lower peak frequency and smaller bandwidth, while the solid divider increased call duration. This is somewhat surprising, as the separation-induced emission of LFVs would have been expected to be mitigated by increased sensory interaction.

Buspirone Treatment Reduces Anxiety but Does Not Affect Low-Frequency Vocalizations in Mice, Indicating Anxiety Is Not the Primary Cause

Low-frequency vocalizations, as observed in our experiments, have previously been reported primarily in aversive contexts involving restraint or low temperature environments (Grimsley et al., 2016 [↗](#); Yamauchi et al., 2022 [↗](#)). Based on this, we aimed to investigate whether treatment with the anxiolytic buspirone would alter the call repertoire of separated mice to vocalizations of higher peak frequency and diminish the use of low-frequency vocalizations. Buspirone was shown to cause an anxiolytic effect in various behavior tests without the sedative effect known from benzodiazepines such as diazepam (Onofre-Campos et al. 2023). To test this, we compared vocalizations emitted after buspirone treatment to a control vehicle (saline) condition. Each mouse received both vehicle and buspirone treatments in a counterbalanced order, with a recovery period between sessions to control for potential carryover effects.

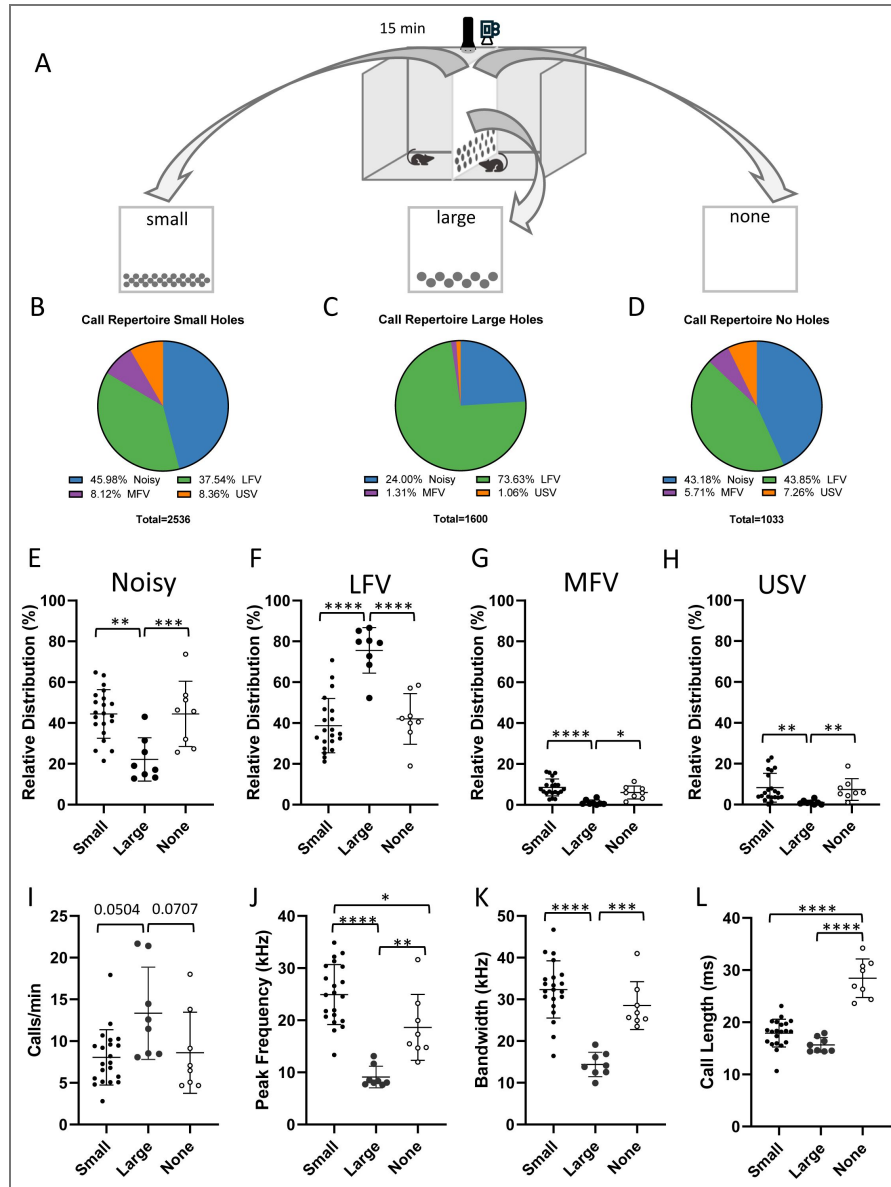


Figure 7. Influence of hole-diameter in the divider on call repertoire and acoustic call features.

(A) Schematic depiction of the experimental design and the different types of dividers used: hole diameter = 0.5 cm (small), hole diameter = 1 cm (large), and no holes (none). (B–D) Pie charts displaying total call repertoire during separation of same-sex pairs utilizing a divider with small holes (B), large holes (C), and no holes (D). Total number of recorded calls is displayed at the bottom of each pie chart. Call classes are color coded: Noisy (blue), low frequency vocalizations (LFV, green), middle frequency vocalizations (MFV, purple), and ultrasonic vocalizations (USV, orange). (E–H) Quantification of the relative distribution of Noisy (E), LFV (F), MFV (G), and USV (H) calls during separation with dividers carrying different diameter bore holes. Small diameter holes = small black dots, large diameter holes = large black dots, no holes = white dots with black border. Each dot represents one same-sex dyad. Noisy, LFV, and MFV data was analyzed using one-way ANOVA with Tukey’s multiple comparisons. USV data was analyzed using Kruskal-Wallis test with Dunn’s multiple comparisons. Noisy ratio: $F_{(2, 34)} = 9.758$, $P = 0.0004$, LFV ratio: $F_{(2, 34)} = 25.39$, $P < 0.0001$, MFV ratio: $F_{(2, 34)} = 12.53$, $P < 0.0001$, USV ratio: $H_{(2)} = 14.92$, $P = 0.0006$. (I–L) Quantification of spectro-temporal properties: call rate (I), peak frequency (J), bandwidth (K), and call length (L) of same-sex mouse dyads during separation with either small-diameter hole carrying divider (small), large diameter hole carrying divider (large), or no hole carrying divider (none). Each dot represents the average parameter for one same-sex dyad. Small: $n = 21$, large: $n = 8$, none: $n = 8$. Call rate was analyzed using Kruskal-Wallis test, while peak frequency, bandwidth, and call length was analyzed using one-way ANOVA with Tukey’s multiple comparisons. Call rate: $H_{(2)} = 6.785$, $P = 0.0336$, peak frequency: $F_{(2, 34)} = 25.86$, $P < 0.0001$, bandwidth: $F_{(2, 34)} = 25.92$, $P < 0.0001$, call length: $F_{(2, 34)} = 54.62$, $P < 0.0001$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$.

Vocal behavior of female same-sex dyads was impacted by the repeated exposure to the apparatus, but not the pharmacological treatment (Fig. 8 – Suppl. 1 [↗](#)), thus, female dyads were excluded from the analysis. In contrast, vocal behavior of male dyads appeared unaffected by the repeated exposure. Interestingly, treatment with buspirone did not seem to affect the separation-induced call repertoire, compared to vehicle treatment (Fig. 8B, C [↗](#)). However, including data of previously used untreated male same-sex dyads (Fig. 4 [↗](#)) revealed untreated mice appeared to have emitted fewer Noisy calls, more MFV and USV calls (Fig. 8D [↗](#)). The quantitative analysis of anxiolytic treatment effects on the four call classes supported this observation. Buspirone administration had no effect on the call distribution compared to vehicle treatment (Fig. 8E-H [↗](#)). Compared to untreated mice, however, vehicle treated animals emitted more Noisy calls ($52.9 \pm 13.73\%$ vs $38.8 \pm 12.11\%$, $t_{(14)} = 2.064$, $P = 0.0580$), fewer MFVs ($3.2 \pm 0.89\%$ vs $8.2 \pm 4.62\%$, $t_{(14)} = 2.368$, $P = 0.0328$; Fig. 8G [↗](#)) and USVs ($2.8 \pm 1.02\%$ vs $8.1 \pm 6.83\%$, $U = 9$, $n_1 = 5$, $n_2 = 11$, $P = 0.0380$; Fig. 8H [↗](#)).

When investigating the spectro-temporal parameters, we did not observe any effect of either buspirone or vehicle treatment on peak frequency, bandwidth or call length. However, following the vehicle administration dyads emitted about 14 calls/min, while the same dyads emitted about 5 calls/min after buspirone treatment ($W = 15.00$, $P = 0.0625$; Fig. 8I [↗](#)). The call rate of vehicle treated male dyads also exceeded that of untreated male dyads (~ 7 calls/min; $t_{(14)} = 2.637$, $P = 0.0195$; Fig. 8I [↗](#)).

Together, these results suggest that the low-frequency call classes observed during separation are unlikely to be driven primarily by anxiety, as the administration of the anxiolytic buspirone did not affect the call repertoire. However, the increased call rate observed in vehicle-treated mice indicates that the injection process itself may induce mild discomfort, which is mitigated by buspirone treatment.

Discussion

The prevention of direct physical interaction of stranger same-sex mouse dyads through a divider revealed a separation-induced call repertoire. This repertoire consisted of calls in three frequency bands 1 – 32 kHz (low frequency vocalizations, LFV), 32 – 50 kHz (middle frequency vocalizations, MFV), and > 50 kHz (ultrasonic vocalizations, USV) and an additional class of Noisy calls, which is characterized by their broad bandwidth and warbled spectral appearance. Noisy calls and LFVs dominated the separation-induced call repertoire, while MFVs and USVs only accounted for a small portion. Both male and female FVB dyads used the same call repertoire, however, the females' calls bandwidth being larger and the duration longer than that of the males. Furthermore, we observed a tendency towards a higher call rate in female dyads. Three different mouse lines commonly used in vocalization experiments were able to produce all four call classes, but emitted them at different proportions during separation. When separated the two mice were most likely to emit calls when they were less than one body length apart, facing one another. Once united the same mice would display normal social behavior including anogenital sniffing, pursuit and fleeing behavior. They adjusted their vocal repertoire according to the degree of direct interaction. Both dividers with no holes and with small holes resulted in a very similar call repertoire, while dyads encountering large-hole dividers emitted a vocal repertoire predominantly consisting of LFVs with an elevated call rate and fewer Noisy calls. Overall, the separation-induced call repertoire seemed not to be anxiety-related, as the anxiolytic buspirone had no effect on the repertoire, call rate, and spectro-temporal properties.

Noisy mouse calls

Noise in mouse calls – feature, not artifact

Noisy calls were one of the two dominant call classes in the separation-induced call repertoire in our study. They pose a particular challenge with respect to their spectrogram-based identification as calls and differentiation from noise of non-vocal origin. However, we found evidence suggesting mice emit Noisy calls voluntarily in a context-dependent manner:

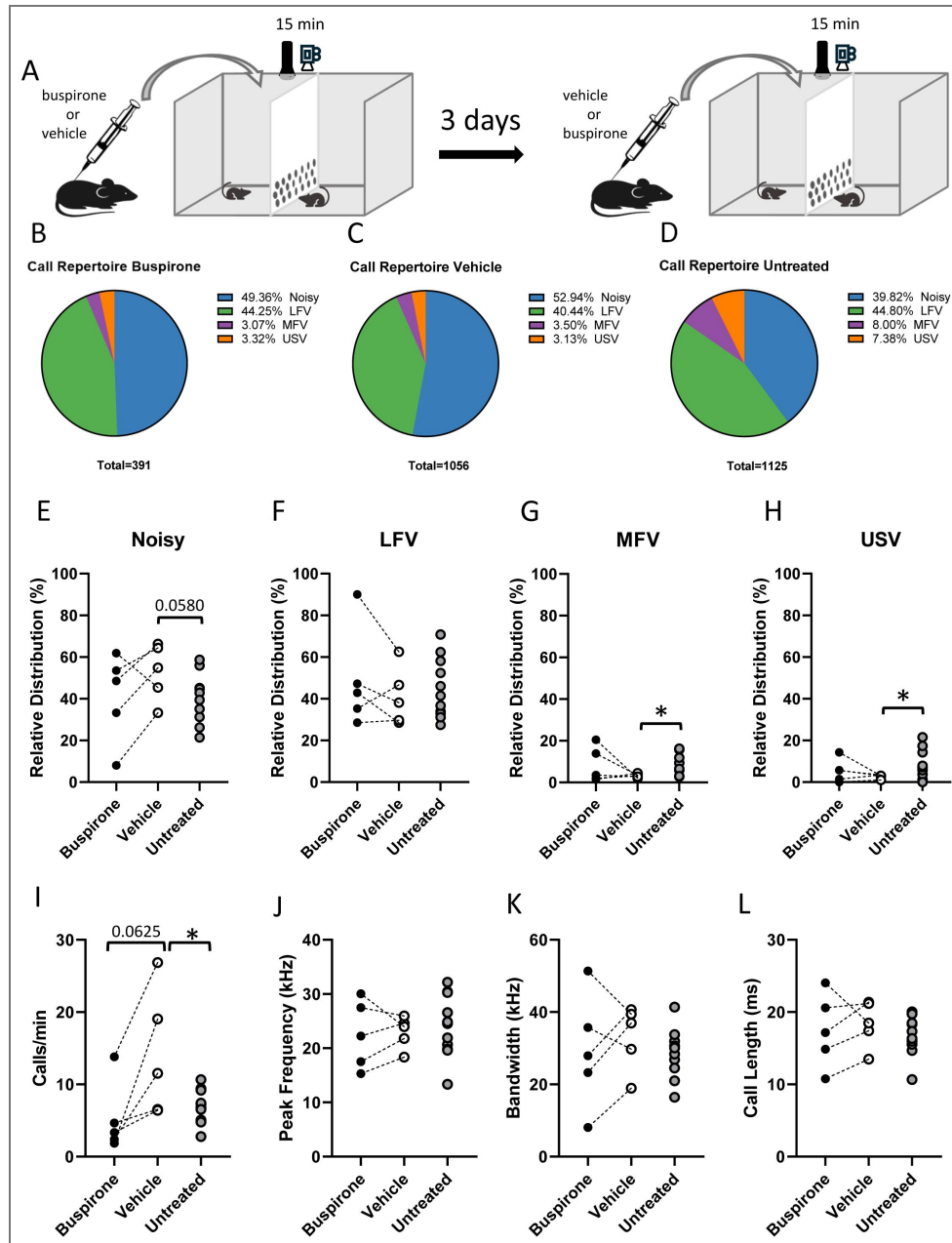


Figure 8. Treatment with the anxiolytic buspirone had no effect on both separation-induced call repertoire and spectro-temporal call properties.

(A) Schematic depiction of the separation context of experiment design. (B – D) Pie charts display total call repertoire during physical separation of male same-sex mouse dyads after treatment with buspirone (B) or vehicle (physiological saline; C) and untreated male same-sex dyads (D). The total number of recorded calls is displayed at the bottom of the pie charts. Call classes are color coded: Noisy = blue, low frequency vocalizations (LFV) = green, middle frequency vocalizations (MFV) = purple, ultrasonic vocalizations (USV) = orange. (E–H) Quantification of the relative distribution of Noisy (E), LFV (F), MFV (G), and USV (H) calls during the physical separation of same-sex mouse dyads after treatment with buspirone ($n = 5$) or vehicle ($n = 5$), or of untreated dyads ($n = 11$). Each dot represents the proportion of the respective call class of one same-sex dyad. Noisy distribution: $t_{(14)} = 2.064$, $P = 0.0580$, MFV distribution: $t_{(14)} = 2.368$, $P = 0.0328$, USV distribution: $U = 9$, $n_1 = 5$, $n_2 = 11$, $P = 0.0380$. (I–L) Quantification of spectro-temporal call properties: call rate (I), peak frequency (J), bandwidth (K), and call length (L). Each dot represents the average of one same-sex dyad of the respective parameter. Treatments are color coded, buspirone = black dots, vehicle = white dots, and untreated = grey dots. Paired data points are connected by a dashed line. The differences between buspirone and vehicle treatment were analyzed using either paired t -test or Wilcoxon signed rank test. Comparisons between buspirone-treated and not treated mice, as well as between vehicle-treated and not treated mice were made using either unpaired t -test or Mann-Whitney-U test. Call rate: $t_{(14)} = 2.637$, $P = 0.0195$. * $p < 0.05$

Firstly, 63.7% of Noisy call spectrograms contained a clearly visible tonal component. This could be explained by the use of the two distinct vocalization production mechanisms, vocal cord vibration and whistle, which are found in USV-producing rodent species (Mahrt et al., 2016; J. Park et al., 2024; Pasch et al., 2017; Riede et al., 2022; Roberts, 1975).

Secondly, most Noisy calls (77.2%) were emitted in bouts. In fact, mice have been shown to emit the majority of their USVs in bouts, both in courtship (Castellucci et al., 2018; Chabout et al., 2015) and in same-sex context (Hoier et al., 2016). The emission of vocalizations in bouts has also been described in other rodent species such as *Scotinomys teguina*, *Onychomys sp.*, and *Peromyscus sp.* (Okobi et al., 2019; Pasch et al., 2017; Riede et al., 2022). In our study, bouts consisting purely of Noisy calls were temporally highly regular with a median ICI of around 80 ms, suggesting that one call was emitted during one exhalation (Sirotin et al., 2014). This aligns with the ICI durations described for USVs produced in bouts (Castellucci et al., 2018; Chabout et al., 2015).

Thirdly, changing context parameters such as the diameter of holes in the divider resulted in a call repertoire containing considerably fewer Noisy calls meaning mice adapted their separation call repertoire as a consequence of the increased degree of sensory exchange through the divider.

Attracting attention through Noisy calls

Based on the observations that Noisy calls make up 43% of all calls in isolated male mice in an open field and Noisy calls spanning nearly the entire mouse hearing range, a role as seeking calls has been suggested (Grimsley et al., 2016). Adult CBA/CaJ mice have been reported to emit Noisy calls which spectrograms have a warbled, noisy, broadband appearance likely corresponding to “deterministic chaotic elements” (Grimsley et al., 2011), much like the spectrograms of our Noisy calls. Indeed, non-linear phenomena (NLP), such as biphonations or deterministic chaos, are a common component in mammalian vocalizations and are in some species associated with a heightened arousal state, e.g. in infant elephants (Stoeger et al., 2011), big brown bats (Gadziola et al., 2012), and vervet monkeys (Mercier et al., 2019). Furthermore, NLP in vocalizations have been shown to “grab” the listener’s attention in red deer and koalas (Charlton et al., 2017; Massenet et al., 2025; Reby and Charlton, 2012). Thus, the Noisy calls emitted in our experiments might serve to attract the attention of conspecifics in mice, too.

In our study, facilitated exchange of visual cues and somatosensory interaction through large holes in the divider (e.g., social whisking) reduced the proportion of Noisy calls. Whisking, together with both auditory and olfactory interactions between same-sex conspecifics are necessary in discriminating a familiar from an unfamiliar conspecific (de la Zerda et al., 2022). Indeed, in a resident-intruder context Noisy calls are being emitted during the social whisking preceding fight onset (Gourbal et al., 2004). A temporal coordination between whisking and USV emission has been shown in rats with the concomitant emission of a particular call repertoire at an increased call rate while in whisker-to-whisker contact. Not only the USV emission is linked to whisking in rats, but during whisker-to-whisker contact the response of regularly spiking neurons in primary auditory cortex to USVs is modulated (Rao et al., 2014).

Our data show that Noisy calls were particularly prevalent when direct physical interaction was prevented or strongly restricted and both mice were in close proximity at the divider. Facilitating direct physical interaction through large holes in the divider resulted in a reduction of Noisy call emission, while unrestricted physical interaction saw a cessation of Noisy call emission. These findings, together with the broad bandwidth and warbled spectral properties, support the notion that Noisy calls serve to attract conspecifics’ attention in situations where other forms of direct physical interaction are hampered or impeded.

Putative communicational role of LFVs beyond the vocal expression of negative affective states

Besides Noisy calls, the separation-induced call repertoire was dominated by LFVs, which presented with an average peak frequency of about 12 kHz and spectrograms mostly without harmonics. These LFVs are being produced by the same neural circuit that gives rise to USVs,

encompassing neurons in the dorsolateral periaqueductal gray (PAG) projecting to neurotensin-positive neurons in nucleus retroambiguus (Nts^+ RAM neurons) (Veerakumar et al., 2023), from which brain stem motor neurons controlling the vocal cords are being innervated (Chen et al., 2021; J. Park et al., 2024; Tschida et al., 2019; Veerakumar et al., 2023; Ziobro et al., 2024). While neuronal activity of USV-eliciting PAG neurons scales the amount of USVs and their loudness (Chen et al., 2021), Nts^+ RAM neuron activity scales the dominant frequency of mouse calls (Veerakumar et al., 2023). This dominant frequency scaling constitutes a stepwise transformation induced by crossing a spike frequency threshold. Optogenetic stimulation of Nts^+ RAM neurons in the range of 15 to 20 Hz reliably evoked the emission of LFVs, while stimulation frequencies > 25 Hz led to the emission of USVs (Veerakumar et al., 2023).

LFVs (corresponding to MFVs published by Grimsley et al. 2016) have been reported to vocally express despair and/or distress, e.g. during restraint (either by jacket, tube, or headpost) and/or cold stress (Grimsley et al., 2016; Yamauchi et al., 2022). However, the established, best-known example for calls expressing a negative affective state in mice are squeaks (or broadband vocalizations, or low frequency harmonics). They are characterized by a fundamental frequency of about 3 kHz, an average of three to five harmonics, often with co-occurring NLP such as subharmonics (Finton et al., 2017; Lupanova and Egorova, 2015; Wang et al., 2008). Mice emit squeaks to express pain, despair and/or distress, for instance during tail snipping, when being bitten (Gourbal et al., 2004; Williams et al., 2008), or during courtship with concomitant defensive behavior (Finton et al., 2017; Lupanova and Egorova, 2015; Sugimoto et al., 2011; Wang et al., 2008). A link between anxiety and squeaks is suggested, as squeaks evoked by a tail-suspension test in mice bred for high anxiety-related behaviors, but not CD-1, BALB/c, DBA, or B6N, are sensitive to diazepam treatment (Ruat et al., 2022).

In contrast, a link between anxiety and LFV emission is not clear. While pharmacological manipulation of anxiogenic neural circuits by the anxiolytic benzodiazepine midazolam or the putative anxiolytic δ opioid receptor agonist KNT-127 causes an overall decrease of calls emitted by animals experiencing restraint and/or cold stress, midazolam has no effect on and KNT-127 even elevates the proportion of LFVs emitted (Yamauchi et al., 2022). However, playing back LFVs to mice that had prior restraint experience leads to an increase in intra-amygdalar acetylcholine concentration, as well as flinching (Ghasemahmad et al., 2024). Cholinergic signaling in the basolateral amygdala has been shown to be important for the durability of fear memory in rats (Crimmins et al., 2023).

In our study, separating same-sex mouse dyads with a divider was sufficient to evoke the predominant emission of LFVs together with Noisy calls. Treatment with the anxiolytic 5-HT_{1A} receptor agonist buspirone reduced the call rate compared to saline treatment, however, the call rate after buspirone treatment was not different from that of completely untreated mice, suggesting buspirone treatment would normalize the effect of the injection rather than alleviating anxiety caused by separation. Furthermore, we did not observe any effect of buspirone treatment on the proportion of LFVs or any other call class.

Squeaks are clearly used by mice to express a negative affective state provoked by another organism. LFVs have, so far, only been reported in mice during considerable restriction of their freedom of movement, suggesting they are also used to express a negative state. However, we have used a gentle separation-method allowing animals at least limited freedom of movement, that is used routinely e.g. for the exposure of naïve male mice to female mice (Klaus et al., 2025; Matsumoto and Okanoya, 2018; Musolf et al., 2010; Zala et al., 2017). Yet, we have observed LFVs to be one of the two dominant (about 40% of all calls) call classes used in this context. Even more so, once direct sensory (and/or tactile) interaction was facilitated with larger holes, LFVs became the dominant call class in the call repertoire with about 75% of all calls. Hence, LFVs might not be limited to the expression of an aversive state, but may encompass a broader communicational role when mice are restricted in their direct interaction, but yet free to move around the test arena.

Communicating at distance using Noisy calls and LFVs

Until recently, it was agreed upon that mice vocalize mostly at a frequency range from 40 kHz and above with the exception of squeaks (Lupanova and Egorova, 2015 [↗](#); Portfors, 2007 [↗](#); Venkatraman et al., 2024 [↗](#)). However, most vocalization studies in adult mice allow them to interact directly with one another. Our data clearly show that preventing this direct interaction leads mice to produce predominantly calls with a low dominant frequency. Considering the low-pass filter properties of the atmosphere (Lawrence and Simmons, 1982 [↗](#)), it seems reasonable to use calls with more energy in the low frequency components (Noisy calls and LFVs) as these calls propagate a farther distance than those with more energy in the high frequency components, and also cost less energy to produce. Moreover, mice show the lowest hearing thresholds for tone frequencies ranging from about 10 to 20 kHz (Ohlemiller et al., 2010 [↗](#); C. R. Park et al., 2024 [↗](#)). Recently, it was highlighted that both Noisy calls and LFVs (referred to as MFVs in Tehrani et al., 2014) elicit widespread neuronal activity throughout the IC and along its tonotopic axis compared to a more limited neuronal activity in response to USVs (Tehrani et al., 2024 [↗](#)). The presentation of noise (non-stationary noise, white noise or natural stationary noise) increases neuronal activity in most sites of the auditory midbrain of both guinea pig and rat (Hosseini et al., 2021 [↗](#); Mishra et al., 2021 [↗](#)). However, organization of the auditory midbrain into zones with different response properties as well as the higher energy of non-stationary noise in lower frequencies compared to higher frequencies has to be considered (Chen et al., 2012 [↗](#); Hosseini et al., 2021 [↗](#)).

Taken together, the auditory sensitivity of mice in the 10 – 20 kHz frequency range, the wide spread activity along the tonotopic axis in the auditory midbrain, and the putatively lower energetic production cost suggest a key role for Noisy calls and LFVs in the vocal communication of mice when interaction along other sensory modalities is not feasible.

Few and far between – MFVs and USVs in separation-induced acoustic communication

In all our separation experiments we encountered a small proportion of calls with peak frequencies between 32 and 50 kHz (MFVs). They formed a distinct peak in the peak frequency distribution and their spectrograms appeared tonal and differed from the low frequency component of step USVs. Hence, we regard MFVs as a separate call class. Reports on mouse calls in the MFV peak frequency range are rare, however, so far, they are restricted to mixed-sex interactions. Male mice have been shown to emit calls in the MFV range during ejaculatory mounts (White et al., 1998 [↗](#)), while female mice emit 40 kHz calls to initiate pup retrieval in the pup's father (Liu et al., 2013 [↗](#)). Based on the exemplary spectrograms provided, a proportion of copulatory calls would likely be identified to be the low frequency component of step USVs (Arriaga and Jarvis, 2013 [↗](#); Klaus et al., 2025 [↗](#); Scattoni et al., 2008 [↗](#); Wang et al., 2008 [↗](#)). The 40 kHz pup retrieval calls likely express an agitated state, as an elevated corticosterone plasma level has been reported in dams that were removed from their litter (Moles et al., 2008 [↗](#)). To our knowledge, this is the first time MFVs have been reported to occur during same-sex vocal communication, scarcely, yet consistently. Unfortunately, we were unable to elucidate their role for mouse social behavior any further.

USVs are the best researched class of mouse call, being emitted both during same-sex (de Chaumont et al., 2021 [↗](#); Moles et al., 2007 [↗](#); Panksepp et al., 2007 [↗](#)) and mixed-sex interactions (Hanson and Hurley, 2012 [↗](#); Panksepp et al., 2007 [↗](#); Schleidt, 1951 [↗](#); White et al., 1998 [↗](#)). Surprisingly, in our study separated mice emitted only few, short USVs of lower peak frequency compared to those emitted during direct same-sex interaction (Caruso et al., 2022 [↗](#); de Chaumont et al., 2021 [↗](#); Ferhat et al., 2016 [↗](#)).

Conclusion

Preventing direct physical interaction of same-sex mouse dyads through a divider revealed a call repertoire consisting mainly of Noisy calls and LFVs. In light of the auditory sensitivity, widespread IC response, and dominant frequencies with greater propagation range, Noisy calls

and LFVs appear ideal for communication when e.g., whisking is prevented. In this situation Noisy calls could serve the purpose to attract the attention of other mice, which declines with the concomitant rise of direct interaction possibility. During unrestricted direct interaction (e.g., in a resident-intruder scenario), however, Noisy calls might serve a different role. Until now LFVs have only been described in aversive contexts, however, our study, using a gentle separation design, extends the putative communicational role of LFVs beyond the expression of aversive states.

Taken together, the communication of mice at frequencies below 30 kHz seems to play a much larger role than previously thought, and may even, in certain situations, be the predominant form of mouse vocalisation. As such, research into the vocal communication of mice should extend its scope beyond USVs to obtain a complete picture.

Materials and Methods

Animals

Experimental procedures were approved by the State Office for Health and Social Affairs Berlin under the animal license numbers “G0045/22” and “G0276/18”. The FVB.129P2-*Pde6b*⁺ *Tyr*^{c-*chl*}/*AntJ* mouse strain (strain no. 004828; Jackson Laboratories, USA) was chosen since these mice do not show retinal degeneration and do not exhibit early onset high frequency hearing loss, making it particularly suitable for behavioral vocalization experiments (Errjgers et al., 2007 [↗](#); Garcia-Pino et al., 2017 [↗](#); Ho et al., 2014 [↗](#)). Founding mice were sourced from Jackson Laboratories and bred in-house at the animal facility of Freie Universität Berlin. C57BL/6J (B6J) mice arrived from the Max Rubner laboratory (German Institute of Human Nutrition, Potsdam, Germany). CBA/J (CBA) mice were obtained from the Department of Otolaryngology - Head & Neck Surgery, University of Tübingen Medical Center. Both B6J and CBA mice were allowed to acclimatize to the housing facility for at least 14 days before entering experiments. Mice were housed in standardized home cages (Type II L), with group sizes of two to four individuals per cage. The animals were housed on a 12:12 light-dark cycle (lights on at 3 a.m.) and the facility temperature kept at 22±1°C with relative humidity of 45-65%. Food and water were provided *ad libitum*. The experiments were conducted on mice aged between P52 and P124 (median: P82).

Experimental Setup

Behavioral experiments were conducted in a custom-built arena with a square area of 930 cm² and a wall height of 30 cm, constructed from Makrolon® and divided into two equally sized compartments by a transparent Plexiglas® wall. No bedding was used during the experiment. The divider featured a perforated section (8.5 cm x 27 cm (HxB)) at the bottom. For the majority of experiments this section featured holes of either 0.5 cm diameter (small holes) or 1 cm diameter (large holes), or was not perforated (no holes). Hence, the two mice could engage with each other using auditory, visual, and olfactory cues, while the exchange of somatosensory cues was severely limited or not possible at all (Hoier et al., 2016 [↗](#); Pessoa et al., 2022 [↗](#)). The arena was placed within a sound-attenuated chamber (METRIS-Systems, Hoofddorp, Netherlands) during the experiments. Video recordings were captured at 30 fps using EthoVision XT software (Noldus, Wageningen, Netherlands) and a Brio Ultra HD Pro Webcam (Logitech, Apples, Switzerland) mounted 35 cm above the arena. Audio signals were recorded using a microphone (CM16 Avisoft-Bioacoustics) positioned 30 cm above the arena. The microphone was connected to an CMPA40-5V amplifier (Avisoft Bioacoustics) and the signal was digitized with a sample rate of 384 kHz using an ADI-2-Pro analog-to-digital converter (RME, Haimhausen, Germany) and the Avisoft-RECORDER software (Avisoft Bioacoustics, Glienicke, Germany).

Experimental procedures

In total 48 pairs of FVB mice (24 male and 24 female pairs), 5 pairs of B6 mice (all male), 5 pairs of CBA mice (2 male and 3 female pairs), and 5 mixed-sex FVB mouse pairs were employed in the physical separation experiment.

All experiments were conducted at dusk ('lights on' in the animal facility) to align with the crepuscular activity of mice. Mice underwent a two-day habituation protocol prior to the experiments. On the first day, the animals were transferred to the experimental room for one hour while remaining in their home cages, with food and water provided *ad libitum*. On the second day, the animals were again habituated to the experimental room for one hour before being individually placed into one of the arena compartments for a 20-minute habituation period. After this, they were returned to their home cages.

Before the experiment, the animals were habituated again to the experimental room for one hour in their home cages. Following this, two individuals were transferred to the test arena and placed each in one of the compartments. The arena was then placed inside the sound-attenuated chamber, and video and audio recordings were collected continuously for 15 minutes. After this recording session, the Plexiglas® divider (small holes) separating the two compartments was removed, allowing the mice to physically interact. Video and audio recordings continued for an additional 5 minutes. In the case of dividers without holes, no unification phase was initiated, as the arena contained a lid in order to minimize physical, olfactory and vocal communication between the respective mice.

For the investigation of buspirone's effect on the separation-induced vocal behavior, the experiments were conducted as described above with the exception that animals received either physiological saline or buspirone hydrochloride (4 mg/kg at 0.1 ml/10g body weight; abcr GmbH, Karlsruhe, Germany, art.no. AB348846) 20 min before the start of the experiment. After the experiment mice returned to their home cage and were transferred back to the animal facility. The experiment was repeated after 3 days, with the same mice now receiving the respectively other substance, be it saline or buspirone. This way the effect of the experimental sequence could be accounted for. A total of 5 male and 5 female same-sex FVB mouse pairs were used for in these experiments.

Vocalization Analysis

Audio recordings were analyzed -first by creating spectrograms from the original wav files using DeepSqueak 3.1.0, a MATLAB-based platform for detecting and visually illustrating bioacoustic signals (Coffey et al., 2019). Vocalizations were initially detected using the Mouse Detector YOLO2 convolutional neural network, which effectively identifies ultrasonic vocalizations (USVs) but is less sensitive to detect lower-frequency calls. All automatically detected vocalizations were manually reviewed and corrected. Detection boxes were redrawn to precisely match the vocalization contours, ensuring they covered the entire spectral morphology. Vocalizations missed by automatic detection were manually added by drawing detection boxes around the call contours in the spectrogram. For noisy vocalizations without clear spectral morphology, and vocalizations of low frequency (<32 kHz) with multiple harmonics, detection boxes were drawn around the entire signal. To distinguish vocalizations from movement-induced noise, we used multiple verification criteria: presence of tonal call contours in the spectrogram, regular production patterns in bouts similar to previously observed vocal sequences, and audio playback verification using DeepSqueak's down sampling feature (factor of 20). This way low-frequency vocalizations were additionally verified by having similar acoustic features such as downward and upward sweeps or frequency steps similar to USVs.

For classification, the spectral and temporal parameters (Peak Frequency, Call Length) of the detected vocalizations were exported from DeepSqueak. Bandwidth was extracted by modifying the DeepSqueak CalculateStats.m function to extract the frequency bounds of the user-drawn box surrounding the call contours. Vocalizations were classified based on their peak frequency into Low-Frequency Vocalizations (LFVs; <32 kHz), Middle-Frequency Vocalizations (MFVs; 32–50 kHz), and Ultrasound Vocalizations (USVs; >50 kHz). Noisy vocalizations, were manually classified based on their spectral appearance and large bandwidth.

Smooth example spectrograms were created by converting the recordings through fast Fourier transformation (nfft: 4096 samples, Hamming window: 512 samples, overlap: 256 samples) in MatLab.

Call spectrogram feature extraction using convolutional neural network (CNN) and clustering

All computational steps were made using Python (v. 3.10.10). Single calls were extracted from recorded audio WAV files using the timestamps obtained by call detection as described above. The calls' spectrograms (nfft: 256 samples, Hanning window: 256 samples, overlap: 128, linear detrending; matplotlib 3.10.1) were plotted, and the resulting images were rescaled to 224 by 224 pixel size. Default VGG16 image preprocessing was used before feeding the images into the CNN.

We employed a keras (v. 3.9.0) implementation of VGG16 neural network model (Simonyan and Zisserman, 2014) that achieved 92.7% accuracy in the ImageNet Large Scale Visual Recognition Challenge 2014 (ILSVRC2014) outperforming other state of the art CNN models for computer vision such as Clarifai. VGG16's architecture consists of 13 convolutional layers, 5 max-pooling layers, 3 fully connected layers (fc), and 1 soft-max layer, allowing for extraction of complicated image features. To extract image features from our call spectrograms, we used VGG16 with the weights obtained by previous training on the ImageNet dataset (<https://www.image-net.org>). A similar approach of clustering images using VGG16 - extracted image features has been used successfully in the past (Cohn and Holm, 2021; Lachmann et al., 2022). To extract features, the network was truncated at the second fully connected layer (fc2), resulting in a 4096-dimensional feature vector for each image. Images were clustered using UMAP (umap-learn library, v 0.5.7) based on the obtained features. To optimize the UMAP projection, a grid search was conducted to identify optimal values for the 'n_neighbors' and 'metric' parameters. These parameters significantly influence the resulting embedding by controlling the local and global structure of the data. The number of neighbours (5) and Manhattan distance were chosen based on their ability to effectively cluster the data points while preserving the underlying manifold structure, assessed through parameters such as peak frequency, bandwidth and call duration of calls and the visual similarity of spectrograms projected on the embedding. Additionally, the effect of individual animals emitting the call was checked in order to make sure it does not affect the clustering by forming separate, individual-specific clusters.

Behavioral analysis

Videos were analyzed using DeepLabCut V2.2.1 (Mathis et al., 2018). Nine body parts were defined for each mouse: snout, left ear, right ear, left shoulder, right shoulder, left hip, right hip, tail base, tail tip. The network (dlcrnet_ms5 with multi-animal-imgaug and ellipse tracking method) was retrained on 950 frames from 38 videos each featuring the same setup as for the experimental videos (left mouse = mouse in left compartment, right mouse = mouse in right compartment). Following automatic body part detection, outlier frames were extracted using DeepLabCut's 'jump' algorithm and were corrected manually by adjusting body point markers in the automatically selected frames. The dataset was updated accordingly. The obtained coordinates were filtered and interpolated using SARIMA algorithm (AR: 1, MA: 1, cut-off p: 0.01). The filtered x-y-coordinates were exported from DeepLabCut in the form of a CSV file.

We analyzed the proximity of the snouts of the two mice at the time of call emission (snout-to-snout distance). Snout-to-snout distance was calculated by applying Pythagorean theorem: the length of the direction vector between the snout of the left and of the right mouse were calculated using the x-y-coordinates obtained from DeepLabCut. Then the frame number was translated into time information. The snout-to-snout distance time stamps were then compared to the time stamps marking the beginning of detected vocalizations. Frames with the smallest time difference to call onset were selected for analysis. To determine the mouse body length, the length of the distance vector between snout coordinates of each mouse and the coordinates of their own tail base was calculated.

Furthermore, to visualize whether certain snout-to-snout distances coincided with call production more often than others, we calculated the call probability by dividing the amount of calls at respective snout-to-snout distances by the overall occurrence of this snout-to-snout distance

during the experiment. For line plots, data was smoothed using a moving average with a span of 0.025.

Additionally, snout-to-snout angles (during separation phase) as well as snout-to-tail base angles (during unification phase) were calculated as follows, with the head center being defined as the half distance between left and right ear (between-ear-point, B) for each mouse:

$$\text{Snout-to-snout angle: } \alpha = \cos^{-1} \left(\frac{\overline{B_L S_L} \cdot \overline{B_L S_R}}{|\overline{B_L S_L}| |\overline{B_L S_R}|} \right)$$

$\overline{B_L S_L}$ = vector between B of left mouse (B_L) and snout of the same mouse (S_L)

$\overline{B_L S_R}$ = vector between B of left mouse (B_L) and snout of right mouse (S_R)

$$\text{Snout-to-tail base angle: } \alpha = \cos^{-1} \left(\frac{\overline{B_1 S_1} \cdot \overline{B_1 T_2}}{|\overline{B_1 S_1}| |\overline{B_1 T_2}|} \right)$$

$\overline{B_1 S_1}$ = vector between B of one mouse (B_1) and snout of the same mouse (S_1)

$\overline{B_1 T_2}$ = vector between B of one mouse (B_1) and tail base of other mouse (T_2)

Snout-to-tail base angles were calculated for both the left mouse' snout to the right mouse' tail base and the right mouse' snout to left mouse' tail base. The smaller of the two angles and its corresponding distance were used for analysis.

Data Analysis

All data are presented as the mean \pm standard deviation of the mean. Data fulfilling the requirements for parametric statistics was analyzed statistically using two-way repeated measures ANOVA (2-way RM ANOVA) for comparisons among three or more groups, with call type as within-subject factor and event as between-subject factor. In the case that 2-way RM ANOVA could not be calculated due to missing values, a restricted maximum likelihood (REML) mixed-effects analysis was employed. If ANOVA results were significant, Sidak's multiple comparison tests were performed. For comparisons between strains and hole sizes a one-way ANOVA was employed. When parametric statistics requirements were not met Kruskal-Wallis test was employed to examine both strain and hole differences. Sex differences and differences between mice receiving treatment (buspirone or vehicle) and mice not receiving any treatment were analyzed using unpaired t-tests, when requirements for parametric statistics were met. In case the data did not meet these requirements, Mann-Whitney-U test was used for analysis. For comparisons between buspirone and vehicle treatment a paired t-test was used if requirements for parametric statistics were met. If these requirements were not met, Wilcoxon signed rank test was used. Correlations between snout-to-snout distance and angle were analyzed using Spearman correlation analysis. Statistical computations were performed using GraphPad Prism (V. 8, GraphPad Software Inc, U.S.A).

Figure supplements



Figure 4—figure supplement 1. Call repertoires are adapted when a mixed-sex dyad is separated by a divider. Pie chart shows the call repertoire of separated mice of opposite sex. Call classes are color coded: Noisy (blue), low frequency vocalizations (LFV, green), middle frequency vocalizations (MFV, purple), and ultrasonic vocalizations (USV, orange). The total number of calls recorded under the respective condition is displayed at the bottom of each pie chart. Opposite-sex dyads: n = 5.

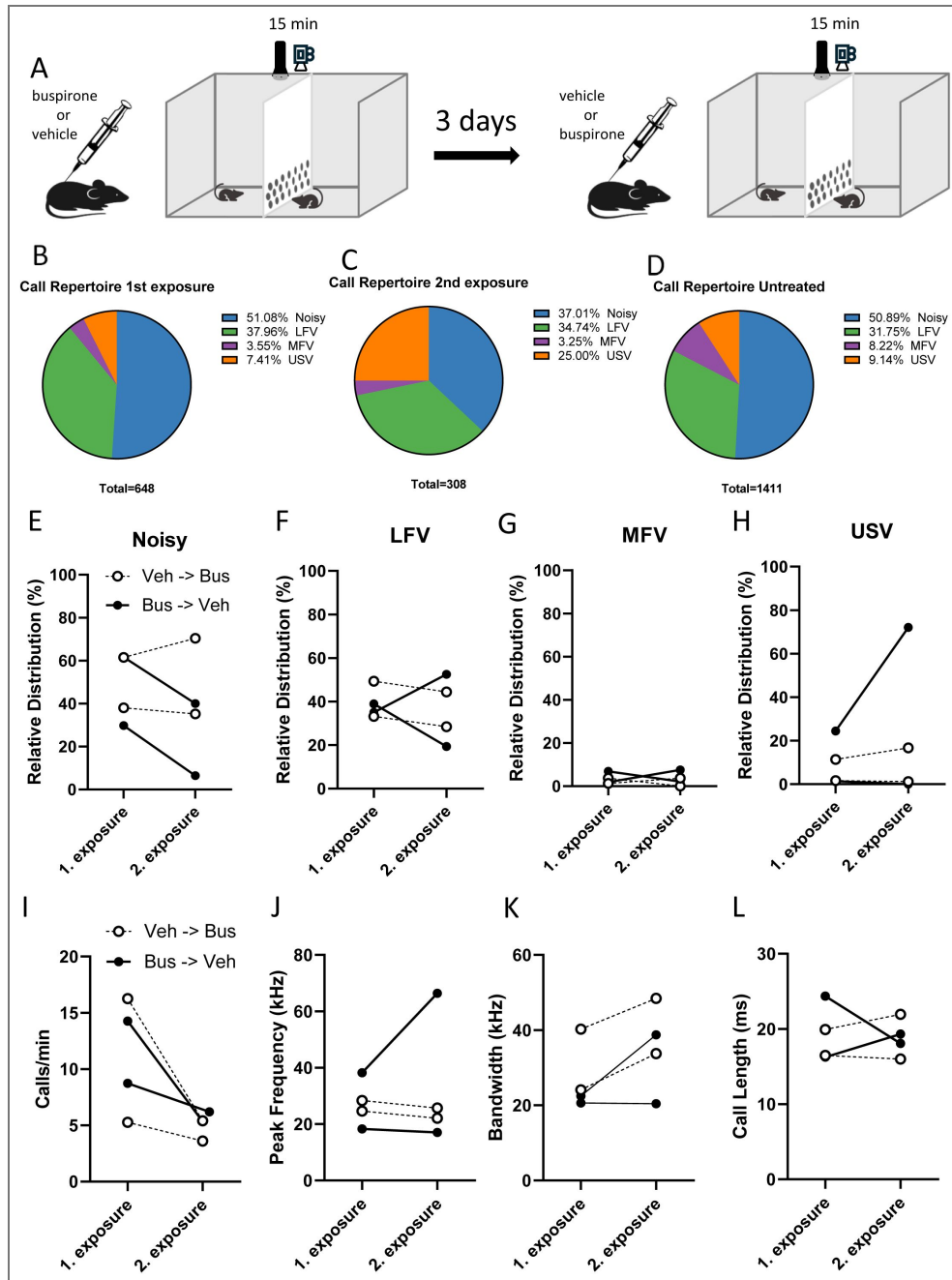


Figure 8—figure supplement 1. Repeated exposure to the experimental context affects the call repertoire used by separated female same-sex dyads

(A) Schematic depiction of the separation context of experiment design. (B–D) Pie charts display the total call repertoire of separated female same-sex dyads treated with either vehicle or buspirone recorded during their first exposure to the experimental context (B), during their second exposure to the experimental context (C), and of untreated female same-sex dyads’ first and only exposure (D). The total number of recorded calls is displayed at the bottom of each pie chart. Noisy calls (blue), low frequency vocalizations (LFV, green), middle frequency vocalizations (MFV, purple), and ultrasonic vocalizations (USV, orange). (E–H) Quantification of call class distribution during first and second exposure to the experimental context for Noisy (E), LFV (F), MFV (G), and USV (H) calls. Female dyads that were treated with vehicle before the first exposure and buspirone before the second exposure are represented by white dots. Female dyads that were treated first with buspirone and with vehicle before the second exposure are represented by black dots. Each dot represents the proportion of the respective call class of one female same-sex dyad. (I–L) Quantification of spectro-temporal call parameters: call rate (I), peak frequency (J), bandwidth (K), and call length (L). Each dot represents the average of the respective spectro-temporal call parameter of one female same-sex dyad. Vehicle → buspirone dyads: n = 2, buspirone → vehicle dyads: n = 2.

Data availability

All single calls and their spectrograms are deposited at <https://zenodo.org/records/17581239>. All data used for mouse-to-mouse distances and body angles are deposited at <https://zenodo.org/records/17491957>. All data used for VGG16-feature extraction from spectrograms and UMAP are deposited at <https://zenodo.org/records/17493153>. Data will be publicly available. The code for VGG16-feature extraction is available at <https://github.com/tmb404/MouseCallClustering>. The code for analysis of mouse-to-mouse distances and body angles is available at <https://github.com/tmb404/MouseBehav>. All other data generated or analyzed during this study are included in the manuscript and supporting files; source data and code files have been provided for all figures.

Acknowledgements

We thank Virginia M. Baatz, Johanna M. Kube, Julia Freitag, and Luna Reimer for assistance with experimentation. This work was supported by the German Center for the Protection of Laboratory Animals (Bf3R) under award number 60-0102-01.P615. M. Wojcik was awarded the Elsa Neumann Scholarship.

Additional information

CRedit authorship contribution statement

Daniel Breslav: Conceptualization, Methodology, Data Curation, Investigation, Formal Analysis, Visualization, Writing – Original Draft, Writing – Review & Editing

Michal Wojcik: Formal Analysis, Visualization, Writing – Original Draft, Writing – Review & Editing, Funding Acquisition

Ursula Koch: Conceptualization, Methodology, Writing – Review & Editing, Supervision, Project Administration, Resources

Thorsten Becker: Conceptualization, Methodology, Investigation, Formal Analysis, Data Curation, Visualization, Writing – Original Draft, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition

Funding

Funder	Grant reference number	Author
German Center for the Protection of Laboratory Animals (Bf3R)	60-0102-01.P615	Thorsten Becker
DRS-NaFöG (Elsa Neumann Stipendium)		Michal P Wojcik

Author ORCID iDs

Daniel Breslav: <https://orcid.org/0009-0008-4171-185X>

Michal Wojcik: <https://orcid.org/0000-0001-9464-3266>

Ursula Koch: <https://orcid.org/0000-0002-7550-1622>

Thorsten Becker: <https://orcid.org/0000-0002-4814-3794>

Additional files

Source data 1. Archive containing .xlsx files for each figure.

Source code 1. MatLab script used for the generation of line plots in Fig. 5.

References

- Arriaga G, Jarvis ED** (2013) Mouse vocal communication system: are ultrasounds learned or innate?. *Brain Lang* **124**:96-116 <https://doi.org/10.1016/j.bandl.2012.10.002> | [PubMed](#)
- Burke K, Screven LA, Dent ML** (2018) CBA/Caj mouse ultrasonic vocalizations depend on prior social experience. *PLoS ONE* **13**:e0197774 <https://doi.org/10.1371/journal.pone.0197774> | [PubMed](#)
- Caruso A, Marconi MA, Scattoni ML, Ricceri L** (2022) Ultrasonic vocalizations in laboratory mice: strain, age, and sex differences. *Genes Brain Behav* **21**:e12815 <https://doi.org/10.1111/gbb.12815> | [PubMed](#)
- Castellucci GA, Calbick D, McCormick D** (2018) The temporal organization of mouse ultrasonic vocalizations. *PLoS ONE* **13**:e0199929 <https://doi.org/10.1371/journal.pone.0199929> | [PubMed](#)
- Chabout J, Sarkar A, Dunson DB, Jarvis ED** (2015) Male mice song syntax depends on social contexts and influences female preferences. *Front Behav Neurosci* **9**:76 <https://doi.org/10.3389/fnbeh.2015.00076> | [PubMed](#)
- Chabout J, Serreau P, Ey E, Bellier L, Aubin T, Bourgeron T, Granon S** (2012) Adult male mice emit context-specific ultrasonic vocalizations that are modulated by prior isolation or group rearing environment. *PLoS ONE* **7**:e29401 <https://doi.org/10.1371/journal.pone.0029401> | [PubMed](#)
- Charlton BD, Watchorn DJ, Whisson DA** (2017) Subharmonics increase the auditory impact of female koala rejection calls. *Ethology* **123**:571-579 <https://doi.org/10.1111/eth.12628>
- Chen C, Rodriguez FC, Read HL, Escabí MA** (2012) Spectrotemporal sound preferences of neighboring inferior colliculus neurons: implications for local circuitry and processing. *Front Neural Circuits* **6**:62 <https://doi.org/10.3389/fncir.2012.00062> | [PubMed](#)
- Chen J, Markowitz JE, Lilascharoen V, Taylor S, Sheurpukdi P, Keller JA, Jensen JR, Lim BK, Datta SR, Stowers L** (2021) Flexible scaling and persistence of social vocal communication. *Nature* <https://doi.org/10.1038/s41586-021-03403-8> | [PubMed](#)
- Cohn R, Holm E** (2021) Unsupervised Machine Learning Via Transfer Learning and k-Means Clustering to Classify Materials Image Data. *Integr Mater Manuf Innov* **10**:231-244 <https://doi.org/10.1007/s40192-021-00205-8>
- Crimmins BE, Lingawi NW, Chieng BC, Leung BK, Maren S, Laurent V** (2023) Basal forebrain cholinergic signaling in the basolateral amygdala promotes strength and durability of fear memories. *Neuropsychopharmacology* **48**:605-614 <https://doi.org/10.1038/s41386-022-01427-w> | [PubMed](#)
- de Chaumont F, Lemièrre N, Coqueran S, Bourgeron T, Ey E.** (2021) LMT USV Toolbox, a Novel Methodological Approach to Place Mouse Ultrasonic Vocalizations in Their Behavioral Contexts-A Study in Female and Male C57BL/6j Mice and in Shank3 Mutant Females. *Front Behav Neurosci* **15**:735920 <https://doi.org/10.3389/fnbeh.2021.735920> | [PubMed](#)
- de la Zerda SH, Netser S, Magalnik H, Briller M, Marzan D, Glatt S, Abergel Y, Wagner S.** (2022) Social recognition in laboratory mice requires integration of behaviorally-induced somatosensory, auditory and olfactory cues. *Psychoneuroendocrinology* **143**:105859 <https://doi.org/10.1016/j.psyneuen.2022.105859> | [PubMed](#)
- Errijgers V, Van Dam D, Gantois I, Van Ginneken CJ, Grossman AW, D'Hooge R, De Deyn PP, Kooy RF.** (2007) FVB.129P2-Pde6b(+) Tyr(c-ch)/Ant, a sighted variant of the FVB/N mouse strain suitable for behavioral analysis. *Genes Brain Behav* **6**:552-557 <https://doi.org/10.1111/j.1601-183X.2006.00282.x> | [PubMed](#)
- Ferhat A-T, Torquet N, Le Sourd A-M, de Chaumont F, Olivo-Marin J-C, Faure P, Bourgeron T, Ey E.** (2016) Recording Mouse Ultrasonic Vocalizations to Evaluate Social Communication. *Journal of Visualized Experiments* <https://doi.org/10.3791/53871> | [PubMed](#)
- Finton CJ, Keesom SM, Hood KE, Hurley LM** (2017) What's in a squeak? Female vocal signals predict the sexual behaviour of male house mice during courtship. *Animal Behaviour* **126**:163-175 <https://doi.org/10.1016/j.anbehav.2017.01.021>

- Gadziola MA**, Grimsley JMS, Faure PA, Wenstrup JJ (2012) Social vocalizations of big brown bats vary with behavioral context. *PLoS One* **7**:e44550 <https://doi.org/10.1371/journal.pone.0044550> | [PubMed](#)
- Garcia-Pino E**, Gessele N, Koch U (2017) Enhanced Excitatory Connectivity and Disturbed Sound Processing in the Auditory Brainstem of Fragile X Mice. *J Neurosci* **37**:7403-7419 <https://doi.org/10.1523/JNEUROSCI.2310-16.2017> | [PubMed](#)
- Ghasemahmad Z**, Mrvelj A, Panditi R, Sharma B, Perumal KD, Wenstrup JJ (2024) Emotional vocalizations alter behaviors and neurochemical release into the amygdala. *eLife* **12**:RP88838 <https://doi.org/10.7554/eLife.88838> | [PubMed](#)
- Goffnet J**, Brudner S, Mooney R, 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> | [PubMed](#)
- Gourbal BEF**, Barthelemy M, Petit G, Gabrion C (2004) Spectrographic analysis of the ultrasonic vocalisations of adult male and female BALB/c mice. *Naturwissenschaften* **91**:381-385 <https://doi.org/10.1007/s00114-004-0543-7> | [PubMed](#)
- Grimsley JMS**, Monaghan JJM, Wenstrup JJ (2011) Development of social vocalizations in mice. *PLoS ONE* **6**:e17460 <https://doi.org/10.1371/journal.pone.0017460> | [PubMed](#)
- Grimsley JMS**, Sheth S, Vallabh N, Grimsley CA, Bhattal J, Latsko M, Jasnow A, Wenstrup JJ (2016) Contextual Modulation of Vocal Behavior in Mouse: Newly Identified 12 kHz “Mid-Frequency” Vocalization Emitted during Restraint. *Front Behav Neurosci* **10**:38 <https://doi.org/10.3389/fnbeh.2016.00038> | [PubMed](#)
- Hanson JL**, Hurley LM (2012) Female presence and estrous state influence mouse ultrasonic courtship vocalizations. *PLoS ONE* **7**:e40782 <https://doi.org/10.1371/journal.pone.0040782> | [PubMed](#)
- Ho MK**, Li X, Wang J, Ohmen JD, Friedman RA (2014) FVB/NJ mice demonstrate a youthful sensitivity to noise-induced hearing loss and provide a useful genetic model for the study of neural hearing loss. *Audiol Neurotol Extra* **4**:1-11 <https://doi.org/10.1159/000357770> | [PubMed](#)
- Hoier S**, Pfeifle C, von Merten S, Linnenbrink M. (2016) Communication at the Garden Fence--Context Dependent Vocalization in Female House Mice. *PLoS ONE* **11**:e0152255 <https://doi.org/10.1371/journal.pone.0152255> | [PubMed](#)
- Holy TE**, Guo Z (2005) Ultrasonic songs of male mice. *PLoS Biol* **3**:e386 <https://doi.org/10.1371/journal.pbio.0030386> | [PubMed](#)
- Hood KE**, Hurley LM (2023) Listening to your partner: serotonin increases male responsiveness to female vocal signals in mice. *Front Hum Neurosci* **17**:1304653 <https://doi.org/10.3389/fnhum.2023.1304653> | [PubMed](#)
- Hood KE**, Long E, Navarro E, Hurley LM (2023) Playback of broadband vocalizations of female mice suppresses male ultrasonic calls. *PLoS One* **18**:e0273742 <https://doi.org/10.1371/journal.pone.0273742> | [PubMed](#)
- Hosseini M**, Rodriguez G, Guo H, Lim HH, Plourde É. (2021) The effect of input noises on the activity of auditory neurons using GLM-based metrics. *J Neural Eng* **18** <https://doi.org/10.1088/1741-2552/abe979> | [PubMed](#)
- Klaus T**, Wernisch B, Zala SM, Penn DJ (2025) Courtship vocalizations of wild house mice show highly dynamic changes and correlate with male copulatory success. *Animal Behaviour* **220**:123024 <https://doi.org/10.1016/j.anbehav.2024.11.002>
- Lachmann M**, Rippen E, Rueckert D, Schuster T, Xhepa E, von Scheidt M, Pellegrini C, Trenkwalder T, Rheude T, Stundl A, et al. (2022) Harnessing feature extraction capacities from a pre-trained convolutional neural network (VGG-16) for the unsupervised distinction of aortic outflow velocity profiles in patients with severe aortic stenosis. *Eur Heart J Digit Health* **3**:153-168 <https://doi.org/10.1093/ehjdh/ztac004> | [PubMed](#)

- Lahvis GP, Alleva E, Scattoni ML (2011) Translating mouse vocalizations: prosody and frequency modulation. *Genes Brain Behav* **10**:4-16 <https://doi.org/10.1111/j.1601-183X.2010.00603.x> | PubMed
- Lawrence BD, Simmons JA (1982) Measurements of atmospheric attenuation at ultrasonic frequencies and the significance for echolocation by bats. *J Acoust Soc Am* **71**:585-590 <https://doi.org/10.1121/1.387529> | PubMed
- Liu H-X, Lopatina O, Higashida C, Fujimoto H, Akther S, Inzhutova A, Liang M, Zhong J, Tsuji T, Yoshihara T, et al. (2013) Displays of paternal mouse pup retrieval following communicative interaction with maternal mates. *Nat Commun* **4**:1346 <https://doi.org/10.1038/ncomms2336> | PubMed
- Lupanova AS, Egorova MA (2015) Vocalization of sex partners in the house mouse (*Mus Musculus*). *J Evol Biochem Phys* **51**:324-331 <https://doi.org/10.1134/S0022093015040080>
- MacDonald A, Hebling A, Wei XP, Yackle K (2024) The breath shape controls intonation of mouse vocalizations. *eLife* **13**:RP93079 <https://doi.org/10.7554/eLife.93079> | PubMed
- Mahrt E, Agarwal A, Perkel D, Portfors C, Elemans CPH (2016) Mice produce ultrasonic vocalizations by intra-laryngeal planar impinging jets. *Curr Biol* **26**:R880-R881 <https://doi.org/10.1016/j.cub.2016.08.032> | PubMed
- Massenet M, Mathevon N, Anikin A, Briefer EF, Fitch T, Reby D (2025) Nonlinear phenomena in vertebrate vocalizations: mechanisms and communicative functions. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences* **380**:20240002 <https://doi.org/10.1098/rstb.2024.0002> | PubMed
- Mathis A, Mamidanna P, Cury KM, Abe T, Murthy VN, Mathis MW, Bethge M (2018) DeepLabCut: markerless pose estimation of user-defined body parts with deep learning. *Nat Neurosci* **21**:1281-1289 <https://doi.org/10.1038/s41593-018-0209-y> | PubMed
- Matsumoto YK, Okanoya K (2018) Mice modulate ultrasonic calling bouts according to sociosexual context. *R Soc Open Sci* **5**:180378 <https://doi.org/10.1098/rsos.180378> | PubMed
- Mehrabian A (1971) *Silent messages* Belmont: Calif: Wadsworth Pub. Co.
- Mercier S, Déaux EC, van de Waal E, Bono AEJ, Zuberbühler K. (2019) Correlates of social role and conflict severity in wild vervet monkey agonistic screams. *PLoS One* **14**:e0214640 <https://doi.org/10.1371/journal.pone.0214640> | PubMed
- Mishra AP, Peng F, Li K, Harper NS, Schnupp JWH (2021) Sensitivity of neural responses in the inferior colliculus to statistical features of sound textures. *Hear Res* **412**:108357 <https://doi.org/10.1016/j.heares.2021.108357> | PubMed
- Moles A, Costantini F, Garbugino L, Zanettini C, D'Amato FR (2007) Ultrasonic vocalizations emitted during dyadic interactions in female mice: a possible index of sociability?. *Behav Brain Res* **182**:223-230 <https://doi.org/10.1016/j.bbr.2007.01.020> | PubMed
- Moles A, Sarli C, Bartolomucci A, D'Amato FR (2008) Interaction with stressed mothers affects corticosterone levels in pups after reunion and impairs the response to dexamethasone in adult mice. *Psychoneuroendocrinology* **33**:462-470 <https://doi.org/10.1016/j.psyneuen.2008.01.003> | PubMed
- Musolf K, Hoffmann F, Penn DJ (2010) Ultrasonic courtship vocalizations in wild house mice, *Mus musculus musculus*. *Animal Behaviour* **79**:757-764 <https://doi.org/10.1016/j.anbehav.2009.12.034>
- Neunuebel JP, Taylor AL, Arthur BJ, Egnor SER (2015) Female mice ultrasonically interact with males during courtship displays. *eLife* **4** <https://doi.org/10.7554/eLife.06203> | PubMed
- Ohlemiller KK, Dahl AR, Gagnon PM (2010) Divergent aging characteristics in CBA/J and CBA/CaJ mouse cochleae. *J Assoc Res Otolaryngol* **11**:605-623 <https://doi.org/10.1007/s10162-010-0228-1> | PubMed
- Okobi DE, Banerjee A, Matheson AMM, Phelps SM, Long MA (2019) Motor cortical control of vocal interaction in neotropical singing mice. *Science* **363**:983-988 <https://doi.org/10.1126/science.aau9480> | PubMed

- Oliveira-Stahl G**, Farboud S, Sterling ML, Heckman JJ, Van Raalte B, Lenferink D, Van Der Stam A, Smeets CJLM, Fisher SE, Englitz B. (2023) High-precision spatial analysis of mouse courtship vocalization behavior reveals sex and strain differences. *Sci Rep* **13**:5219 <https://doi.org/10.1038/s41598-023-31554-3> | [PubMed](#)
- Panksepp JB**, Jochman KA, Kim JU, Koy JJ, Wilson ED, Chen Q, Wilson CR, Lahvis GP (2007) Affiliative behavior, ultrasonic communication and social reward are influenced by genetic variation in adolescent mice. *PLoS ONE* **2**:e351 <https://doi.org/10.1371/journal.pone.0000351> | [PubMed](#)
- Park CR**, Willott JF, Walton JP (2024) Age-related changes of auditory sensitivity across the life span of CBA/Caj mice. *Hear Res* **441**:108921 <https://doi.org/10.1016/j.heares.2023.108921> | [PubMed](#)
- Park J**, Choi S, Takato J, Zhao S, Harrahill A, Han B-X, Wang F (2024) Brainstem control of vocalization and its coordination with respiration. *Science* **383**:ead18081 <https://doi.org/10.1126/science.adi8081> | [PubMed](#)
- Pasch B**, Tokuda IT, Riede T (2017) Grasshopper mice employ distinct vocal production mechanisms in different social contexts. *Proc Biol Sci* **284**:20171158 <https://doi.org/10.1098/rspb.2017.1158> | [PubMed](#)
- Pessoa D**, Petrella L, Martins P, Castelo-Branco M, Teixeira C (2022) Automatic segmentation and classification of mice ultrasonic vocalizations. *The Journal of the Acoustical Society of America* **152**:266-280 <https://doi.org/10.1121/10.0012350> | [PubMed](#)
- Portfors CV** (2007) Types and functions of ultrasonic vocalizations in laboratory rats and mice. *J Am Assoc Lab Anim Sci* **46**:28-34 [PubMed](#)
- Rao RP**, Mielke F, Bobrov E, Brecht M (2014) Vocalization-whisking coordination and multisensory integration of social signals in rat auditory cortex. *eLife* **3**:e03185 <https://doi.org/10.7554/eLife.03185> | [PubMed](#)
- Reby D**, Charlton BD (2012) Attention grabbing in red deer sexual calls. *Anim Cogn* **15**:265-270 <https://doi.org/10.1007/s10071-011-0451-0> | [PubMed](#)
- Riede T**, Kobrina A, Bone L, Darwaiz T, Pasch B (2022) Mechanisms of sound production in deer mice (*Peromyscus* spp.). *J Exp Biol* **225**:jeb243695 <https://doi.org/10.1242/jeb.243695> | [PubMed](#)
- Roberts LH** (1975) The rodent ultrasound production mechanism. *Ultrasonics* **13**:83-88 [https://doi.org/10.1016/0041-624x\(75\)90052-9](https://doi.org/10.1016/0041-624x(75)90052-9) | [PubMed](#)
- Ronald KL**, Zhang X, Morrison MV, Miller R, Hurley LM (2020) Male mice adjust courtship behavior in response to female multimodal signals. *PLoS ONE* **15**:e0229302 <https://doi.org/10.1371/journal.pone.0229302> | [PubMed](#)
- Ruat J**, Genewsky AJ, Heinz DE, Kaltwasser SF, Canteras NS, Czisch M, Chen A, Wotjak CT (2022) Why do mice squeak? Toward a better understanding of defensive vocalization. *iScience* **25**:104657 <https://doi.org/10.1016/j.isci.2022.104657> | [PubMed](#)
- Sainburg T**, Thielk M, Gentner TQ (2020) Finding, visualizing, and quantifying latent structure across diverse animal vocal repertoires. *PLoS Comput Biol* **16**:e1008228 <https://doi.org/10.1371/journal.pcbi.1008228> | [PubMed](#)
- Sangiameo DT**, Warren MR, Neunuebel JP (2020) Ultrasonic signals associated with different types of social behavior of mice. *Nature Neuroscience* <https://doi.org/10.1038/s41593-020-0584-z> | [PubMed](#)
- Scattoni ML**, Crawley J, Ricceri L (2009) Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. *Neurosci Biobehav Rev* **33**:508-515 <https://doi.org/10.1016/j.neubiorev.2008.08.003> | [PubMed](#)
- Scattoni ML**, Gandhi SU, Ricceri L, Crawley JN (2008) Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. *PLoS One* **3**:e3067 <https://doi.org/10.1371/journal.pone.0003067> | [PubMed](#)
- Schleidt WM** (1951) [Tones of high frequency in mice]. *Experientia* **7**:65-66 <https://doi.org/10.1007/BF02153830> | [PubMed](#)

- Simonyan K, Zisserman A.** (2014) Very Deep Convolutional Networks for Large-Scale Image Recognition. *arXiv* <https://doi.org/10.48550/ARXIV.1409.1556>
- Sirotin YB, Costa ME, Laplagne DA** (2014) Rodent ultrasonic vocalizations are bound to active sniffing behavior. *Front Behav Neurosci* **8**:399 <https://doi.org/10.3389/fnbeh.2014.00399> | [PubMed](#)
- Stoeger AS, Charlton BD, Kratochvil H, Fitch WT** (2011) Vocal cues indicate level of arousal in infant African elephant roars. *J Acoust Soc Am* **130**:1700-1710 <https://doi.org/10.1121/1.3605538> | [PubMed](#)
- Sugimoto H, Okabe S, Kato M, Koshida N, Shiroishi T, Mogi K, Kikusui T, Koide T** (2011) A role for strain differences in waveforms of ultrasonic vocalizations during male-female interaction. *PLoS ONE* **6**:e22093 <https://doi.org/10.1371/journal.pone.0022093> | [PubMed](#)
- Tehrani M, Shanbhag S, Huyck JJ, Patel R, Kazimierski D, Wenstrup JJ** (2024) The Mouse Inferior Colliculus Responds Preferentially to Non-Ultrasonic Vocalizations. *eNeuro* **11**:ENEURO.0097-24.2024 <https://doi.org/10.1523/ENEURO.0097-24.2024> | [PubMed](#)
- Tschida K, Michael V, Takatoh J, Han B-X, Zhao S, Sakurai K, Mooney R, Wang F** (2019) A Specialized Neural Circuit Gates Social Vocalizations in the Mouse. *Neuron* **103**:459-472.e4. <https://doi.org/10.1016/j.neuron.2019.05.025> | [PubMed](#)
- Veerakumar A, Head JP, Krasnow MA** (2023) A brainstem circuit for phonation and volume control in mice. *Nat Neurosci* **26**:2122-2130 <https://doi.org/10.1038/s41593-023-01478-2> | [PubMed](#)
- Venkatraman A, Bretl M, Kim S-I, Christensen L, Kelm-Nelson CA, Ciucci MR, Thibeault SL** (2024) Stress-Induced Ultrasonic Vocalization in Laboratory Rats and Mice: A Scoping Review. *Brain Sci* **14**:1109 <https://doi.org/10.3390/brainsci14111109> | [PubMed](#)
- Wang H, Liang S, Burgdorf J, Wess J, Yeomans J** (2008) Ultrasonic vocalizations induced by sex and amphetamine in M2, M4, M5 muscarinic and D2 dopamine receptor knockout mice. *PLoS One* **3**:e1893 <https://doi.org/10.1371/journal.pone.0001893> | [PubMed](#)
- White NR, Prasad M, Barfield RJ, Nyby JG** (1998) 40- and 70-kHz vocalizations of mice (*Mus musculus*) during copulation. *Physiol Behav* **63**:467-473 [https://doi.org/10.1016/s0031-9384\(97\)00484-8](https://doi.org/10.1016/s0031-9384(97)00484-8) | [PubMed](#)
- Williams WO, Riskin DK, Mott AKM** (2008) Ultrasonic sound as an indicator of acute pain in laboratory mice. *J Am Assoc Lab Anim Sci* **47**:8-10 [PubMed](#)
- Yamauchi T, Yoshioka T, Yamada D, Hamano T, Ohashi M, Matsumoto M, Iio K, Ikeda M, Kamei M, Otsuki T, et al.** (2022) Cold-Restraint Stress-Induced Ultrasonic Vocalization as a Novel Tool to Measure Anxiety in Mice. *Biol Pharm Bull* **45**:268-275 <https://doi.org/10.1248/bpb.b21-00776> | [PubMed](#)
- Zala SM, Reitschmidt D, Noll A, Balazs P, Penn DJ** (2017) Sex-dependent modulation of ultrasonic vocalizations in house mice (*Mus musculus musculus*). *PLoS One* **12**:e0188647 <https://doi.org/10.1371/journal.pone.0188647> | [PubMed](#)
- Ziobro P, Woo Y, He Z, Tschida K** (2024) Midbrain neurons important for the production of mouse ultrasonic vocalizations are not required for distress calls. *Curr Biol* **34**:1107-1113.e3. <https://doi.org/10.1016/j.cub.2024.01.016> | [PubMed](#)
- Breslav D, Wojcik M, Koch U, Becker T** (2025) Separation slang - Laboratory mice use low-frequency call repertoire during physical separation - Data for mouse-to-mouse distance and body angles. Zenodo. <https://doi.org/10.5281/zenodo.17491956>
- Breslav D, Wojcik M, Koch U, Becker T** (2025) Separation slang - Laboratory mice use low-frequency call repertoire during physical separation - VGG16-extracted call spectrogram features and UMAP plot. Zenodo. <https://doi.org/10.5281/zenodo.17493152>
- Breslav D, Wojcik M, Koch U, Becker T** (2025) Separation slang - Laboratory mice use low-frequency call repertoire during physical separation - Audio recordings and spectrograms of single calls. Zenodo. <https://doi.org/10.5281/zenodo.17581239>

Peer reviews

Reviewer #1 (Public review):

Summary:

Adult laboratory mice produce ultrasonic vocalizations during free social interactions, as well as lower-frequency, voiced calls (squeaks) during aversive contexts. The question of whether mice possess a more complex repertoire of vocalizations has been of great interest to scientists studying rodent vocal behavior. In the current study, the authors analyze the rates and acoustic features of vocalizations produced by pairs of mice that are allowed to interact across a barrier, which prevents direct physical interaction. In this context, they find that same-sex (but not opposite-sex) pairs of mice produce vocalizations that are lower in frequency than the typical 70 kHz ultrasonic vocalizations produced during free interactions and that are also distinct from squeaks. These lower frequency vocalizations were observed in both male-male and female-female pairs, as well as in same-sex pairs from multiple mouse strains. The authors also report that call rates and acoustic features are not affected in male-male pairs that have been treated with the anxiolytic drug buspirone, suggesting that anxiety is not a major driver of vocalization in this behavioral context.

Strengths:

- (1) The observation that same-sex pairs of mice produce lower frequency (<70 kHz) vocalizations in this behavioral context is novel.
- (2) The consideration of multiple types of pairs (female-female, male-male, and female-male), as well as the inclusion of multiple strains of mice and barriers with different hole diameters, are all strengths of the study.
- (3) The authors include detailed analyses of vocalization acoustic features, as well as detailed tracking of mouse positions relative to the barrier.

Weaknesses:

The categorization applied to vocalizations based on their mean frequencies is poorly supported and ignores the distinction in laryngeal production mechanism between voiced and ultrasonic vocalizations. Specifically, the authors are likely lumping together voiced and ultrasonic vocalizations into their "low frequency" (< 30 kHz) category, while they reserve the term "ultrasonic" exclusively for the subset of ultrasonic vocalizations with the highest mean frequencies (> 50 kHz). This categorization scheme also does not align well with past work on lower frequency rodent vocalizations, which complicates the comparison of the present findings to that past work.

In some analyses, the authors report that different groups of mice produce different relative proportions of vocalization types (as defined by mean frequency) but then compare acoustic features of vocalizations between groups after pooling all vocalizations together. The analyses of acoustic features conducted in this way may be confounded by the different proportions of vocalization types across groups.

<https://doi.org/10.7554/eLife.109605.1.sa2>

Reviewer #2 (Public review):

Summary:

In this manuscript, the authors examine vocal communication during same-sex dyadic interactions in mice, comparing periods of physical separation (with limited sensory access) to direct social contact. They report that separation dramatically alters the vocal repertoire, shifting it away from canonical ultrasonic vocalizations (USVs) toward low-frequency vocalizations (LFVs) and broadband "noisy" calls. While LFVs and noisy calls have been described previously, largely in aversive contexts, this study provides a detailed, systematic characterization of these vocalizations during social interactions, thereby extending prior work.

The authors explore several experimental manipulations and analyses, including divider hole size, strain and sex differences, anxiolytic drug treatment, and correlations with spatial proximity, to infer potential functions of these call types. Although the dataset is rich, the results are largely descriptive, and many conclusions remain tentative. Several experimental variables are not fully controlled, and in some cases, the interpretation exceeds what the data can clearly support. Nonetheless, with improved experimental framing, additional analyses of existing data, and a clearer discussion of limitations, this work has the potential to make a valuable contribution by broadening the field's focus beyond USVs to understand a wider vocal repertoire relevant to social context.

Strengths:

Much work on mouse vocal communication focuses almost exclusively on USVs. This manuscript convincingly demonstrates that non-USV vocalizations (LFVs and noisy calls) are prominent and systematically modulated by social context, highlighting an underappreciated dimension of mouse communication. Furthermore, the authors employ several experimental manipulations, including sensory access, strain, sex, and pharmacological treatment, to assess changes in vocalization repertoire. This provides a valuable resource for the field and reveals robust context dependence of vocalization. The discussion is thoughtful and integrative, particularly in its consideration of potential communicative roles of LFVs and noisy calls and their relationship to sensory constraints and signal propagation, although these ideas will require further experimental validation.

Weaknesses:

There are several concerns regarding experimental design and data interpretation that could be addressed to strengthen the manuscript.

(1) The terminology used for vocalization types is confusing and needs better clarification. The authors refer to Grimsley et al. (2016) multiple times, yet they use the same names for their vocalizations while applying different definitions. This makes it very difficult to compare the two papers. Since this study and Grimsley et al. use different mouse strains (FVB vs CBA), a direct comparison of absolute frequencies may also not be appropriate. Please explicitly clarify the definitions of the call types (e.g., frequency range, voiced vs. USV) and explain how they relate to those in the previous study earlier in the manuscript.

(2) In the initial experiment, mice always experience separation first (15 minutes), followed by unification (5 minutes), using novel same-sex dyads. Multiple factors besides physical contact could influence vocalization across this sequence, including habituation to the arena, reduced anxiety over time, or increasing familiarity with the partner despite physical separation. It is unclear whether the authors have tested the reverse order (unification first, followed by separation). If not, this limitation should be explicitly acknowledged. In addition, examining whether vocalizations or behaviors change over the course of the 15-minute separation period, for example, by comparing early vs late phases, could help disentangle effects of habituation from those of physical separation per se.

(3) The conclusion that separation-induced LFVs are unlikely to be anxiety-driven may

overinterpret the buspirone experiment (Figure 8). Vehicle injections themselves produced large changes in call rate and call-type distribution, raising concerns about stress or arousal induced by the injection procedure. Comparisons between buspirone-treated animals and untreated animals are therefore problematic, as these groups differ in their experimental histories, including the number of exposures. The manuscript would benefit from independent measures confirming the anxiolytic efficacy of buspirone compared to vehicle injection in this paradigm, such as behavioral readouts of anxiety. In addition, the experimental design requires a clearer description. It is not always clear whether the same dyads were tested twice, or how social familiarity, contextual familiarity, and habituation to injections were handled. Male data comparing first and second exposures should also be included as supplementary figures to allow direct comparison with the excluded female dataset.

(4) The idea that noisy calls function to attract conspecific attention is intriguing. However, in Figure 5, all call types, including LFVs and USVs, are most likely to occur when mice are already in close proximity during separation, which seems inconsistent with a long-distance signaling role. Analyses of the temporal relationship between vocalizations and behavior would strengthen this claim. For example, it would be informative to test whether bouts of noisy calls precede approach behavior or a reduction in inter-animal distance. Examining whether calls occur before, during, or after orientation toward the partner could further clarify whether these vocalizations actively modulate social behavior.

(5) The effects of divider hole size on vocal repertoire are striking but difficult to interpret. Unexpectedly, small holes and no holes yield similar call distributions, whereas large holes produce a markedly different profile dominated by LFVs, which also differs from free interactions. If large holes allow greater tactile or close-range interaction, the reduction in USVs and MFV is counterintuitive. Incorporating behavioral metrics such as distance, orientation, or specific interaction types alongside call classification would greatly aid interpretation and help link vocal output to interaction quality rather than divider type alone.

(6) Throughout the study, vocalizations are pooled across both animals in the dyad. Because the arena is neutral rather than a home cage, either animal could be initiating vocalization. Assigning calls to individuals, where possible, using spatial or acoustic cues, would substantially strengthen functional interpretations. Even limited analyses, e.g., identifying which animal vocalizes first or whether calls precede approach by the partner, could provide important insight into the communicative role of different call types.

<https://doi.org/10.7554/eLife.109605.1.sa1>

Author Response:

Public Reviews:

Reviewer #1 (Public review):

Summary:

Adult laboratory mice produce ultrasonic vocalizations during free social interactions, as well as lower-frequency, voiced calls (squeaks) during aversive contexts. The question of whether mice possess a more complex repertoire of vocalizations has been of great interest to scientists studying rodent vocal behavior. In the current study, the authors analyze the rates and acoustic features of vocalizations produced by pairs of mice that are allowed to interact across a barrier, which prevents direct physical interaction. In this context, they find that same-sex (but not opposite-sex) pairs of mice produce vocalizations that are lower in frequency than the typical 70 kHz ultrasonic vocalizations

produced during free interactions and that are also distinct from squeaks. These lower frequency vocalizations were observed in both male-male and female-female pairs, as well as in same-sex pairs from multiple mouse strains. The authors also report that call rates and acoustic features are not affected in male-male pairs that have been treated with the anxiolytic drug buspirone, suggesting that anxiety is not a major driver of vocalization in this behavioral context.

Strengths:

(1) The observation that same-sex pairs of mice produce lower frequency (<70 kHz) vocalizations in this behavioral context is novel.

(2) The consideration of multiple types of pairs (female-female, male-male, and female-male), as well as the inclusion of multiple strains of mice and barriers with different hole diameters, are all strengths of the study.

(3) The authors include detailed analyses of vocalization acoustic features, as well as detailed tracking of mouse positions relative to the barrier.

Weaknesses:

The categorization applied to vocalizations based on their mean frequencies is poorly supported and ignores the distinction in laryngeal production mechanism between voiced and ultrasonic vocalizations. Specifically, the authors are likely lumping together voiced and ultrasonic vocalizations into their "low frequency" (< 30 kHz) category, while they reserve the term "ultrasonic" exclusively for the subset of ultrasonic vocalizations with the highest mean frequencies (> 50 kHz). This categorization scheme also does not align well with past work on lower frequency rodent vocalizations, which complicates the comparison of the present findings to that past work.

We thank the reviewer for their assessment. Firstly, we did not use mean frequencies, but peak frequencies of each single call.

The distinction between 'voiced' and 'whistled' vocalizations based on their spectral-temporal features is hardly possible. While evidence in form of audio recordings made from both deer mouse and grasshopper mouse in helium-enriched air suggests vocalizations with lower fundamental frequency being 'voiced' (Pasch et al., 2017; Riede et al., 2022), a computational model considering the laryngeal anatomy of *Mus musculus* estimates fundamental frequencies of vocalizations at subglottal phonation threshold pressures usual for USVs to be in the range of 1 – 5 kHz and approaching 10 kHz for higher subglottal pressures usually found in the production of 'voiced' vocalizations (Pasch et al., 2017). Furthermore, a recent study in the singing mouse (*Scotinomys teguina*) found minimal fundamental frequencies of single song notes, produced by a whistle mechanism, to be about 4 kHz (Zheng et al., 2025). Thus, the presence of low fundamental (peak) frequencies in mouse vocalizations alone appears to be insufficient for deducing the production mechanism of these vocalizations.

We did not observe differences in acoustic features clearly separating our 'LFV' calls into two groups suggestive of different production mechanisms. Thus, we cannot rule out that our 'LFV' class contains vocalizations produced by different mechanisms. However, we did not observe any squeaks in our experiments and can therefore rule out that this prominent type of 'voiced' call is lumped together with other calls in the 'LFV' calls.

While the questions regarding production mechanism, the neurocircuitry involved, and the context-dependent choice of which mechanism to use is intriguing/enticing, the distinction between 'voiced' and 'whistled' vocalizations lies beyond the scope of our manuscript. Instead, the neurocircuitry involved in mouse vocalization production, particularly USVs and squeaks has been revealed by other laboratories. Optogenetical activation of RAM Nts

neurons elicited emission of both audible vocalizations (fundamental frequencies of 10 kHz and below) and USVs in awake mice in a stimulus-dependent manner (Veerakumar et al., 2023). Furthermore, optogenetical activation of RAM-vocalization neurons led to immediate measurable adduction of vocal folds and emission of canonical USVs (Park et al., 2024). While different populations of PAG neurons are responsible for the production both squeaks and USVs (Ziobro et al., 2024), the two input streams seem to converge on RAM vocalization neurons, as silencing the output of these neurons abolished both squeak and USV emission completely (Park et al., 2024). Thus, while near complete closing of the vocal folds is necessary for the production of canonical USVs (Mahrt et al., 2016; Park et al., 2024), it is not clear which degree of vocal fold opening would result in what fundamental frequencies.

We will add a paragraph on this issue to the discussion in the next version of the manuscript.

In some analyses, the authors report that different groups of mice produce different relative proportions of vocalization types (as defined by mean frequency) but then compare acoustic features of vocalizations between groups after pooling all vocalizations together. The analyses of acoustic features conducted in this way may be confounded by the different proportions of vocalization types across groups.

We displayed the relative distribution of the different call classes demonstrating that 80% of the call repertoire during the separation consisted of noisy calls and 'LFV'. Thus, the per individual averaged acoustic features e.g. peak frequency would be predominantly shaped by the features of these two call classes. However, we agree with the reviewer's criticism and will provide a more detailed display and analysis of the acoustic features of each call class.

Reviewer #2 (Public review):

Summary:

In this manuscript, the authors examine vocal communication during same-sex dyadic interactions in mice, comparing periods of physical separation (with limited sensory access) to direct social contact. They report that separation dramatically alters the vocal repertoire, shifting it away from canonical ultrasonic vocalizations (USVs) toward low-frequency vocalizations (LFVs) and broadband "noisy" calls. While LFVs and noisy calls have been described previously, largely in aversive contexts, this study provides a detailed, systematic characterization of these vocalizations during social interactions, thereby extending prior work.

The authors explore several experimental manipulations and analyses, including divider hole size, strain and sex differences, anxiolytic drug treatment, and correlations with spatial proximity, to infer potential functions of these call types. Although the dataset is rich, the results are largely descriptive, and many conclusions remain tentative. Several experimental variables are not fully controlled, and in some cases, the interpretation exceeds what the data can clearly support. Nonetheless, with improved experimental framing, additional analyses of existing data, and a clearer discussion of limitations, this work has the potential to make a valuable contribution by broadening the field's focus beyond USVs to understand a wider vocal repertoire relevant to social context.

Strengths:

Much work on mouse vocal communication focuses almost exclusively on USVs. This manuscript convincingly demonstrates that non-USV vocalizations (LFVs and noisy calls) are prominent and systematically modulated by social context, highlighting an underappreciated dimension of mouse communication. Furthermore, the authors employ several experimental manipulations, including sensory access, strain, sex, and pharmacological treatment, to assess changes in vocalization repertoire. This provides a valuable resource for the field and reveals robust context dependence of vocalization.

The discussion is thoughtful and integrative, particularly in its consideration of potential communicative roles of LFVs and noisy calls and their relationship to sensory constraints and signal propagation, although these ideas will require further experimental validation.

Weaknesses:

There are several concerns regarding experimental design and data interpretation that could be addressed to strengthen the manuscript.

(1) The terminology used for vocalization types is confusing and needs better clarification. The authors refer to Grimsley et al. (2016) multiple times, yet they use the same names for their vocalizations while applying different definitions. This makes it very difficult to compare the two papers. Since this study and Grimsley et al. use different mouse strains (FVB vs CBA), a direct comparison of absolute frequencies may also not be appropriate. Please explicitly clarify the definitions of the call types (e.g., frequency range, voiced vs. USV) and explain how they relate to those in the previous study earlier in the manuscript.

The existence and use of various distinct classification systems for mouse vocalizations is well known and the need to agree on a common classification system is consensus in the field. Thus, it was not our intention to complicate mouse call classification even more.

Grimsley et al. (2016) reserve the ‘low frequency’ band solely for squeaks (or “low frequency harmonics”). Hence, it appears straight forward to name mouse calls with “mean dominant frequencies” falling between squeaks and USVs, “mid-frequency tonal vocalizations (MFVs)” (Grimsley et al., 2016). We did not observe the emission of squeaks in our experiments, but instead we observed tonal vocalizations in a peak frequency spectrum encompassing both squeaks and Grimsley and colleagues’ ‘MFVs’, representing the lowest peak frequencies we observed (< 32 kHz). Furthermore, we observed vocalizations in the range of 32 – 50 kHz (which were not low frequency components of canonical USVs) and of > 50 kHz (corresponding to canonical USVs). Leaning on the terminology of Grimsley and colleagues (2016), we thought it to be straightforward to name these call classes according to their location on the frequency spectrum: low frequency vocalizations (LFVs; up to 32 kHz), encompassing squeaks, but also Grimsley and colleagues’ MFVs, middle frequency vocalizations (MFVs; 32 – 50 kHz), and finally canonical USVs (> 50 kHz). Admittedly, choosing ‘MFVs’ for mouse calls with different acoustic features than those described by Grimsley and colleagues (2016) has caused unnecessary confusion. We therefore consider adapting our classification scheme for the next version of the manuscript.

Regarding the comparison of call classes between different mouse strains, strain differences of spectral-temporal features of call classes have been described for canonical USVs (e.g. Scattoni et al., 2008). However, the acoustic features as well as call repertoire are still quite comparable. Furthermore, we have additionally tested both CBA/J and C57BL/6J mice in our study confirming the presence of both noisy calls, ‘LFVs’, ‘MFVs’, and ‘USVs’ in the vocal repertoire of these two strains.

We will provide a more detailed display and analysis of the acoustic features of the call classes with the next version of the manuscript.

(2) In the initial experiment, mice always experience separation first (15 minutes), followed by unification (5 minutes), using novel same-sex dyads. Multiple factors besides physical contact could influence vocalization across this sequence, including habituation to the arena, reduced anxiety over time, or increasing familiarity with the partner despite physical separation. It is unclear whether the authors have tested the reverse order (unification first, followed by separation). If not, this limitation should be explicitly

acknowledged. In addition, examining whether vocalizations or behaviors change over the course of the 15-minute separation period, for example, by comparing early vs late phases, could help disentangle effects of habituation from those of physical separation per se.

We had not tested mice in the reverse order, beginning with 5 minutes of unification followed by 15 minutes of separation. Therefore, we acknowledge this limitation of our study and will address it explicitly in the next version of our manuscript. We appreciate the reviewer's note regarding the inclusion of vocalizations over time and aim to provide this analysis in the next version of the manuscript.

(3) The conclusion that separation-induced LFVs are unlikely to be anxiety-driven may overinterpret the buspirone experiment (Figure 8). Vehicle injections themselves produced large changes in call rate and call-type distribution, raising concerns about stress or arousal induced by the injection procedure. Comparisons between buspirone-treated animals and untreated animals are therefore problematic, as these groups differ in their experimental histories, including the number of exposures. The manuscript would benefit from independent measures confirming the anxiolytic efficacy of buspirone compared to vehicle injection in this paradigm, such as behavioral readouts of anxiety. In addition, the experimental design requires a clearer description. It is not always clear whether the same dyads were tested twice, or how social familiarity, contextual familiarity, and habituation to injections were handled. Male data comparing first and second exposures should also be included as supplementary figures to allow direct comparison with the excluded female dataset.

We agree with the reviewer's point that the injection procedure itself appeared to have an impact on vocalization behavior. In fact, we had included the 'untreated' cohort in Fig. 8 despite their different experimental history to appreciate the potential impact of injection onto vocal behavior.

Furthermore, we appreciate the reviewer's point of confirming the anxiolytic effect of buspirone treatment with further behavioral readouts and aim to provide such analysis in the next version of the manuscript.

Regarding the reviewer's query for clearer experimental design description, the same dyads were tested twice. All mice lived in groups in their home cage, however, they had not met the individual they would face during the experiment before the first experiment. We will improve the description of the experimental design addressing the reviewer's points in the next version of the manuscript.

(4) The idea that noisy calls function to attract conspecific attention is intriguing. However, in Figure 5, all call types, including LFVs and USVs, are most likely to occur when mice are already in close proximity during separation, which seems inconsistent with a long-distance signaling role. Analyses of the temporal relationship between vocalizations and behavior would strengthen this claim. For example, it would be informative to test whether bouts of noisy calls precede approach behavior or a reduction in inter-animal distance. Examining whether calls occur before, during, or after orientation toward the partner could further clarify whether these vocalizations actively modulate social behavior.

We appreciate the reviewer's remarks regarding the apparent inconsistencies between noisy calls as conspecific attraction calls and their occurrence in close mouse-to-mouse proximity. We must concede that the size of our testing arena limited the maximum distances mice could achieve. Thus, we aim to provide a more extensive analysis including approach behavior and changes of inter-animal distances for resubmission of the manuscript as suggested by the reviewer.

(5) *The effects of divider hole size on vocal repertoire are striking but difficult to interpret. Unexpectedly, small holes and no holes yield similar call distributions, whereas large holes produce a markedly different profile dominated by LFVs, which also differs from free interactions. If large holes allow greater tactile or close-range interaction, the reduction in USVs and MFV is counterintuitive. Incorporating behavioral metrics such as distance, orientation, or specific interaction types alongside call classification would greatly aid interpretation and help link vocal output to interaction quality rather than divider type alone.*

We agree with the reviewer that the interpretation of the divider-hole-size-experiment are difficult and following this reviewer's input, aim to provide additional behavioral analysis for the effect of divider hole size with the next version of the manuscript.

(6) *Throughout the study, vocalizations are pooled across both animals in the dyad. Because the arena is neutral rather than a home cage, either animal could be initiating vocalization. Assigning calls to individuals, where possible, using spatial or acoustic cues, would substantially strengthen functional interpretations. Even limited analyses, e.g., identifying which animal vocalizes first or whether calls precede approach by the partner, could provide important insight into the communicative role of different call types.*

We agree with the points raised by the reviewer regarding the importance of assigning recorded calls to the respective individual for deciphering the communicative role of different call types. Unfortunately, our system was only equipped with one condenser microphone therefore we are not able to assign calls to individual mice.

Literature:

Grimsley, J. M. S., Sheth, S., Vallabh, N., Grimsley, C. A., Bhattal, J., Latsko, M., Jasnow, A., & Wenstrup, J. J. (2016). Contextual Modulation of Vocal Behavior in Mouse: Newly Identified 12 kHz „Mid-Frequency“ Vocalization Emitted during Restraint. *Frontiers in Behavioral Neuroscience*, 10, 38. <https://doi.org/10.3389/fnbeh.2016.00038>

Mahrt, E., Agarwal, A., Perkel, D., Portfors, C., & Elemans, C. P. H. (2016). Mice produce ultrasonic vocalizations by intra-laryngeal planar impinging jets. *Current Biology: CB*, 26(19), R880–R881. <https://doi.org/10.1016/j.cub.2016.08.032>


Park, J., Choi, S., Takatoh, J., Zhao, S., Harrahill, A., Han, B.-X., & Wang, F. (2024). Brainstem control of vocalization and its coordination with respiration. *Science (New York, N.Y.)*, 383(6687), eadi8081. <https://doi.org/10.1126/science.adi8081>


Pasch, B., Tokuda, I. T., & Riede, T. (2017). Grasshopper mice employ distinct vocal production mechanisms in different social contexts. *Proceedings. Biological Sciences*, 284(1859), 20171158. <https://doi.org/10.1098/rspb.2017.1158>

Riede, T., Kobrina, A., Bone, L., Darwaiz, T., & Pasch, B. (2022). Mechanisms of sound production in deer mice (*Peromyscus* spp.). *The Journal of Experimental Biology*, 225(9), jeb243695. <https://doi.org/10.1242/jeb.243695>

Scattoni, M. L., Gandhi, S. U., Ricceri, L., & Crawley, J. N. (2008). Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. *PloS One*, 3(8), e3067. <https://doi.org/10.1371/journal.pone.0003067>

Veerakumar, A., Head, J. P., & Krasnow, M. A. (2023). A brainstem circuit for phonation and volume control in mice. *Nature Neuroscience*, 26(12), 2122–2130. <https://doi.org/10.1038/s41593-023-01478-2>

Zheng, X. M., Harpole, C. E., Davis, M. B., & Banerjee, A. (2025). Vocal repertoire expansion in singing mice by co-opting a conserved midbrain circuit node. *Current Biology: CB*, 35(23), 5762-5778.e6. <https://doi.org/10.1016/j.cub.2025.10.036> 

Ziobro, P., Woo, Y., He, Z., & Tschida, K. (2024). Midbrain neurons important for the production of mouse ultrasonic vocalizations are not required for distress calls. *Current Biology: CB*, 34(5), 1107-1113.e3. <https://doi.org/10.1016/j.cub.2024.01.016> 

<https://doi.org/10.7554/eLife.109605.1.sa0>