What matters to a mouse? Effects of internal and external context on male vocal response to female squeaks

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Abstract

Individuals plastically adjust their signaling behavior depending on the social context of an interaction, but which aspects of context elicit the strongest responses from individuals is often difficult to determine. To explore how internal and external contextual factors influence the communication of house mice, we employed a split-cage playback assay to assess how dominant and subordinate male mice differed in their ultrasonic vocalization (USV) production in response to playback of broadband vocalizations (BBVs, or squeaks). We assessed how individuals altered their behavior depending on the odors present during the assay: either just female odors (Fem condition) or female odors in addition to the odors of potential competitors, other males (Fem+Male condition). Using this repeated-measures design, we evaluated two distinct cohorts of individuals: males exposed to the odor of familiar competitors in the Fem+Male condition (Familiar odor cohort), and males exposed to the odor of unfamiliar competitors in the Fem+Male condition (Unfamiliar odor cohort). While dominants and subordinates did not differ in their baseline vocal production or response to squeaks, all groups reduced their vocal production in response to BBV playback, and the addition of unfamiliar male odor prompted mice to decrease their baseline levels of calling and decrease the complexity of their vocal repertoire. Importantly, this effect of additional male odor was not present when individuals were presented with male odor familiar to them. These findings suggest that mice alter their vocal behavior during courtship interactions in response to cues that indicate the presence of potential competitors, and this response is modulated by the familiarity of these competitor cues.

Introduction

The ability to process and respond appropriately to conspecific signals is crucial for animals as they navigate social interactions [1–3]. How an individual processes and responds to signals may heavily depend on the context of the situation [4–6], and the role of context in vocal communication in particular is highly documented across taxa [7–11]. Research on how contextual influences such as the presence of competitors and potential mates, seasonality, changes in steroid hormones, and resource abundance affect song production and song composition in birds has revealed that many aspects of communication depend on context, and prompted advances and questions on how context can influence the signaling in other species [12,12–16]. These include laboratory mice, studied not only as a model of rodent communication signals, but also for their potential applicability to human communication disorders [17,18].

House mice (*Mus musculus*), produce a variety of vocalizations that can vary in usage, structure, and meaning across different contexts [1,10,11,19,20]. Vocal communication in mice is prominent during courtship interactions, in which calls are used to communicate receptivity and sexual motivation [1,10,21–24]. One type of call, ultrasonic vocalizations (USVs), are high-frequency calls (30-110 kHz) produced by both males and females during social interactions [1,20,24–26]. These calls are produced in large numbers by males during opposite-sex interactions, and are often referred to as “courtship calls” because of their prosocial function to facilitate mating [1,24,27] (Fig 1A). Male USV production thus may indicate male courtship effort and motivation [22]. Mouse vocal repertoires, or the composition of different types of syllables individuals use, are complex and can vary depending on factors such as age, genetics, and behavioral situation [10,28–30]. One type of USV in particular, 50 kHz harmonic USVs, tend to be

longer in duration and more complex structurally than other classes of USVs. These calls are closely associated temporally with incidents of male mounting behavior [10] (Fig 1A).

Fig 1: Representative spectrograms of (A) Ultrasonic vocalizations (USVs) and (B) Broadband vocalizations (BBVs). The circled vocalization in (A) is an example of a 50 kHz

Another type of call, broadband vocalizations (BBVs), or squeaks, are lower-frequency calls with a broader frequency range than USVs, featuring many harmonics in (Fig 1B). Both male and female mice produce BBVs across varying contexts, including during distress, handling, and conspecific interactions [26,31–33]. When produced by females during the early stages of courtship interactions, these calls are closely correlated with females performing physical rejection behaviors like kicking, lunging, and attempted escape [11,34]. Thus, during courtship, female-produced BBVs may be negatively-valenced, and function to express a lack of female receptivity to sexual advances by males. In accordance with the connection of squeaks to physical rejection, male mice reduce their USV output in response to BBV playback during limited interactions with female mice [35]. However, little is known about what sorts of contextual factors may influence this response to BBVs, and which levels of context may “matter” the most to mice as they interact.

The goal of this study was to explore the effects of both internal and external contextual factors on male vocal production and vocal repertoire in response to female squeaks. As an internal contextual factor, we evaluated dominance status in all of our individuals. Some calls are more associated with the production of behaviors classified as “more dominant” such as aggression, and courting [20]. Dominant and subordinate mice tend to differ in their vocal responses to female cues, though the direction of this difference varies across studies. For example, one study found that dominant male mice call more quickly and more often compared

to subordinate males in response to female stimuli [36], while in contrast, another found that subordinates called the most when interacting with a female [7]. However, this was only true when subordinates were tested in the experimental cage first; if subordinates were recorded in the same arena in which a dominant male had previously interacted with a female, their calling was sharply reduced, indicating that some cues from the dominant males were able to stunt the courtship behavior of subordinate mice. The same effect did not occur when dominants were tested after subordinates [7]. Thus, the effects of dominance status on vocal production in male mice appear to be highly-context dependent, and worthy of further investigation.

In addition to the playback of BBVs, the external contextual factor of interest to us was the presence of non-vocal cues that may alter male response to BBVs: specifically, odor from other male mice. Audience effects, in which individuals adjust their behavior in the presence of additional individuals, are well-documented examples of context-dependent communication [37]. Studies in songbirds and domesticated fowl have revealed that the presence of audiences during courtship signaling can influence not only the acoustic properties of song and song repertoire, but also the motivation to interact with their partner [38–41]. Previous studies have established that certain strains of mice are susceptible to audience effects when responding to female cues [37,42]. For example, Seagraves et al. (2016) found that increases in vocal production in response to live and anesthetized male audience members may be due to signalers shifting their vocalizations to be directed to males, potentially in a competitive display. Thus, the presence of an audience may cause mice to adjust their vocal output and the function of their calling may change, similar to the strategic changes influenced by audiences across other contexts [37,41,43–45].

In order to assess the effects additional male cues and social status on male behavioral response to BBVs, we employed a recently established paradigm [35] that allows for the decoupling of BBVs and their frequently associated physical rejection behaviors. We conducted two types of trials using this paradigm that varied based on the odors presented to the focal male during the trial: a trial in which the male was exposed to female odor only (Fem odor condition) and a trial in which the male was exposed to both female odor and odor from additional males (Fem+Male odor condition). We predicted that the addition of male odor during these interactions would increase the degree by which males downregulated their vocal output in response to BBV playback, and that this effect would be dependent on social status. It has been reported that dominant mice are more sensitive to social challenges than subordinate mice [46–48], so we predicted that dominant males would alter their behavior in response to a simulated social competitor more than subordinates by showing a larger decrease in USV production during BBV playback if additional male odor was present. For the first part of this study, these assays were conducted using male odor that was familiar to the stimulus animals.

The second half of this study included an additional cohort of animals who differed from the first cohort in the familiarity of the mice that provided the additional odor during the trials to assess the effects of novel vs familiar “audience members”. For this familiar odor cohort, the odor from the familiar male would come from a mouse that the focal male had interacted with (and with whom they had an established hierarchy). This was of particular interest to us because identity and status of audience members has been shown to alter behavior during signaling in response to female cues [49]. We predicted that more robust differences between dominants and subordinates would be seen in the familiar odor cohort because dominants would be

exposed to the odor of an individual known to be subordinate to them, and subordinates would be exposed to the odor of an individual known to be dominant over them, rather than the odor of completely unknown male mice. Surprisingly, we found that dominance status did not affect vocal behavior, while the presence of additional male odor dampened vocal production, but only when the odor came from a male unfamiliar to the focal individual.

Methods

Animals

Focal subjects consisted of seven-week-old male CBA/J mice (N = 23) (The Jackson Laboratory, Bar Harbor, ME). Mice were housed in a 14:10 light:dark cycle with food and water *ad libitum*. All mice were housed in pairs from at least one week prior to behavioral assays until the end of the experiments in order to allow for the establishment and maintenance of a social hierarchy within the cage. Dominance status was evaluated using multiple measurements of a standardized tube test [35,50]. All housing pairs underwent 3-4 tube tests with no more than one test per day, and with tests occurring no more than 4 days prior to the trial dates. Almost all mice were consistent in their dominance status across all of their tests, aside from two pairings that switched dominance status after the first test, but were consistent for the final three tests. At least two days prior to experimental trials, all mice were given opposite-sex experience with novel female CBA/J mice (The Jackson Laboratory, Bar Harbor, ME) through multiple 10 minute interactions across the span of several days [11]. All mice received approximately the same

amount of social experience prior to trials. All stimulus animals for experimental trials were sexually experienced adult female CBA/J house mice novel to the focal mouse.

Behavioral assay and experimental conditions

A split-cage playback paradigm previously established in our lab [35] was employed to evaluate the effects of vocal playback on male mouse vocal behavior. In this paradigm, the focal mouse and stimulus female are placed on opposite sides of a 12 inch x 6 inch x 6 inch cage with a plexiglass barrier in the middle with a small hole at the bottom of the barrier that allows for limited nose-to-nose social investigation. Dirty bedding containing urine and feces from the stimulus female and her cage mate was placed on the focal male's side of the cage to promote robust vocalizations [35,36], while clean bedding was placed on the female's side of the cage. A microphone (CM16/CMPA, Avisoft Bioacoustics, Glienicke/Nordbahn, Germany) and Canon VIXIA video camera were placed above the cage on the focal male's side to allow for recording of ultrasonic vocalizations and non-vocal behavior, respectively, during the interaction. The entire cage and recording setup were located in a sound-proof chamber (IAC Acoustics, Naperville, IL). Mice were placed in this cage for 15-minute playback trials that consisted of 5 minutes of silence (a baseline period), followed by 5 minutes of exemplar BBV playback through an ultrasonic speaker powered by Avisoft next to the cage, followed by another five minutes of silence (a recovery period). The segment of BBV playback was created using five repeated 1-minute segments of BBVs collected from a previous direct interaction between a male and a female during a period of frequent physical rejection and BBV vocalization [35]. Each of the calls during this period were replaced with a single exemplar BBV with a duration, peak frequency, and

161 percentage of non-linear segments within one standard deviation of the mean for all BBVs in the
162 interaction in order to eliminate possible differences in behavior over the course of the playback
163 due to variation in BBV structure [35]. The BBV segment was played at a loudness calibrated to
164 be approximately matched to the intensity that male mice would experience from BBVs during
165 natural interactions with females, with a maximum of 104.9 dB [35].

166 Previous use of this paradigm in our lab has shown that males decrease their USV
167 production during BBV playback relative to the baseline and increase back to baseline levels
168 during the recovery. This response to BBV playback when males are physically separated from
169 the stimulus female indicates that playback of BBVs can alter male courtship vocal behavior even
170 when the correlated physical rejection behaviors are absent [35]. For the current study, a
171 modified version of this paradigm was created to allow for two distinct experimental conditions
172 that altered the available olfactory cues: a female-scent-only condition in which only bedding
173 from the stimulus female's home cage was placed on the focal animal's side of the cage (Fem
174 condition) and a female scent + male scent condition in which the focal male was exposed to
175 equal parts bedding from the stimulus female as well as bedding from a cage of male mice
176 (Fem+Male condition; Fig 2).

Fig 2: Experimental assay and design.
(A) Split cage paradigm used to evaluate the effects of only BBV playback on male vocal behavior, modified to create the Fem+Male condition for this study. Male and female symbols present on the male mouse's side of the cage represent the presence of dirty bedding from male and female mice. (B) Experimental groups broken up by familiarity cohort, dominance status, and odor condition.

177 Both assay conditions were applied to the two separate experiments with separate
178 cohorts of animals described in the following sections. These experiments differed based on the
179 origin of the male bedding used in the Fem+Male condition, described below, while the Fem

condition trials in both experiments were identical in design. All individuals in both experiments/cohorts underwent both a Fem condition trial and a Fem+Male condition trial to account for individual differences in vocal behavior, and the order of the trials was counterbalanced across all individuals.

Experimental design

Experiment 1 - Unfamiliar male odor cohort: In the Fem+Male condition, individuals in the unfamiliar male odor cohort (n=12) were exposed to equal amounts of dirty bedding from the stimulus female's cage and dirty bedding that came from a cage of males unfamiliar to them. These "odor donations" were from the other cages of males used in this experiment. No males from separate cages ever interacted with one another, thus all of the "odor donations" came from males novel to the focal individuals. The Fem+Male odor condition in this experiment was designed this way to simulate the presence of an unfamiliar male competitor during the limited interaction with the female.

Experiment 2 - Familiar male odor cohort: In a subsequent experiment, a new cohort (n=16) was established as the familiar male odor cohort. For these individuals, in the Fem+Male condition they were exposed to equal amounts of dirty bedding from the stimulus female's cage and dirty bedding from their own home cage, in order to simulate the presence of their familiar cage mate: their social hierarchical partner.

Audio analysis

USVs from all of the interactions were counted individually by trained individuals using Avisoft SasLab Pro software (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany). A total of 74,859 USVs were counted manually during the course of this study. Durations of the USVs were automatically calculated by Avisoft. In order to assess specific components of the individuals' vocal repertoires, all USVs were manually categorized as either non-harmonic or 50 kHz harmonic USVs (Fig 1). 50 kHz harmonic USVs were specifically denoted because they are highly correlated to male mouse mounting behavior and could potentially be used to promote receptivity in females during courtship interactions [10,35].

Non-vocal behavior analysis

Non-vocal behavior was analyzed using Behavioral Observation Research Interactive Software (BORIS) [51]. The odor condition and cohort of the focal individual was blinded to the analyst for each trial. The duration of time both the focal male and stimulus female spent at the hole in the plexiglass divider was scored for all individuals. Investigation of the window was defined as the mouse placing their nose at the entrance of (< 1 cm away from the entrance) or through the opening in the barrier.

Statistical analysis

Statistical analyses were performed in IBM SPSS Statistics 28. A Pearson correlation was used to evaluate the relationship between the baseline USVs produced in the Fem condition and the baseline USVs produced in the Fem+Male condition across all individuals to assess consistency in baseline vocal production. Four Pearson correlations were used to evaluate the relationship between the investigative behavior of the focal male and the stimulus female in each cohort, for each odor condition. Due to the difference in the Fem+Male conditions across the two cohorts, distinct models were used for each cohort for analysis of vocal and non-vocal behavior.

To assess the effects of BBV playback across time bins and odor treatment on number of non-harmonic calls, a three-way repeated measures ANOVA was used for the unfamiliar odor cohort, and an identical three-way repeated measures ANOVA was used for the familiar odor cohort (within-subjects factors: time bin (baseline, playback, or recovery) and odor treatment (Fem or Fem+Male); between-subjects factor: dominance status (dominant or subordinate)). Analyses on the effects of odor condition and dominance status on baseline number of USVs in each interaction and the duration of baseline USVs produced were conducted using identical two-way mixed ANOVAs for the unfamiliar odor cohort and familiar odor cohort (within subjects factor: odor treatment (Fem or Fem+Male); between-subjects factor: social status (dominant or subordinate)).

In order to investigate how the composition of vocal repertoires responded to our manipulations, we characterized the proportion of harmonic USVs out of all USVs in each time bin of the interaction. To assess the effects of BBV playback across time bins and odor treatment on proportion of harmonic calls produced, proportions of harmonic calls across bins were calculated as: number of harmonic calls in bin/number all calls in bin. These proportions were

then compared using identical binary logistic regressions for each cohort [52–54] (within-subjects factors: time bin (baseline, playback, or recovery) and odor treatment (Fem or Fem+Male); between-subjects factor: dominance status (dominant or subordinate)). Binary logistic regressions were also used to evaluate the effects of odor condition and dominance status on the baseline proportion of harmonic USVs for the unfamiliar odor cohort and familiar odor cohort (within subjects factor: odor treatment (Fem or Fem+Male); between-subjects factor: social status (dominant or subordinate)).

To assess the effects of BBV playback across time bins and odor treatment on non-vocal behavior, two identical three-way repeated measures ANOVAs were used, one for each cohort (within-subjects factors: time bin (baseline, playback, or recovery) and odor treatment (Fem or Fem+Male); between-subjects factor: dominance status (dominant or subordinate)).

The following datasets violated the ANOVA assumption of normality and thus were normalized using square root transformations in order to run analyses of variance: USVs across time bins in the unfamiliar odor cohort, and female investigative behavior in the unfamiliar odor cohort. Small violations of the homogeneity of variance requirement were noted, but as these models were the best fit aside from this fact, and because ANOVAs are robust to small violations of this assumption when sample sizes are equal, we proceeded with ANOVAs [55]. Benjamini-Hochberg corrections (Q value = 0.05) were conducted in order to account for multiple comparisons [56], and Bonferroni tests were performed as posthoc analyses.

Results

Individual baseline vocal production in both odor conditions is positively correlated

All individuals underwent both a Fem odor condition trial, in which focal individuals were only exposed to the odor of the female stimulus animal, and a Fem+Male odor condition trial, in which focal individuals were exposed to both odor from the female stimulus and odor from an additional male conspecific (Fig 2B). A Pearson Correlation revealed that the baseline vocal production across both trials was strongly positively correlated across individuals in both the familiar odor cohort and unfamiliar odor cohort ($r = 0.735$, $p < 0.001$) (Fig 3).

Figure 3: Correlation between vocalizations produced in the Fem condition and the Fem+Male condition for all individuals. Individuals in both cohorts are consistent in vocal output across both odor conditions, as evidenced by a positive Pearson's Correlation ($r = 0.735$, $p < 0.001$). Red data points represent individuals in the familiar odor cohort, blue data points represent individuals in the unfamiliar odor cohort. Dominants are represented by X's, and subordinates are represented by circles.

Social status does not alter behavior across cohorts or odor conditions

Social hierarchies for each cage consisted of a dominant individual and a subordinate individual, and the placement of individuals within these hierarchies remained stable throughout multiple rounds of tube tests. Out of 14 hierarchy pairings, only 2 pairings changed status at all throughout the tube tests. Both of these switches occurred during the second test and the pairings were subsequently stable throughout the rest of the three tests. When incorporating social status in our statistical models as a between-subjects factor, there were no significant effects of social status in any behavioral measures (Tables 1-3).

Table 1. Output of models assessing the effects of BBV playback, Fem or Fem+Male odor condition, and dominance status on total USV production and harmonic USV production in both cohorts.

		Number of USVs in Unfamiliar odor cohort [a]		Proportion of 50 kHz Harmonics in Unfamiliar odor cohort [b]	
Effect	***df***	***F***	***p***	***F***	***p***
Time Bin	2	50.538	**<.001**	4.925	**0.011**
Odor Condition	1	12.026	**0.006**	8.429	**0.005**
Dominance Status	1	0.019	0.892	0.702	0.406
Bin x Odor Condition	2	0.223	0.802	2.236	0.116
Bin x Dominance Status	2	0.614	0.551	0.131	0.877
Odor Condition x Dominance Status	1	0.742	0.409	0.022	0.882
Odor Condition x Bin x Dominance Status	2	1.203	0.321	1.093	0.342

[a] Nonharmonic USVs were assessed with three-way mixed ANOVAs.

[b] Proportion of 50 kHz harmonic USVs were assessed using binary logistic regressions.

Table 2. Output of two-way mixed ANOVAs assessing the effects of Fem or Fem+Male odor condition and dominance status on baseline USV production, baseline harmonic USV production, and duration of baseline USVs in both cohorts.

		Baseline number of USVs in Unfamiliar odor cohort		Baseline 50 kHz Harmonics in Unfamiliar odor cohort		Duration of ba USVs in Unfami cohort	
Effect	***df***	***F***	***p***	***F***	***p***	***F***	***p***
Odor Condition	1	10.005	**0.01**	10.752	**0.004**	5.285	0.04
Dominance Status	1	0.04	0.846	0.036	0.852	0.051	0.82
Odor Condition x Dominance Status	1	0.919	0.36	2.101	0.163	0.576	0.46

* p value is not statistically significant after correcting for multiple comparisons.

Table 3. Output of three-way mixed ANOVAs assessing the effects of BBV playback, Fem or Fem+Male odor condition, and dominance status on male and female investigation of the divider window in both cohorts.

		Male investigation in Unfamiliar odor cohort		Female investigation in Unfamiliar odor cohort			Male Famil
Effect	***df***	***F***	***p***	***F***	***p***	***df***	***F***
Time Bin	2	3.965	0.037*	3.841	0.041*	2	4.391

Odor Condition	1	0.958	0.353	5.437	0.045*	1	0.034
Dominance Status	1	3.315	0.102	0.549	0.478	1	0.665
Bin x Odor Condition	2	0.421	0.663	0.254	0.779	2	0.93
Bin x Dominance Status	2	0.732	0.495	4.109	0.034*	2	2.8
Odor Condition x Dominance Status	1	1.962	0.195	0.059	0.814	1	0.062
Odor Condition x Bin x Dominance Status	2	0.585	0.567	0.493	0.619	2	0.063

* p value is not statistically significant after correcting for multiple comparisons.

Additional male odor during limited social interactions decreases baseline vocal output depending on the familiarity of male odor

In order to assess how contextual manipulations affected baseline vocal behavior, vocal behavior during BBV playback, and vocal behavior after playback ended, we employed a fifteen minute interaction in which the first five minutes consisted of silence, followed by five minutes of BBV playback, followed by a final five minutes of silence [35]. The addition of unfamiliar male odor during the interaction (Fem+Male condition) caused significantly lower baseline levels of USVs compared to when only female odor was present (Fem condition) (Two-way mixed ANOVA: $F_{1,10} = 10.005$, $p = 0.01$). The addition of familiar male odor however produced no significant difference in baseline USV levels compared to when only female odor was present (Two-way mixed ANOVA: $F_{1,14} = 0.084$, $p = 0.776$) (Fig 4A) (Table 2).

Figure 4: Properties of vocal repertoires. (A) Baseline USVs produced in the Fem condition vs the Fem+Male condition. (B) Average duration of baseline USVs in seconds. (C) Baseline proportion of harmonic USVs (# baseline harmonics/# baseline USVs). Proportion of baseline harmonic USVs was calculated as baseline harmonic USVs /(baseline harmonic USVs + baseline nonharmonic USVs). Blues represent the unfamiliar odor cohort, reds represent the familiar odor cohort. Averages from dominants and subordinates are pooled due to the lack of effect of social status. Individual points of data noted with dominants as X's and subordinates as circles.

Because USVs can vary widely in duration [1], we measured the duration of all baseline USVs (both harmonic and nonharmonic). Males produced significantly longer baseline USVs in the Fem condition compared to the Fem+Male condition in unfamiliar male cohort (Two-way mixed ANOVA: $F_{1,10} = 5.285$, $p = 0.044$), though this result was not significant after correcting for multiple comparisons (Table 2). No effects of odor condition on duration of baseline USVs were observed in the familiar male odor cohort (Two-way mixed ANOVA: $F_{1,14} = 0.005$, $p = 0.945$) (Fig 4B).

Additional male odor during limited social interactions decreases baseline usage of 50 kHz harmonic USVs depending on the familiarity of male odor

50 kHz harmonic USVs are calls highly correlated to mounting behavior in male mice [11]. In this study, harmonic USVs made up a small proportion of all calls; on average, harmonic USVs made up 7.31% (SD = 0.0701) of all USVs in the interaction. In order to assess whether the frequency of harmonic USV usage differed across conditions, for each individual we evaluated the proportions of harmonic USVs produced in each time bin out of the total number of all USVs in that time bin. The addition of unfamiliar male odor during the interaction (Fem+Male condition) caused mice to use a significantly lower proportion of 50 kHz harmonic USVs during the baseline period compared to when only female odor was present (Fem condition) (Binary logistic regression: $F_{1,10} = 10.752$, $p = 0.004$), though this effect was not present in the familiar male odor cohort (Binary logistic regression: $F_{1,14} = 0.035$ $p = 0.853$) (Fig 4C) (Table 2).

Male mice decrease USV production in response to BBV playback across all conditions

In both the unfamiliar odor cohort and the familiar odor cohort, male mice reduced their calls during playback of BBVs, and then increased calling rates after playback ceased in both Fem and Fem+Male trials (Three-way mixed ANOVAs: Significant main effect of bin $F_{2,20} = 50.538$, $p < 0.001$ and $F_{2,28} = 34.635$, $p < 0.001$ for the unfamiliar and familiar cohorts, respectively). This subsequent increase in calling after the playback period did not fully recover to levels similar to baseline, however, but rather increased to a level between that observed in the baseline and the recovery. In the unfamiliar odor cohort, individuals produced more USVs in the Fem odor condition compared to the Fem+Male odor condition (Significant main effect of odor condition: $F_{1,10} = 12.026$, $p = 0.006$) (Fig 5A), though this effect was not present in the familiar odor cohort ($F_{1,14} = 0.454$, $p = 0.511$) (Fig 5B). No interaction effects between odor and bin were present in either the unfamiliar odor cohort or the familiar odor cohort ($F_{2,20} = 0.223$, $p = 0.802$, and $F_{2,30} = 0.734$, $p = 0.489$) (Table 1).

Figure 5: Number of USVs produced across time bins (A) in the unfamiliar odor cohort, and (B) in the familiar odor cohort. Average USV production of dominants is represented by the solid lines, and average USV production of subordinates is represented by dashed lines. Lighter lines denote average USV production of individuals in the Fem condition, while darker lines denote average USV production of individuals in the Fem+Male condition.

Because there was no interaction effect between time bin and any other factor in these models, our data showed that differences across odor conditions and familiarity cohorts can be attributed to differences in baseline levels of USV production, rather than differences in vocal response to BBV playback.

349

350 **Male mice increase usage of 50 kHz harmonics after BBV playback ends**

351 **when exposed to unfamiliar male odor**

352 In the unfamiliar male odor cohort, the proportion of 50 kHz harmonic calls used relative to the
353 total number of calls produced did not change in response to BBV playback, however after
354 playback ended, males produced a greater proportion of harmonic calls in the recovery period
355 (Binary logistic regression: Significant main effect of bin $F_{2,20}$ = 4.925, p = 0.011). However,
356 posthoc pairwise comparisons revealed this increase in harmonic usage only occurred in trials
357 with unfamiliar male odor added (t_{55} = 2.344, p = 0.023) (Fig 6A). Overall, in the unfamiliar male
358 odor cohort, males produced a greater proportion of harmonics in Fem trials compared to
359 Fem+Male trials (Binary logistic regression: Significant main effect of odor condition $F_{1,10}$ = 8.429,
360 p = 0.005). In the familiar male odor cohort, no significant effects of any factor were observed
361 (Table 1) (Fig 6B).

Figure 6: Proportion of harmonic USVs across time bins out of total USVs (A) in the unfamiliar odor cohort and (B) in the familiar odor cohort. Average harmonic USV production of dominants is represented by the solid lines, and average harmonic USV production of subordinates is represented by dashed lines. Lighter lines denote average harmonic USV production of individuals in the Fem condition, while darker lines denote average harmonic USV production of individuals in the Fem+Male condition. Proportion of harmonic USVs for each time bin was calculated as harmonic USVs in time bin/(harmonic USVs + nonharmonic USVs in time bin). The significance bar in 5A) showing increased usage of proportion of harmonic USVs in the recovery period compared to the baseline and playback period is dark blue, representing that it only applies to the Fem+Male conditions, not the Fem conditions.

362

Male and female investigative behavior does not significantly differ across conditions, cohorts, nor time bins

In the unfamiliar odor cohort, male investigation of the divider window appeared to slightly decrease during BBV playback, and investigative behavior did not recover after BBV playback, with no effects of dominance status or odor condition (Three-way mixed ANOVA: $F_{2,18} = 3.965$, $p = 0.037$) (Fig 7A). In contrast, in the familiar odor cohort, male investigation of the divider window appeared to slightly decrease during BBV playback and subsequently recover after playback, with no effects of dominance status or odor condition (Three-way mixed ANOVA: $F_{2,28} = 4.391$, $p = 0.022$) (Fig 7C). However, neither of these results were significant after correcting for multiple comparisons.

Figure 7: Duration of time the focal male (A and C) or the stimulus female (B and D) spent investigating the divider window across time bins in the unfamiliar odor cohort (A and B) and in the familiar odor cohort (C and D). Average duration of investigation in trials with dominant males is represented by the solid lines, and average duration investigation in trials with subordinates is represented by dashed lines. Lighter lines denote average investigation during trials in the Fem condition, while darker lines denote average investigation in the Fem+Male condition.

Stimulus females paired with males in the unfamiliar odor cohort tended to investigate the divider window more during the Fem+Male condition trials ($F_{1,9} = 5.437$, $p = 0.045$), and this investigation actually increased during BBV playback, and decreased back to baseline levels after playback ended ($F_{2,18} = 3.841$, $p = 0.041$) (Fig 7B). This effect of playback was dependent on dominance status in that female investigative behavior was only affected by playback when females were paired with subordinate males, and there was no effect when they were paired with dominant males ($F_{2,18} = 4.109$, $p = 0.034$). When stimulus females were paired with males in the familiar odor cohort, they decreased their investigative behavior during BBV playback, and

this behavior increased back to baseline levels when playback ended ($F_{2,28} = 3.949$, $p = 0.031$) (Fig 7D), with no effect of odor condition or dominance status. However, again, none of these results were significant after correcting for multiple comparisons.

Additional male odor alters the relationship between male and female investigative behavior

In the Fem condition trials, the total duration of male investigative behavior did not correlate to the total duration of female investigation behaviors in either the unfamiliar odor cohort or the familiar odor cohort (Pearson correlation: $r = -0.373$, $p = 0.259$; $r = 0.235$, $p = 0.381$ respectively) (Fig 8A-B). In the Fem+Male condition trials, the total duration of male investigative behaviors negatively correlated to the total duration of female investigative behaviors for those in the unfamiliar odor cohort (Pearson correlation $r = -0.625$, $p = 0.04$) (Fig 8C), though this effect was not significant after correcting for multiple comparisons. In contrast, in the Fem+Male odor condition trials conducted with the familiar odor cohort, the total duration of male investigation behaviors positively correlated to the total duration of female investigative behaviors (Pearson correlation $r = 0.839$ $p < 0.001$) (Fig 8D), and this effect remained significant after correcting for multiple comparisons.

Figure 8: Correlation between female investigation and male investigation behavior in the (A) unfamiliar odor cohort: Fem condition, (B) familiar odor cohort: Fem condition, (C) unfamiliar odor cohort: Fem+Male condition, and (D) familiar odor cohort: Fem+Male condition. Dominant individuals denoted as X's and subordinates as circles.

Discussion

Behavioral decisions during courtship are highly influenced by the interplay between environmental cues, internal factors, and signals from communicating conspecifics [4]. Thus, by evaluating how individuals respond to signals in various contexts, we can begin to decipher how much weight a given factor may have on such decisions. In this study, when interactions included odors cues from unfamiliar, but not familiar, male conspecifics, male mice exhibited reduced courtship motivation compared to interactions when only female odors were present. This decrease in courtship motivation was evidenced by shifts in multiple aspects of male courtship vocalizations: decreases in call production, call durations, and variety of calling repertoires. Male mice thus may be evaluating their environment for potential competitors, discerning the identity of these competitors, and scaling their courtship activities in response to these cues. The interplay between signals not only from courtship partners but also from competitors as well as social recognition of those signals is instrumental in motivating behavioral decisions during mating interactions.

Unfamiliar male odor during courtship decreases male courtship motivation

Vocal signaling can often reflect motivational state across taxa [57–62]. USV production is a well-established proxy for courtship motivation in male mice, as these calls function to encourage female sexual receptivity and mating [18,21,22,24,63,64]. Furthermore, out of the possible measurements for courtship motivation in mice, these signals are particularly important as they can convey information about male fitness, identity, and reproductive state,

which can in turn influence female mate choice [22,26,65].Changes in courtship motivation can be spurred by exposure to social cues [58,59]. Olfactory cues can perform a variety of functions during social interactions for both males and females. Because these cues are so significant in defining the context of an interaction, particularly opposite-sex interactions, individuals may plastically adjust their behavior in response to cues like these odors, potentially to avoid expending unnecessary time and energy engaging with individuals that are not sexually receptive [66–72]. In this study, we manipulated the context of courtship interactions by including odor cues from additional males not present during the interaction in order to assess how the focal males would behave compared to when no additional male odor cues are present. While many courtship assays used in laboratory mice are conducted with a single male and a single female [73], because of the propensity of mice to live in a wide range of social densities, it is likely that males in natural settings are often confronted with the odors of other potentially competing males [74,75]. Thus, investigating how these odor cues that simulate a potential competitive audience may affect how mice produce and respond to signals gives important insight to how behavioral decisions are made during courtship, and what information is most impactful to individuals as they choose how to navigate social interactions. It is well-established that birdsong exhibits signal flexibility in response to social context [76]. For example, male cowbirds will modulate the attractiveness of their songs in response to the presence of competitors, though how they modulate their songs may depend on factors like whether or not the competitors can easily access the focal individual and if the individual can gauge the motivational state of the competitor [77,78].

As mice can use chemical components within urine to discriminate between individual identity, familiarity, and dominance [79–82], odor cues can be used to simulate different audiences, and investigate how different identity characteristics alter behavior during sexual interactions. We found that whether males responded to this odor was dependent on whether or not the focal male had social contact with the odor's source. When confronted with unfamiliar male odor during the interaction, males produced fewer baseline courtship calls (Fig 4A), the calls produced during the interaction prior to the playback of BBVs, and the average length of these calls showed a trend in that they shorter than when they interacted with a female with no additional male odor present (Fig 4B). These responses may indicate a decrease in courtship motivation and effort. Not only is number of calls produced indicative of motivational state, but types of calls produced can also reveal information about motivation. Across taxa, increasing vocal production through the expression of complex syllables and longer calls is associated with greater energetic costs [22,42,64,83–85]. Longer duration of vocalizations have also been associated with increased courtship display effort in singing mice, and female rodents tend to prefer males that produce longer "higher-effort" songs [64,86,87]. Thus, male mice increasing the usage of more complex and longer-duration elements in songs, like the 50 kHz harmonics assessed in this study, may reflect increased mating effort.

When males were confronted with unfamiliar male odor during the courtship interaction, they also shifted the composition of the different types of syllables they used, their vocal repertoire [29], to produce a lower proportion of 50 kHz harmonic calls during the baseline period when unfamiliar male odor is present (Fig 4C). These calls are categorized by exhibiting a harmonic frequency band at 50 kHz, and are longer and more structurally complex than USVs

464 that do not contain this band (Fig 1). Importantly, these calls are positively correlated with bouts
465 of mounting [10,11]. That males specifically decrease the use of these calls in their vocal
466 repertoire when unfamiliar competitor odors are present reflects the intentional function of
467 these calls to promote mating, and that these competitive odors are significantly affecting
468 numerous aspects of the male courtship vocal repertoire. Calling less and producing less complex
469 and shorter calls thus could indicate decreased reproductive motivation.

470 It may be logical for individuals to show decreased courtship motivation, through the
471 reduction of and simplification of calling behaviors, as cues that indicate the presence of potential
472 reproductive competitors can decrease sexual motivation and induce rapid behavioral plasticity
473 [49,88]. This is supported by the theory of sperm competition and its subsequent behavioral
474 consequences, in that males may "budget" copulatory efforts depending on factors like risk of
475 competition [89–95]. Across taxa, male precopulatory courtship behaviors can be reduced as the
476 density of competitors increase [89,93]. This could be due to adaptive changes in reproductive
477 strategies by the males, or sexual interference from the competitors [89,93]. Our results are
478 consistent with this interpretation. This propensity to quickly adjust one's behavior in response
479 to the socio-sexual environment is especially relevant to house mice, as in nature they can exist
480 in a wide range of population densities, and are able to be successful at both very high and low
481 densities [96–99].

482 Importantly, the specific presence of male odors during sexual interactions can also
483 influence female mate preferences [100–102] and impact competition tactics of males across
484 taxa [103,104]. Thus, it could be important for male mice to recognize when other male odors
485 are present, and "hedge their bets" when it comes to interacting with a female that has multiple

mate options, as to not invest heavily in courting a female that may prefer another male, or may have even already mated with another male [89,90,103,105].

A second cohort of individuals were exposed to the odor of their social hierarchy partner during the limited courtship interaction, and interestingly, when the odor was familiar to the focal individual, their vocal behavior did not differ from when no odor was present at all. We predicted that familiar male competitor odor would emphasize the vocal response differences in dominant and subordinate males, as dominants were confronted with the smell of their subordinate during the interaction, and subordinates with their dominant. Not only was there no difference in dominant and subordinate individuals (to be discussed in more detail in subsequent sections), but number of calls, duration of calls, and repertoire of calls were not affected by the familiar competitor odor at all. This important distinction between the responses of individuals to familiar compared to unfamiliar competitor odors could have occurred due to various reasons. Mice can identify individuals they are familiar with through chemical cues in urine [79]. After frequent exposure to odor cues from conspecifics, mice can habituate to these odors, and thus have a less pronounced response to them [103,106,107]. The dear enemy hypothesis predicts that familiar competitors should elicit less of a reaction from territory owners than unfamiliar competitors, due to recognition and knowledge of the benefits and costs of defending against those particular individuals [108–110]. Our results are consistent with the dear enemy hypothesis in that the cues from familiar competitors elicit less of a response (less of a decrease in calls) than cues from unfamiliar competitors. The familiarity of the male cues present could determine whether or not males pay more attention to the male cues, or ignore them and shift focus to the female. A potential way for future studies to explore if, in this context, the decrease in vocal

behavior in response to unfamiliar competitor odor is paired with an increase in behaviors aimed to signal to the competitor could be to assess competitive scent marking behavior of the focal males when confronted with familiar vs unfamiliar odor [106,111,112]. If males confronted with unfamiliar odor are shifting their efforts from courting the females to focusing on competing with potential competitors, we would expect to see an increase in marking behavior is correlated with a decrease in vocal behavior.

The relationship between male and female investigative behavior changes direction depending on familiarity of the competitor odor

Any apparent small changes in male and female investigation of the divider window, through which limited access of the other individual was possible, were not statistically significant after correction for multiple comparisons (Fig 7; Table 3). However, unexpectedly, when investigating how male and female investigative behavior related to one another across the different odor conditions and familiarity cohorts, we found that when trials included odor from familiar males, there was a positive correlation between male and female investigative behavior (Fig 8D), and we found no correlation between male and female investigative behavior in any other conditions (Fig 8A-C). It is possible that the Fem+Male trials having odor from two different males available to the females would prompt more investigation from the female, though this was not the case (Fig 7B, 7D). Overall, our data revealed that the relationship between male and female investigative behavior in the presence of varying social odor conditions is not straightforward as one might expect, and worthy of future investigation.

BBVs spur decreased courtship effort in male mice

All experimental groups in this study decreased calling in response to playback of broadband vocalizations, BBVs, and no other factors measured affected this response (Fig 5). While the function of BBVs across contexts has been largely unexplored, evidence suggests that in certain courtship contexts, BBVs serve to communicate a negative affective state of the signaler [26,34,113,114]. Surprisingly, we found that when individuals were exposed to the odor of an unfamiliar competitor, the proportion of 50 kHz harmonic USVs they used increased in the recovery period after exposure to BBV playback, and it was in this period that they used the highest proportion of harmonics in their calling, even more so than in the baseline period (Fig 6A). More in depth research will have to explore the intricacies of why the discontinuation of BBV playback might spur a rapid increase in harmonic usage only when cues from an unfamiliar potential competitor are present, though this could potentially reflect habituation to the competitor cues as the trial goes on. This result, though difficult to interpret, does however support that the cessation of BBV playback changes the trajectory of syllable usage in male mice when cues indicating the presence of an unfamiliar competitor are present.

BBVs may not always produce a negative reproductive response, and are sometimes correlated with increases in mounting and calling [11,114,115]. It is possible that the potential different functions of BBVs manifest when BBVs are paired with other nonvocal behaviors, and the isolated vocal characteristics of the BBVs may indicate a negative valence, and thus promote decreased courtship effort in receivers without access to additional cues from the female.

Our results exemplify that these signals are significant and attention-grabbing to male mice, seemingly regardless of certain identity and environmental factors. BBVs and their correlated behavioral responses remain a largely untapped line of research; and very few studies have explored the effects of BBV playback isolated from the associated physical rejection behaviors often correlated with BBV production. Greater research devoted to this area of mouse communication could help elucidate how different internal and external factors may affect responses to these signals.

Lack of an effect of dominance status

In this study, dominance status did not significantly affect any vocal or nonvocal behavioral measurements in either the unfamiliar odor cohort or the familiar odor cohort. This was not what we predicted, as it is generally accepted that dominant male mice produce more USVs than subordinate individuals in response to a novel female [116,117], though USVs are not a standard way to measure dominance as they do not directly measure access to mates, but rather reflect mating effort [22]. Because USV production is highly dependent on other contextual factors, the relationship between USV production and dominance is likely influenced by finetuned differences in social history and situational context rather than being simply and strictly linear [118]. For example, certain studies have found the relationship between dominance and increased USV production depends on the types of cues present and the recency of social defeat, and can even reverse [7,19,36]. Interestingly, in this study, the individual with the highest number of baseline calls in all conditions (both odor cohorts in both the Fem and Fem+Male conditions) was subordinate (Fig 4A), though there was no overall statistically significant effect of dominance

on any measurements. Despite observations that this strain of mice may not exhibit overt severe dominance their hierarchies [119], all housed pairs in this experiment showed consistent and reliable dyadic hierarchy relationships through multiple rounds of the tube test. Thus, depending on the context of an interaction, dominance may affect behaviors such as offensiveness and aggression during tube tests, while not manifesting in indirect courtship assays like that used in this study.

Dominant and subordinate males also tend to differ in their response to social challenges [120–122]. Dominant rodents tend to be more susceptible to negative health consequences and behavioral impairment following social challenges compared to subordinate males [46,47,123,124], however this is most often exhibited during direct interactions with social competitors. In this study, dominant males and subordinate males were not differentially affected by odors of competitor males during courtship, thus odors alone may not be sufficient to induce this hierarchy effect. Salience of cues, interaction context, social history, and genetic factors may all contribute to how the effects of dominance manifest during courtship [116,122,125–127], and more research is needed to tease out which of these factors are most influential on determining the effects of dominance.

Conclusions

By looking at how these multiple factors intertwine to affect behavioral decisions during courtship, we can begin to assess what “matters” to a mouse as they navigate mating and when and where to exert effort during courtship. This study supports the potential negative valence of a relatively ill-explored female signal: broadband vocalizations, or squeaks, in certain contexts.

We determined that while males robustly adjust their behavior in response to these calls, the degree of this behavioral response was not altered by any of the internal or external factors we manipulated. Furthermore, this study posits that cues indicating the presence, or "audience", of potential competitors can alter male mouse courtship motivation and behavior, but this effect is dependent on social history with the audience members. Furthermore, our results support that certain identity factors, like dominance status, do not affect the relationship between these cues and the observed behavioral response.

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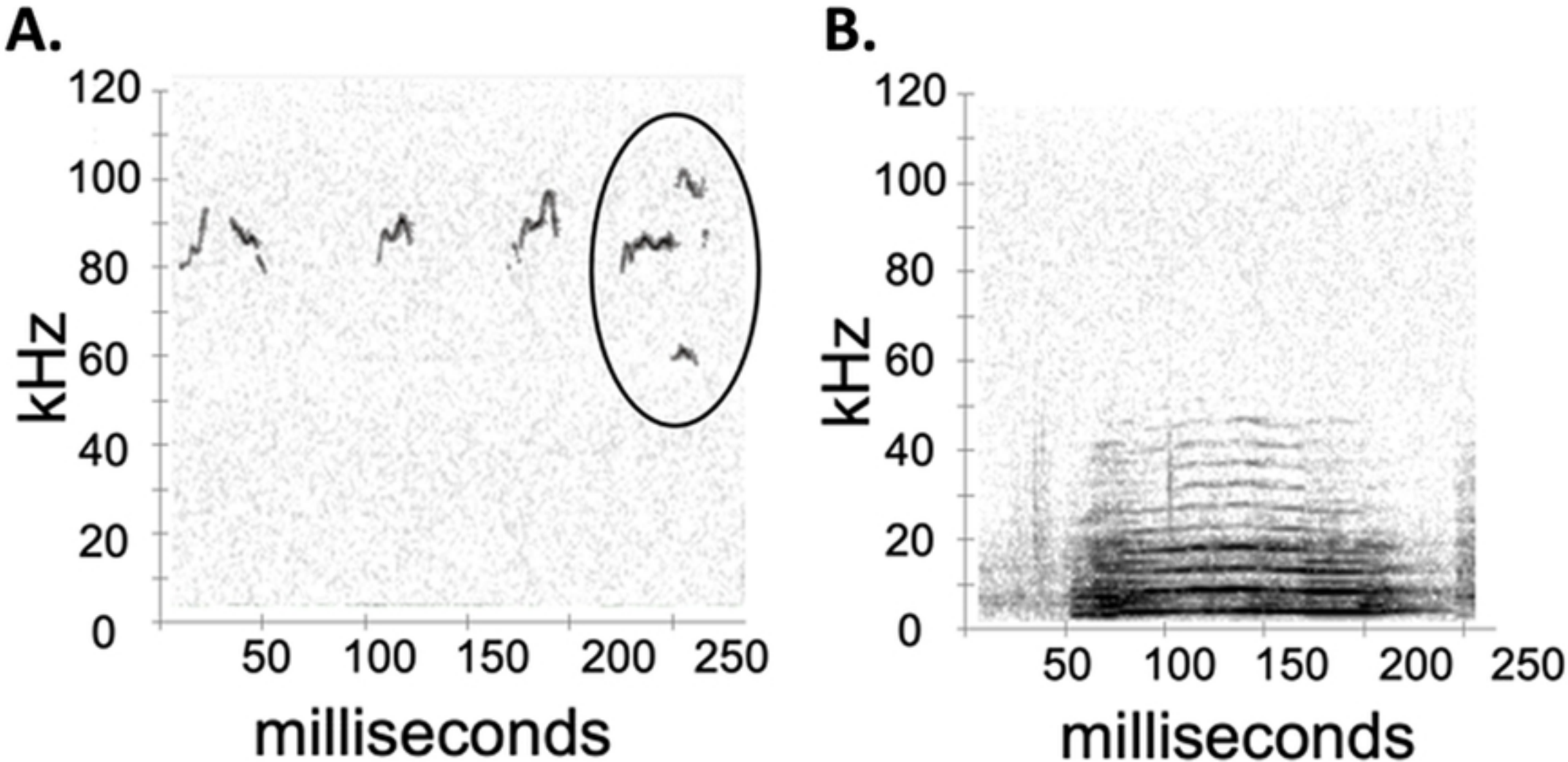
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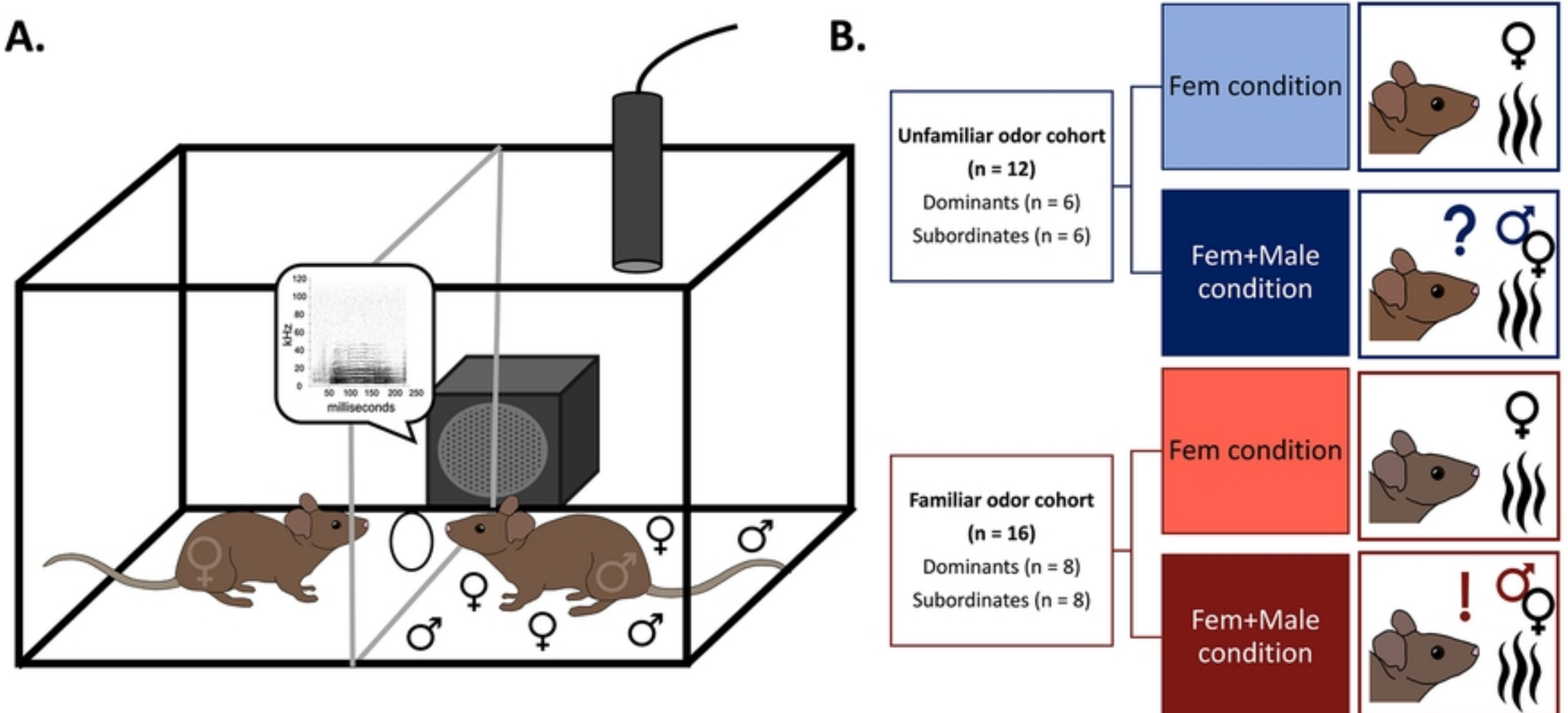
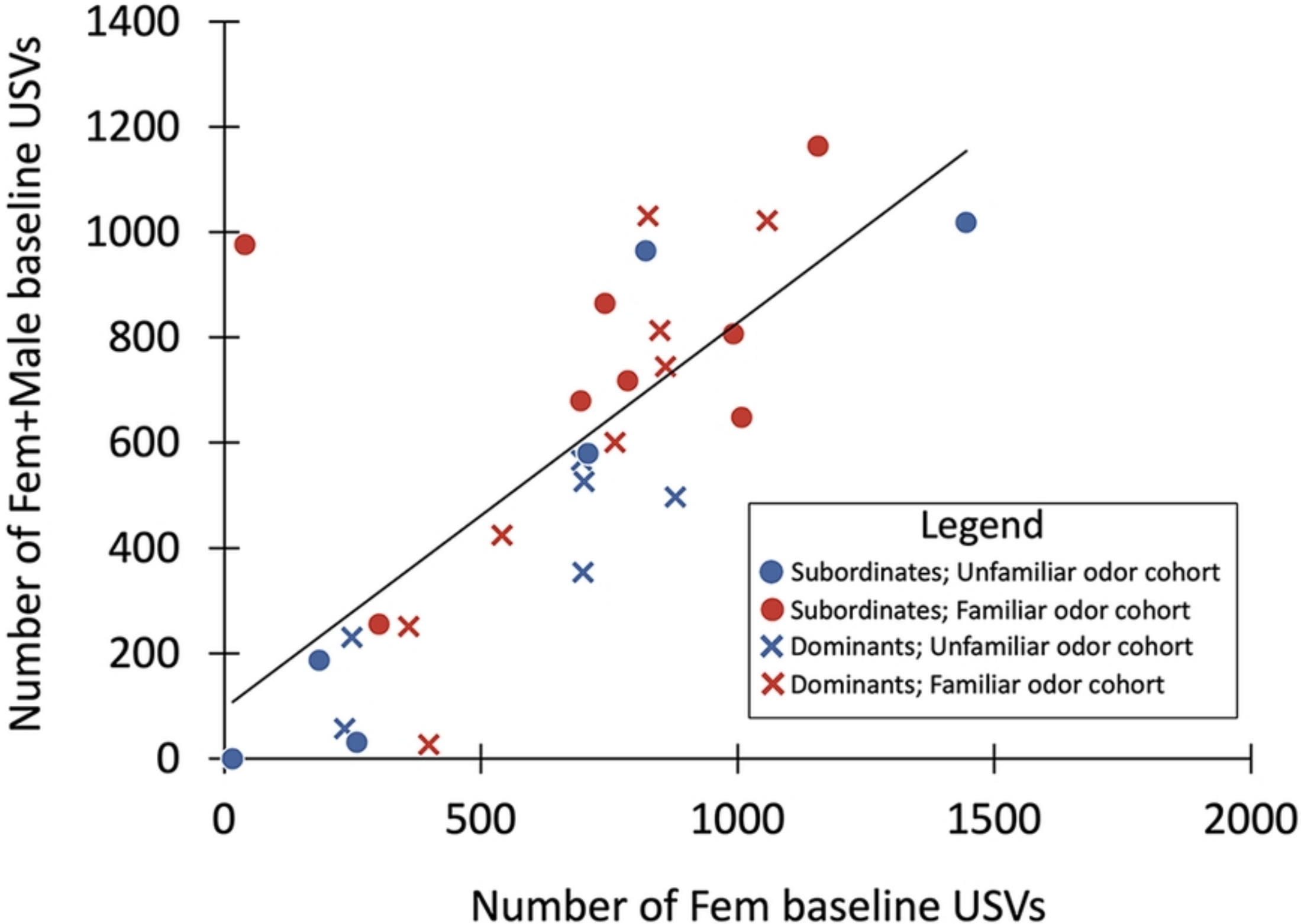
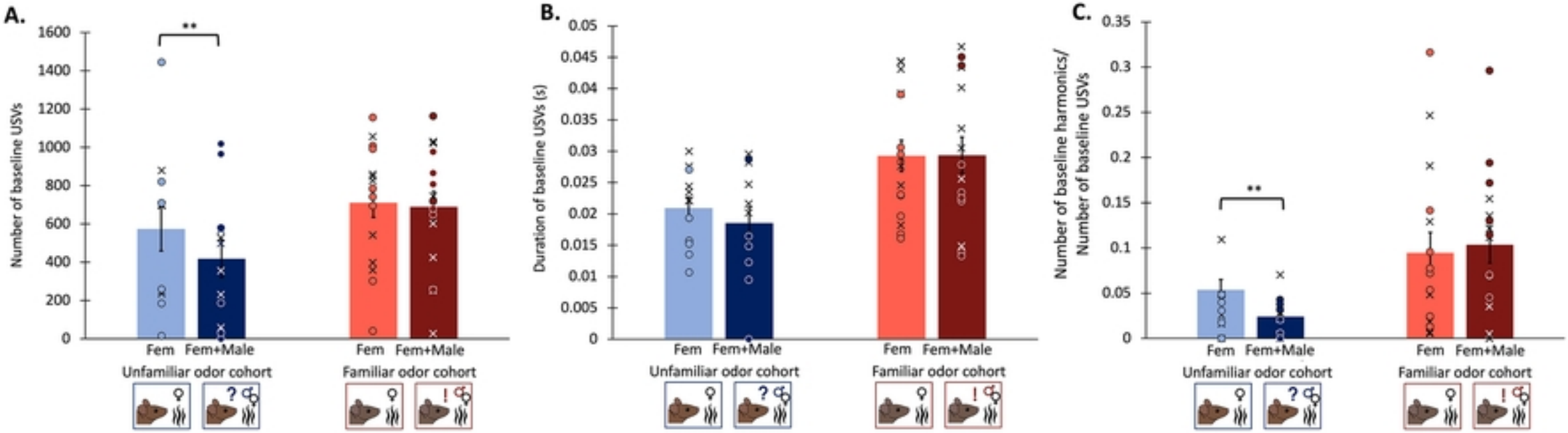
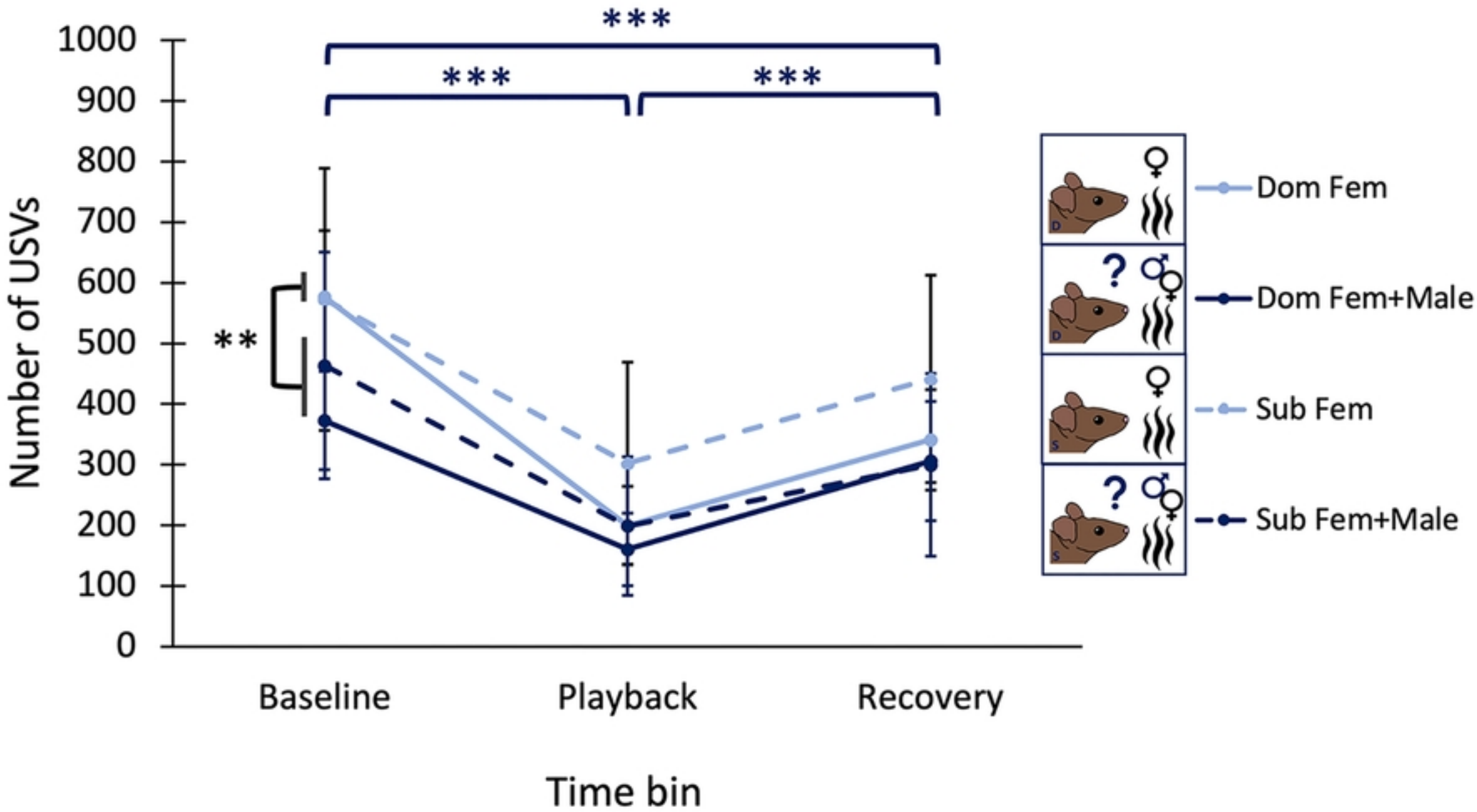

A.
B.
kHz
milliseconds
Unfamiliar odor cohort
(n = 12)
Dominants (n = 6)
Subordinates (n = 6)
Fem condition
Fem+Male condition
Familiar odor cohort
(n = 16)
Dominants (n = 8)
Subordinates (n = 8)
Fem condition
Fem+Male condition

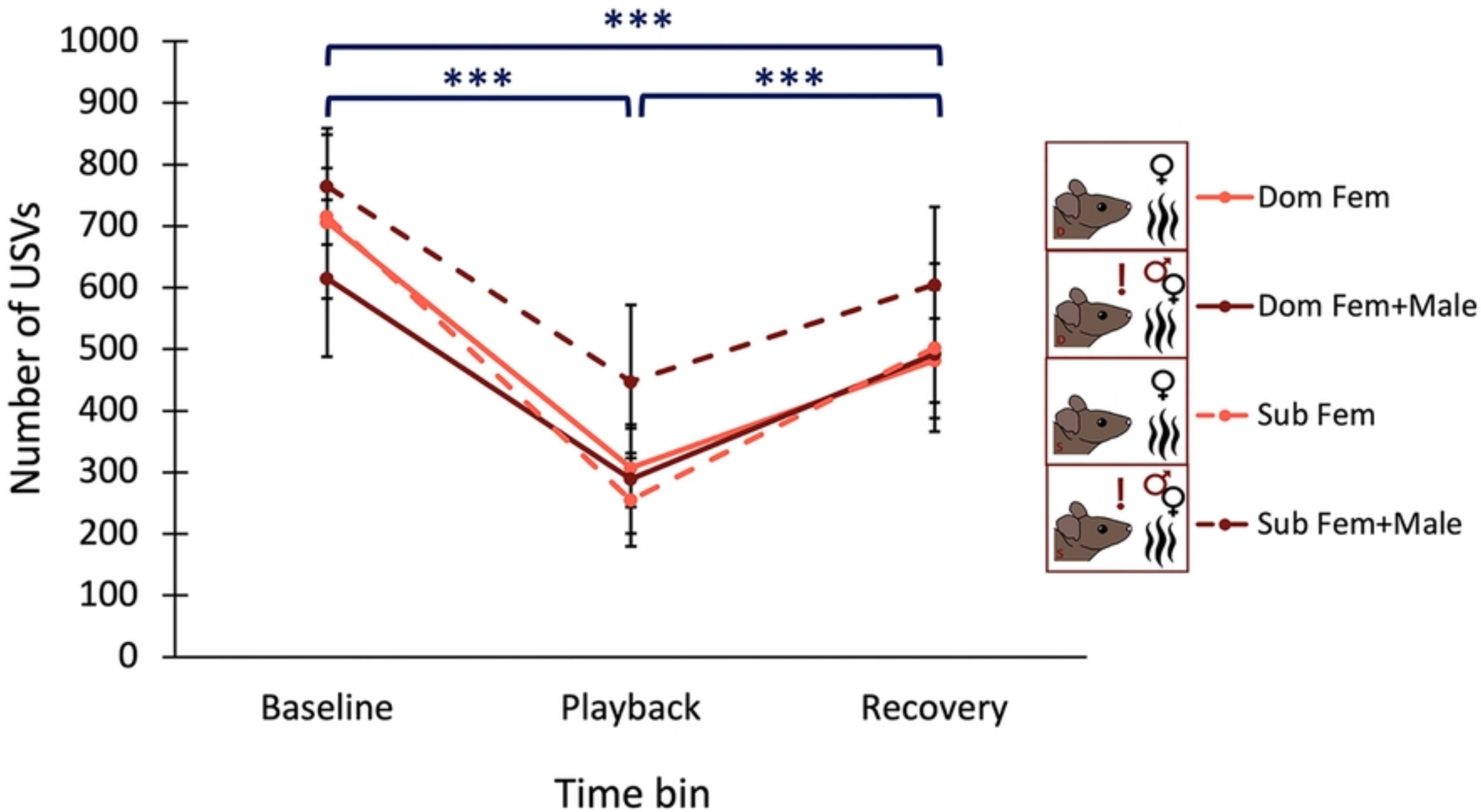

Number of Fem+Male baseline USVs
1400
1200
1000
800
600
400
200
0
0
500
1000
1500
2000
Number of Fem baseline USVs
Legend
Subordinates; Unfamiliar odor cohort
Subordinates; Familiar odor cohort
Dominants; Unfamiliar odor cohort
Dominants; Familiar odor cohort


A.
Number of baseline USVs
1600
1400
1200
1000
800
600
400
200
0
**
Fem
Fem+Male
Fem
Fem+Male
Unfamiliar odor cohort
Familiar odor cohort
B.
Duration of baseline USVs (s)
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Fem
Fem+Male
Fem
Fem+Male
Unfamiliar odor cohort
Familiar odor cohort
C.
Number of baseline harmonics/
Number of baseline USVs
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
**
Fem
Fem+Male
Fem
Fem+Male
Unfamiliar odor cohort
Familiar odor cohort

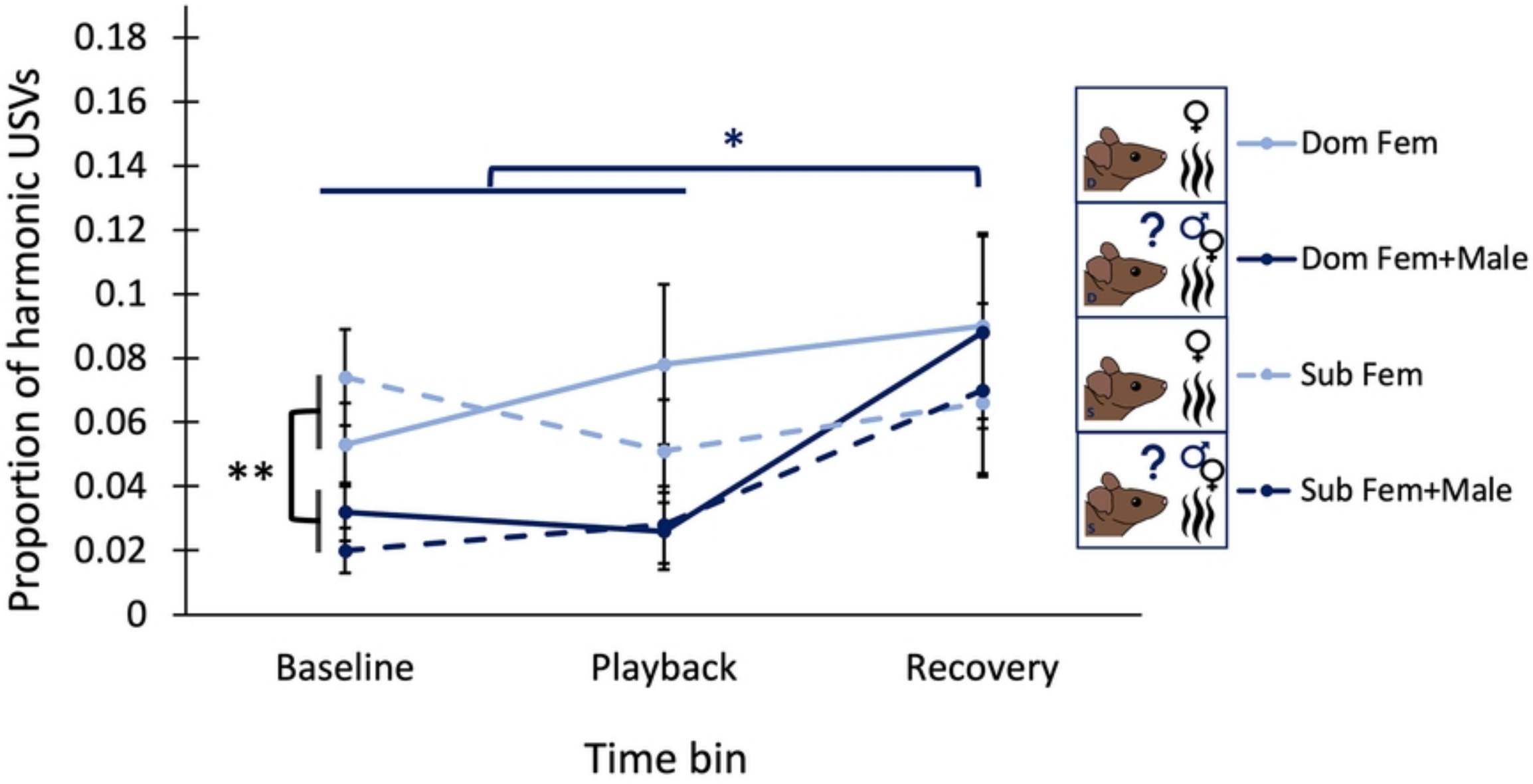
A. Unfamiliar odor cohort



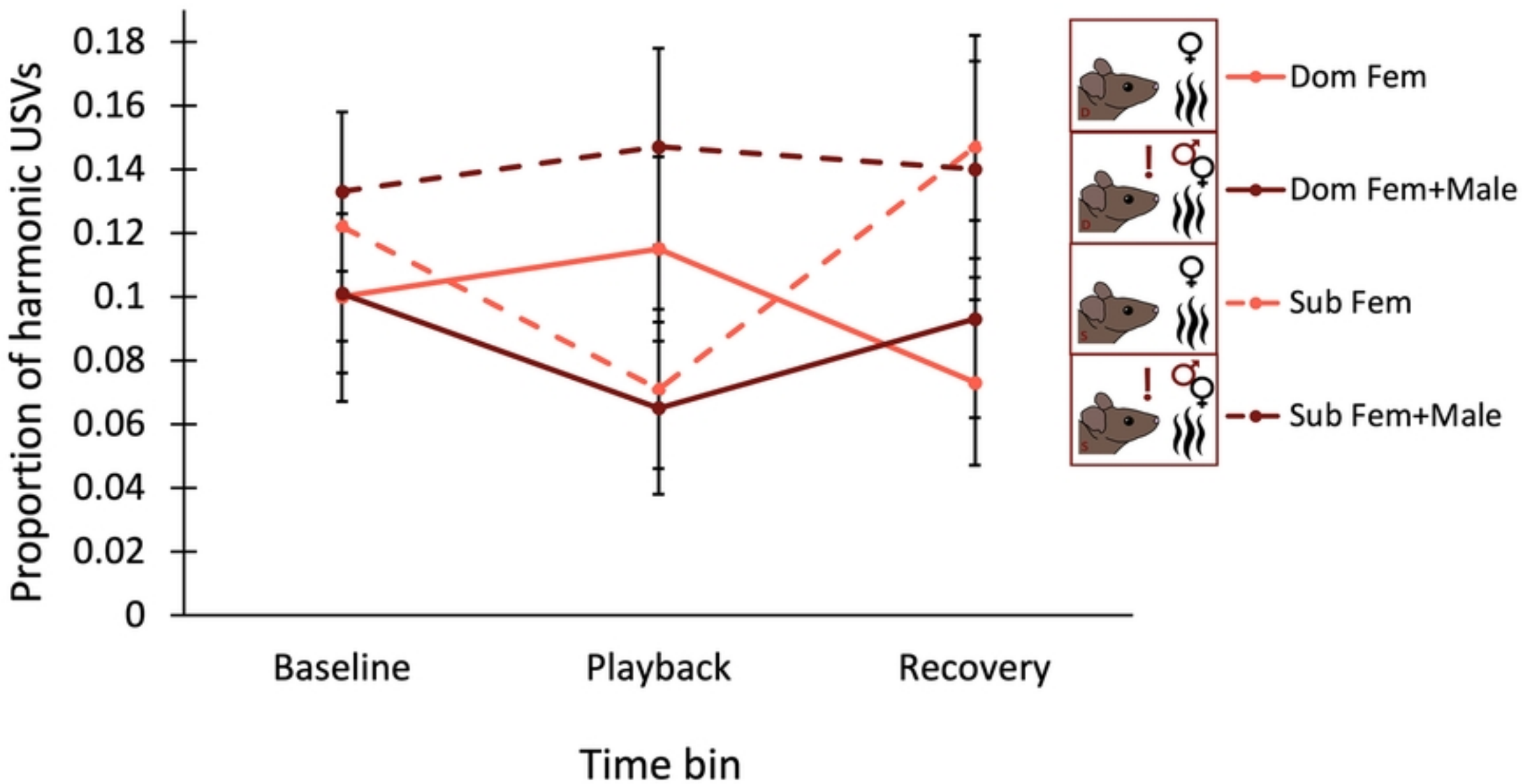
B. Familiar odor cohort

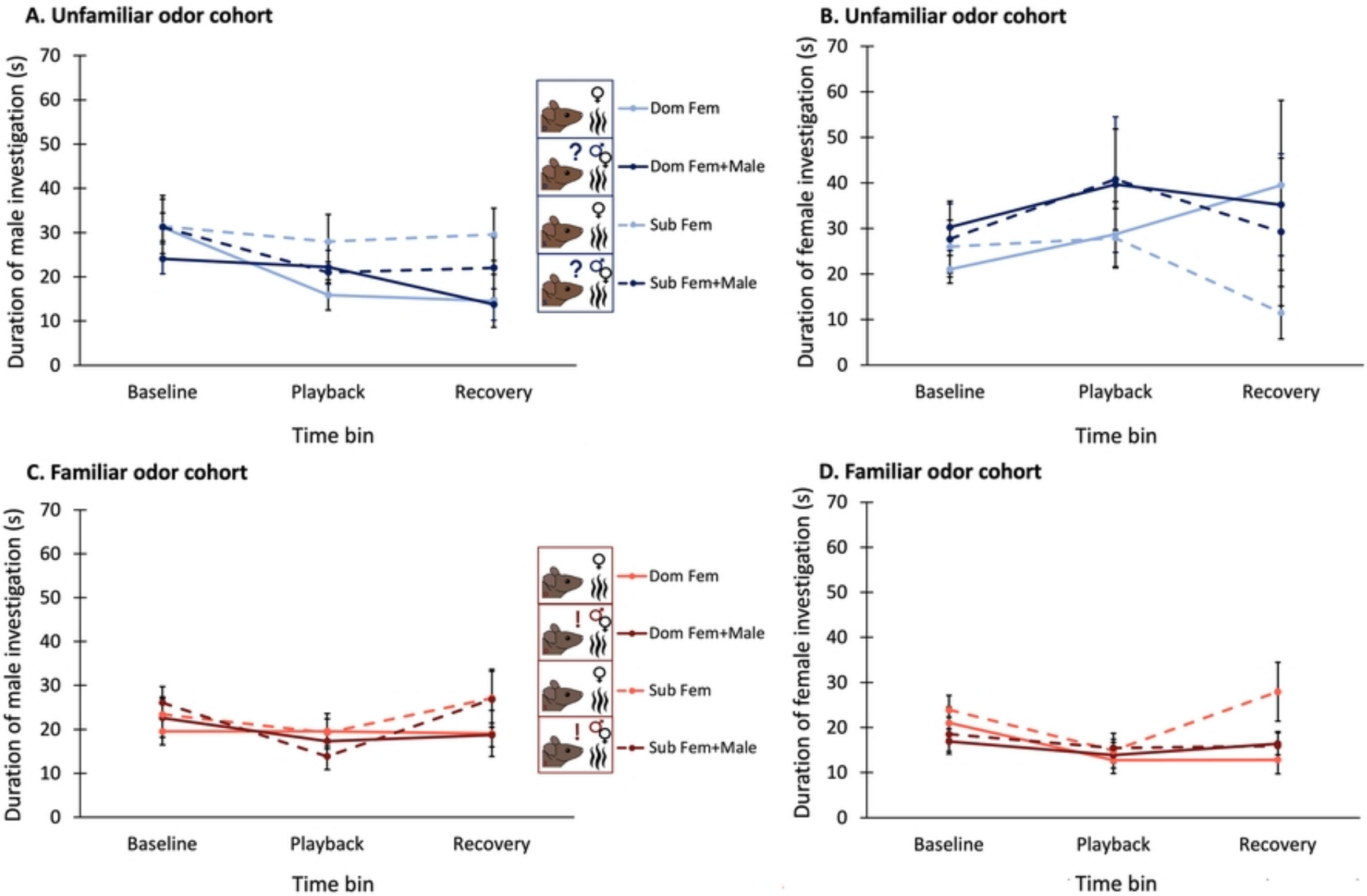


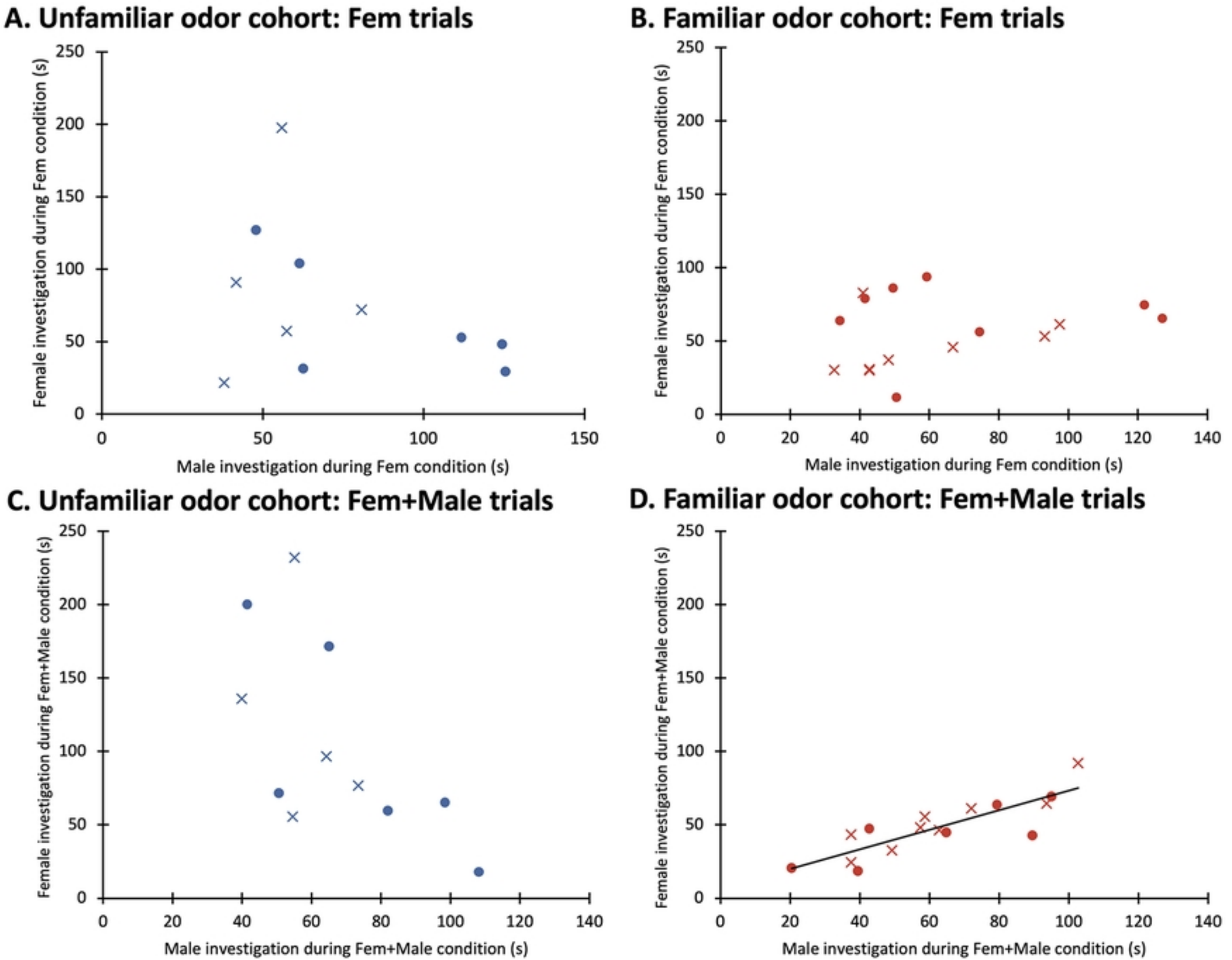
A. Unfamiliar Odor Cohort



B. Familiar Odor Cohort




A. Unfamiliar odor cohort
Duration of male investigation (s)
70
60
50
40
30
20
10
0
Baseline
Playback
Recovery
Time bin
Dom Fem
Dom Fem+Male
Sub Fem
Sub Fem+Male
B. Unfamiliar odor cohort
Duration of female investigation (s)
70
60
50
40
30
20
10
0
Baseline
Playback
Recovery
Time bin
C. Familiar odor cohort
Duration of male investigation (s)
70
60
50
40
30
20
10
0
Baseline
Playback
Recovery
Time bin
Dom Fem
Dom Fem+Male
Sub Fem
Sub Fem+Male
D. Familiar odor cohort
Duration of female investigation (s)
70
60
50
40
30
20
10
0
Baseline
Playback
Recovery
Time bin


A. Unfamiliar odor cohort: Fem trials
Female investigation during Fem condition (s)
Male investigation during Fem condition (s)
B. Familiar odor cohort: Fem trials
Female investigation during Fem condition (s)
Male investigation during Fem condition (s)
C. Unfamiliar odor cohort: Fem+Male trials
Female investigation during Fem+Male condition (s)
Male investigation during Fem+Male condition (s)
D. Familiar odor cohort: Fem+Male trials
Female investigation during Fem+Male condition (s)
Male investigation during Fem+Male condition (s)