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Research Article

Developmental trajectories of isolation-induced ultrasonic vocalizations in rat pups exposed to early life stress

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ABSTRACT

Rodent infants emit ultrasonic vocalizations (USVs) when separated from the nest, producing a communicative signal that elicits maternal care. These calls are considered as a sign of isolation distress, and call emission is acutely altered by stressful early life experiences. However, the selective developmental trajectory of these stress-induced USV alterations remains unknown and may have implications for neurodevelopmental processes underlying social dysfunction. Here, we employed a 3 h/day maternal separation (MS) procedure in Wistar rat pups (male and female) and recorded isolation-induced USVs every third day between the postnatal days (PND) 2–14. We observed systematic changes in both the quantity and spectro-temporal acoustic features of USVs, including shifts in frequency, duration and temporal emission patterns. Clustering of acoustic features revealed two USV types emerging after PND 8: short, high-frequency calls and highly frequency-modulated calls, alongside earlier flat, low-frequency calls. MS reduced overall USV emission on PND 8, selectively reduced and shortened flat low-frequency calls on PND 11, and shortened both low- and high frequency calls on PND 14. MS also disrupted the temporal organization of USVs including inter-vocalization intervals and bout structure across development. In contrast, the developmental emergence and diversification of high-frequency and high frequency-modulated call types were largely preserved under MS. These findings suggest a maturation process in rat pup vocalizations between PND 8–11, characterized by a rapid increase in acoustic complexity and changes in temporal structure, while early life stress exerts selective, time-specific effects on particular call categories, notably reducing emission and shortening flat low-frequency calls.

1. Introduction

Ultrasonic vocalizations (USVs) are species-specific signals with a communicative and physiological value in rodents [1–5]. Infant rats emit USVs in the pre-weaning period [2], typically in response to isolation distress [4,6]. These isolation-induced calls function to elicit maternal care including search and retrieval of the separated pup [5,7,8]. Emission rates of infant USVs have been linked to the anxiety level of the pups [9,10], and their expression is modulated by early life stress [11–13] and pharmacological treatments [14–16]. Early developmental USV patterns have been associated with several behavioral outcomes, including conditional fear responses [17], social interaction in adolescence [13] and anxiety-like behavior in adulthood [18]. Given that early USV patterns are linked to later behavioral outcomes [17,18], clarifying how early life stress influences their postnatal maturation is essential for understanding the role of these vocalizations in stress-related neurodevelopment [19]. Studying how early life stress affects the maturation of communicative signals across mammalian species may provide insight into the neurodevelopmental mechanisms that contribute to social dysfunction and vulnerability to neuropsychiatric disorders [20–22]. In particular, it remains unknown whether stress broadly alters the developmental progression of vocal signals in rat pups or if it exerts selective effects on specific acoustic properties. Here, we track infant USVs during the first two weeks of life in Wistar rats subjected to repeated maternal separation (MS) stress. We investigated and characterized various properties of USVs and identified MS-induced changes in acoustic features emerging along the developmental trajectory.

Isolation-induced rat infant USVs are dynamic and vary systematically across early postnatal development. These calls are not uniform or necessarily reflexive, and they exhibit structured changes in acoustic and temporal features such as frequency, duration, and frequency

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modulation that emerge at different developmental stages [4,23–25]. Low-frequency, long-duration calls tend to dominate the early postnatal period, while high-frequency and frequency-modulated calls emerge later in development [26], indicating increasing complexity in vocal production. These changes likely reflect the maturation of laryngeal, respiratory, and neural systems involved in vocal-motor control [27–29]. The timing and structure of rodent USVs may also align with the development of sensory systems, such as auditory tuning in dams and pups, which modulate the communicative salience and effectiveness of calls [30,31]. Importantly, the developmental profile of USVs is not fixed; it is shaped by external influences such as maternal care, environmental conditions, and early life stress [32]. Immediate sensory (thermal, olfactory, tactile) experiences modulate USV emission rates [33–35], whereas experimental manipulations such as MS or pharmacological exposures have been shown to modify rodent isolation-induced USV emission rates, onset timing, spectral characteristics, and the emergence of specific call categories [11,36–40]. While some of these effects have been described in terms of broad acoustic shifts, it remains unclear whether early life stress disrupts the maturation and diversification of infant USV subtypes or instead exerts effects on select acoustic parameters during postnatal development. Understanding how and when early life stress, which underlies alterations in later social behavior [41–43] as well as neural, cognitive, and affective outcomes [44–48], influences the developmental profile and structural characteristics of infant USVs requires detailed acoustic and temporal analyses across postnatal development.

This study investigated isolation-induced pup USVs in Wistar rats following repeated MS within the first two weeks of life. We aimed to characterize the normative developmental trajectory of infant vocalizations and determine whether early life stress alters specific components of this trajectory. In addition to assessing overall USV emission rates and within-session temporal patterns, we examined detailed spectro-temporal features across development, analyzed frequency distributions, and separately evaluated low- and high-frequency calls emerging after postnatal day (PND) 11. Finally, using clustering based on acoustic features [49], we identified three distinct USV types to assess whether maternal separation affects the emergence or structural properties of specific call categories.

2. Methods

2.1. Subjects

Pregnant Wistar rats were housed individually under standard laboratory conditions (21 ± 1°C, 40–60% humidity, 12:12 day/night) with *ad libitum* access to food and water. The lights were turned on at 08:00 and all the procedures were conducted in the daytime. The cages were randomly assigned to experimental and control conditions after parturition (PND 0). We tested 57 and 51 pups in the control and MS conditions, respectively (5 litters each, control litter sizes: 9, 11, 12, 12, 13; MS litter sizes: 8, 8, 11, 12, 12), with both male and female animals included in all procedures (N = 108 animals, comprising 28 females and 29 males in the control condition, and 23 females and 28 males in the MS condition). All animal protocols were approved by the Boğaziçi University Institutional Ethics Committee for the Use of Animals in Experiments.

2.2. Maternal separation and isolation

The MS procedure was conducted for 3 h/day between PND 2 and 13. The timing of MS was randomized across days (08:00–20:00). Each day, the mother was removed from the nest and placed in a temporary cage that was moved to another room to eliminate olfactory and auditory cues. Until PND 11, each pup was placed in individual paper cups (diameter: 50 mm, height: 70 mm) with fresh bedding in an incubator set at 32–34°C to maintain their body temperature [50]. Between PND 11 and 14, the pups were separated into individual cages (21 ± 1 °C). After the 3-h isolation period, they were reunited in their home cage, and the mother was returned to complete the litter. The control group was left undisturbed except for a brief handling period during the marking procedure and USV recordings. On days when individual USV recordings were obtained, MS was carried out after all pups in the cage had already been tested.

2.3. USV recordings and analyses

Starting on PND 2/3 and continuing every three days (PND 5, 8, 11, and 14), isolation-induced USV emissions of the pups were recorded for subsequent analysis. Before each recording session, the mother rat was moved to another room and brought back only after testing was completed for all pups in the nest. Recordings were performed for three minutes on each recording day (21 ± 1 °C, 40–60% humidity). The pups were labeled each day using a non-toxic marker to allow individual identification and longitudinal tracking of USV data over two weeks of testing. During the first 10 days, pups were marked on the back using a non-toxic permanent marker. Starting on PND11, the tail was marked to maintain individual identification. The markings were renewed daily to ensure reliable individual identification throughout the longitudinal testing period. Each pup was returned to the litter in their home cage once their recording, weighing, and marking procedures were completed. Testing order of the pups was randomized each day. Testing order was not significantly associated with the number of USVs after accounting for litter (Supplementary Fig. 1). USVs were first recorded prior to MS, 54–66 h after birth. Given variability in the exact time of birth across mothers, this time point was designated as PND 2/3.

We used an ultrasonic recording device (UltraSoundGate 116 H, Avisoft Bioacoustics, Germany) to capture USVs. A condenser ultrasound-sensitive microphone (CM16/CMPA, Avisoft Bioacoustics), placed approximately 15 cm above the pups, was used with the Avisoft-Recorder software (version 4.2) at a sampling rate of 250 kHz (frequency range: 0–250 kHz). Individual USVs were automatically detected and analyzed via DeepSqueak 2.0 software [51] run on MATLAB. All automatically detected contours were manually validated and corrected by a trained observer who was blind to the experimental conditions. The lower cut-off frequency was set at 20 kHz to exclude sonic noise. USV call duration, principal frequency, and frequency modulation (delta frequency) was automatically calculated using DeepSqueak. Call duration was defined as the time between the onset and offset of the extracted contour. Principal frequency was defined as the median frequency of the contour, while mean frequency was defined as the average of its highest and lowest frequencies. The extent of the frequency modulation was calculated by subtracting the lowest frequency of the contour from the highest. The USV onset latency was defined as the onset time of the first USV emitted by a pup in any given recording session. Validated vocalization information for each recording session were exported as Excel files for further analyses.

For frequency-based call category analyses, we set a 60 kHz threshold to differentiate low- and high-frequency calls based on the distribution of calls on PND 11 (see Fig. 3A), where the differentiation was observed most clearly. For cluster type analyses (as previously described by Schwarting and Wöhr [49]), we used mean frequency, frequency modulation, and call duration as the descriptor variables. Inspection of the distribution of calls in this 3-dimensional feature space revealed 3 clearly distinct clusters (see Fig. 4). The first two clusters were characterized by low (<60 kHz, Type 1) and high (>60 kHz, Type 2) frequencies, both with low frequency modulation (<20 kHz). Type 1 calls typically had longer call duration than Type 2 calls ($t(32551) = 341$, $p < 0.001$). The third call type was characterized by a high frequency modulation (>20 kHz extent of frequency modulation, Type 3) and consisted mostly of fragmented segments.

For temporal structure analyses, validated USV timestamps exported from DeepSqueak were analyzed in Python and GraphPad Prism.

Within-session dynamics were assessed by binning 3-min recordings into three consecutive 1-minute intervals and, additionally, by computing Gaussian-filtered 60 s moving averages of call rates. Inter-vocalization intervals (IVIs), or call repetition periods, were calculated as offset-to-onset intervals between consecutive calls. Bout structure was determined using day-specific maximum IVI thresholds derived from kernel density estimates (99th percentile around the first IVI peak), with bouts defined as two or more calls separated by intervals shorter than this threshold. We quantified the number of bouts, calls per bout, and inter-bout intervals (calculated as the offset-to-onset interval between two consecutive bouts).

2.4. Statistical analyses

Statistical analyses and figure preparation were done in GraphPad Prism (9.5.1) and Python (Jupyter Notebook). For datasets spanning multiple postnatal days (PND 5–14), we applied two-way or three-way (when sex is included) mixed-design ANOVA to examine the effects and interactions of experimental groups and PND, with Greenhouse-Geisser corrections to account for violations of sphericity. Independent samples *t*-tests were performed for specific group comparisons. We conducted post-hoc analyses following significant main effects or interactions. Sidak correction was applied to post hoc comparisons following ANOVAs to control the family-wise error rate. Statistical significance was defined as $p < 0.05$, and error bars in the figures depict standard error of the mean (SEM).

3. Results

The timeline of USV recordings and MS procedures is shown in Fig. 1A. Across the 5 recording days over the examined developmental period (PND 2–14), we recorded and analyzed a total of 132,225 USVs from 108 animals. This large dataset enabled a detailed characterization of both developmental changes and stress-induced modulation of vocal behavior. We first examined whether pup sex influenced key measures, including weight (Supplementary Fig. 2A), call number (Supplementary Fig. 2B), average principal frequency (Supplementary Fig. 2C), call length (Supplementary Fig. 2D), frequency modulation (Supplementary Fig. 2E), and call latency (Supplementary Fig. 2F), using three-way ANOVAs with sex, group, and recording day as factors. Across all measures, there were no significant main effects of sex, nor interactions with the group or recording day (all ps >.05; Table S1). These results indicate that the developmental trajectories and MS effects observed in this study were consistent across male and female pups. Therefore, sex was not included as a factor in subsequent analyses.

We tracked animal weight across recording days. As expected, body weight increased with age ($F(1.712, 178.9) = 3103$, $p < 0.001$; $\eta_p^2 = 0.967$; 2×5 two-way mixed ANOVA; Fig. 1B), confirming normal somatic development. MS induced a change in weight ($F(1, 106) = 3.990$, $p = 0.048$; $\eta_p^2 = 0.036$), and a significant MS x postnatal day interaction was observed ($F(4, 418) = 5.111$, $p < 0.001$; $\eta_p^2 = 0.046$). However, pairwise comparisons at individual days revealed no significant group differences in any of the recording days (Table 1). These results indicate that differences in somatic development are unlikely to account for the effects of MS on USV production across PND 2–14.

3.1. USV emission across days

We first analyzed the number of calls emitted across days and groups. USV rates changed across recording sessions ($F(2.53, 264.68) = 93.062$, $p < 0.001$; $\eta p^2 = 0.471$; 2×5 two-way Mixed ANOVA; Fig. 1C), indicating a substantial decrease on PND 14 (Table 1). This trajectory suggests that isolation-induced vocal activity is maintained during the first two postnatal weeks, likely corresponding to their communicative function in early development. Notably, animals exposed to MS over this period displayed lower call rates overall ($F(1, 106) = 6.327$, $p = 0.013$, $\eta_p^2 = 0.056$), indicating that early life stress can reduce overall vocal activity. A significant MS x postnatal day interaction was also observed ($F(4, 418) = 4.831$, $p < 0.001$, $\eta_p^2 = 0.044$), with significantly lower USV emission on PND 8 (Control: $M = 324.09$, $SD = 126.30$; MS: $M = 255.14$, $SD = 129.84$; $t(101.94) = 2.765$, $p = 0.033$, Cohen's $d = 0.538$, Sidak corrected *t*-test; Fig. 1C). This pattern suggests that the impact of MS on vocal behavior is developmentally specific rather than uniform across the early postnatal period.

The principal frequency of isolation-induced USVs changed across postnatal development ($F(1.831, 181.8) = 63.226$, $p < 0.001$; $\eta_p^2 = 0.396$; 2×5 two-way mixed ANOVA; Fig. 1D), reflecting the dynamic maturation of the spectral characteristics of pup vocalizations. This trajectory was not linear but occurred in two distinct phases. An initial shift was observed between PND 2/3 and PND 5, characterized by a decrease in average principal frequency (Table 1), likely contributing to the predominance of low-frequency flat calls on PND 5 and 8. A second, more pronounced developmental transition emerged between PND 8 and PND 11, characterized by a sharp increase in principal frequency (Table 1). This shift was accompanied by a marked rise in the standard error of the mean on PND 11 and 14 (Fig. 1D), indicating greater inter-individual variability in vocal output during this later developmental phase. These changes represent a substantial restructuring of vocal production, consistent with a critical window of rapid acoustic maturation. Notably, MS significantly increased overall call frequency ($F(1, 106) = 14.523$, $p < 0.001$, $\eta p^2 = 0.120$). This effect also interacted with developmental stage ($F(4, 397) = 5.995$, $p < 0.001$, $\eta p^2 = 0.057$), driven by higher average call frequency in MS pups specifically on PND 11 while it did not reach significance on PND 14 (Table 1). These results indicate that early life stress selectively shifts vocal frequency during the later phase of vocal maturation.

Analysis of average call length revealed a progressive shortening of isolation-induced vocalizations across postnatal development ($F(2.51, 249.18) = 90.402$, $p < 0.001$; $\eta_p^2 = 0.477$; 2×5 two-way mixed ANOVA; Fig. 1E). However, this trajectory was not linear. Call duration remained relatively stable between PND 2/3 and PND 5, followed by a pronounced reduction across the subsequent recording days (PND 5–11), after which it stabilized again between PND 11 and 14 (Table 1). This pattern suggests that the major refinement of temporal call structure occurs primarily during the mid-phase of the second postnatal week. There was an overall main effect of MS on call length ($F(1, 106) = 12.763$, $p = 0.002$; $\eta_p^2 = 0.107$); and it significantly interacted with recording day ($F(4, 397) = 22.731$, $p < 0.001$; $\eta_p^2 = 0.186$; Fig. 1E). Day-specific comparisons revealed that MS pups emitted significantly shorter calls than controls both on PND 11 (Control: $M = 0.068$, $SD = 0.027$; MS: $M = 0.051$, $SD = 0.020$; $t(102.23) = 3.778$, $p = 0.001$, Cohen's $d = 0.731$, Sidak corrected *t*-test) and PND 14 (Control: $M = 0.097$, $SD = 0.049$; MS: $M = 0.041$, $SD = 0.042$; $t(82.57) = 5.619$, $p < 0.001$, Cohen's $d = 1.226$, Sidak corrected *t*-test; Fig. 1E), whereas no group differences were observed on earlier days (Table 1). These findings suggest that early life stress selectively alters call duration at a later stage of vocal maturation rather than exerting a consistent effect across development.

We also tested whether individual pups maintained consistent vocalization rates across development and observed positive correlations between several pairs of days (Fig. 1F). Specifically, the number of USVs emitted showed positive associations between PND 5 and PND 8 ($r = 0.369$, $p = 0.005$) as well PND 8 and 11 for the control group ($r = 0.310$, $p = 0.020$). In the MS group, significant correlations were noted for PNS 2/3 and 5 ($r = 0.279$, $p = 0.047$), PND 8 and 11 ($r = 0.291$, $p = 0.040$), and PNS 11 and 14 ($r = 0.344$, $p = 0.018$). Because all significant correlations were positive, the data further suggests that pups with higher early vocal output tended to maintain relatively higher calling rates on later days, although the exact associations differed between groups. Altogether, these observations show that the calling tendencies of individual pups are relatively consistent across development.

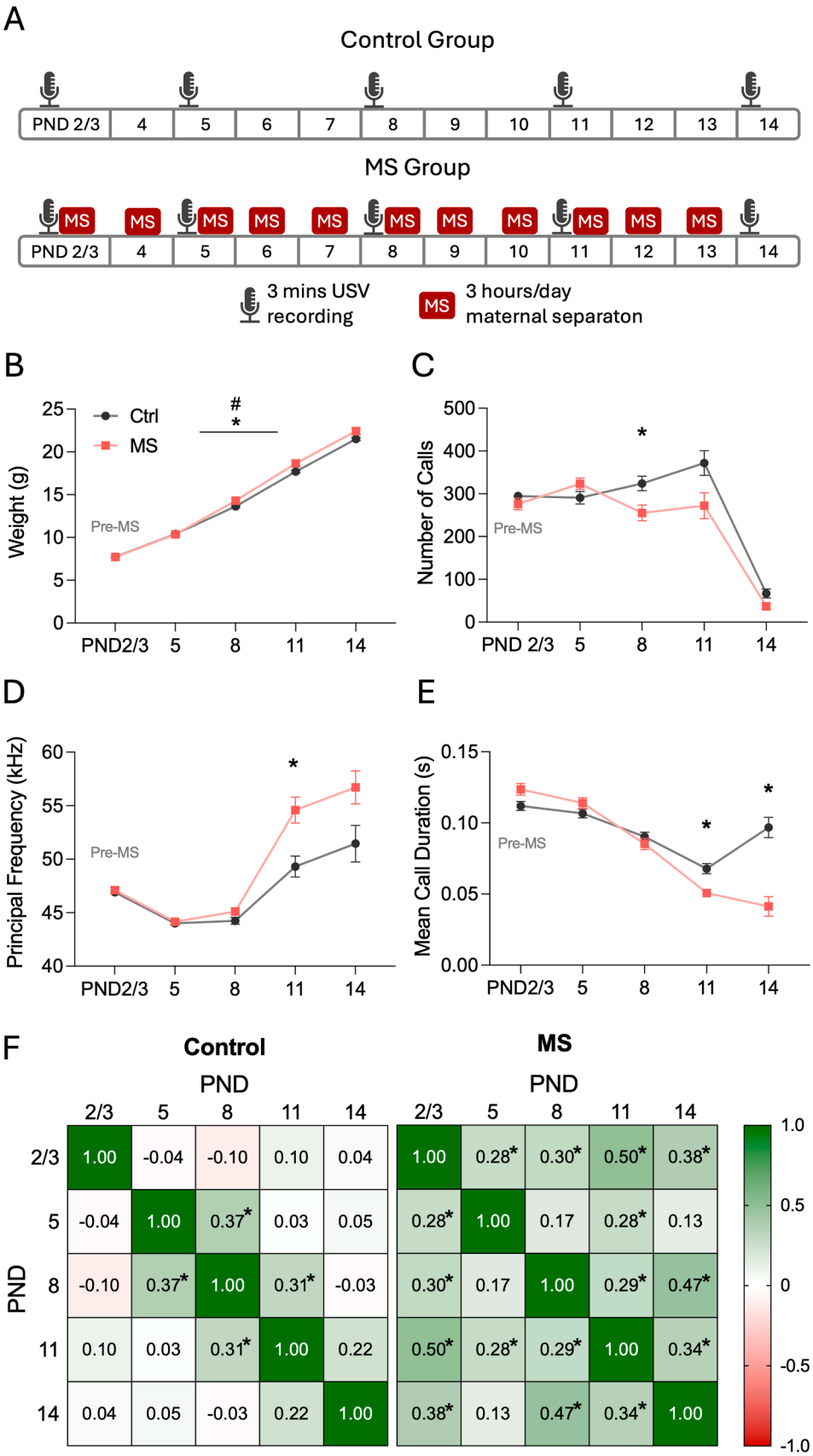

Fig. 1. Experimental design and USV properties across days. (A) The illustration of the experimental procedures indicating the timeline of MS and USV recordings. (B) The changes in the body weights of the animals. (C) The total number of emitted calls, (D) the average principal frequency, (E) the mean duration of infant calls across days ($n = 57$ for control and 51 for MS groups). (F) The correlation matrix for the number of USVs emitted by control and MS animals in each day, with the numbers indicating r values. Error bars represent SEM. The asterisks (*) depict significant post-hoc and correlation test results ($p < .05$).

Table 1
Statistical results of additional tests and follow-up comparisons.

Variable	Comparison	Test	F/t	df	p	Variable	Comparison	Test	F/t	df	p
Weight	Ctrl vs. MS	Post-hoc PND 2/3	0.042	99.10	> .999	Number of Bouts	Ctrl - MS	Post-hoc PND 11	1.207	55.35	0.739
		Post-hoc PND 5	0.197	81.44	> .999			Post-hoc PND 14	0.825	23.91	0.930
		Post-hoc PND 8	2.383	76.16	0.950		PND 2/3–5	Post-hoc	3.692	104	0.004
		Post-hoc PND 11	2.543	70.25	0.640		PND 5–8		2.112	100	0.316
		Post-hoc PND 14	1.896	62.13	0.276		PND 8–11		7.136	101	< 0.001
Number of Calls	PND 2/3–5	Post-hoc	1.664	107	0.648		PND 11–14		8.298	66	< 0.001
	PND 5–8		1.018	105	0.976	Number of Calls	Group	Two-way	6.838	1106	0.010
	PND 8–11		1.592	105	0.703	per Bout	PND	ANOVA	18.15	1.87,175.0	< 0.001
	PND 11–14		13.150	103	< 0.001		Group*PND		3.868	4374	0.004
	Ctrl vs. MS	Post-hoc PND 2/3	1.149	89.61	0.768		Ctrl - MS	Post-hoc PND 2/3	0.282	94.42	> 0.999
		Post-hoc PND 5	1.686	105.83	0.392			Post-hoc PND 5	0.064	94.61	> 0.999
		Post-hoc PND 8	2.764	101.94	0.033			Post-hoc PND 8	1.951	95.45	0.242
		Post-hoc PND 11	2.386	104.87	0.090			Post-hoc PND 11	2.642	81.98	0.048
		Post-hoc PND 14	2.222	100.31	0.135			Post-hoc PND 14	3.834	64.22	0.001
Principal Frequency	PND 2/3–5	Post-hoc	15.300	107	< 0.001	Inter Bout	Group	Two-way	12.21	1106	< 0.001
	PND 5–8		2.282	104	0.220	Interval	PND	ANOVA	24.550	2.72,246.50	< 0.001
	PND 8–11		9.391	103	< 0.001		Group*PND		6.651	4363	< 0.001
	PND 11–14		2.017	83	0.381		Ctrl - MS	Post-hoc PND 2/3	0.622	71.33	0.978
	Ctrl vs. MS	PND 2/3	0.5296	105	0.989			Post-hoc PND 5	2.167	62.32	0.159
		PND 5	0.5824	101.60	0.984			Post-hoc PND 8	2.032	70.05	0.210
		PND 8	1.892	101.20	0.271			Post-hoc PND 11	3.219	55.40	0.011
		PND 11	3.409	98.38	0.005			Post-hoc PND 14	1.548	27.99	0.510
		PND 14	2.286	83	0.118	Frequency	PND 2/3–5	Post-hoc	0.127	106	> 0.999
Mean Call Duration	PND 2/3–5	Post-hoc	2.635	107	0.093	Modulation	PND 5–8		3.470	103	0.008
	PND 5–8		7.095	104	< 0.001		PND 8–11		4.956	102	< 0.001
	PND 8–11		8.788	103	< 0.001		PND 11–14		2.048	79	0.362
	PND 11–14		2.479	83	0.142	**PND 11**					
	Ctrl vs. MS	Post-hoc PND 2/3	2.313	95.86	0.109	LF Call Number	Ctrl - MS	Unpaired *t*-test	2.967	106	0.004
		Post-hoc PND 5	1.489	98.62	0.529	LF Call Duration	Ctrl - MS	Unpaired *t*-test	2.844	105	0.005
		Post-hoc PND 8	0.990	94.68	0.859	LF Frequency Modulation	Ctrl - MS	Unpaired *t*-test	0.411	103	0.682
		Post-hoc PND 11	3.777	102.23	0.001	HF Call Number	Ctrl - MS	Unpaired *t*-test	1.607	106	0.111
		Post-hoc PND 14	5.619	82.57	< 0.001	HF Call Duration	Ctrl - MS	Unpaired *t*-test	0.887	99	0.377
PND 2/3 Number of Calls	Bin 1–2	Post-hoc	17.446	107	< 0.001	HF Frequency Modulation	Ctrl - MS	Unpaired *t*-test	0.809	100	0.421
	Bin 1–3		19.876	107	< 0.001	**PND 14**					
	Bin 2–3		6.231	107	< 0.001	LF Call Number	Ctrl - MS	Unpaired *t*-test	2.786	102	0.006
PND 5 Number of Calls	Bin 1–2	Post-hoc	19.050	106	< 0.001	LF Call Duration	Ctrl - MS	Unpaired *t*-test	4.508	80	< 0.0001
	Bin 1–3		19.940	106	< 0.001	LF Frequency Modulation	Ctrl - MS	Unpaired *t*-test	1.603	77	0.113
	Bin 2–3		6.103	106	< 0.001	HF Call Number	Ctrl - MS	Unpaired *t*-test	1.155	102	0.251
PND 8 Number of Calls	Bin 1–2	Post-hoc	15.270	104	< 0.001	HF Call Duration	Ctrl - MS	Unpaired *t*-test	2.205	60	0.031
	Bin 1–3		13.920	104	< 0.001	HF Frequency Modulation	Ctrl - MS	Unpaired *t*-test	0.363	57	0.718
	Bin 2–3		4.154	104	< 0.001	Type 1 Calls	Group	Two-way	9.989	1, 106	0.002
	Ctrl - MS	Bin 1 Post-hoc	1.725	95.57	0.241		PND	ANOVA	77.690	2.66, 263.30	< 0.001
		Bin 2 Post-hoc	3.072	102.92	0.008		Group*PND		7.053	4395	< 0.001
		Bin 3 Post-hoc	2.110	101.91	0.108		PND 2/3–5	Post-hoc	1.636	105	0.670

(*continued on next page*)

Table 1 (*continued*)

Variable	Comparison	Test	F/t	df	p	Variable	Comparison	Test	F/t	df	p
PND 11 Number of Calls	Bin 1–2	Post-hoc	7.585	106	< .001		PND 5–8		1.542	202	0.877
	Bin 1–3		5.871	106	< .001		PND 8–11		0.367	103	> 0.999
	Bin 2–3		0.518	106	0.939		PND 11–14		10.640	83	< 0.001
	Ctrl - MS	Bin 1 Post-hoc	2.196	104.39	0.088		Ctrl - MS	Post-hoc PND 2/3	1.117	88.66	0.789
		Bin 2 Post-hoc	2.782	104.02	0.019			Post-hoc PND 5	1.820	103.90	0.311
		Bin 3 Post-hoc	2.021	101.30	0.131			Post-hoc PND 8	2.880	102.80	0.024
PND 14 Number of Calls	Bin 1–2	Post-hoc	6.263	82	< .001			Post-hoc PND 11	3.193	105	0.009
	Bin 1–3		5.533	82	< .001			Post-hoc PND 14	3.302	71.18	0.007
	Bin 2–3		1.951	82	0.155	Type 2 Calls	Group	Two-way	4.709	1, 106	0.032
	Ctrl - MS	Bin 1 Post-hoc	2.679	55.31	0.029		PND	ANOVA	42.770	1.32, 130.60	< .001
		Bin 2 Post-hoc	2.285	78.59	0.073		Group*PND		2.153	4395	0.138
		Bin 3 Post-hoc	1.230	70.07	0.530		PND 2/3–5	Post-hoc	0.884	105	0.991
Call Latency	PND 2/3–11	Post-hoc	3.941	105	0.001		PND 5–8		4.179	102	< .001
	PND 2/3–14		11.210	81	< 0.0001		PND 8–11		6.578	103	< .001
	PND 5–11		3.201	104	0.015		PND 11–14		6.111	83	< .001
	PND 5–14		10.510	81	< 0.0001		Ctrl - MS	Post-hoc PND 2/3	0.939	74.38	0.884
	PND 8–11		4.844	102	< 0.0001			Post-hoc PND 5	0.570	89.18	0.985
	PND 8–14		11.340	79	< 0.0001			Post-hoc PND 8	2.210	72.87	0.143
	PND 11–14		9.762	79	< 0.0001			Post-hoc PND 11	1.636	95.23	0.427
IVI	ctrl - MS	Post-hoc PND 2/3	1.685	103.40	0.393			Post-hoc PND 14	1.462	44.9	0.558
		Post-hoc PND 5	2.668	102.90	0.044	Type 3 Calls^	Group	Two-way ANOVA	1.384	1, 501	0.240
		Post-hoc PND 8	2.739	57.32	0.040		PND		18.230	2.13, 266.10	< 0.001
		Post-hoc PND 11	3.317	94.76	0.006		Group*PND		1.382	4501	0.253
		Post-hoc PND 14	0.646	32.10	0.975		PND 2/3–5	Post-hoc	1.387	105	0.842
Number of Bouts	Ctrl - MS	Post-hoc PND 2/3	0.518	102.50	0.990		PND 5–8		2.912	102	0.043
		Post-hoc PND 5	0.317	97.67	> 0.999		PND 8–11		3.240	103	0.016
		Post-hoc PND 8	0.543	97.63	0.988		PND 11–14		4.183	83	< 0.001

Note. All post-hoc tests are Sidak-corrected. ^Subject factor was excluded from the model as it has random effect with zero SD.

3.2. Temporal properties of USVs

We investigated the temporal structure of pup USVs across development by focusing on four main features. First, we examined the within-session trajectory of USV rates and how these changed across development. We then analyzed call latency, call repetition periods, defined as inter-vocalization intervals (IVIs), and the bout structure of pup calls. Call emission rates for each animal within recording sessions are shown as heatmaps in Fig. 2A.

To assess within-session changes, we binned call rates into three 1-minute periods and analyzed their progression within each session. We observed significant within-session reductions in vocalization rates on PND 2/3 ($F(1.79, 190.04) = 283.627$, $p < 0.001$; $\eta_p^2 = 0.728$; 2 × 3 two-way mixed ANOVA; Fig. 2B), PND 5 ($F(1.69, 177.142) = 305.258$, $p < 0.001$; $\eta_p^2 = 0.744$) and PND 8 ($F(1.53, 157.92) = 166.462$ $p < 0.001$; $\eta_p^2 = 0.618$). These effects were further supported by significant pairwise comparisons between bins (Table 1). In contrast, the direction of this bin effect reversed on later developmental days, with a gradual increase in vocalization rates across the 3-minute testing period on PND 11 ($F(1.63, 170.88) = 31.370$, $p < 0.001$; $\eta_p^2 = 0.230$) and PND 14 ($F(1.516, 122.8) = 25.420$, $p < 0.001$; $\eta_p^2 = 0.239$). This pattern was also supported by significant increases from the first to the third minute of the session (Table 1).

These findings suggest a progressive shift in the pattern of USV emission density, with calls initially concentrated in the first minute during early development and becoming increasingly concentrated toward the end of the session over consecutive recording days. MS significantly reduced call rates on PND 8 ($F(1, 103) = 6.358$, $p = 0.013$, $\eta_p^2 = 0.058$; 2 × 3 two-way mixed ANOVA), and this effect was observed specifically in the second time bin (Table 1). The same pattern also appeared on PND 11 ($F(1, 105) = 6.668$, $p = 0.011$, $\eta_p^2 = 0.060$; 2 × 3 two-way mixed ANOVA); while MS-induced decrease in call rates on PND 14 ($F(1, 81) = 4.561$, $p = 0.036$, $\eta_p^2 = 0.053$; 2 × 3 two-way mixed ANOVA) revealed itself in the first time bin (Table 1). Furthermore, we calculated Gaussian-filtered smoothed 60-s moving averages (Supplementary Fig. 3), which recapitulated the results of the binned analysis. Altogether, these results point to a gradual developmental change in USV rates, with a shift in within-session dynamics occurring between PND 8 and PND 11. MS interacted with this process both before and after the temporal flip from decelerating to accelerating USV production.

We observed a gradual delay in the initial vocalization onset across days ($F(1.580, 154.8) = 103.900$, $p < 0.001$, $\eta p^2 = 0.515$; 2 × 5 two-way mixed ANOVA; Fig. 2C), showing an increased latency to emit

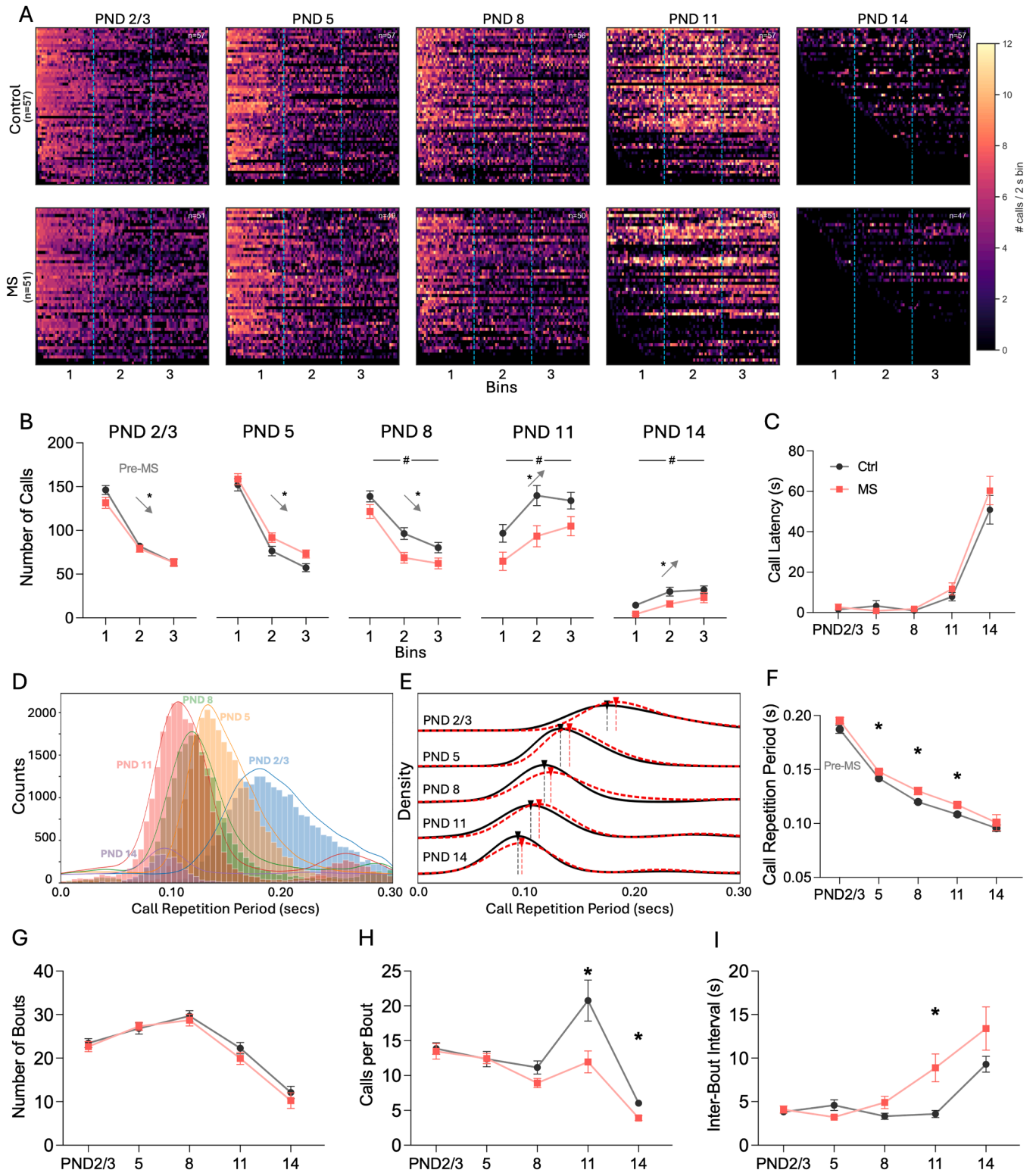

Fig. 2. Within-session temporal dynamics of isolation-induced USVs. (A) Heatmaps showing the number of USVs emitted by individual animals (rows) during each recording session across days. Dashed lines (*cyan*) indicate the boundaries of 1-minute bins within each session. (B) Line plots indicating the number of USVs per bins for each test day. Grey arrows point to the direction of significant changes across consecutive bins. (C) The latency to emit calls during test sessions. (D) Distribution of call repetition periods (inter-call intervals) pooled across animals for each developmental day. Kernel density estimates are overlaid on histograms. Colored shading indicates density per age group. (E) Group-wise comparison of the same repetition period distributions shown in (D), plotted separately for Control (black) and MS (red dashed) groups across days. Lines reflect group-level density estimates. (F) Average call repetition periods across days. (G) The total number of bouts in each recording session, (H) the average calls per bout, and (I) the inter-bout intervals across days. Error bars represent SEM. The asterisks (*) depict significant post-hoc tests, and hashtags (#) refer to the group main effect.

calls within sessions. Consistent with the aforementioned results, this finding shows an overall slowing of initial vocalization following separation from the litter. Pairwise comparisons further showed that onset latencies on PND 11 and PND 14 were significantly longer than on all preceding recording days (Table 1). This pattern is expected, as separation-induced vocalization rates rapidly decline after approximately PND 15. ANOVA results showed that MS did not affect the call onset latencies ($F(1, 106) = 1.684$, $p = 0.197$; interaction: $F(4, 392) = 1.006$, $p = 0.404$).

Call repetition periods, defined as offset-to-onset inter-vocalization intervals, were analyzed based on their cumulative counts (Fig. 2D) and density distributions (Fig. 2E) across days. Statistical analyses based on the mean IVIs of individual pups revealed a significant decrease across development ($F(2.66, 244.9) = 335.9$, $p < 0.001$; $\eta_p^2 = 0.$ 0.785; 2 × 5 two-way mixed ANOVA; Fig. 2F). Moreover, the MS group exhibited overall longer repetition periods across days ($F(1, 106) = 15.450$, $p < 0.001$; $\eta_p^2 = 0.127$), with significantly longer inter-vocalization intervals on PND 5, 8 and 11 (Table 1).

To better understand the changes in temporal structure of the vocalizations, we analyzed the bout structures of the USVs produced by the control and MS groups. To determine the maximum allowable gaps between intervals, we used kernel density estimates to identify the 99th percentile values of the normal distribution of IVIs around the first peak (PND 2/3: 307 ms, PND 5: 235 ms, PND 8: 206 ms, PND 11: 188 ms, PND 14: 161 ms). Using these empirically defined thresholds, we calculated the number of bouts consisting of at least two vocalizations with IVIs shorter than the corresponding threshold.

The number of bouts varied across recording days, indicating a shift

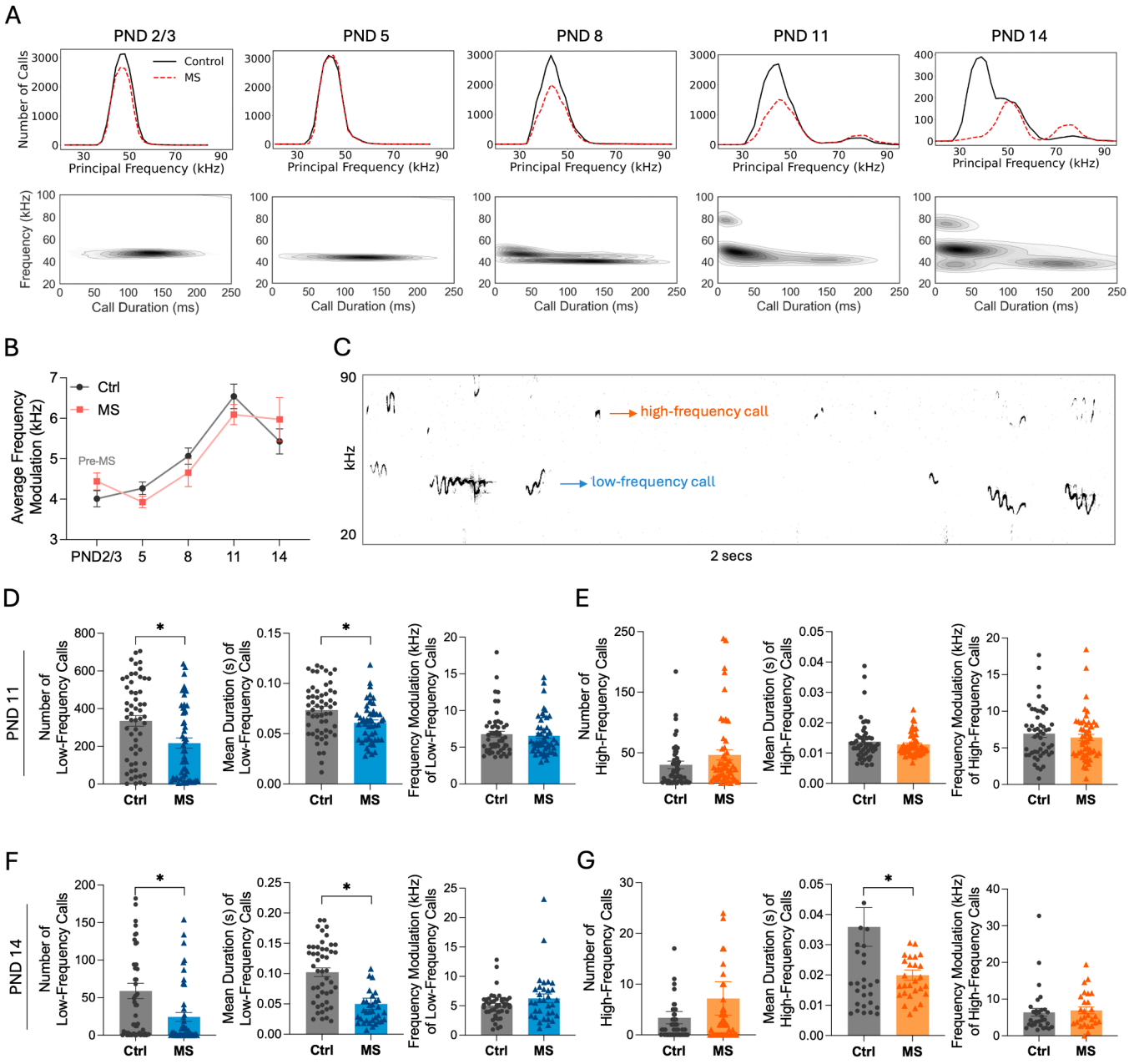

Fig. 3. Frequency-related properties and classification of USVs. (A) Top: the distribution of infant calls from all animals based on principal frequency across days; Bottom: heatmaps of infant calls from all animals based on principal frequency and call durations across days. (B) A line graph showing the average of frequency changes in calls across days. (C) A spectrogram from PND11, indicating examples of low- (<60 kHz, *blue*) and high-frequency (≥ 60 kHz, *orange*) USVs. (D-G) The number, mean duration and frequency modulation rates of (D, F) the low- and (E, G) high-frequency calls from (D-E) PND 11 and (F-G) PND 14. Error bars represent SEM. The asterisks (*) depict significant test results ($p < .05$).

from an increase to decrease on PND 8 ($F(3.71, 347) = 52.650$, $p < 0.001$, $\eta_p^2 = 0.360$; 2 × 5 two-way mixed ANOVA; Fig. 2G). MS did not influence the number of bouts ($F(1, 106) = 1.444$, $p = 0.232$; MS x PND: $F(4, 374) = 0.446$, $p = 0.776$) We next examined the number of USVs per bout and identified a significant interaction of MS and the testing day ($F(4, 374) = 3.868$, $p = 0.004$, $\eta_p^2 = 0.040$; 2 × 5 two-way mixed ANOVA; Fig. 2H), driven by a significant reduction in calls per bout on PND 11 and 14 (Table 1). In addition, MS significantly increased inter-bout intervals ($F(1, 106) = 12.210$, $p < 0.001$; $\eta_p^2 = 0.103$, 2 × 5 two-way mixed ANOVA; Fig. 2I), leading to sparser bout onsets in the MS group, which reached significance on PND 11 (Table 1). Altogether, these results suggest that MS-induced reduction in USV emission is not simply due to a decrease in the number of bouts, but mostly driven by the emission of fewer calls per bout.

Overall, analyses of the temporal structure of isolation-induced rat pup USVs indicate that early postnatal vocal behavior is shaped not only by call rates and acoustic properties, but also by how USVs are organized over time. Stress-induced changes in call repetition periods and bout structure suggest that MS disrupts temporal patterns of USV production, with effects most pronounced after PND 8. Thus, early life stress may interfere with maturation of vocal pacing during a sensitive developmental window.

3.3. Frequency-based characteristics and classification

The distribution of calls across principal frequency and duration (Fig. 3A) indicated developmental changes in vocal structure in both groups during the second postnatal week. Frequency modulation rates, defined as the kilohertz range between the highest and lowest frequency points within a single USV, were analyzed as averages across calls for each animal. This analysis revealed a significant effect of recording day ($F(3.12, 305.87) = 31.133$ $p < 0.001$, $\eta_p^2 = 0.241$; 2 × 5 two-way mixed ANOVA; Fig. 3B), with modulation rates progressively increasing from PND 5, reaching their highest levels on PND 11, and remaining at similar levels on PND 14 (Table 1). Thus, acoustic modulation peaking at PND 11, suggesting a permanent increase in vocal complexity. Maternal separation did not significantly affect frequency modulation ($F(1, 106) = 0.050$, $p = 0.822$) nor did it alter its developmental trajectory ($F(3.12, 305.87) = 1.853$, $p = 0.135$).

To separately evaluate acoustic properties of low- and high-frequency calls between the groups on the last two recording days (as seen in Fig. 3A), USVs were classified as low-frequency (< 60 kHz) and high-frequency (≥ 60 kHz; Fig. 3C). On both PND 11 and 14, MS group emitted fewer numbers of low-frequency calls (PND 11, Control: M = 334.68, SD = 213.46, MS: M = 217.10, SD = 196.41; PND 14, Control: M = 59.96, SD = 76.67, MS: M = 24.36, SD = 40.66), which also appears to be also shorter in duration (PND 11, Control: M = 0.07, SD = 0.03, MS: M = 0.06, SD = 0.02; PND 14, Control: M = 0.10, SD = 0.05, MS: M = 0.05, SD = 0.05; Table 1; Fig. 3D, F). For high-frequency calls, only a significant reduction in call length was noted for USVs emitted on PND 14 (Control: M = 0.04, SD = 0.04, MS: M = 0.02, SD = 0.01; Fig. 3G), but no other MS-induced changes were observed (Table 1; Fig. 3E, G). No stress-induced differences were observed in frequency-modulation rates for any call type (Table 1; Fig. 3D-G). Overall, these findings indicate that maternal separation produced substantial alterations in low-frequency call number and structure by the end of the second postnatal week, potentially reflecting early stress-related changes in communicative signaling.

3.4. Call type clustering

Three clusters were defined based on call frequency and modulation rates, as described in the Methods section (Fig. 4A, B). To visualize call distributions and developmental changes in production rates, we generated 3D scatter plots of pooled pup USVs for each recording day (N = 132,225 calls), plotting average frequency, call duration, and frequency modulation for each call (Fig. 4C). Type 1 calls made up almost all USVs emitted on PND 2/3 (~99.6%), PND 5 (~99.7%) and PND 8 (~97.2%), which were characterized by low frequency and low modulation. The proportion of Type 1 calls decreased on subsequent days, but still remained as many as ~85.0% on PND 11 and ~84.2% PND 14, respectively. Type 2 calls, defined by higher frequencies above 60 kHz and low modulation, were rare on early days (~0.15% on PND 2/3, ~0.18% on PND 5 and ~1.10% on PND 8), but rapidly increased on subsequent days (~11.00% on PND 11 and ~11.46% on PND 14). A similar pattern was also observed for Type 3 calls, characterized by their high frequency modulation. Type 3 calls accounted for ~3.97% of calls on PND 11 and ~4.30% on PND 14, while remaining nearly absent on earlier days. Consistent with previous findings, the period between PND 8 and PND 11 represents a critical window during which early infant USVs diversify into more mature call types through acoustic changes.

In line with the low-frequency call emission rate changes on and after PND 8, MS significantly altered the production of Type 1 calls ($F(1, 106) = 9.989$, $p = 0.002$; $\eta_p^2 = 0.081$; 2 × 5 two-way mixed ANOVA), with a marked group difference specifically on PND 8, 11 and 14 (Table 1; Fig. 4D). Notably, MS significantly increased the overall number of Type 2 calls across the recording period ($F(1, 106) = 4.709$, $p = 0.032$; $\eta_p^2 = 0.043$), with no interaction effect or pairwise group differences at any individual recording day. In contrast, Type 3 calls were not modulated by MS (Table 1; Fig. 4D). However, they were influenced only by developmental stage, with both Type 2 ($F(1.323, 130.6) = 42.770$, $p < 0.001$; $\eta_p^2 = 0.302$; 2 × 5 two-way mixed ANOVA) and Type 3 calls ($F(2.125, 266.1) = 18.230$, $p < 0.001$; $\eta_p^2 = 0.127$; 2 × 5 two-way mixed ANOVA) showing significant increases in emission rates between PND 8 and PND 11 (Table 1). These findings indicate that infant USVs diversify around PND 8–11, and that early MS exposure selectively reduces production of Type 1 calls with flat, low-frequency profiles starting on PND 8, coinciding with the onset of vocal maturation and diversification. MS slightly increased the emergence of high-frequency calls but did not disrupt highly frequency-modulated calls, suggesting a strong stress effect that suppresses early simple vocalizations immediately before development of a more complex vocal repertoire.

4. Discussion

We showed that isolation-induced USVs in infant rats follow a structured and dynamic developmental trajectory during the first two weeks of life. Between PND 2 and 14, we observed systematic changes in both the quantity and spectro-temporal acoustic features of USVs, including shifts in frequency, duration, and temporal emission patterns. The emergence of distinct call types followed a consistent developmental pattern. These changes suggest a maturation process in rat pup vocalizations, characterized by a rapid increase in acoustic complexity and changes in temporal structure, particularly between PND 8 and 11. Early life stress in the form of repeated MS did not change the overall developmental trajectory in the vocal complexity, but altered the USV emission rate specifically driven by a reduction in flat low frequency calls after PND8. MS also suppressed the call emission partly through changes in bout structure, in addition to shortening the call duration on PND 11 and 14. These findings point to a sensitive period between PND 8 and 14 during which the vocal repertoire rapidly diversifies within a narrow developmental window vulnerable to early adversity.

USV emission rates of rat pups declined over the second postnatal week, consistent with previous findings [4,32]. Alongside this decrease, we observed a developmental increase in the overall principal frequency of calls and a reduction in average call duration. These patterns reflect the emergence of high-frequency, short-duration USVs after PND 8 as revealed by subsequent clustering analyses. Tracking individual animals across PND 2 and 14 revealed a positive correlation in USV rates over time, showing that pups who vocalized more on early testing days tended to do so in the following tests. This within-subject consistency aligns with prior studies that used early life USV rates to predict later

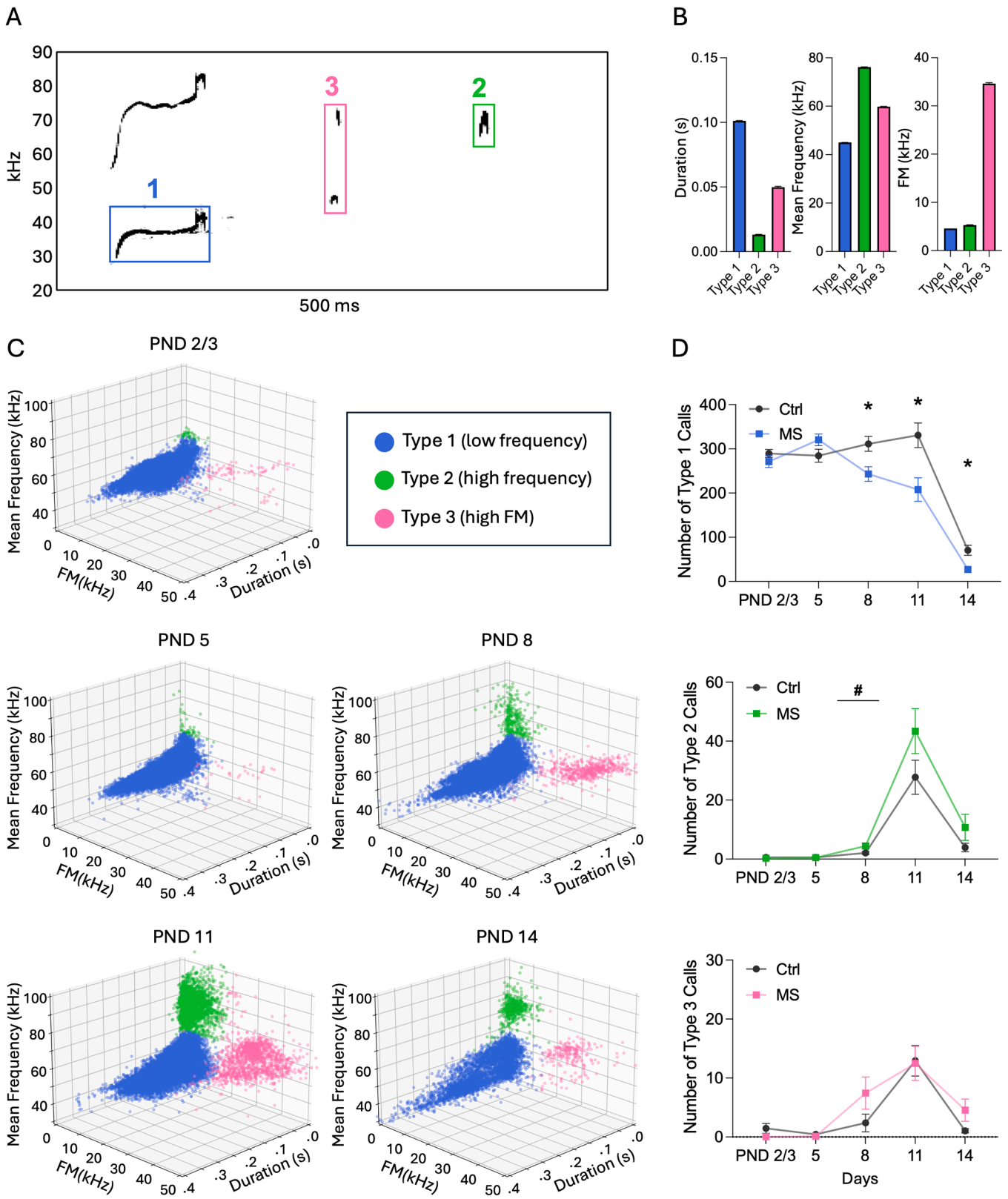

Fig. 4. Call type clustering over time. (A) A spectrogram from PND 11, showing examples of each type of USVs. (B) The average acoustic properties of call types in terms of duration, mean frequency and frequency modulation (FM). (C) The visualization of the total number of infant calls on each recording day, plotted across the same three acoustic variables. (D) The line graphs depicting the number of USVs emitted by each experimental group for Type 1 (*top*), Type 2 (*middle*), and Type 3 (*bottom*) calls separately. Error bars represent SEM. The asterisks (*) depict significant post-hoc tests, and hashtags (#) refer to the group main effect.

behavioral and affective phenotypes [13,17,18] and with research on rat lines selectively bred for high vs. low USV emission showing differential outcomes in adult behavior [52]. Thus, individual infant call rates may serve as semi-stable early life behavioral markers.

To better characterize the developmental diversification of infant USVs, we classified calls into three types based on three acoustic parameters: mean frequency, frequency modulation, and call duration using the call typification method adapted from Schwarting and Wöhr [49]. Visual inspection of the 3D feature space revealed three empirically distinct clusters that were consistently observed across days. Type 1 calls, defined by low frequency (<60 kHz) and low modulation (<20 kHz), dominated the early postnatal days and remained the most prevalent throughout the recording period. Type 2 calls, characterized by higher frequency (>60 kHz) but low modulation, began to emerge around PND 11. Type 3 calls were distinguished by high frequency modulation (>20 kHz), often appearing as fragmented or spectrally complex vocalizations. Notably, both Type 2 and Type 3 calls showed a marked increase between PND 8 and 11, highlighting a developmental shift in vocal repertoire complexity during this period. The refinement of acoustic features during the second postnatal week may reflect coordinated development of vocal and sensory systems, as well as a learned adaptation to produce more diverse calls that reduce masking by the vocalizations of other pups, as previously hypothesized in mice [30,53]. In rats, the alignment of vocal change with the maturation of auditory sensitivity may serve to optimize the communicative salience of distress calls to the dam, via enhancing maternal responsiveness and reinforcing dyadic interactions [6,8]. If altered call timing or spectral properties render the signals more salient to maternal perception [8,54] this may possibly increase the likelihood or speed of maternal retrieval, facilitating early affiliative interactions critical for emotional development.

Experimental manipulations such as early life stress models [55–58] and prenatal conditions [59–61] can modulate different pup USV features. In our work, MS suppressed overall USV emission on PND 8, which consisted almost exclusively of flat, low-frequency vocalizations, and was accompanied by changes in specific acoustic and temporal properties of infant calls. In a previous study, rat pups exposed to MS increased their high-frequency calls (~60 kHz) in a second isolation period on PND 12, while no difference was reported for the first isolation [12]. However, a reduction in complex USV types, according to the sonographic classification by Brudzynski [4], was also observed following the low bedding paradigm [13]. These detailed comparisons can help illuminate how such models influence the developmental trajectory of pup USVs, particularly in shaping the diverse call structures that constitute a mature vocal repertoire [4]. Here, we conducted a frequency-based classification based on a previous model [49] and showed that MS enhanced the emergence of the second call type characterized by higher frequency on PND11, but did not alter the emergence of the third call type which is characterized by high frequency-modulation. However, similar to the reduction in overall USV emission on PND 8, MS decreased the rates of low-frequency calls on PND 11 and 14. In addition, even though the number of calls emitted on PND 14 was reduced compared to the preceding days, the call duration was shorter in the MS group on both days, which was restricted again to low-frequency calls. Interestingly, a similar effect was also observed in high-frequency calls on PND 14. Sprague-Dawley rat pups exposed to a similar MS procedure exhibited a comparable pattern with reduced call length on PND 12 [12]. Overall, these findings indicate that MS alters specific features of pup USVs and interferes with the developmental maturation and modulates diversification of calls over time. USV diversity, which may reflect underlying affective or communicative states [49], emerges via an experience-sensitive process during a narrow developmental window. Given that early life USV patterns can predict later socio-emotional and cognitive phenotypes [18,62], the MS-induced alterations reported here may serve as early indicators of long-term developmental trajectories.

Between PND 2 and 14, we observed a gradual increase in the latency to emit the first isolation-induced vocalization. This delay may reflect the maturation of stress and arousal regulatory systems, including the HPA axis, which develops progressively during the early postnatal period and likely raises the threshold for distress vocalizations in older pups [32,63]. Reduced maternal dependence due to improved thermoregulation and motor control [24] may also decrease the urgency to vocalize upon brief separation. Here, we did not measure body temperature during the isolation period, which limits our ability to determine whether developmental changes in thermoregulation predict the observed temporal changes across development. The increased latency may also arise from experience-based habituation to isolation, as vocal responses in rodents are known to decline with repeated exposure [6]. The changes in call latency may indicate heightened affective reactivity or a failure to develop inhibitory regulatory mechanisms that normally delay vocal response especially in older pups [64,65]. We did not detect any difference in call onset in pups exposed to MS. However, pups subjected to daily MS produced fewer vocalizations per bout after PND 8, despite emitting a comparable number of bouts, and showed overall changes in both call repetition periods and interbout intervals across days. Such disruption of vocal timing or acoustic features aligns with the enhanced behavioral sensitivity of MS-exposed animals [17,66], and may reflect lasting alterations in affective circuits that mediate threat evaluation.

We also identified a shift in within-session vocal dynamics across development. Between PND 2/3 and 8, USV rates peaked immediately after separation and declined over the 3-minute recording period, suggesting a reflexive and short-lived response to isolation. However, in older pups, vocalizations gradually increased over time within the testing session, indicating a developmental reversal in temporal emission patterns. This transition may reflect changes in arousal regulation, as older pups appear to take longer to escalate into a distress state, potentially due to more integrated sensory processing or delayed activation of stress-related circuits [23,63]. It may also indicate a shift from reflexive to more organized or context-dependent vocal behavior emerging with increased control over vocal-motor output. In several mouse strains, overall vocal development follows a developmental trajectory comparable to that observed here in rats [53,67–69]. Moreover, developmental transitions in mice pup USV temporal dynamics are marked by a clear reduction in variability and the crystallization of call types into stereotyped frequency-duration clusters over development, alongside a shortening of repetition periods between successive calls [30]. Our rat data revealed partially parallel dynamics: we observed the emergence of distinct call-type clusters alongside a gradual developmental reduction in repetition intervals over days. Whether this shift in repetition periods reflects affective intensity or motor coordination remains to be clarified. Altogether, these findings show that pup vocalizations change markedly during early postnatal development, and that early life stress can disrupt the timing of these transitions. Dynamic vocal measures beyond average call rates and frequencies may serve as sensitive markers of neurodevelopment and its disruption by early adversity.

Higher isolation-induced vocal activity of male pups compared to females has been reported during the first two weeks of life [70]. Male pups have been reported to emit more USVs with lower average frequencies than females [71]; however, we did not observe sex effects in any primary USV variables, consistent with other studies that failed to replicate robust sex differences across USV features [13,72]. Additional mechanisms, such as maternal behavior, may also contribute to reported sex effects in isolation-induced USVs. Dams have been shown to preferentially retrieve male pups before females, potentially responding to sex-linked variations in vocal features or olfactory cues [71]. This is consistent with earlier observations of increased maternal licking and grooming of male pups [73,74], which could further shape sex-biased developmental trajectories even when they are not readily observable in early vocal behaviors.

Rodent pup USVs trigger mother rats for retrieval and maternal care

[7,75–78], and, in turn, maternal behavior influences the rate of USV emission in infant rodents [12,17,79]. Furthermore, brief or prolonged MS may also alter maternal behaviors regarding infant care [80–82]. This bidirectional relationship highlights the complexity of interpreting infant call rates and the importance of considering maternal care dynamics. In this study, the lack of direct measures of maternal behavior limits our ability to fully assess this interplay. Future research should examine dynamic changes in maternal behavior and the indirect effects of early life stress on fine-scale developmental trajectories of acoustic and temporal features of early vocalizations.

5. Conclusion

Isolation-induced rodent pup vocalizations provide a translationally relevant and quantifiable index of psychosocial and neural development. Defining normative developmental trajectories and identifying sensitive windows of vulnerability may help bridge preclinical models of early adversity with mechanisms underlying later dysfunctions. Accordingly, time-window and feature-specific alterations in early vocal output may serve as early markers of altered circuit maturation. Here, we demonstrate that infant rats undergo substantial acoustic and temporal reorganization of their vocal repertoire during the second postnatal week, with USVs transitioning from predominantly flat low-frequency calls to a more diverse distribution that includes high-frequency and high frequency-modulated types. This developmental trajectory aligns with previous findings demonstrating age-dependent increases in acoustic maturity and the emergence of more structured vocal patterns in both rats and mice [4,27,30,53,72,83,84]. These observations suggest that vocal development follows a temporally constrained and programmatic course. This species-specific developmental pattern is likely associated with the later emergence of the adult rat vocal repertoire, which ultimately differentiates into two primary types: 22 kHz and 50 kHz calls [85–88]. We also showed that MS selectively influences the emission and duration of flat, low-frequency calls, which constitute the predominant vocalization type early in development and precede maturation of the vocal repertoire in infant rat USVs. Together, these findings indicate the presence of a sensitive developmental window around PND 8–11, during which the vocal characteristics of infant rats diversify across multiple acoustic and temporal dimensions. Early life adversity, such as MS, can interfere with this process by selectively disrupting call-type expression in a feature-specific manner.

CRediT authorship contribution statement

Bahar Yuksel: Writing – original draft, Investigation, Conceptualization. **Cem Sevinc:** Writing – original draft, Investigation, Conceptualization. **Tuana Demirhan:** Investigation, Writing – original draft. **Gunes Unal:** Writing – review & editing, Project administration, Conceptualization.

Ethics approval

Boğaziçi University Ethics Committee for the Use of Animals in Experiments (approval no: 2024–08).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2026.116472.

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