Acoustic features of and behavioral responses to emotionally intense mouse vocalizations

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Keywords:

acoustic communication, mating, mouse, restraint, syllables, vocalization playback

ABSTRACT

Social vocalizations contain cues that reflect the motivational state of a vocalizing animal. Once perceived, these cues may in turn affect the internal state and behavioral responses of listening animals. Using the CBA/CAJ mouse model of acoustic communication, this study examined acoustic cues that signal intensity in male-female interactions, then compared behavioral responses to intense mating vocal sequences with those from another intense behavioral context, restraint. Experiment I in this study examined behaviors and vocalizations associated with male-female social interactions. Based on several behaviors, we distinguished more general, courtship-type interactions from mating interactions involving mounting or attempted mounting behaviors. We then compared vocalizations between courtship and mating. The increase in behavioral intensity from courtship to mating was associated with altered syllable composition, more harmonic structure, lower minimum frequency, longer duration, reduced inter-syllable interval, and increased sound intensity. We then used these features to construct highly salient playback stimuli associated with mating. In Experiment II, we compared behavioral responses to playback of these mating sequences with responses to playback of aversive vocal sequences produced by restrained mice, described in previous studies. Subjects were females in estrus and males. We observed a range of behavioral responses. Some (e.g., Attending and Stretch-Attend) showed similar responses across playback type and sex, while others were context dependent (e.g., Flinching, Locomotion). Still other behaviors showed either an effect of sex (e.g., Self-Grooming, Still-and-Alert) or an interaction between playback type and sex (Escape). These results demonstrate both state-dependent features of mouse vocalizations and their effectiveness in evoking a range of behavioral responses, independent of contextual cues provided by other sensory stimuli or behavioral interactions.

INTRODUCTION

Humans and other species use vocal communication for expressing thoughts, internal state, and emotions during social interactions in order to affect the thoughts, internal states, or behaviors of listeners. Acoustic cues that shape the listener's responses are generally represented in the segmental features of vocal communication (syllables, words) and suprasegmental features that contribute to prosody, including spectral, temporal, and amplitude cues. Both the choice of syllables and the prosodic features of sounds can be affected by internal state and emotions of the sender (S. M. Brudzynski et al., 2011; Ehret, 2013; Gadziola et al., 2012; Litvin et al., 2010). Once perceived, these cues may in turn affect the internal state and behavioral responses of the listening animal. Behavioral reactions to salient and valent vocalizations can further be influenced by the listener's internal state and by previous experience with the vocalizations (Ghasemahmad et al., 2024; Okabe et al., 2010; Petersen et al., 2021; Zala et al., 2020). This study examines acoustic cues that signal intensity in male-female interactions, then compares behavioral responses to intense mating vocal sequences with those from another intense behavioral context, restraint.

In mice, like other species, cues such as syllable types and spectrotemporal characteristics convey information linked to the internal affective state of the sender. Ultrasonic vocalizations (USVs), produced during many social interactions, are modulated in spectrotemporal characteristics in context-dependent ways. In mating and in restraint, intense interactions are associated with lower ultrasonic frequencies (Gaub et al., 2016; Giddens et al., 2013; Lahvis et al., 2011; van Mersbergen & Lanza, 2019). Duration is heavily affected by the nature of social interaction: USVs emitted in a mating context are significantly longer than those emitted in restraint or isolation (Grimsley et al., 2016; Lefebvre et al., 2020), and USVs emitted during male fighting are longer than those emitted during fleeing (Sangiamo et al., 2020). Vocalizations in mice also include non-USV categories such as low frequency harmonic (LFH) calls (the mouse squeak), mid-frequency vocalizations (MFVs), and Noisy calls that are used for social communication in several behavioral contexts (Chabout et al., 2015; Finton et al., 2017; Gaub et al., 2016; Grimsley et al., 2011, 2016; Holy & Guo, 2005; Sangiamo et al., 2020). These non-USVs contain frequencies corresponding to the most sensitive part of the mouse audiogram (Ehret, 2006; Green et al., 2019; Radziwon et al., 2009) and are likely to travel further distances due to their longer wavelengths. Like USVs, these also show changes in their acoustic features in a context-dependent manner (Grimsley et al., 2016; Rendon et al., 2015).

The reception of vocal communication signals has been linked to many behavioral responses and internal state changes. For instance, rodent pups emit vocalizations that trigger maternal/paternal nest building, licking, and crouching behaviors that help nursing (S. Brudzynski, 2009; Liu et al., 2013; Scattoni et al., 2009). These calls, when produced under isolation, cause retrieval behavior in mothers (Lahvis et al., 2011; Scattoni et al., 2009; Valtcheva & Froemke, 2019). During mating behavior, females prefer vocalizing male mice over devocalized males (Pomerantz et al., 1983; Portfors & Perkel, 2014) and playback of courtship USVs triggers a female's approach (Hammerschmidt et al., 2009). Further, the patterns of USVs during social interactions can affect the behavior of socially engaged mice but not the behavior of other animals

that observe the interaction (Sangiamo et al., 2020). Such impact of vocalizations on the listener, both physiologically and behaviorally, is shared by non-USVs (Dornellas et al., 2021; Ghasemahmad et al., 2024; Niemczura et al., 2020a; Ronald et al., 2020).

While our work is informed by the extensive studies on sender vocalizations and listener responses to various vocal categories and acoustic cues, this study seeks specifically to understand how vocalizations with opposing valence, carrying prosodic and semantic information in natural sequences, can influence the behavior of the listener. Experiment I in this study distinguishes the features of vocal sequences that reflect lower and higher intensity of interactions between males and females, in order to create highly salient playback stimuli associated with mating. These are used in Experiment II, which evaluates the behavioral responses of male and female mice to highly emotional vocal sequences associated with mating and with an aversive behavioral context (restraint). We hypothesize that oppositely valent vocal sequences, carrying cues reflecting the internal state of vocalizing animals, can change the behavioral responses in a sex- and context-specific manner.

MATERIALS AND METHODS

These experiments share several features with our recently published work (Ghasemahmad et al., 2024) but differ in other ways. Both studies utilized the same mating and restraint vocal exemplars, presented in the same order, during playback experiments. The present study utilized a larger set of male-female pairs to examine vocal features associated with courtship and mating behaviors. While both studies used the same set of behaviors to assess responses to playback, each study utilized a completely different pool of subjects in playback experiments. Unlike subjects in the published work, subjects in this study did not have implanted microdialysis probes during playback.

Animals

Experimental procedures on animal subjects were approved by the Institutional Animal Care and Use Committee at Northeast Ohio Medical University. A total of 48 adult CBA/CaJ mice (Jackson Labs, p90-p180), male and female, were used for this study. Animals were maintained on a reversed dark/light cycle and experiments were performed during the dark cycle. The mice were housed in same-sex groups until the week of the experiments, during which they were singly housed. Food and water were provided *ad libitum* except during the experiment.

The estrous stage of female mice was evaluated based on vaginal smear samples obtained by sterile vaginal lavage. Samples were collected using glass pipettes filled with double distilled water, placed on a slide, stained using crystal violet, and coverslipped for microscopic examination. Estrous stage was determined by the predominant cell type: squamous epithelial cells (estrus), nucleated cornified cells (proestrus), or leukocytes (diestrus) (McLean et al., 2012). To confirm that the stage of estrous did not change during the experiment day, samples obtained prior to and after data collection on the experimental day were compared.

Acoustic methods

Vocalization recording and analysis

To record vocalizations, mice were placed in an open-topped plexiglass chamber (width, 28 cm; length, 28 cm; height, 20 cm), housed within a darkened, single-walled acoustic chamber (Industrial Acoustics, New York, NY) lined with anechoic foam (Grimsley et al., 2016; Niemczura et al., 2020). Acoustic signals were recorded using ultrasonic condenser microphones (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) connected to a multichannel amplifier and A/D converter (UltraSoundGate 416H, Avisoft Bioacoustics). The gain of each microphone was independently adjusted once per recording session to optimize the signal-to-noise ratio (SNR) while avoiding signal clipping. Acoustic signals were digitized at 500 kHz and 16-bit depth, monitored in real time with RECORDER software (Version 5.1, Avisoft Bioacoustics), and Fast Fourier Transformed (FFT) at a resolution of 512 Hz. A night vision camera (VideoSecu Infrared CCTV), centered 50 cm above the floor of the test box, recorded the behaviors synchronized with the vocal recordings (VideoBench software, DataWave Technologies, version 7).

To record mating vocalizations, 16 animals (8 male-female pairs) were used in sessions that lasted for 30 minutes. A male mouse was introduced first into the test box, followed by a female mouse 5 min later. Vocalizations were recorded using two ultrasonic microphones placed 30 cm above the floor of the recording box and 13 cm apart. See below for analysis of behaviors during vocal recordings.

To record vocalizations during restraint, six mice (4 male, 2 female) were briefly anesthetized with isoflurane and then placed in a restraint jacket as described previously (Ghasemahmad et al., 2024; Grimsley et al., 2016). Vocalizations were recorded for 30 minutes while the animal was suspended in the recording box. Since these vocalizations are usually emitted at lower intensity compared to mating vocalizations, the recording microphone was positioned 2-3 cm from the snout to obtain the best SNR.

Vocal recordings were analyzed offline using Avisoft-SASLab Pro (version 5.2.12, Avisoft Bioacoustics) with a hamming window, 1024 Hz FFT size, and an overlap percentage of 98.43. For every syllable the channel with the higher amplitude signal was extracted using a custom-written Python code and analyzed. Since automatic syllable tagging did not allow distinguishing some syllable types such as noisy calls and mid-frequency vocalizations (MFVs) from background noise, we manually tagged the start and end of each syllable, then examined spectrograms to measure several acoustic features and classify syllable types based on Grimsley and colleagues (Grimsley et al., 2011, 2016).

Vocalization playback

Vocalization playback stimuli lasting 20 min were constructed from a sequence of seven repeating stimulus blocks lasting 2:50 min each (Fig. 6B). Each block was composed of a set of vocal sequence exemplars that alternated with an equal duration of background sound (“Silence”) associated with the preceding exemplar. The exemplars were recorded during mating interactions and restraint, and selected based on high SNR, correspondence with behavioral category by video analysis, and representation of the spectrotemporal features of vocalizations emitted during mating and restraint (Ghasemahmad et al, 2024; Grimsley et al., 2016). Mating stimulus blocks contained five exemplars of vocal sequences emitted during mating interactions. These exemplars ranged in duration from 15.0 – 43.6 s. Restraint stimulus blocks included seven vocal sequences, emitted by restrained male or female mice, with durations ranging from 5.7 – 42.3 s. Across exemplars, each stimulus block associated with both mating and restraint included different sets of vocal categories (Fig. 5).

Playback sequences, i.e., exemplars, were conditioned in Adobe Audition CC (2018), adjusted to a 65 dB SNR level, then normalized to 1V peak-to-peak for the highest amplitude syllable in the sequence. This maintained relative syllable emission amplitude in the sequence. For each sequence, an equal duration of background noise (i.e., no vocal or other detected sounds) from the same recording was added at the end of that sequence (Fig. 6B). A 5-ms ramp was added at the beginning and the end of the entire sequence to avoid acoustic artifacts. A MATLAB app (EqualizIR, Sharad Shanbhag; https://github.com/TytoLogy/EqualizIR) compensated and calibrated each vocal sequence for the frequency response of the speaker system. Vocal sequences were

converted to analog signals at 500 KHz and 16-bit resolution using DataWave (DataWave SciWorks, Loveland, CO), anti-alias filtered (TDT FT6-2, fc=125KHz), amplified (HCA-800II, Parasound, San Francisco, CA), and sent to the speaker (LCY, K100, Ying Tai Audio Company, Hong Kong). Each sequence was presented at peak level equivalent to 85 dB SPL.

Behavioral methods

Behaviors during both experience and playback sessions were recorded using a night vision camera (480TVL 3.6mm, VideoSecu), centered 50 cm above the floor of the test box, and SciWorks (DataWave, VideoBench version 7) for video acquisition and analysis.

Male-female interactions

Interactions between male and female mice were video-recorded, then analyzed second-by-second. Among more general courtship interactions, we identified a set of behaviors that were mating-related, as described previously (Gaub et al., 2016; Heckman et al., 2016; Ghasemahmad et al., 2024). All vocal sequences selected as exemplars for playback of “mating” vocalizations were recorded in association with these male mating behaviors: head-sniffing, attempted mounting, or mounting (Table 1). Vocalizations during these behaviors included chevron, stepped, and complex USVs emitted with longer durations and higher repetition rates, and more LFH calls (Gaub et al., 2016; Ghasemahmad et al, 2024; Hanson & Hurley, 2012).

Experience and playback sessions

Prior to playback experiments, each animal underwent 90-min sessions on two consecutive days (Days 1 and 2) that provided both mating and restraint experiences (Fig. 6A). The order of sessions was presented in a counterbalanced pattern across subjects. For the mating experience, mounting or attempted mounting was required for the animal to be included in the remainder of the experiment. However, we did not record detailed behaviors or track estrous stage during the mating experience session.

On Day 6, the day of the playback experiment, mice were randomly assigned to either restraint or mating vocal playback groups. Animals were placed within the experimental chamber and allowed to habituate for three hours.

Behaviors were video-recorded for 40 mins, beginning 10 min before vocal playback (pre-stimulation, Pre), continuing for 20 min during playback (Vocal Playback), and including another 10 min after playback ended (post-stimulation, Post) (Fig. 6B). From the video recording, several behaviors (Table 2) were analyzed in 10-second intervals but reported in 2:50 min intervals associated with the blocks of vocal sequences. Videos were further analyzed automatically using the video tracker within VideoBench (DataWave Technologies, version 7) for speed of locomotion, distance traveled, and time spent in the periphery and center of the test arena. All video analysis was performed blind to the sex of the animal and the context of vocalizations.

Data analysis

All statistical analyses were performed using SPSS (IBM, V. 26 and 27). To examine vocalizations in different stages of mating, we used a linear mixed model to analyze the

changes of interval, duration, peak-to-peak amplitude and minimum frequency (dependent variables) of vocalizations based on mating interaction intensity, harmonicity, syllable types and the interaction of these (fixed effect). For behavioral analyses in playback experiments we used the General linear model with repeated measures for statistical comparisons of the behaviors. We used a full-factorial model of: intercept + sex + context + sex*context. Where Mauchly's test indicated that the assumption of sphericity had been violated, degrees of freedom were corrected using Greenhouse Geisser estimates of sphericity. All multiple comparisons were corrected using Bonferroni post hoc testing.

For behavioral analyses, we tested the hypotheses that the number of behaviors was differently modulated by the vocal playback type (mating and restraint), by sex of the animals, or the interaction of these two. The GLM model for testing these hypotheses used time as a within-subject factor, with vocalization context, sex, and the interaction of these and time as the between-subject factors. Dependent (response) variables included the numbers of behaviors as previously described in the behavioral analysis section.

To increase the analysis power, we focused the statistical analyses of the behaviors to the last 2:50 min block of pre-sound, first 2:50 block of vocal playback, and first 2:50 min of post playback. We chose a 2:50 analysis window based on the duration of a unique vocalization block and pre and post vocal playback windows were also chosen to match this window. Other time windows of behavioral observations, from other stimulus blocks, are shown in the figures but not used in statistical analysis.

RESULTS

The overall goal of these experiments was to examine the acoustic features of mouse social vocalizations associated with different, highly salient behavioral contexts—mating and restraint—and the behaviors in response to them. Vocalizations associated with restraint have been described previously (Ghasemahmad et al., 2024; Grimsley et al., 2016) (see Figure 1A), and the development of restraint vocal sequences as stimuli was straightforward. Vocalizations associated with courtship and mating have also been extensively described (Gaub et al., 2016; Grimsley et al., 2011a; Hanson & Hurley, 2012), but this study required the development of vocalization stimuli typical of mating interactions. Experiment 1 describes the acoustic features of vocalizations emitted during mating, distinguished from less intense male-female courtship interactions. Some aspects of this analysis have been described previously (Ghasemahmad et al., 2024). Experiment 2 describes the behavioral responses to mating and restraint vocal sequences.

Experiment 1: Acoustic features associated with intense mating interactions

We recorded behaviors and vocalizations (n = 11,822 syllables) of CBA/CaJ mice during 30-minute interactions between a male mouse and female mouse (n = 8 male-female pairs). We first categorized behaviors between the male-female pairs, separating these into categories associated with low intensity interactions ("courtship") and high intensity interactions associated with attempted mounting ("mating") (see Materials and Methods; (Gaub et al., 2016; Ghasemahmad et al., 2024; Heckman et al., 2016b). Briefly, when female or male mouse engaged in mutual sniffing the vocalizations and behaviors were not intense (courtship), but when male sniffed female's head, attempted to mount the female or had successful mounting, the behavior and the emitted vocalizations represented more intense interaction (mating). These behavioral classifications are shown in Table1. We then analyzed the acoustic features of vocalizations emitted during these two behavioral categories. Example sequences and acoustic features are shown in Figures 1B and 1C. During courtship interactions, syllables consisting of mostly USVs were emitted in brief, relatively infrequent sequences, with long intervals between sequences (Fig. 1B). Vocal sequences emitted during mating consisted of both USVs, most likely emitted by the male, and low frequency harmonic (LFH) calls likely emitted by the female. Compared to the courtship sequence, the mating sequence included more stepped and complex USVs, more fundamental harmonic components, lower minimum frequencies, longer syllable duration, and higher emitted sound levels (Fig. 1C). These general features of the vocalizations associated with these behavioral categories are documented in Figures 2-4. Both of these sequences differ substantially from those emitted during restraint (Fig. 1A).

Figure 2 compares syllable composition and harmonic content for vocalizations emitted during courtship and mating interactions. In this analysis, we include both USVs and non-USVs, whether or not the syllables overlapped, to represent the proportion of emitted syllables. During courtship interactions, the most common syllables were, in order: up-FM, flat, down-FM, noisy, and chevron (Fig. 2A). The most common syllables emitted during mating interactions were mostly the same (chevron, up-FM, flat, complex, and down-FM) but in a different order and very different proportions. Overall,

mating vocalizations included more complex, stepped, and chevron syllables and fewer FM, flat, short, and noisy syllables, compared to courtship vocalizations. The mating syllables were much more likely to include at least one additional harmonic element, especially the chevron, complex, and stepped syllables (Fig. 2B). Syllable complexity thus increased during mating behavior, in agreement with previous studies (Egnor & Seagraves, 2016; Gaub et al., 2016; Hanson & Hurley, 2012). To perform further analyses on spectral, temporal, and amplitude features of syllables, we removed from consideration all overlapping syllables, as occur when a male-emitted USV overlaps with a female-emitted LFH (Fig. 1B). The results of these analyses are shown in Figures 3 and 4.

Previous work showed that the minimum frequency of syllables varies with the emotional state of the caller (Gaub et al., 2016; Grimsley et al., 2016; Hanson & Hurley, 2012). We found that, in male-female interactions, such context-related changes for USVs depended on syllable type and harmonicity (significant three-way interaction of mating state, syllable type, and harmonicity, n=4590 syllables, 9 USVs, $F_{(7, 4582.2)} = 4.0$, $p<0.001$, Linear mixed model, Fig. 3). Thus, most of the syllables that were emitted more frequently during mating behavior (e.g., stepped and chevron syllables) and had multiple harmonics during mating also showed the most significant harmonicity-dependent reductions in minimum frequency (for instance one-stepped syllables t=10.63, $p<0.001$, chevron: t=2.5, $p=0.01$, or ≥2step syllables t=8.0, $p<0.001$ Linear mixed model; Fig. 3). In contrast, other syllables that were emitted primarily in non-harmonic forms, such as up-FM and down-FM, showed little change in minimum frequency (e.g. up-FM: t = 1.1, $p=0.3$, down-FM: t=1.4, p=0.16, Linear mixed model; Fig. 3). The decreased minimum frequency that occurred with increased harmonicity appears to be due to the presence of the fundamental harmonic of these USVs (see Fig. 1B).

Temporal and amplitude features of vocalizations differed significantly with intensity of males-female interactions. Figure 4A shows a marked reduction in the inter-syllable interval (ISI) during mating interactions for all male-female pairs (significant main effect of state, n=8 mouse pairs and 10,189 syllable intervals: $F_{(1, 10180.2)} = 13.1$, *** $p<0.001$, Linear mixed model). Syllable duration increased substantially during mating interaction in each male-female pair (n=8 mouse pairs, 10,717 syllables; $F_{(1,10715)}=83.0$, ***$p<0.001$, Linear mixed model; Fig. 4B). Sound amplitude also increased during mating; Figure 4C shows that peak-to-peak amplitude of all syllables increased across all mating pairs (n=8 mouse pairs, 10,313 syllables, $F_{(1,10307.0)}=35.6$, ***$p<0.001$, Linear mixed model). We excluded LFHs from amplitude analysis as these syllables are usually specific to the mating state and have significantly higher amplitude than other syllable types (Mean±SD in volts: LFH (0.48±0.55), MFV (0.04±0.01), Noisy (0.06±0.04), Chevron (0.1±0.09)).

Overall, our data suggest that during vocal communication in mice, the intensity of male-female interactions affects syllable composition (including USV, LFH, MFV, and noisy syllables), spectral features (harmonicity, minimum frequency), temporal features (e.g. interval and duration), and syllable intensity. Together with our previous study (Grimsley et al, 2016), these findings indicate that the change in state of vocalizing

animals, represented in the intensity of social and sexual interactions, is reflected in the acoustic characteristics of their vocal signals.

Based on these findings and previous work by Grimsley et al. (Grimsley et al., 2016), we constructed vocal stimuli to be used in Experiment 2. Samples of these sequences are shown in Figure 5. Each spectrogram shows a 2-second sample of a longer sequence that contributed to the acoustic stimuli. Mating sequences (Fig. 5A) included many USVs with harmonics, steps, and complex structure, as well as LFH calls. Restraint vocalizations (Fig. 5B) include many USVs, MFVs, LFHs, and Noisy calls. These vocalizations were described previously in our related study on neuromodulator release (Ghasemahmad et al., 2024).

Experiment 2: Emotional vocalizations result in behavioral changes in mice

In this experiment, we asked whether the emotionally charged vocalizations illustrated in Figure 5, representing a sender's internal state in their syllable composition and spectrotemporal features, produced changes in the behavior of listening animals. We hypothesized that the high salience of our vocal stimuli would likely evoke some similar behaviors for both stimuli, while other behaviors would depend on the valence or behavioral context of the vocal stimuli. Further, since one context involves mating behavior, we hypothesized that there are sex-based differences in responses to these vocalizations. To test these hypotheses, we presented vocal stimulus blocks consisting of exemplars recorded either during the high-arousal stage of mating or during restraint, then monitored several behaviors of listening mice ($n_{\text{female mating}}= 9$, $n_{\text{male mating}}= 8$, $n_{\text{female restraint}}=11$, $n_{\text{male restraint}}= 8$).

Subjects were sexually naïve before undergoing mating and restraint experiences on two consecutive days (counterbalanced design) prior to the playback experiment (Fig. 6A). On the day of the playback experiment, mice acclimated to the experimental chamber for a period of three hours, after which we recorded their behaviors for 40 minutes during the following playback periods: Pre (10 mins) with no playback, Vocal Playback (20 mins) including playback of the entire group of mating or restraint sequences, and Post (10 mins) with no playback (Fig. 6B). Behavioral observations were collected continuously but are represented and analyzed in 2:50 min intervals equal to the duration of one mating or restraint stimulus block. In the following figures, we display behavioral measures throughout the 40 min recording period, in order to show the time course of behaviors before, during (six stimulus blocks), and after playback. However, our statistical analyses focus on three time points: 1) the last Pre interval, the first Vocal Playback interval, and the first Post interval.

In response to these two groups of contextual vocal sequences, mice displayed several patterns of behavioral responses. In one pattern, behavioral responses to playback were similar across both playback type and sex and were only observed during the playback periods. For instance, both male and female mice increased the number of attending behaviors (significant effect of sound: $F_{(1.37,43.76)} =115.0$, $p<0.001$; n=0.8) with vocalization playback regardless of the context (mating or restraint) associated with these stimuli (Vocal Playback vs Pre:, mean ± SD: female mating: +8.45± 1.4, restraint: +8.0± 4.8, male mating: +9.4.0± 3.0, restraint: +11.6± 5.1, Fig. 7A). Similarly, both male and female mice increased stretch-attend posture, a risk-assessment behavior

associated in rodents with detection and analysis of possible threats (Blanchard et al., 2011; Hager et al., 2014). This increase was significant in both sexes and contexts, except for males in response to mating playback calls (significant effect of sound: $F_{(1.07, 34.35)} = 44.4$, $p<0.001$; n=0.6. see Fig7B). Overall females showed more increase in stretch-attend posture compared to males in mating playback), while this increase was equally observed in both male and female during restraint vocal playback. Both behaviors showed very low levels prior to the beginning of playback, with a sharp increase that was consistently observed during the first playback block (Vocal Playback vs Pre: female mating: +6.4 ±5.3, male mating: +2.75 ±3.5, female restraint: + 7.5 ±4.5, male restraint: + 6.25 ±5.0, Fig. 7B.). The behaviors were observed at lower levels in subsequent playback intervals and returned to baseline levels once playback ended (Fig.7).

Other behaviors seemed to be significantly shaped by the sex of the listening animal. For instance, females more than males showed reductions in self-grooming with start of vocal playback (significant main effect of sex: $F(1,32) = 4.28$, $p=0.04$, Fig. 8A). Similarly, rearing decreased in females, accompanied by the increase of still-and-alert, a behavior signified by gradual reduction of activities (Fig. 8B, C). These behaviors displayed wider variation across individuals before, during, and after playback, suggesting that they were less closely tied to vocalization responses.

Vocal playback also resulted in some behaviors that were shaped by the vocalization context. For instance, male and female mice flinched more during restraint playback, but not during mating playback (Fig. 9A) (significant interaction of time and context: $F_{(1.02, 32.63)} = 17.2$, $p<0.001$; n=0.0234). This behavior was generally absent prior to vocal playback and followed a sound-dependent patterns in which the behavior diminished throughout playback duration. Locomotion behavior showed a significant increase during mating Vocal Playback (significant main effect of context: $F_{(1, 32)} = 8.0$, $p=0.008$; n=0.2) (Fig. 9B), with males generally showing more locomotion than females (Vocal Playback vs Pre: female mating: +1.2 ±1.5, male mating: +5.62 ±2, female restraint: +3.1 ± 4.4, male restraint: + 0.9 ± 0.1 , Fig. 7).

Among the observed behaviors in these groups of animals, escape behavior showed a significant interaction between sex of the listener and the context of vocalizations, such that both mating and restraint vocalizations resulted in increased escape behavior in female mice, while males only exhibited such behavior during restraint vocalization playback ($F_{(1.02, 32.6)} = 5.0$, $p=0.03$; n=0.13). Furthermore, this behavior in females showed more gradual decrease after the sound onset while in males was more abrupt after the first vocal playback block (Fig. 10).

Overall, these behavioral findings show that salient vocalizations affect the behavior of listening mice. Further, the changes for some behaviors were independent of the context of vocalizations, but in other cases depended on the sex of listening animals, the context of vocalizations, or an interaction of these factors.

DISCUSSION

This study describes behavioral responses to emotional vocalizations produced by mice under very different behavioral contexts. The first experiment identified acoustic features associated with an appetitive social interaction, courtship and mating, that presumably reflect the internal state of vocalizing animals. We used multichannel audio and simultaneous video recordings to relate vocalizations to the intensity of male-female interactions. The behavioral observations provided the basis to distinguish vocal features associated with “courtship” and “mating”, then developed a series of vocal exemplars for use in the second (playback) experiment. With playback of highly salient, emotional vocalizations associated with mating and restraint, we observed a range of behavioral responses, some generalized to both types of stimuli, others sex-dependent, and others displaying context-by-sex interactions. Some of the behaviors were strongly linked to vocal playback while others appeared minimally linked. Clearly, vocalization-induced behavioral responses do not uniformly indicate an internal state change in the listening animal.

Vocalizations in courtship and mating

Vocalizations are valuable indicators of emotions or internal state that contribute to the affective component of social communication (Zimmermann et al., 2013). During vocal communications, both syllable type and spectrotemporal features are affected by a sender’s affective state. Change in fundamental frequency, duration, and rate of syllable emission have been reported in different species as they are affected by the internal state (Gadziola et al., 2012; Gaub et al., 2016; Grimsley et al., 2016; Knutson et al., 1998; Moles et al., 2007; Rendall & Owren, 2010; Soltis et al., 2011).

Motivated by the need to identify highly emotional vocal sequences associated with mating, we used previously described behaviors of male-female mouse pairs (Gaub et al., 2016; Heckman et al., 2016b) to identify two levels of intensity of these interactions, “courtship” and “mating”. We then compared the acoustic features of a large number of vocalizations, nearly 12,000 syllables from 8 mouse pairs, that were produced during these two behavioral interactions. The mice emitted USVs and three lower frequency/broadband vocal categories. Our results show several changes in vocalizations as the intensity of the interaction increased. Large scale changes in vocalization sequences included increased repetition rate and sound level. There was also a change in overall syllable composition, marked by increased complexity of syllable types: more chevron, complex, and stepped calls, fewer FM, flat, and short calls). Especially for syllables that increased during the mating interactions, the duration and harmonic number increased while the minimum frequency decreased.

Our findings demonstrate that acoustic characteristics and syllable composition change with the intensity of male-female interactions in mice, presumably reflecting changes in the underlying internal state of the animals. These findings (change of syllable composition and their spectro-temporal features) are in general agreement with previous studies in other contexts (Grimsley et al., 2016) or similar context but different mouse models (Gaub et al., 2016; Wang et al., 2008).

During mating interactions, chevrons were the dominant USV syllable, with increased proportion of syllables with frequency steps (40 %vs 8%). These proportions differ from

previous studies, only a few of which have identified chevron calls (Grimsley et al., 2011, 2016; Hanson & Hurley, 2012; Matsumoto & Okanoya, 2016). Instead, other studies report that syllables with frequency jumps are the main category emitted during the mating (Chabout et al., 2015; Gaub et al., 2016; Holy & Guo, 2005).

There are several possible explanations for this discrepancy. For instance, different mouse strains may utilize syllable types in different proportions (Gaub et al., 2016; Holy & Guo, 2005; Matsumoto & Okanoya, 2016; Wang et al., 2008). Further, our recording techniques may have allowed us to detect chevron calls more sensitively. We used a multichannel recording setup for vocalization recordings. For every syllable, the channel with highest SNR was chosen to determine syllable type, rather than the single microphone recording used by others (Holy & Guo, 2005; Matsumoto & Okanoya, 2016; Wang et al., 2008). With the variations in head position during mating behaviors, a single microphone cannot represent the emitted spectrotemporal features of syllables as accurately, resulting in apparent discontinuities in the spectrograms. Thus, syllables will appear to include multiple elements or frequency steps rather than a continuous chevron shape. Finally, the necessity of removing overlapping syllables from further analysis (Gaub et al., 2016; Grimsley et al., 2011, 2016) may have reduced the observation of chevron syllables, because these syllables are more likely to overlap with LFH calls during mating rather than courtship.

Although harmonicity changes have been reported in emotional vocalizations in various species(Gadziola et al., 2012; Gourbal et al., 2004; Márquez, 1995; Narins et al., 2004; Narins & Smith, 1986; Suthers et al., 2006; Zimmermann et al., 2013), its covariation with other spectrotemporal characteristics of emotional vocalizations is rarely addressed (Gaub et al., 2016; Grimsley et al., 2016). The increase of syllable duration with harmonicity also occurs in vocalizations during aversive contexts in mice (Grimsley et al., 2016).

Our data show increased harmonicity of the major syllables (chevron and stepped calls) of mating, versus courtship. This change of harmonicity may result in the lowered fundamental frequency observed in our data during mating. Mating interactions also feature increased sound level of emitted syllables. Together, the higher sound levels and lower frequencies associated with harmonic syllables in mating will activate many more neurons in mouse Inferior colliculus (Tehrani et al., 2024), increasing the likelihood of responses in higher order regions such as the auditory cortex or amygdala.

Since longer duration syllables are the result of longer exhalations (Castellucci et al., 2018), and since USV production is tightly coordinated with sniffing behavior (Alves et al., 2016; Sirotin et al., 2014), changes in syllable duration and ISIs appear to be the result of a dramatic decrease in sniffing cycle (Castellucci et al., 2018; Sirotin et al., 2014; Wesson et al., 2009). Modulation of spectrotemporal characteristics of vocalizations that result from changes in vocal fold dynamics seem to be under tight control of sympathetic autonomic arousal (Johnstone et al., 2007; Stewart et al., 2013). They are most likely controlled by the central nucleus of amygdala in response to increased arousal (Stewart et al., 2013).

Overall, findings of this experiment help to identify the acoustic feactures that characterize different levels of appetitive male-female interactions leading to mating..

We use these features to develop highly salient vocal stimuli with positive and negative affect that are used to study listeners' behavioral to emotional vocalizations.

Behavioral responses to emotionally intense vocalizations associated with mating and restraint

Responses of mice and other animals to social vocalizations depend substantially on the multi-sensory and behavioral contextual features associated with these vocalizations. For instance, in mice, olfactory cues strongly influence behavioral responses to both USVs and LFH call, i.e., squeaks (Gaub et al., 2016; Grimsley et al., 2013; Hanson & Hurley, 2012). The types of social interactions (e.g., sex-dependency, aggression level) also influence the response to social vocalizations (Premoli et al., 2022; Rendon et al., 2015; Sasaki et al., 2020). Because these multi-sensory and behavioral cues have important effects on acoustic communication, it has been difficult to assess how the vocalization itself shapes behaviors. This and our companion study (Ghasemahmad et al., 2024) utilized playback of highly emotional vocalizations to study how the vocal stimuli alone shape listener's response to vocal signals.

Our study controlled for or analyzed features of the listener that may affect behavioral responses. We ensured that all subjects had previous, well-defined experiences with the behaviors associated with the vocal stimuli since our parallel experiments showed that these experiences are critical to some neurochemical and behavioral responses (Ghasemahmad et al., 2024). To control for hormonal state, only females in estrus were included in the final analysis of the behavioral experiments. We further analyzed results by sex. Overall, the findings revealed some generalized responses that appear to result from enhanced attention and stimulus evaluation (e.g., increased attending–a freezing-like behaviors to stimuli of both contexts by both sexes), some sex-dependent responses (such as increased rearing in males to both vocal categories), and some differentially modulated by context (such as enhanced flinching or escape initiation responses in restraint playback in both sexes). Overall, salient mouse vocalizations that carry context-specific information in their composition and spectrotemporal attributes change the behaviors, and presumably the internal states, of listening mice. Further, the behaviors reveal both generalized responses to salient vocal signals and responses that are sex- or context-specific.

Findings of our current study agree with our previous work (Ghasemahmad et al., 2024). For example, in both studies, we observed an increase in attending in mice regardless of the type of vocal playback. The finer temporal analysis undertaken in the current study suggests that this Attending behavior is the result of the initial assessment of vocal stimuli, regardless of their type. This was revealed by fast decay of attending responses after the first playback period, in both sexes and with both playback types.

Consistent with our previous study, we further observed that the animal's locomotive activity was substantially affected by sex of the listening animals during both behavioral contexts. In females, a response to vocal playback was to remain "still and alert", reducing movements such as locomotion and rearing. In contrast, males generally were more active during vocal playback. This sex-based response to vocalization playback was only observed in playback of affective vocal sequences but not in playback to single syllables (Niemczura et al., 2020a) or behavioral responses to other stimuli

(Bishnoi et al., 2021; Herselman et al., 2023; Kent et al., 2017). Further, females showed increased risk assessment behaviors (stretch-attend posture), suggesting cautious interpretation of vocal sequences. As females in our study were all in estrus, this behavioral response could be due to the amplified attention in estrus females, resulting from increased acetylcholine and potential interaction with dopamine across the estrous cycle (Ghasemahmad et al., 2024).

Interestingly, in both of our studies, males tend to become more exploratory during mating vocal playbacks. In the current study, this is manifested in higher locomotion (exploring different parts of the arena) while in our previous study it appeared as increased rearing during mating vocal playback. Our previous study further suggests that this behavior is related to previous experience with this context, since males without mating experience did not show significant changes in motor responses during presentation of these vocal stimuli. As the mating vocal sequences used in our study included both the male and female vocalizations during mating stage, we cannot conclude which category of these vocalizations is responsible for triggering the observed behaviors. However, since the emotional context in our experiments are established through vocal sequence and syllable types rather than other sensory cues (Ehret, 2013; Grimsley et al., 2013; Ronald et al., 2020), we believe that the natural sequence of mating calls established a potential positive valence for the male mice, resulting in an increase in locomotion and exploratory behaviors throughout the playback period. Such active behaviors further represent males' role during mating interactions, as male mice are overall more exploratory and active in examining the female and vocalizing to engage females in mating behavior (Johansen et al., 2008). Further, males did not exhibit avoidance or aversive behaviors such as escape initiation or flinching confirming the lack of negative cues in such vocalizations for male mice.

Both of our studies demonstrated significant interactions of sex and vocal type, in which vocal stimuli differentially modulated the behavior of male and female mice. In the present study, this response appeared as escape behavior, which males showed only during restraint playback while females showed regardless vocalization type. Our previous study, however, shows that differential influence of vocalization type on the sex of the listening animal can appear in other forms, such as flinching. There, we observed differential modulation of flinching behavior by the internal state of the animal (estrous phase) and vocalization type (Ghasemahmad et al., 2024). Such behaviors could be the result of vocalization type (e.g., responses to restraint vocalizations that were not influenced by restraint experience) or the result of the internal state of the listener (e.g., modulation by female's estrus cycle). Overall, these studies demonstrate that both long terms features, i.e., sex and experience, and short term factors such as hormonal state are critical to the listener's response to these emotional vocalizations.

ACKNOWLEDGMENTS:

We are indebted to Sharad Shanbhag and Daniel Gavazzi for software used to condition vocal sequences and analyze audio and behavioral data, respectively, to Kristin Yeager of the Kent State University Statistical Consulting Center for statistical consulting, and to Sheila Fleming for advice on behavioral assessments. We thank Anthony Zampino, Austin Poth, Debin Lei, and Krish Nair for technical assistance.

FUNDING:

National Institutes of Health grant R01DC00937 (JJW, Alexander Galazyuk)

Kent State University Graduate Student Senate (ZG)

DATA AVAILABILITY:

Software tools used in preparing sounds or analyzing sounds/behaviors are identified in the Materials and Methods and available on provided links to GitHub.

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Figure 1

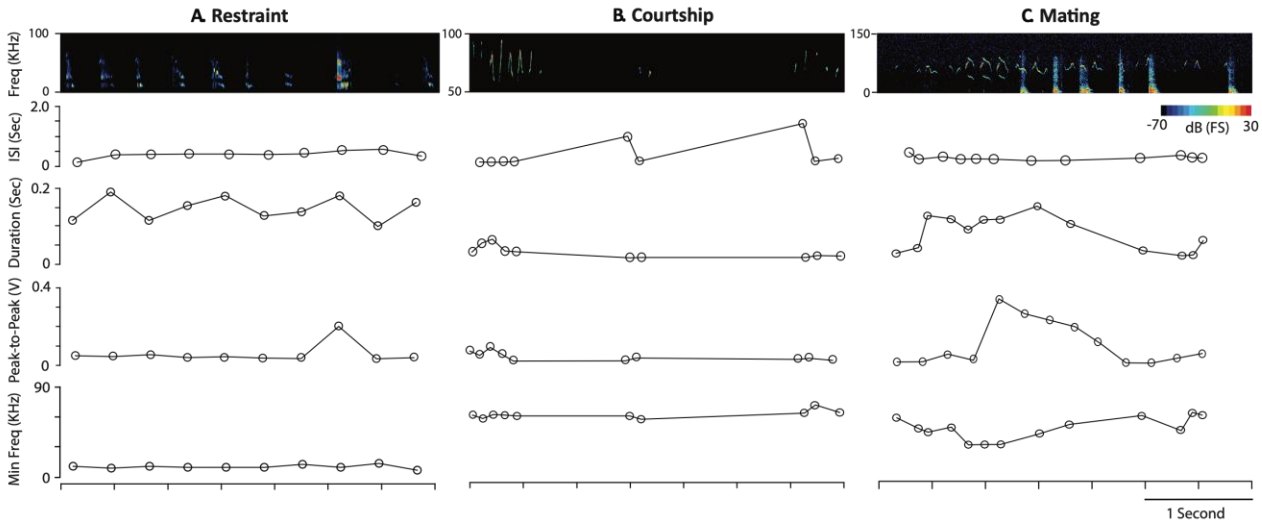

Figure 1. Vocalizations are modulated during different behavioral contexts. Each column displays short sequences of vocalizations and their acoustic features emitted by a single mouse in Restraint **(A)** or by CBA/CaJ male-female pairs in Courtship **(B)** and Mating **(C)**. Sound spectrograms (*top*) show syllables emitted during 3.5 s sequences in each behavior. Note different vertical axis scales for sonograms. Below the sonograms are four acoustic features of the vocal sequences: inter-syllable interval (ISI); duration (sec); sound level measured as peak-to-peak amplitude (volts); and minimum frequency (kHz). During Restraint, mice typically produce longer duration, lower frequency syllables such as the mid-frequency vocalization (MFV) with minimum frequencies below 18 kHz. Note that for Courtship (B) and Mating (C), graphs only show values for USVs. Compared to courtship, the higher intensity mating interaction is associated with more USV syllables and LFH calls, shorter ISI, longer duration, higher level, and lower minimum frequency.

Figure 2

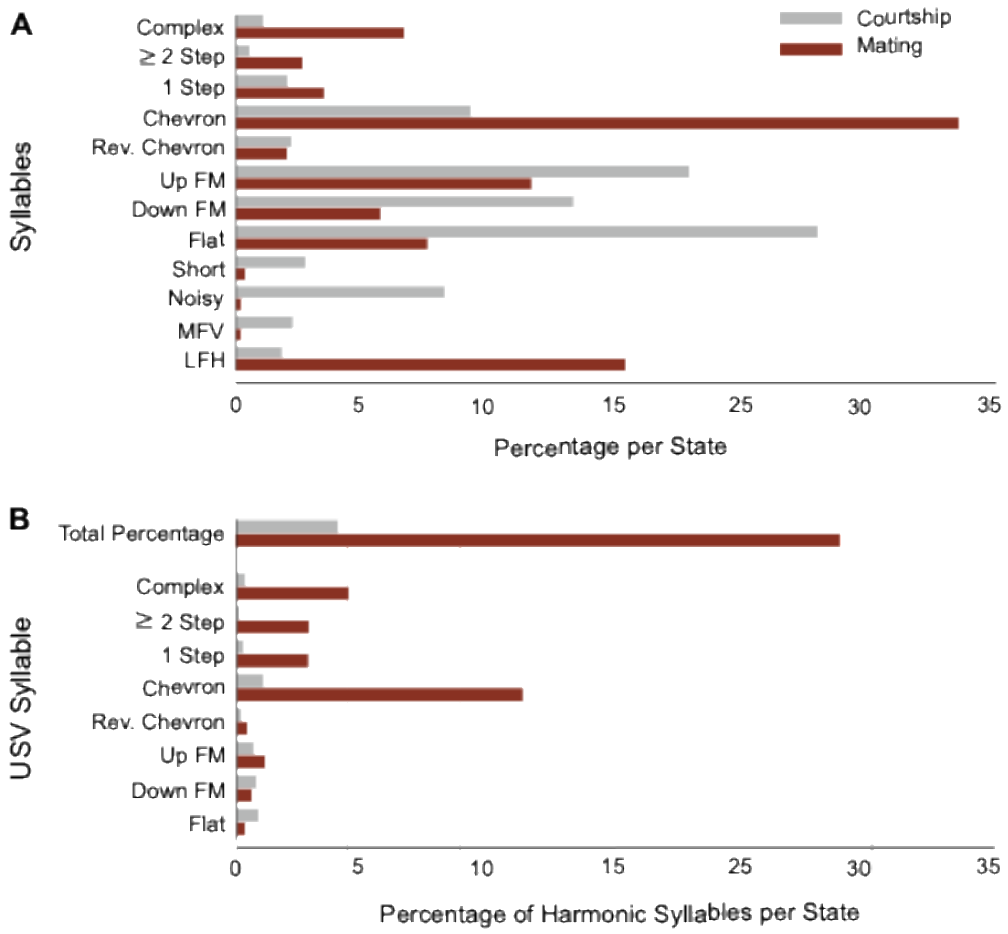

Figure 2. Syllable composition and harmonic structure change with male-female interaction intensity. A. Percentages of each syllable type recorded across courtship (gray) and mating (red) interactions. Note the higher percentage of complex, chevron, and stepped USV syllables and LFH calls emitted during mating. **B.** Frequency of occurrence of harmonics among USV syllables in courtship (gray) and mating (red) interactions. Note that many syllables shown in part A to be emitted preferentially during mating (i.e., complex, stepped, and chevron) have more harmonic components (syllables in courtship, n=5540; syllables in mating, n=5180 syllables).

Figure 3

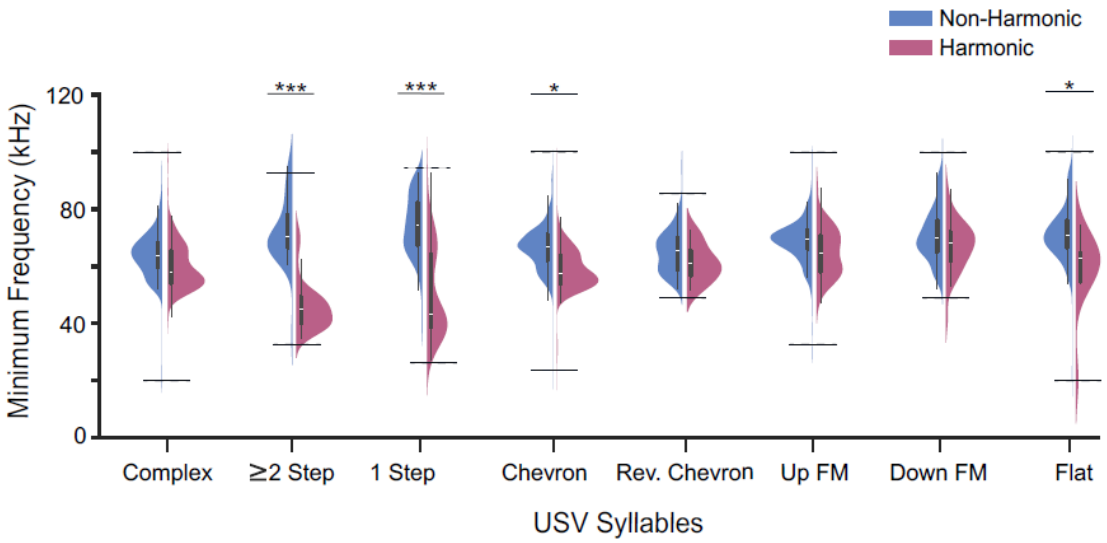

Figure 3. Minimum frequency of syllables decreases with increased intensity of mating interaction as a factor of harmonicity. Minimum frequency by syllable types based on the harmonicity and intensity stage of mating interaction (Significant interaction effect of harmonicity, syllable type, and interaction type on minimum frequency, n=4590 syllables, 9 syllable types, $F_{(1, 4582.2)}$=4.0, *p<0.001*, Linear mixed model). Dashed lines represent the actual range of data for each syllable. Minimum frequency was significantly reduced for harmonic call in Flat (t =2.4, *p=0.02), Chevron (t=2.5, *p=0.01), 1 Step (t=10.63, ****p*<0.001) , and ≥2 Step syllables (t=8.0 , ***p*<0.001), but the effect in other syllables was not significant (e.g. UP FM: t =1.1, *p=0.3*, Down FM: t=1.4, *p*=0.16). Linear mixed model.

Figure 4

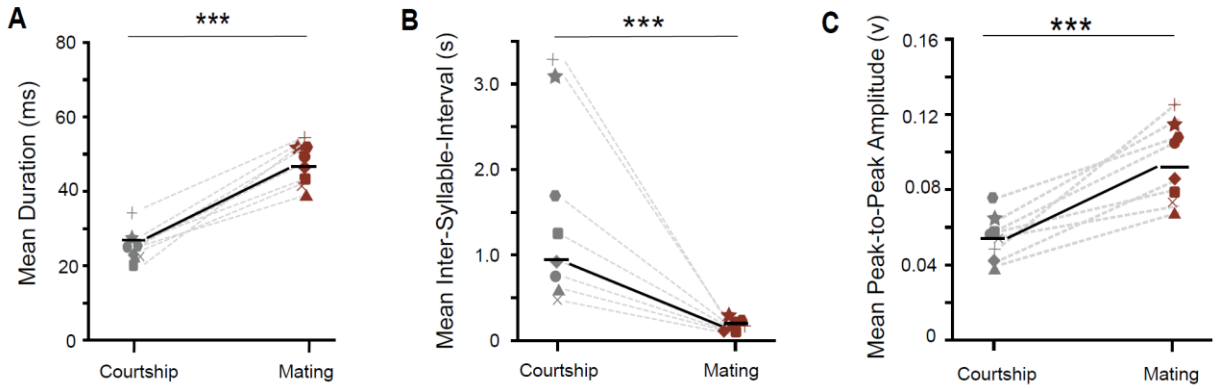

Figure 4. Temporal and amplitude features of syllables differ between courtship and mating interactions. Each symbol pair and connecting dashed line display average values for one mating pair in courtship and mating. Solid black line and symbols display average of all 8 mating pairs. **A.** Syllable duration increases with increased intensity of mating interaction (n=10,717 syllables; F(1,10715)=83.0, ***p<0.001). **B.** Inter-Syllable-Interval (ISI) decreases with increased intensity of mating interactions (significant main effect of mating state, n=10,189 intervals: F(1, 10180.2)=13.1, *** p<0.001). **C.** Peak-to-peak amplitude of emitted syllables increases with mating intensity (n=10,313 syllables, F(1,10307.0)=35.6, ***p<0.001). Linear mixed model.

Figure 5

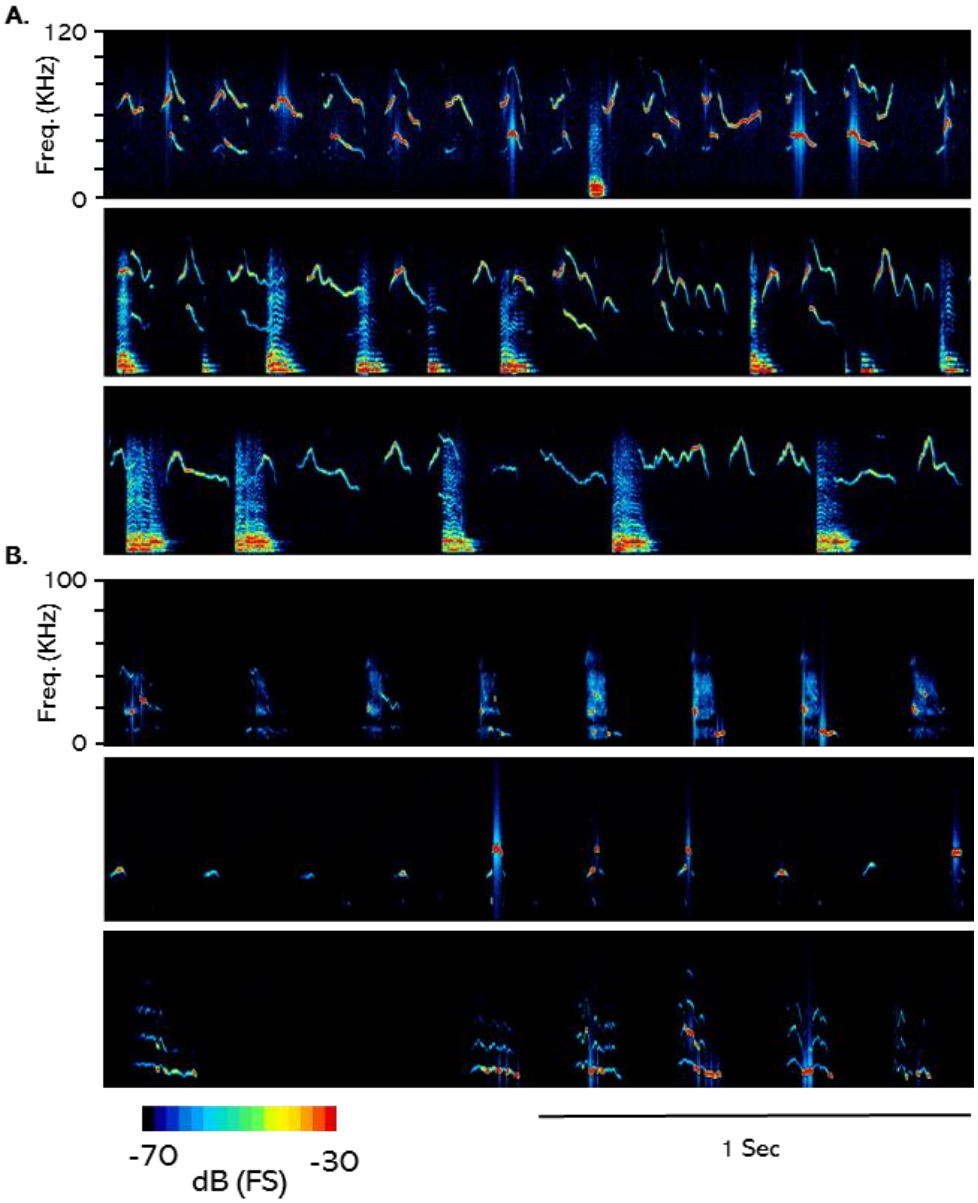

Figure 5. Spectrogram samples of vocal sequences used as stimuli in Experiment 2. A. Mating sequences. The durations of the full sequences were 5 s (top), 17 s (middle), and 7 s (bottom). **B.** Restraint sequences. The duration of the full sequences were 21 s (top), 18.2 s (middle), and 21 s (bottom).

Figure 6

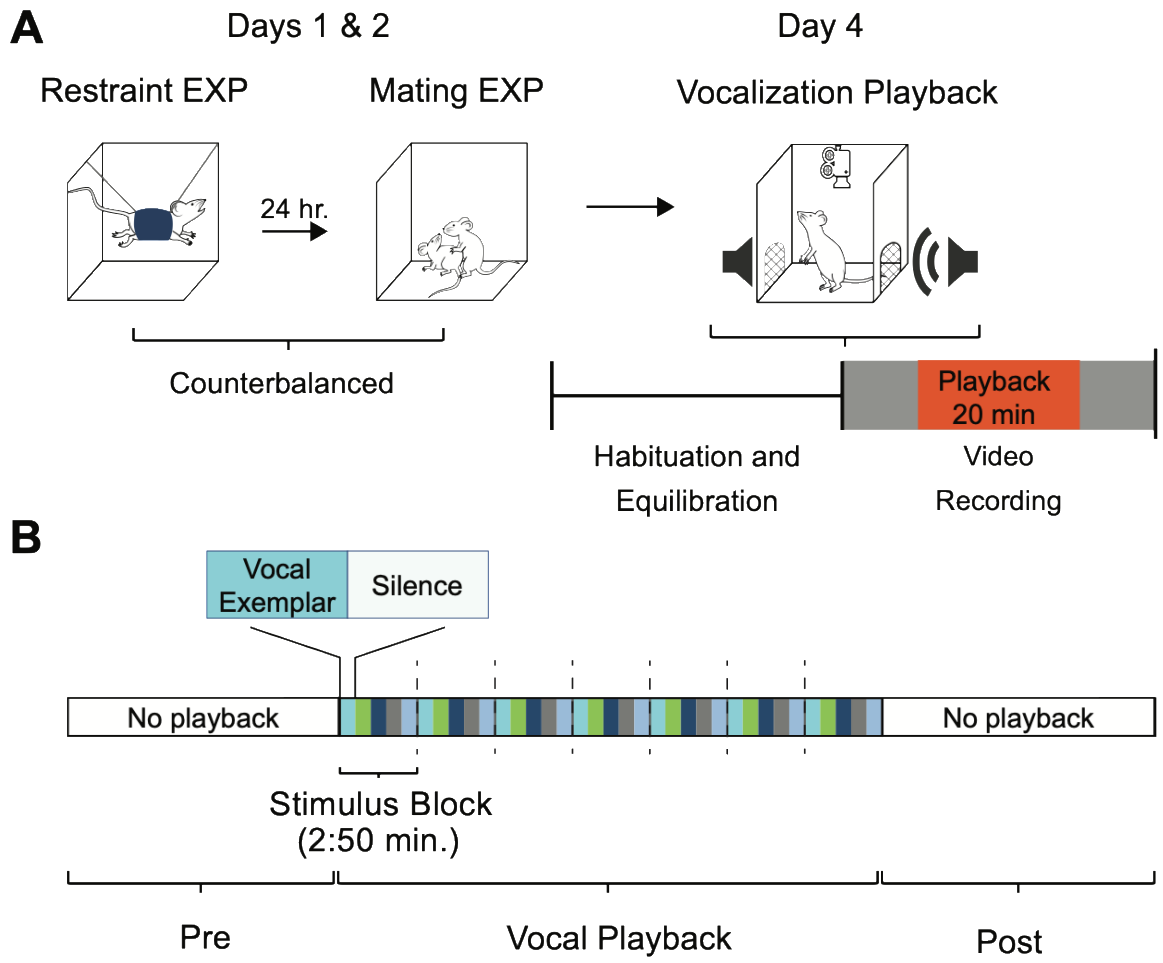

Figure 6. Temporal structures in playback experiment. A. Experimental sequence of experience and playback. On consecutive days, each animal experienced 90 min sessions of restraint and mating. The order of these sessions was counterbalanced across subjects. On the playback day, the subject was placed in the experimental chamber and allowed to habituate for three hours. Behavioral data was video-recorded for 40 mins: 10 mins before playback began, 20 mins during playback, and 10 mins after playback ended. **B.** Schematic illustration of detailed sequencing of vocal stimuli, shown here for mating playback. A 20-min period of vocal playback was formed by seven repeated stimulus blocks of 2:50 mins. The stimulus blocks were composed of five vocal exemplars (each represented by a different color) of variable length, with each exemplar followed by an equal duration of silence. In Figures 7-10, we report behavioral observations for blocks 1, 2, 3, 5, 6,and 7, omitting block 4. See Material and Methods for in-depth description of vocalization playback.

Figure 7

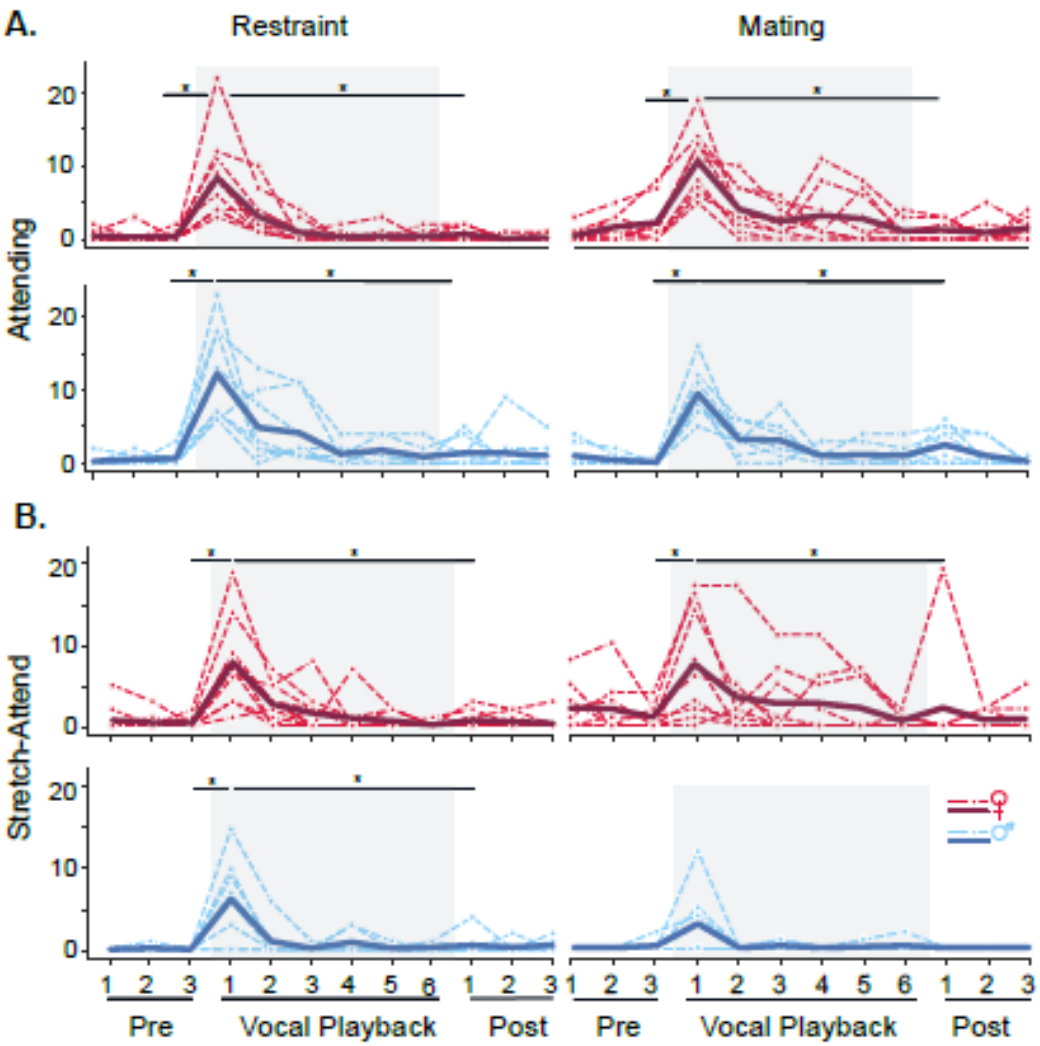

Figure 7. Playback of mating and restraint sequences evokes similar Attend and Stretch-Attend responses in male and female mice. For Figures 7-10, graphs display the number of behaviors during restraint (left) and mating (right) vocalization playback for estrus female (red) and male (blue) mice. Dashed lines represent individual animals; solid blue lines represent average number of the behavior for each group ($n_{female\ mating}$=9, $n_{male\ mating}$=8, $n_{female\ restraint}$=11, $n_{male\ restraint}$=8). **A.** The number of abrupt attending behaviors increased during both mating and restraint playback in both female and male mice (significant effect of vocal playback: $F_{(1.37,43.76)}$ =115.0, *p<0.001*; n=0.8). **B**. Stretch-attend posture in female and male mice similarly increased during restraint and mating playback (significant effect of vocal playback: $F_{(1.07, 34.35)}$ =44.4, *p*<0.001; n=0.6). Statistical tests based on repeated measures GLM with Bonferroni post hoc tests.

Figure 8

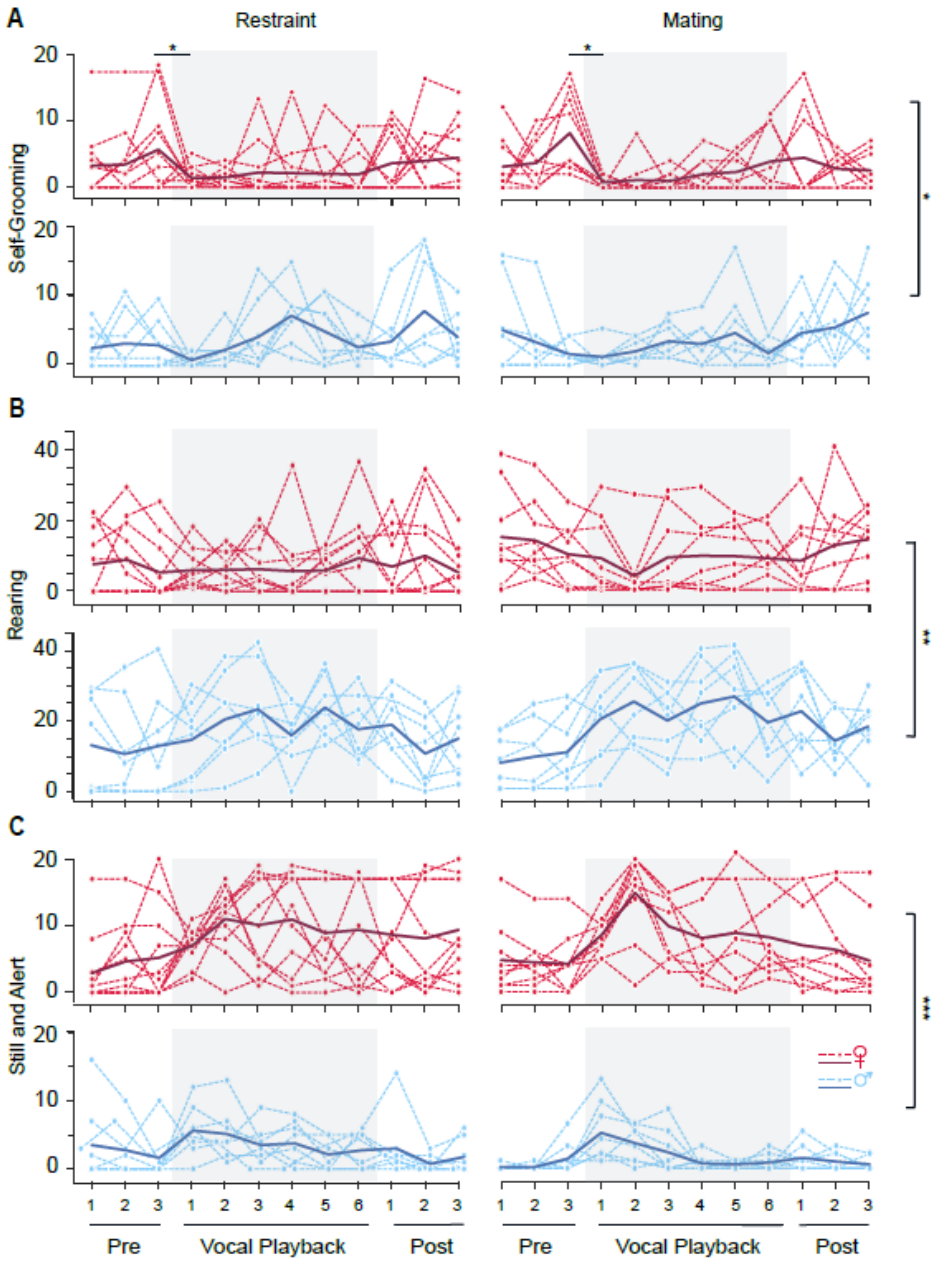

Figure 8. Playback of mating and restraint sequences evokes some behavioral responses that differ between male and female mice. See Figure 7 for protocol. **A.** Self-grooming behavior was reduced during playback in female mice more than males, regardless of vocalization type (significant main effect of sex: $F_{(1,32)}$ =4.3, *P*=0.04; n=0.12). **B.** Rearing increased in males more than females regardless of vocalization type ($F_{(1,32)}$ =10.6, *P*=0.003; n=0.25). **C.** Still and Alert behavior increased more in females than males during playback, regardless of vocalization type ($F_{(1,32)}$ =16.7, *p*<0.001; n=0.34).

Figure 9

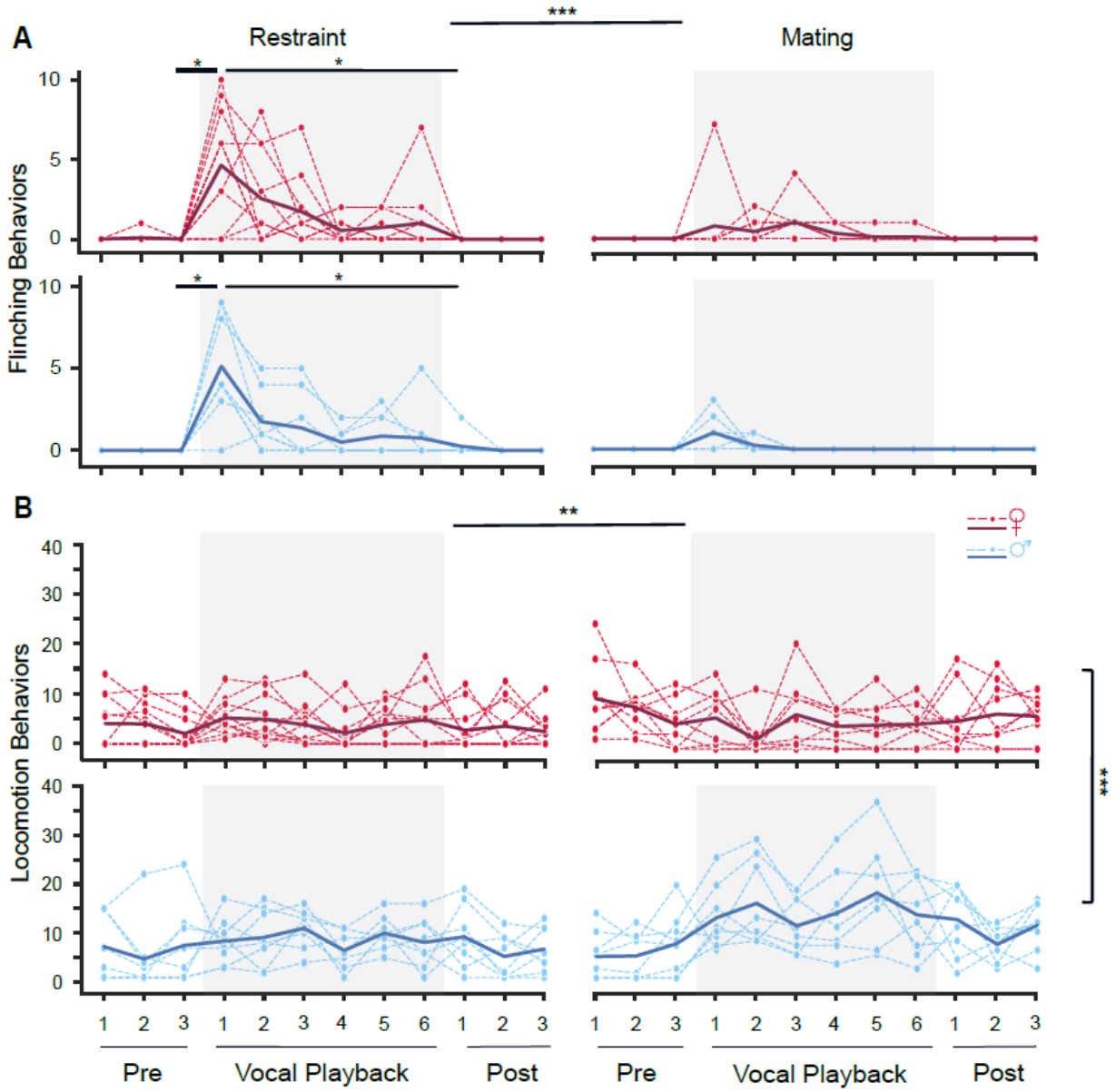

Figure 9. Playback of mating and restraint sequences evokes context-specific Flinching and Locomotion responses. See Figure 7 for protocol. **A.** Flinching behavior increased in both female and male mice during restraint but not in females during mating vocal playback (significant interaction of time and context: $F_{(1.02, 32.63)}$ =17.2, *p*=<0.001; n=0.34). **B.** During mating playback, locomotion increased in male mice more than females (significant main effect of context: $F_{(1,32)}$ =8.0, *p*=0.008; n=0.2).

Figure 10

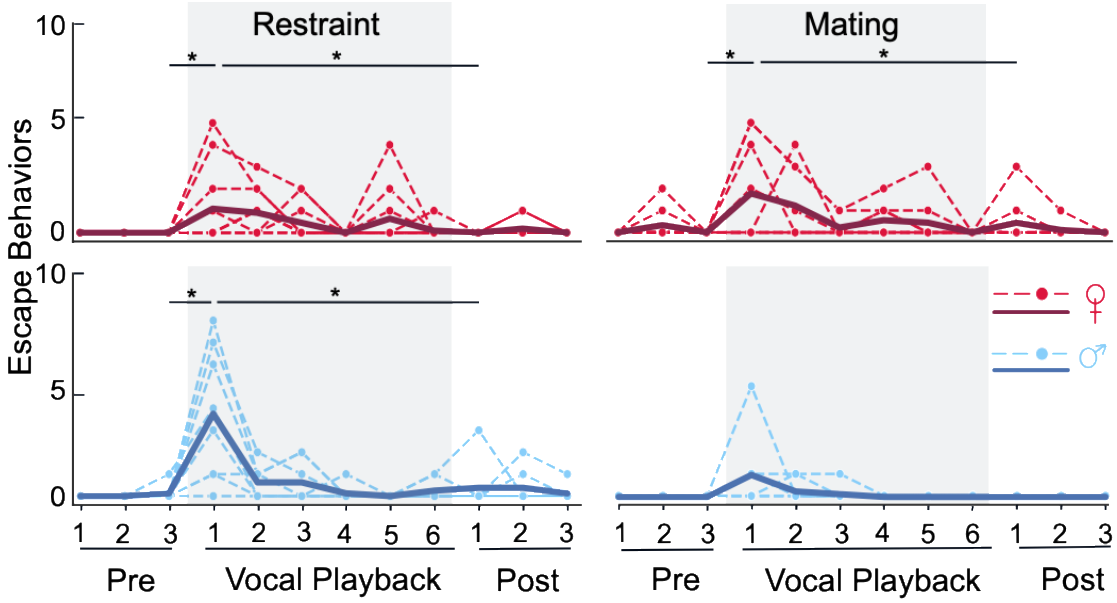

Figure 10. Escape behaviors differed by sex and vocal playback type. See Figure 7 for protocol. There was a significant interaction of time, sex, and context: $F_{(1.02, 32.6)} = 5.0$, $p = 0.03$; n=0.13: repeated measures GLM, Bonferoni post hoc test.

Table 1: Behaviors that distinguish courtship and mating

Interaction Type	Behavior	Definition
Courtship	**Exploring**	Male and female explore the test box in the presence of the other sex. This behavior included rearing and sniffing corners and walls.
	Mutual sniffing	Male and female sniffed each other's head, body or genital area simultaneously.
	Genital sniffing	Male sniffs female's genital area.
	Female sniffing male's head	Female sniffs male's head, snout, or face.
Mating	**Male sniffing female's head**	Male sniffs female's head, snout, or face. The behavior sometimes is followed by grooming of female's head.
	Attempt to mount/mount	Male mouse mounts female followed by copulation behavior characterized by successive movement of his body while holding female with his forelimbs tightly for more than 2 seconds. If this behavior was interrupted by female's avoidance and lasted less than 2 seconds, mounting was considered unsuccessful (attempt to mount). Sniffing or approaching immediately before/after mounting or attempt to mount was considered as part of mounting behavior.

Table 2: Classification of manually evaluated behaviors during vocalization playback*

Behavior	Definition
Abrupt attending	Sudden and quick pause in locomotion followed by abrupt change in head and body position lasting 2+ seconds. This behavior is accompanied by fixation of the eyes and ears during attending. Appears similar to "freezing" behavior described in fear conditioning but occurs in the context of natural response to vocalizations.
Flinch	A short-duration twitch-like movement in response to vocal sequences, occurring at any location within arena. Unlike acoustic startle (Grimsley et al., 2015), this behavior occurs in free-moving animals in response to non-repetitive, variable-level vocal stimuli. Flinching movements are of smaller magnitude than those observed in acoustic startle.
Pull-back posture (escape)	An escape behavior manifested in leaning the body away from the direction of sound, followed by running away from the sound. Animal mostly uses the hindlimbs to lead this behavior.
Locomotion	Movement of all four limbs from one quadrant of the arena to another.
Rearing	A search behavior during which the body is upright, and the head is elevated to investigate more distant locations.
Self-grooming	Licking fur and using forepaws to scratch and clean fur on head. The behavior can extend to other parts of the body.
Still and alert	Gradual reduction or lack of movement for 2+ seconds, during which animal appears to be listening or responding to external stimulus. Distinguished from abrupt attending by gradual onset.
Stretch-attend posture	A risk-assessment behavior during which hind limbs are fixed while the head, forelimbs, and the body are stretched sequentially in different directions.

*Modified from (Ghasemahmad et al., 2024).