Biology of Sex Differences

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A meta-analysis of sex differences in neonatal rodent ultrasonic vocalizations and the implication for the preclinical maternal immune activation model

Alison M. Randell[1*†], Stephanie Salia[1†], Lucas F. Fowler[1,2], Toe Aung[3], David A. Puts[4] and Ashlyn Swift-Gallant[1]

Abstract

As the earliest measure of social communication in rodents, ultrasonic vocalizations (USVs) in response to maternal separation are critical in preclinical research on neurodevelopmental disorders (NDDs). While sex differences in both USV production and behavioral outcomes are reported, many studies overlook sex as a biological variable in preclinical NDD models. We aimed to evaluate sex differences in USV call parameters and determine if USVs are differently impacted based on sex in the preclinical maternal immune activation (MIA) model. Results indicate that sex differences in USVs vary with developmental stage and are more pronounced in MIA offspring. Specifically, developmental stage is a moderator of sex differences in USV call duration, with control females emitting longer calls than males in early development (up to postnatal day [PND] 8), but this pattern reverses after PND8. MIA leads to a reduction in call numbers for females compared to same-sex controls in early development, with a reversal post-PND8. MIA decreased call duration and increased total call duration in males, but unlike females, developmental stage did not influence these differences. In males, MIA effects varied by species, with decreased call numbers in rats but increased call numbers in mice. MIA timing (gestational day ≤ 12.5 vs. > 12.5) did not significantly affect results. Our findings highlight the importance of considering sex, developmental timing, and species in USVs research. We discuss how analyzing USV call types and incorporating sex as a biological variable can enhance our understanding of neonatal ultrasonic communication and its translational value in NDD research.

Plain English Summary

This study looks at how young rodents in their first couple weeks of life communicate using high-pitched sounds called ultrasonic vocalizations (USVs), particularly in the context of when they are separated from their mothers. These vocalizations are often measured in preclinical research aimed at understanding neurodevelopmental disorders (NDDs), such as autism. We evaluated whether there are differences between male and female rodents in how they produce these sounds and how they respond following exposure to an infection while gestating, a model known as maternal immune activation (MIA). Our findings showed that sex differences in vocalizations depend on the age of rodents and are more noticeable in those affected by MIA. In the early days of development, female rodents

†Alison M. Randell and Stephanie Salia co-first authors and contributed equally to this research

*Correspondence:
Alison M. Randell
Full list of author information is available at the end of the article

made longer calls than males, but this pattern reversed as they grew older. For females exposed to MIA, the number of calls decreased, while males showed different patterns depending on whether they were rats or mice. The timing of when the mother experienced immune activation did not significantly change the results. Overall, this research emphasizes the need to consider sex, age, and species when studying these vocalizations. Understanding these factors can help improve preclinical research on early communication in relation to NDDs.

Highlights

Sex modulates ultrasonic vocalizations (USV) in maternal immune activation (MIA).

Developmental stage moderates sex differences in USV call duration.

MIA reduces call numbers in females early (</=PND8), but not later development.

MIA effects in males vary by species: call numbers decrease in rats, increase in mice.

Including sex as a variable enhances translational value in preclinical research.

Keywords Ultrasonic vocalizations, Sex differences, Preclinical models, Maternal separation, Neurodevelopmental disorders, Autism spectrum disorder, Maternal immune activation

Introduction

Communication plays an essential role in survival across diverse mammalian taxa, including whales, primates, mustelids, bats, and rodents, which use high-frequency (i.e., ≤20 kHz) ultrasonic vocalizations (USVs) for conspecific communication [2, 4, 11, 18, 27, 44, 47, 55, 60]. Rodents are highly altricial species born with the inability to see, hear, or thermoregulate, and as such, neonates rely on maternal care [12, 25, 82]. Consequently, rodent pups emit USVs, which intensify in stressful conditions, such as in periods of maternal separation [15, 23, 25, 42]. As the earliest measure of social communication and one of the first feasible behavioral tests for neonates, examining USV production in response to maternal separation is critical in preclinical research of neurodevelopmental disorders (NDDs), such as autism spectrum disorder (ASD).

Zippelius and Schleidt [88] first classified neonate USVs as "whistles of loneliness," expressed in response to maternal separation. Such USVs are believed to be innate signals that improve survivability by eliciting maternal retrieval [25]. The ventral pouch of the larynx, supported by a dorsally bent rostro-ventral component of the thyroid cartilage, contributes to the production of USVs and is developed in utero [70]. Mouse pups emit USVs shortly after birth, although they are born deaf, with the ear canal not opening until PND10-11 [5, 25, 32, 56]. Furthermore, cross-fostering studies show that mice fail to mimic USVs from other genetic strains [46]. Together, this research suggests that pup USVs are inherent with a key role in survival.

Three types of vocalizations have been described in infant pups: (1) low-frequency (below 10 kHz) or 'wiggling calls' that trigger maternal licking and are produced when pups try to reach their mother's nipple [24], (2) broadband or 'pain calls' with frequencies between 4 to 40 kHz inhibit adult biting or injury and are emitted during postpartum cleaning of pups (Haack et al., 1983) and (3) isolation or distress calls (between 30 and 90 kHz) which prompt maternal retrieval and approach behaviours [23]. These isolation-induced USVs have increasingly received attention as a tool for assessing early communication delays in preclinical rodent models of NDDs [74].

Relevance of USVs in NDDs

One of the most well-established preclinical models of ASD is the maternal immune activation (MIA) model, which consists of maternal gestational infection either via a viral mimic (e.g., polyinosinic:polycytidylic acid (poly I:C)) or bacterial agents (e.g., lipopolysaccharide (LPS)). MIA offspring show increases in ASD-like behaviors, including social communication/interaction delays and repetitive behaviors (e.g., [41]). As MIA is a risk factor for ASD in humans [43, 64], and the core symptoms of ASD are recapitulated with this model, it is now one of the most studied environmental (non-genetic based) models of ASD [66].

In preclinical studies, including the MIA model, social communication delays are measured by USV communication during maternal separation; this is one of the earliest viable behavioral assessments for social communication in neonates and often predict later ASD-like

phenotypes [16, 68]. However, there are considerable inconsistencies in the direction of change and interpretation of USV data. For instance, many report that MIA increases USV emissions [17, 48], while others find decreases in MIA offspring (e.g., Carlezon et al. 2019; [54]), and others report no differences (e.g., [50, 77]). Yet, the interpretation is often similar—whether increases or decreases in USV number or duration, these alterations are described as evidence of delays or deficits in neonatal communication.

Similarly, methods for collecting and analyzing USVs vary widely, including differences in analysis metrics (i.e., number, duration, frequency, classifications/call types), the species/strain of rodents used, the embryonic day (E) of MIA induction, the type of MIA (bacterial vs viral), as well as variation in the developmental stage of USV testing. Furthermore, many studies do not consider sex as a biological variable, and instead either only include males, pool the sexes, or do not report the sex of the subjects. In the current meta-analysis, we found that less than one-third of the 32 MIA studies identified for inclusion reported USV data by sex (see Fig. 1A). Given the 4:1 male bias in ASD, it is critical to include sex in preclinical work to improve the translational value of findings. This is especially important in USV research, as there are significant effects of sex and sex-by-environment interactions in rodent USV production.

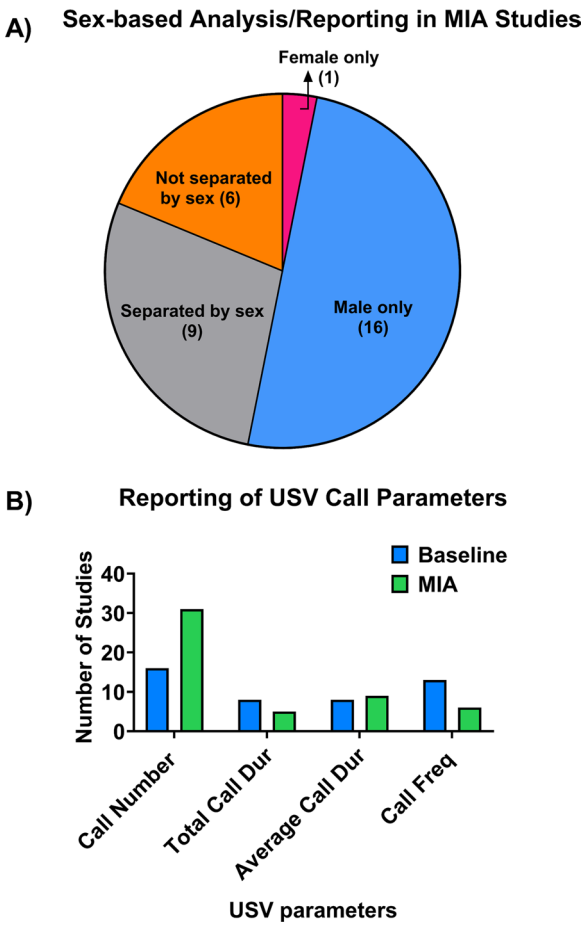

Fig. 1 Lack of integration of sex as a biological variable and limited USV call parameters in MIA research. **A** Less than a third of MIA studies, analyzed USVs by sex, with the majority of studies only including males or pooling the sexes in their analysis. Only 1 study analyzed USVs in females alone. **B** The most commonly reported USV call parameter is call number (49% of papers), while total call duration (10%), average call duration (17%), and call frequency (12%) are each reported in less than a fifth of studies

USV Production Varies by Sex and Environmental Condition

USV production and structure differ between pup sexes, with males generally producing more USVs of longer durations, lower frequencies, and lower amplitudes than females [16, 52]. Such differences may contribute to signal saliency and subsequent maternal retrieval, as dams allocate more attention to male pups than females [3]. For example, all male litters receive more maternal care than all female litters [1], and when in stressful conditions, mothers produce female-biased litters to optimize their fitness [28]. Neuroendocrinological mechanisms, such as hypothalamic-pituitary axis (HPA) activation and co-expression of *FOXP1* and androgen receptors in the striatum, are involved in mediating sex differences in USV variation [8, 21, 30].

Pup USVs are also impacted by their rearing environment and developmental stage [84]. For instance, pups reared in larger, environmentally enriched housing produce fewer USVs with shorter durations and lower frequencies than those in standard, under-stimulating conditions [7, 84]. Moreover, pup USV production gradually rises following birth, peaks at PND8 in mice [15, 74] and PND10 in rats [37], then decreases until stabilizing in puberty. Environmental differences affect HPA activation and can modulate USV deficits in preclinical models of ASD, including the MIA model [7, 21, 84, 87, 89]. Thus, sex and sex-by-environment interactions influence USVs in rodents, and developmental stage, species, and strain may modulate sex differences in USVs. As such, depending on these various factors, sex differences may be overestimated, underestimated, or ignored in experimental research [89], leading to misinterpretations of USV data and underrepresentation of sex as a mediating factor in translational rodent models of NDDs.

Present study objectives

We conducted meta-analyses to assess whether (1) there are sex differences in neonatal isolation-induced USVs, (2) MIA alters neonatal isolated-induced USVs, and (3) USVs of males and females are differentially affected by MIA. Within the meta-analyses assessing these three main questions, we also assessed whether the timing of MIA, type of MIA (viral vs bacterial vs other- e.g., valproic acid), developmental stage (i.e., postnatal day of USV recordings), or species (rats vs mice) were moderators of sex and/or MIA differences in neonatal USV isolation calls.

Methods

Literature search

We conducted a search for studies that evaluated sex differences in neonatal USVs in response to brief maternal separation (referred herein as "baseline studies") and those that assessed neonatal USVs in MIA preclinical models (referred herein as "MIA studies"). The search was performed using two major databases, PubMed, and Google Scholar. For baseline studies, we used keywords such as ("pup ultrasonic vocalizations" OR "neonate ultrasonic vocalizations" OR "isolation-induced ultrasonic vocalization"), combined with ("sex differences" OR "sex"). For MIA studies, additional keywords included ("maternal immune activation" OR "MIA" OR "poly ic" OR "lipopolysaccharide" OR "valproic acid" OR "animal model" OR "ASD"). This systematic review followed PRISMA protocol [63], Fig. 2). The complete search strings are available in the supplementary material.

Criteria for study inclusion

At the screening phase, papers were selected based on the following criteria according to the objectives of this systematic review: (i) Studies with one or more neonatal USV parameters evaluated (e.g., call number, total call duration, average call duration, and call frequency) in male and/or female offspring; (ii) USVs recorded prior to or during the weaning developmental stage (i.e., PND 3—21), and (iii) MIA model studies with intervention occurring during any phase of the gestational period along with appropriate controls (e.g., vehicle injection). The abstracts of all PubMed and Google Scholar records for baseline (n = 368) and MIA studies (n = 410) were evaluated for inclusion in the meta-analysis. Of 778 studies, only 23 baseline and 35 MIA independent studies included information on neonatal isolation-induced USVs recorded in rodent offspring. We excluded 4 of the 23 baseline studies [15], Hahn et al., 1997 & 1998; and Thornton et al., 2005), as sexes were pooled in the analyses and reporting of neonatal USV outcomes.

Extraction of study characteristics and USV parameters data

Study characteristics specific to species, strain, sex of offspring, age at which USVs were recorded, duration of USV recording, type of MIA immunogen used (e.g., poly I:C, LPS, valproic acid), dosage, gestational day of MIA induction, and frequency of administration were extracted. For USV parameters, we extracted the mean, SEM or SD, and sample size for male and female offspring (both treatment and control groups in the case of MIA studies) from each study for Hedges's g calculation to correct for small sample bias [80]. We contacted corresponding authors of studies with insufficient statistical information or unclear data. If authors did not respond or could not provide the requested information, data were extracted from graphs using Webplot Digitizer [71]. Three of the 35 MIA studies [22, 38, 83] were excluded because the descriptive mean and SEM/SD were not provided or data could not be extracted from violin plots. When sample sizes were reported as ranges, the most conservative (i.e., lower value) was used to calculate the effect size.

Meta-analysis

Data for the meta-analysis was analyzed using the "metafor" package in R version 4.2–0 [81]. Some studies provided multiple measures for the same USV parameters (such as call number, mean call duration, total call duration, and call frequency) based on the developmental stage of USV recording. To address this, a three-level multilevel model was used to nest measures from the same study, correcting for the likely correlation between measures from the same study with planned subgroup analyses. First, to investigate potential sex differences in neonatal USVs in response to brief maternal separation, control samples of male and female offspring from MIA studies (n = 9 papers) were combined with the initial 19 baseline studies (total n = 28). Here, the model included species (mice vs. rats) and developmental stages (early vs. late PND) as moderators. The peak of USV production in rodents occurs around PND 8 [74], and as such PND 8 and below were classified as the early neonatal period, and anything above PND 8 as late. Additional moderators included, the gestational timing of MIA induction (early vs. late MIA), with gestational day (GD) 12.5 as the cut-off for early MIA and anything above GD 12.5 as late MIA, and the type of MIA immunogen used (i.e., viral injection: poly I:C vs. bacterial: LPS vs. other).

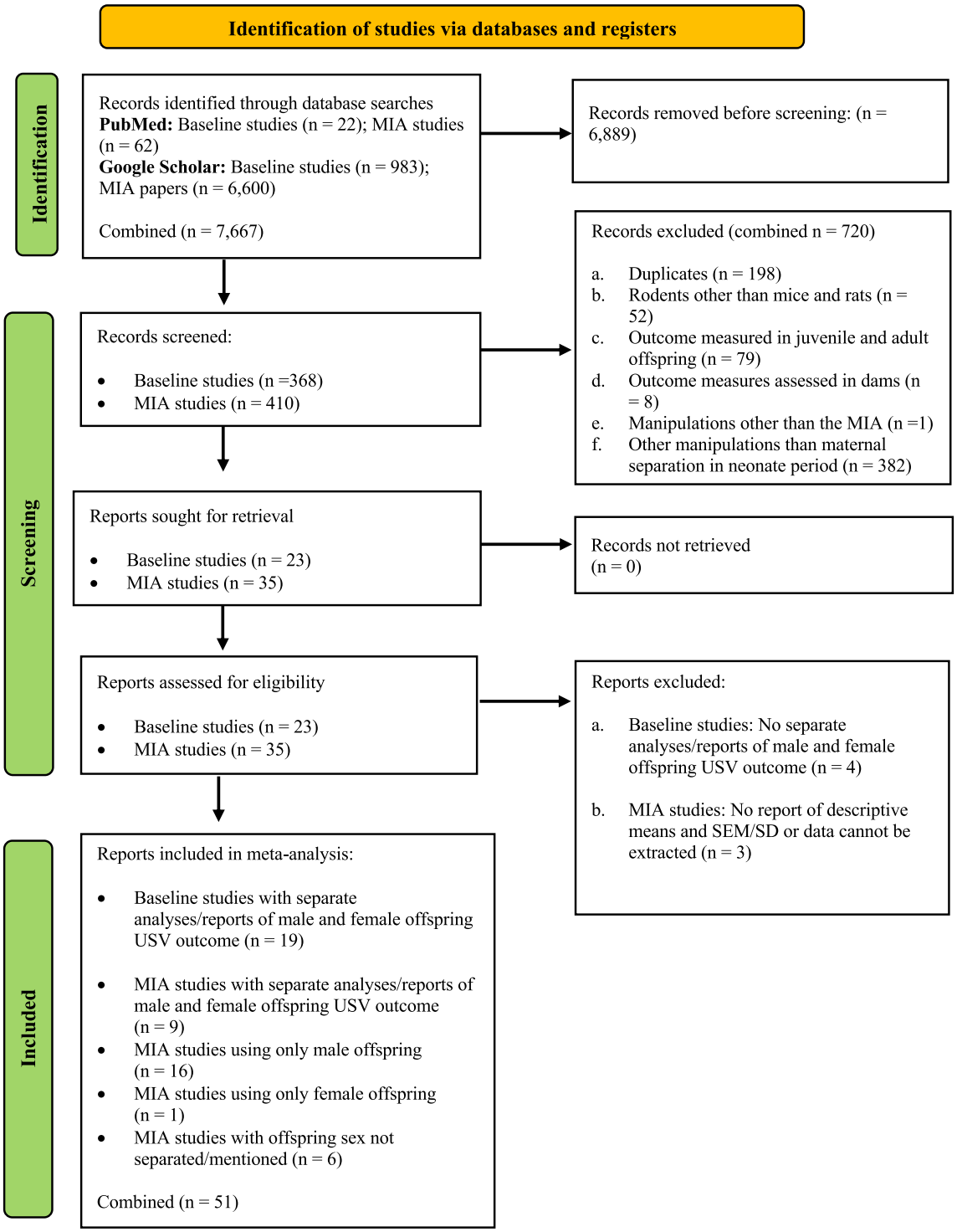

Fig. 2 PRISMA Flowchart detailing the identification and screening of identified records for the systematic review and meta-analysis

Results

Are there sex differences in neonatal USVs in response to brief maternal separation?

In each multilevel meta-analysis model, no significant effect size of sex differences in neonatal USVs in response to brief maternal separation was observed for call number ($g = -0.01$ [−0.13, 0.12], $p = 0.983$; SFig 1), mean call

Table 1 Results from moderator analyses for developmental stage and species

Models	g	se	z	p	Lower CI	Upper CI
Call number						
Intercept	0.06	0.10	0.61	0.545	−0.14	0.26
Late (vs. Early PND)	−0.04	0.12	−0.34	0.731	−0.27	0.19
Rats (vs. Mice)	−0.18	0.15	−1.18	0.236	−0.48	0.12
Mean Call Duration						
Intercept	0.28	0.19	1.47	0.143	−0.09	0.65
Late (vs. Early PND)*	−0.67	0.26	−2.56	**0.010 ***	−1.18	−0.16
Rats (vs. Mice)	0.16	0.27	0.59	0.552	−0.37	0.70
Total Call Duration						
Intercept	0.47	0.19	2.44	0.015	0.09	0.84
Late (vs. Early PND)	−0.47	0.27	−1.77	0.077	−1.00	0.05
Rats (vs. Mice)	−0.44	0.37	−1.18	0.237	−1.17	0.29
Call Frequency						
Intercept	−0.02	0.19	−0.11	0.912	−0.40	0.36
Late (vs. Early PND)	0.39	0.26	1.47	0.143	−0.13	0.90
Rats (vs. Mice)	0.05	0.26	0.20	0.834	−0.45	0.55

* Sub-group analyses were conducted to understand how developmental stage influences sex differences in mean call duration. Females had higher mean call duration than males in early PND groups (g = 0.36 [−0.11, 0.82], $p = .130$), and males had higher mean call duration than females in late PND groups (g = −0.31 [−0.64, 0.02], $p = .067$)

duration (g = −0.03 [−0.30, 0.25], $p = 0.851$; SFig 2), total call duration (g = 0.21 [−0.04, 0.47], $p = 0.098$; SFig 3) and call frequency (g = 0.15 [−0.11, 0.41], $p = 0.259$; SFig 4). We also tested whether sex differences in neonatal USVs in response to brief maternal separation were influenced by developmental stage (early vs. late PND) and species (rats vs. mice). A moderator effect was observed for mean call duration ($Q = 6.57$, $p = 0.037$), with a significant difference between late PND vs. early PND (g = −0.67 [−1.18, 0.16], $p = 0.010$; Table 1).

Sub-group analyses were conducted to understand the significant interaction between developmental stage and sex differences in mean call duration. While these analyses did not reach statistical significance, the direction of the sex difference is reversed depending on developmental stage, with females having a higher mean call duration than males in early PND groups (g = 0.36 [−0.11, 0.82], $p = 0.130$;), but males having higher mean call duration than females in late PND groups, (g = −0.31 [−0.64, 0.02], $p = 0.067$). No other moderators were significant ($p > 0.05$).

Does maternal immune activation (MIA) influence USVs in response to brief maternal separation (irrespective of sex)?

Across studies, MIA significantly influences mean call duration (g = −0.26 [−0.45, −0.07], $p = 0.006$; Fig. 3A) and call frequency (g = 0.37 [0.01, 0.73], $p = 0.043$; Fig. 3B), but not call number (g = −0.19 [−0.50, 0.11], $p = 0.220$; Fig. 4) or total call duration (g = 0.17 [−0.32, 0.66], $p = 0.500$; Fig. 5). The results of potential moderators: developmental stage (Early vs. Late PND), species (Rats vs. Mice), timing of MIA (Early vs. Late MIA), and type of MIA (Viral vs. Bacterial vs. Other) are reported in Table 2: call number ($Q = 10.89$, $p = 0.054$), mean call duration ($Q = 1.99$, $p = 0.851$), total call duration ($Q = 18.95$, $p < 0.001$), and call frequency ($Q = 13.60$, $p = 0.009$).

Sub-group analyses were conducted to understand the influence of significant moderators (see table notes, Table 2). These results suggest that call number is reduced with MIA in early developmental stages, while MIA does not differ from controls in later development. Call duration shows a reversal with age, such that MIA offspring had shorter, but non-significant, total call duration than controls in early PND groups, but had longer, non-significant, total call duration than controls in late PND groups. Species differences suggest that MIA reduces total call duration in rats, but increases total call duration in mice. Subgroup analyses of MIA type (bacteria, viral vs other [i.e., VPA]) indicated that while MIA offspring had significantly higher call frequency than controls in other MIA treatments (g = 0.61 [0.31, 0.90], $p < 0.001$), this difference was non-significant (and in the reverse direction) for viral MIA offspring compared to controls (g = 0.06 [−0.36, 0.47], $p = 0.786$).

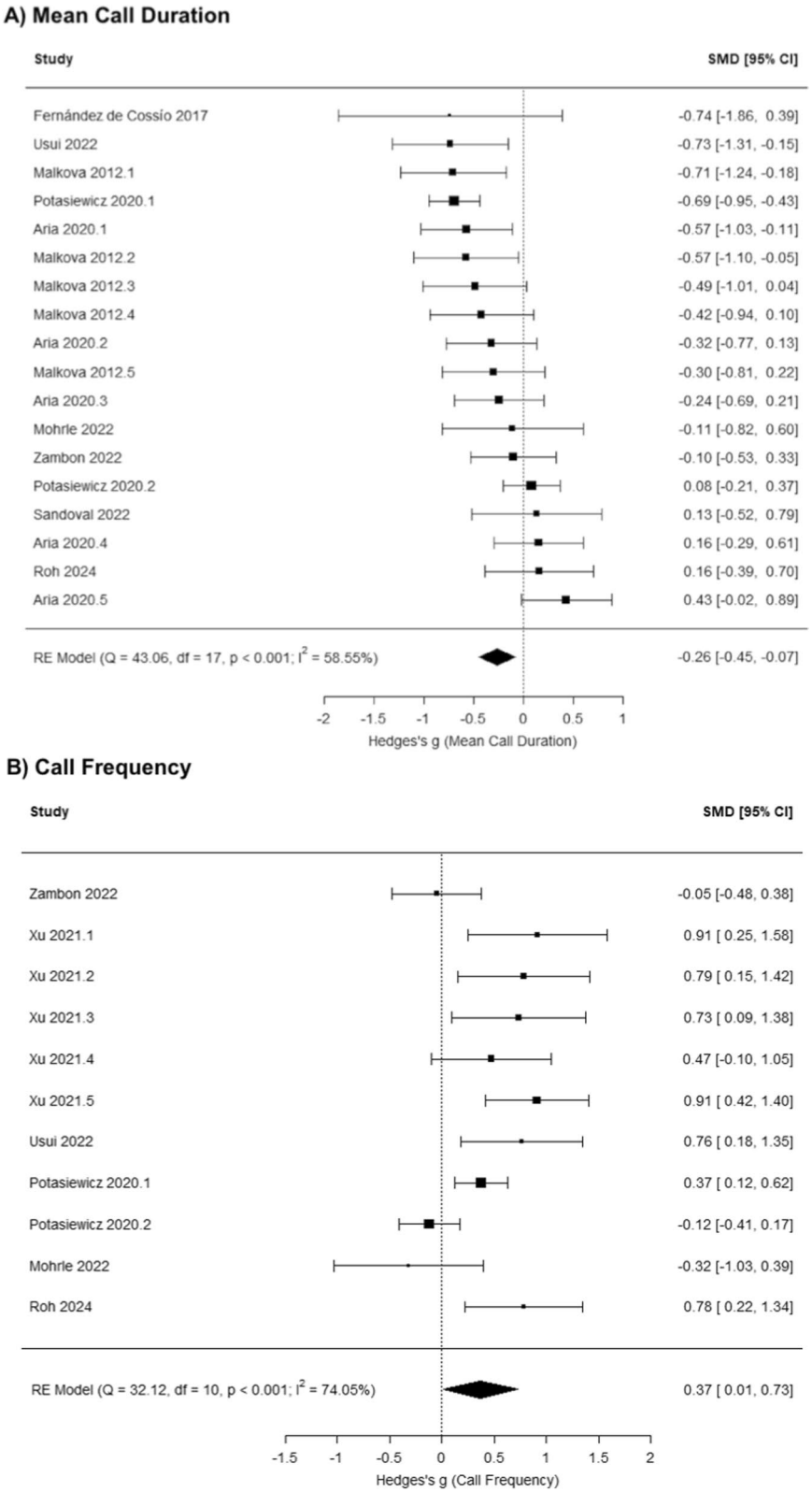

Fig. 3 Meta-analysis results indicate that maternal immune activation (MIA) decreases neonatal USV mean call duration, and increase call frequency in response to maternal separation compared to vehicle controls

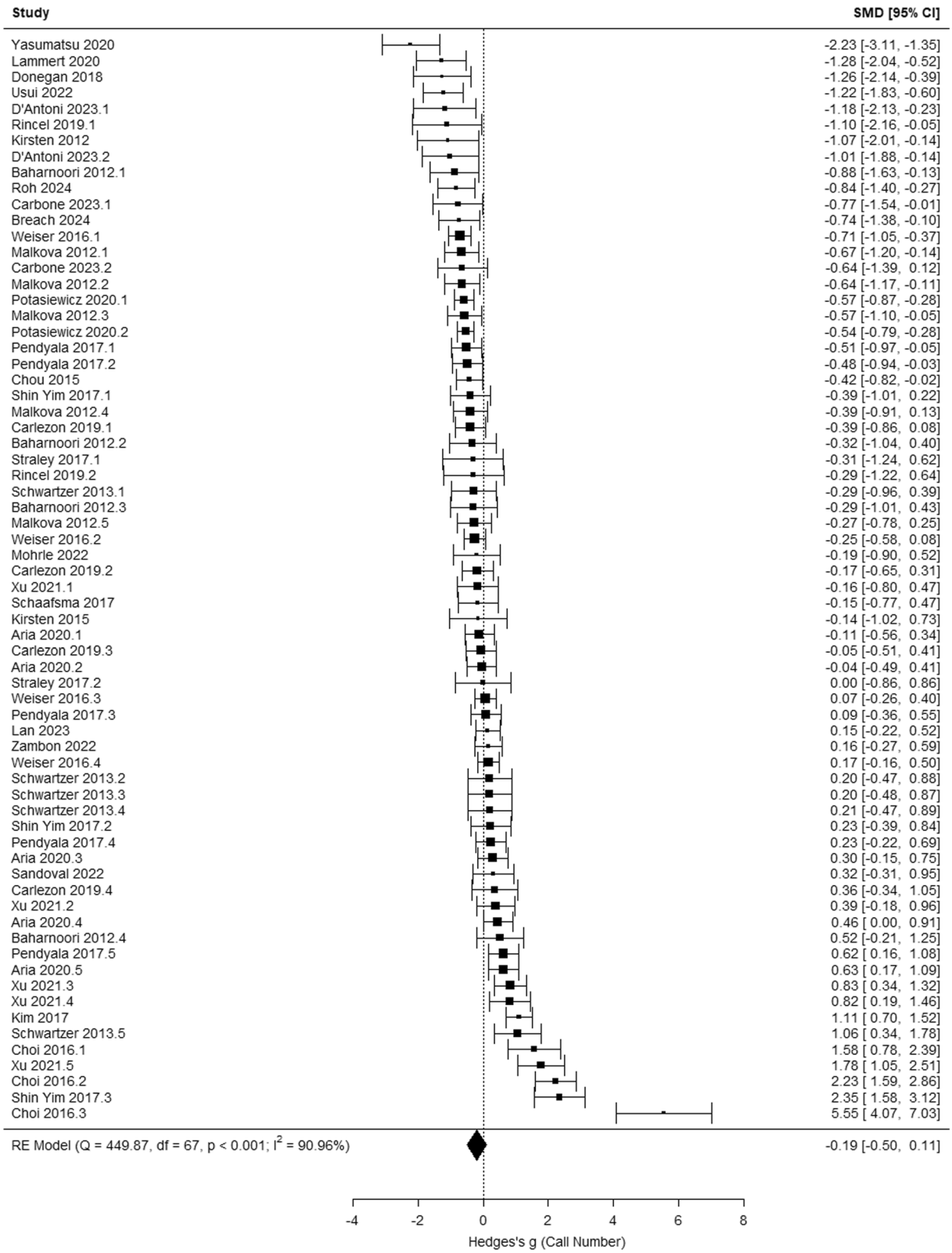

Fig. 4 Meta-analysis results indicate that maternal immune activation does not significantly influence neonatal USV call number

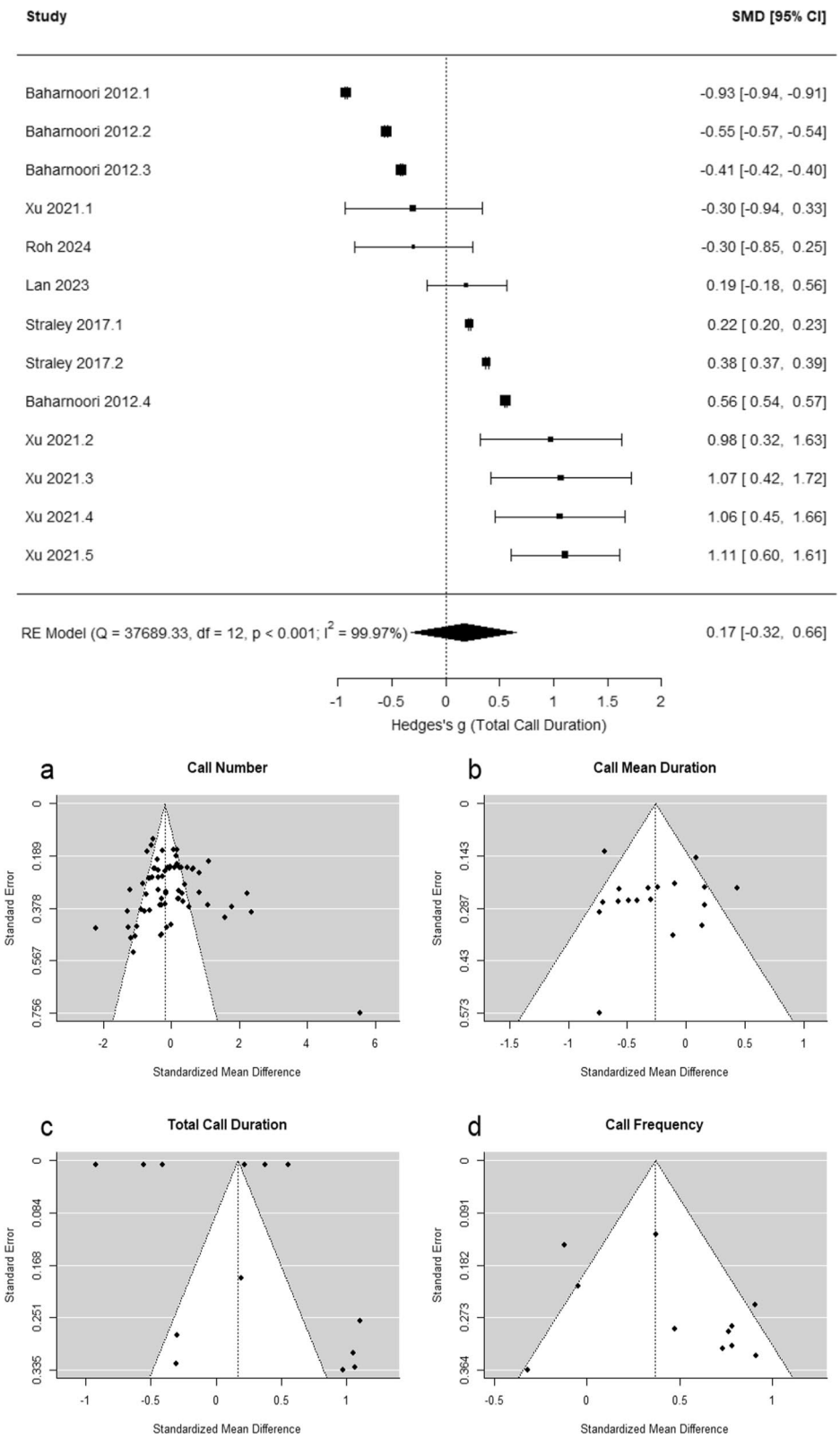

Fig. 5 **A** Meta-analysis results indicate that maternal immune activation does not

Table 2 Results from moderator analyses for developmental stage, species, timing of MIA, and type of MIA for models that include one or both sexes

Models	g	se	z	p	Lower CI	Upper CI
Call Number[+]						
Intercept	−0.24	0.37	−0.65	0.518	−0.97	0.49
Late (vs. Early PND)[*]	0.32	0.15	2.10	**0.036**	0.02	0.63
Rats (vs. Mice)	−0.38	0.33	−1.14	0.253	−1.02	0.27
Late (vs. Early MIA)	−0.39	0.26	−1.47	0.141	−0.91	0.13
Other (vs. Bacterial MIA)[+]	0.04	0.46	0.08	0.934	−0.86	0.93
Viral (vs. Bacterial MIA)	0.28	0.36	0.78	0.436	−0.43	0.99
Mean Call Duration[++]						
Intercept	−0.40	0.56	−0.73	0.468	−1.49	0.69
Late (vs. Early PND)	0.14	0.21	0.65	0.517	−0.27	0.55
Rats (vs. Mice)	0.09	0.43	0.20	0.841	−0.76	0.94
Late (vs. Early MIA)	0.10	0.42	0.24	0.810	−0.72	0.92
Other (vs. Bacterial MIA)	−0.16	0.74	−0.21	0.831	−1.61	1.29
Viral (vs. Bacterial MIA)	0.15	0.50	0.29	0.771	−0.84	1.13
Total Call Duration[+++]						
Intercept	0.07	0.38	0.19	0.848	−0.67	0.81
Late (vs. Early PND)[**]	0.92	0.27	3.41	**0.001**	0.39	1.45
Rats (vs. Mice)[***]	−0.87	0.27	−3.21	**0.001**	−1.40	−0.34
Late (vs. Early MIA)	0.07	0.35	0.21	0.837	−0.61	0.75
Viral (vs. Bacterial MIA)	0.92	0.52	1.77	0.076	−0.10	1.93
Call Frequency[++++]						
Intercept	0.81	0.19	4.30	**0.001**	0.44	1.18
Late (vs. Early PND)	−0.13	0.27	−0.48	0.635	−0.67	0.41
Rats (vs. Mice)	−0.47	0.27	−1.76	0.078	−1.00	0.05
Late (vs. Early MIA)	0.06	0.21	0.27	0.789	−0.36	0.47
Viral (vs. Other MIA)[****]	−0.55	0.20	−2.70	**0.007**	−0.94	−0.15

Note. [+]No significant difference in USV call numbers was observed between Other vs. Viral MIA ($p = .518$). [++]No significant difference in mean call duration was observed between Other vs. Viral MIA ($p = .487$). [+++] Viral MIA was dropped from the model as it was identified as a redundant predictor. [++++] Bacterial MIA was not identified in the data. Sub-group analyses were conducted to understand the influence of significant moderators. *MIA offspring had lower USV call numbers than controls in early PND groups (g = −0.41 [−0.66, −0.16], $p = .001$), while MIA and controls did not differ in call number in late PND groups (g = 0.10 [−0.31, 0.52], $p = .622$). **MIAs had shorter, but non-significant, total call duration than controls in early PND groups (g = −0.16 [−0.74, −0.42], $p = .595$), but had longer, non-significant, total call duration than controls in late PND groups (g = 0.47 [−0.11, 1.05], $p = .109$). ***MIA offspring had shorter (non-significant) total call duration than controls in rats (g = −0.04 [−0.52, 0.43], $p = .859$), but longer (non-significant) total call duration than controls in mice (g = 0.37 [−0.67, 1.41], $p = .489$). **** Subgroup analyses of MIA type (bacteria, viral vs other [i.e., VPA]) indicated that while MIA offspring had significantly higher call frequency than controls in other MIA treatments (g = 0.61 [0.31, 0.90], $p < .001$), this difference was non-significant (and in the reverse direction) for viral MIA offspring compared to controls (g = 0.06 [−0.36, 0.47], $p = .786$)

Potential publication bias was also evaluated via funnel plots (Fig. 5a–d) and tested using Kendall's rank correlations: call number ($\tau = -0.06$, $p = 0.510$), mean call duration ($\tau = -0.20$, $p = 0.260$), total call duration ($\tau = -0.08$, $p = 0.765$), and call frequency ($\tau = 0.16$, $p = 0.542$).

Does MIA affect USVs in males and females differently?

Multilevel meta-analysis models were conducted by comparing neonatal USVs between control and MIA male rodents and by comparing between control and MIA female rodents, allowing us to assess whether MIA influences USVs more in males than females. Among male rodents, MIA significantly influences mean call duration (g = −0.41 [−0.66, −0.17], $p = 0.001$; Fig. 6A) and total call duration (g = 0.78 [0.25, 1.31], $p = 0.004$; Fig. 6B), but not call number (g = −0.27 [−0.63, 0.08], $p = 0.126$; Fig. 7) and call frequency (g = 0.31 [−0.34, 0.95], $p = 0.349$; SFig. 5). Among female rodents, MIA does not significantly influence call number (g = −0.05 [−0.36, 0.27], $p = 0.762$; SFig. 6), mean call duration (g = −0.23 [−0.58, 0.12], $p = 0.203$; SFig. 7), and call frequency (g = 0.05 [−0.28, 0.37], $p = 0.766$; SFig. 8).

We then assessed whether sex was a significant moderator in combined analyses of male and female data.

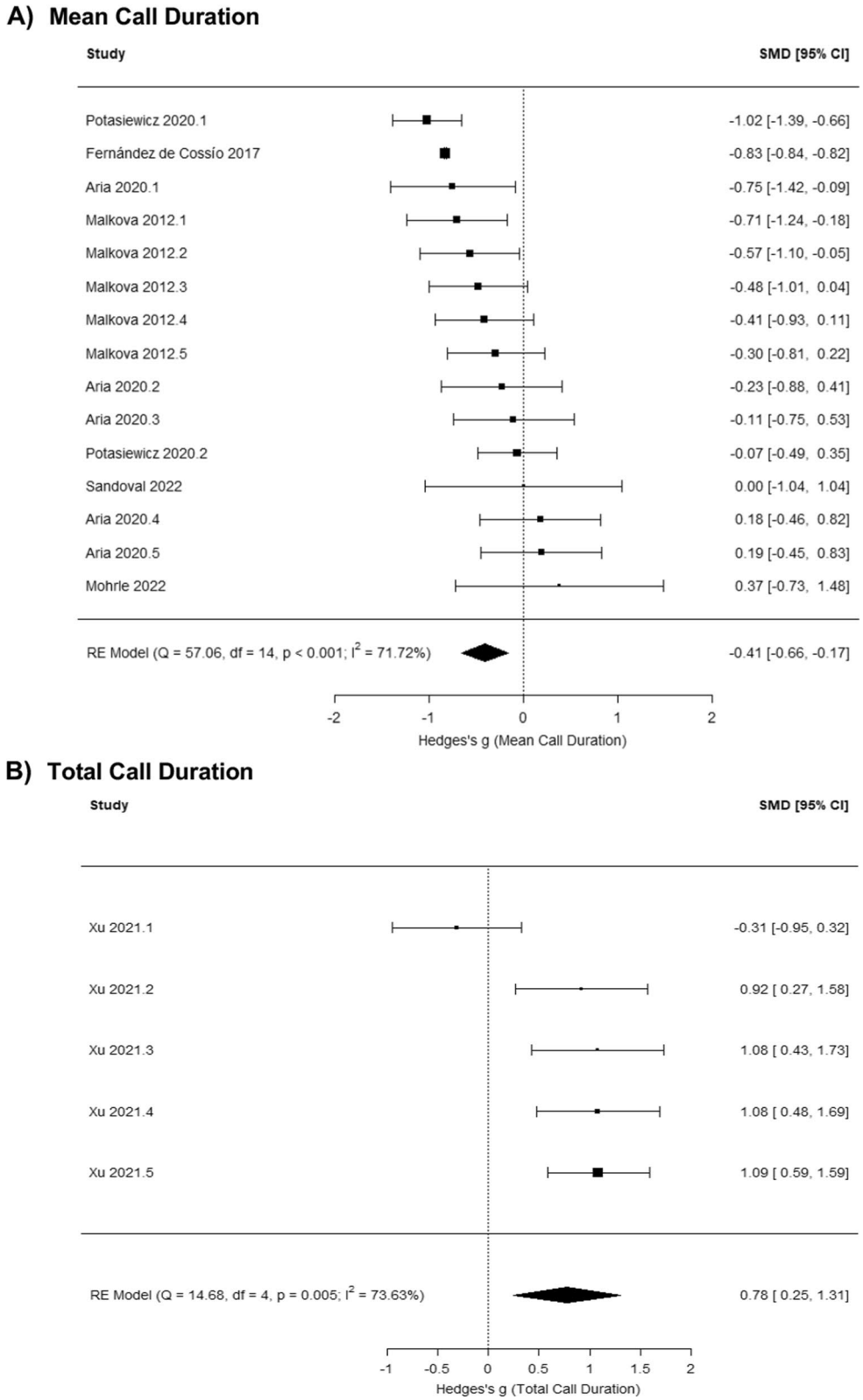

Fig. 6 Meta-analysis results indicate that MIA decreases neonatal USV mean call duration (**A**) and increases total call duration (**B**) among male rodents compared to same-sex controls

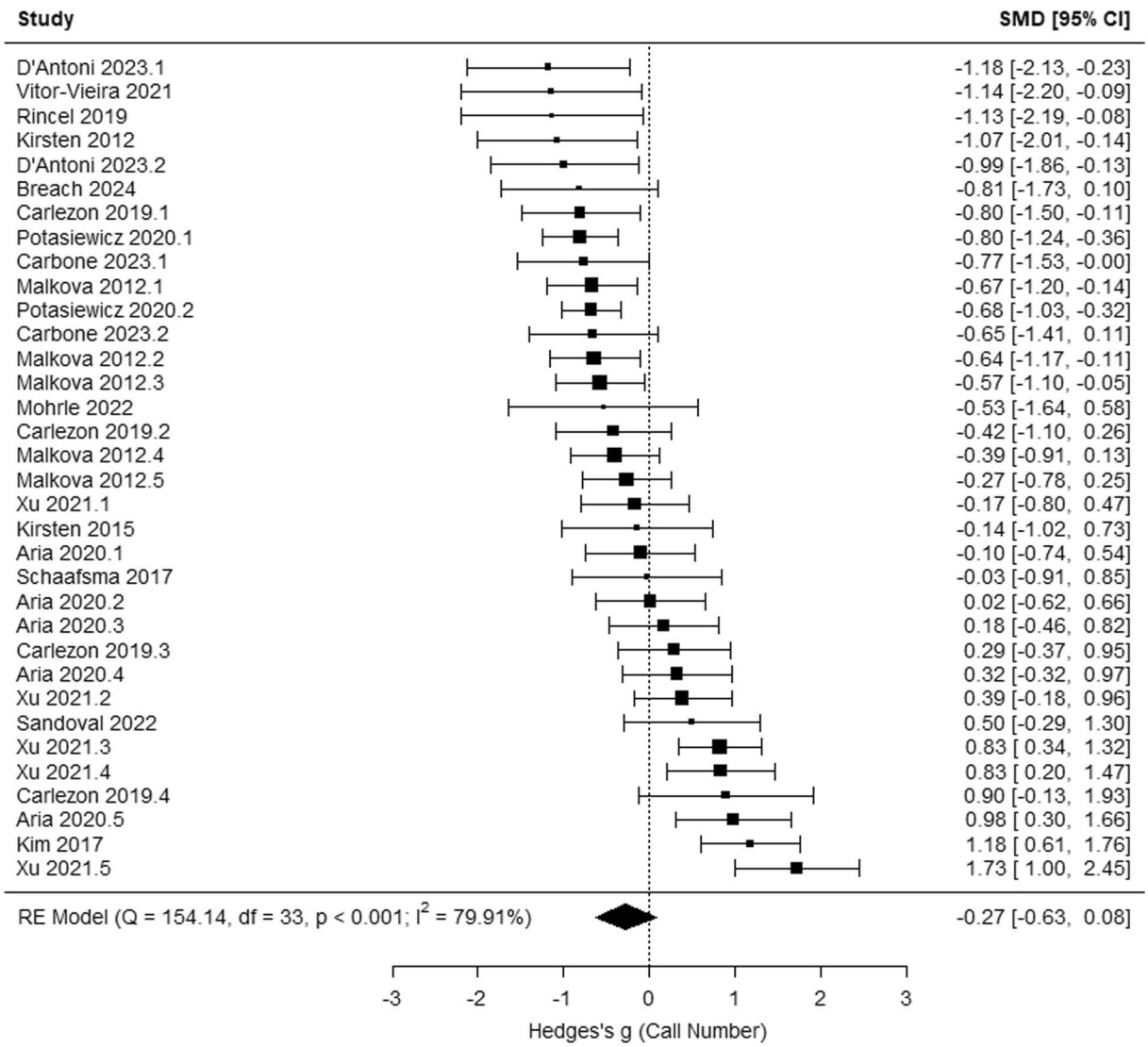

Fig. 7 Meta-analysis results indicate that neonatal USV call number does not significantly differ between control and MIA male rodents

Sex was found to significantly moderate mean call duration (g = 0.06 [0.04, 0.08], $p < 0.001$), but not call number (g = − 0.13 [− 0.34, 0.09], $p = 0.253$) and call frequency (g = 0.19 [− 0.17, 0.55], $p = 0.298$).

The results of potential moderators (Table 3 for males, Table 4 for females): for males, species differences were found, such that MIA reduced call numbers in rats, while in mice MIA increased call numbers compared to controls. For females, MIA reduced USV call number in early development (< / = PND8), but increased call number relative to controls in later development (> PND8).

Discussion

The present meta-analyses revealed that developmental stage is a significant moderator of sex differences in USVs, and sex differences in USVs are more pronounced in the preclinical MIA model. While USVs differed between MIA and control offspring when data were pooled across sexes, these differences should be interpreted with caution: Analyses by sex revealed that sex differences in USVs depend on developmental stage and species. Moreover, the majority of differences were found in mean or total call duration; this is an important consideration for future research, as many studies evaluating early communication delays via USVs report only call number (see Fig. 1B), without any measure of call duration. The differences in mean call duration, but not

Table 3 Results from moderator analyses for developmental stage, species, timing of MIA, and type of MIA for models comparing control and MIA male rodents

Models	g	se	*z*	*p*	Lower CI	Upper CI
Call Numbers[+]						
Intercept	0.13	0.36	0.35	0.728	−0.58	0.83
Late (vs. Early PND)	0.12	0.20	0.59	0.557	−0.27	0.50
Rats (vs. Mice)[*]	−0.99	0.32	−3.06	**0.002**	−1.62	−0.36
Late (vs. Early MIA)	−0.20	0.28	−0.70	0.481	−0.76	0.36
Other (vs. Bacterial MIA)[+]	0.22	0.36	0.61	0.540	−0.48	0.92
Viral (vs. Bacterial MIA)	0.05	0.34	0.16	0.873	−0.61	0.72
Mean Call Duration[++]						
Intercept	−0.09	0.61	−0.14	0.889	−1.28	1.11
Late (vs. Early PND)	0.11	0.19	0.60	0.551	−0.25	0.48
Rats (vs. Mice)	0.49	0.50	0.97	0.331	−0.50	1.48
Late (vs. Early MIA)	−0.45	0.55	−0.82	0.414	−1.52	0.62
Other (vs. Bacterial MIA)	−1.43	0.82	−1.74	0.083	−3.04	0.18
Viral (vs. Bacterial MIA)	−0.03	0.45	−0.06	0.951	−0.92	0.86
Total Call Duration[+++]						
Intercept	0.38	0.40	0.95	0.342	−0.40	1.16
Late (vs. Early PND)	0.66	0.51	1.29	0.196	−0.34	1.65
Call Frequency[++++]						
Intercept	0.29	0.66	0.44	0.662	−1.00	1.57
Late (vs. Early PND)	−0.13	0.29	−0.45	0.654	−0.69	0.43
Rats (vs. Mice)	0.24	0.68	0.35	0.724	−1.10	1.58
Late (vs. Early MIA)	0.58	0.61	0.94	0.346	−0.62	1.78
Viral (vs. Other MIA)	−1.17	0.60	−1.95	0.051	−2.35	0.00

Note. [+]No significant difference in USV call numbers was observed between Other vs. Viral MIA ($p = .623$). [++]A significant difference in mean call duration was observed between Other vs. Viral MIA ($g = 1.40$; $p = .015$). [+++] Only 1 level of species, timing of MIA, and type of MIA were identified in the data, and predictors were not included in the model. [++++] Bacterial MIA was not identified in the data. Call number ($Q = 11.59$, $p = .041$), mean call duration ($Q = 13.80$, $p = .017$), total call duration ($Q = 1.67$, $p = .196$), and call frequency ($Q = 15.12$, $p = .005$). Sub-group analyses were conducted to understand how species influences USV call numbers in MIA male rodents. MIA male rodents had lower USV call numbers than control males in rats ($g = -0.74$ [−0.95, −0.53], $p < .001$), and had higher USV call numbers than control males in mice ($g = 0.14$ [−0.30, 0.58], $p = .534$)

always in total call duration or call number, may also suggest that there are group differences in call types (i.e., flat, two-step, chevron, etc., see Fig. 8A). As such, we recommend that in studying USVs in MIA (or other preclinical models), researchers consider developmental stage, species, sex, and expand their analysis to include additional USV parameters, including call type. Below, we detail the significance of these findings, as well as provide recommendations for non-invasive visual sexing of neonates, and for automated scoring software that allows for accurate and relatively quick analyses of USV call parameters and call type classification.

We found that at baseline (i.e., controls, without MIA), developmental stage was a significant moderator of sex differences in USV mean call duration. While subgroup analyses were non-significant, the sign of the effect sizes are reversed, indicating that females emit longer calls earlier on (≤PND8), but that this sex difference reverses in later development (>PND8). However, it is noteworthy that the majority of studies in the present meta-analyses either excluded females or pooled the sexes (see Fig. 1A), and few studies reported on mean call duration when compared to call number (see Fig. 1B). Given that the strength of meta-analyses is limited by the availability and quality of existing research, more systematic studies are essential to better understand the effects of sex and developmental timing on USV emissions. Nevertheless, the present meta-analysis suggests that researchers should consider developmental timing when studying USVs generally and in translational NDD models, as PND can modulate sex differences in USV emission. It is also notable that how the day of birth is defined varies: PND 0, 0.5 or 1. Yet, the majority of papers do not report how they define the day of birth, which introduces another potential source of variation, when assessing USVs and developmental stage, that should be accounted for in future research.

When assessing the effects of MIA across all studies (i.e., pooling sexes), MIA reduced mean call duration and increased call frequency, and when developmental

Table 4 Results from moderator analyses for developmental stage, species, timing of MIA, and type of MIA for models comparing control and MIA female rodents

Models	g	se	z	p	Lower CI	Upper CI
Call Numbers[+]						
Intercept	−0.15	0.37	−0.40	0.687	−0.87	0.57
Late (vs. Early PND)[*]	0.58	0.28	2.03	**0.043**	0.02	1.13
Rats (vs. Mice)	0.05	0.49	0.10	0.917	−0.90	1.01
Late (vs. Early MIA)	−0.07	0.35	−0.21	0.832	−0.76	0.61
Other (vs. Bacterial MIA)[+]	−0.27	0.64	−0.42	0.674	−1.53	0.99
Viral (vs. Bacterial MIA)	−0.13	0.43	−0.31	0.758	−0.99	0.72
Mean Call Duration[++]						
Intercept	−1.09	1.00	−1.09	0.275	−3.05	0.87
Late (vs. Early PND)	0.42	0.42	1.01	0.315	−0.40	1.24
Rats (vs. Mice)	0.05	1.14	0.04	0.967	−2.19	2.29
Late (vs. Early MIA)	0.60	0.93	0.65	0.518	−1.22	2.42
Other (vs. Bacterial MIA)	0.60	1.99	0.30	0.764	−3.31	4.51
Viral (vs. Bacterial MIA)	0.67	1.31	0.52	0.606	−1.89	3.23
Call Frequency[+++]						
Intercept	0.26	0.19	1.39	0.164	−0.11	0.62
Late (vs. Early MIA)	−0.01	0.52	−0.03	0.977	−1.03	1.00
Viral (vs. Other MIA)	−0.38	0.51	−0.75	0.454	−1.38	0.62

Total call duration data were not identified for analysis. [+]No significant difference in USV call numbers was observed between Other vs. Viral MIA ($p = .713$). [++]No significant difference in mean call duration was observed between Other vs. Viral MIA ($p = .936$). [+++] Only 1 level of developmental stage and species were identified in the data and were not included in the model. Bacterial MIA was not identified in the data. [*]Sub-group analyses were conducted to understand how developmental stage influences USV call numbers in MIA female rodents. MIA female rodents had lower USV call numbers than control females in early PND groups (g = −0.25 [−0.49, −0.01], $p = .038$), and had higher USV call numbers than control females in late PND groups (g = 0.36 [−0.21, 0.93], $p = .217$). Call number ($Q = 6.51$, $p = .260$), mean call duration ($Q = 2.88$, $p = .718$) and call frequency ($Q = 2.25$, $p = .325$)

stage is considered, call number reductions were found to be restricted to early development. Species was also a significant moderator, such that call duration is reduced in MIA rats but increased in MIA mice. However, when sex is considered as a biological variable, it can be discerned that many of these effects are driven by males *or* females. For example, MIA decreased mean call duration, but increased total call duration in males, but not in females. However, when developmental stage is considered for females, MIA reduced USV call numbers in early development (≤ PND8) relative to controls, but increased in later development (> PND8). Moreover, species differences were found only for males, such that MIA reduced call numbers in rats, while in mice MIA increased call numbers compared to controls. These findings highlight that USVs are affected by MIA in both males and females, but are moderated by developmental stage and species.

Of note, sex differences in the presentation of USV delays underscores the necessity of considering sex as a biological variable in USV and NDD research, especially in light of the sex difference in the clinical manifestations of these conditions. For instance, in ASD, there is a known gender bias in diagnosis, where females who meet the criteria of ASD are less likely to receive a clinical diagnosis [53] or receive a diagnosis later in life [6, 79]. This diagnostic disparity may partly result from variations in symptom presentation. This bias extends into preclinical research, where studies frequently focus on males or fail to account for sex differences, leading to incomplete results and skewed conclusions (e.g., [49, 50, 54, 77]). The present study highlights that neonatal USV production in response to MIA is affected in both males and females, but additional research is necessary to gain a comprehensive understanding of the effects of MIA in the understudied sex—females.

Indeed, most studies in the present meta-analysis were single-sex (17 of 32), and 40% of studies reporting both sexes pooled male and female data (6 of 15 studies) for assessing USVs; although, we note that pooling of the sexes was not always the case for other juvenile behavioral measures in these studies. We hypothesize this may be due to unfamiliarity in sexing neonatal pups or the invasive nature of genotyping and/or tracking into adolescence. However, accurate sexing of neonates, and even fetuses at embryonic day 18, via visual inspection alone is feasible. Our data show fetal sexing accuracy is > 93% (n = 39 of 43), and sexing from PND 4 is > 97% accurate (n = 481 of 492), in C57BL/6 mice. As illustrated in Fig. 8B, males and females show distinct anogenital differences by late embryonic development: males have a

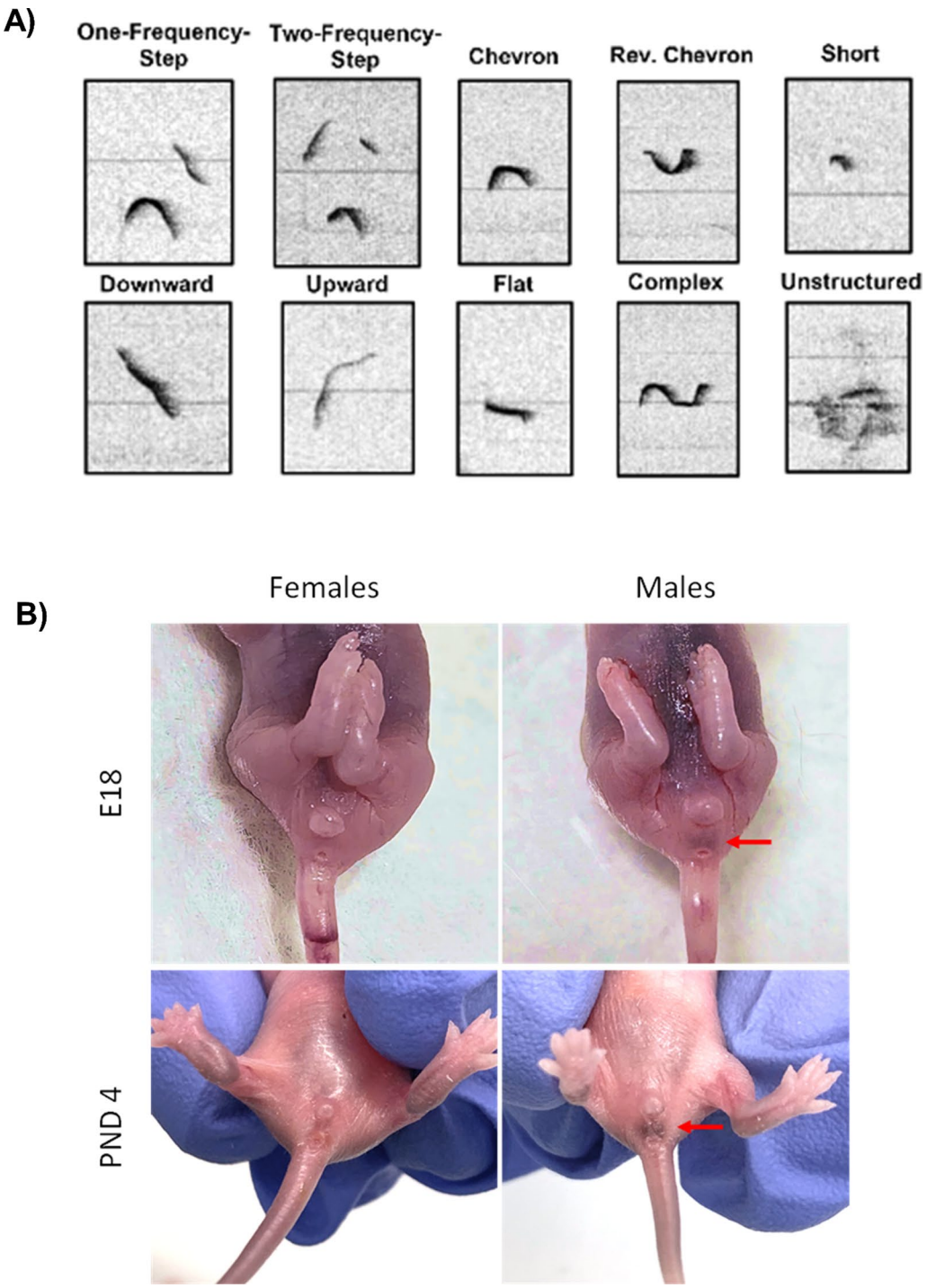

Fig. 8. **A** Call type classification (reproduced from [13]). **B** Sexing fetal and neonatal rodents can be accomplished by visual inspection of anogenital region to identify the dark pigmented spot in the scrotal region of male pups (indicated by red arrows)

noticeable dark spot in the scrotal region, which is absent in females. This pigmentation is the result of higher melanin concentration linked to androgen exposure [85]. It is visible from birth in several dark-fur mouse strains [86] and Long-Evans rats [85], although white-furred strains lack this pigmentation (e.g., A/J, 129X1/SvJ, and C57Bl/6 J-Chr 7A/NaJ, Wolterink-Donselaar et al. Thus, we recommend visual inspection for sexing

in dark-furred rodent strains, without genotyping and/or tattooing for tracking, and urge reviewers and editors to accept this method as valid, to promote the inclusion of sex as a biological variable in neonatal rodent studies.

In addition to including sex, expanding USV analysis to include the wide repertoire of USV call types emitted by pups may provide insight into the characteristics of pups' ultrasounds associated with NDD-like phenotypes. Indeed, USV call types, categories or classifications, since their description by Sales and Smith [72], have become an integral part of qualitative analysis of rodent vocalizations. These spectrographic analyses are based on the frequency modulation and duration of acoustic signals [10] and may provide additional information for quantitative USV parameters, such as call number, duration, and frequency [10, 15, 75]. Before Scattoni et al. [75], USVs were classified into five categories: constant frequency, modulated frequency, frequency steps, composite, and short [10, 72]. Scattoni's taxonomy expanded this framework to include additional call types—complex, harmonics, two-syllable, upward, downward, flat, chevron, short, composite, and frequency steps (Fig. 8A)—allowing for the detection of more subtle differences [15, 75]. Functionally, specific USV call types have been suggested to correlate with later social behavior. For example, infant isolation-induced calls, such as flat and short calls, have been found to correlate with the frequency of social interactions during adolescence [33]. This indicates that analyzing USV call type repertoire in pups can be an additional tool for quantifying the extent of ASD-like traits in rodent models during early development [57].

Several automated software programs (e.g., Avisoft analysis, VocalMat, DeepSqueak, Kaleidoscope Pro) can automatically segment rodent audio files into calls and apply classification algorithms to label vocalizations (for detailed reviews see [19, 29] and [67]). Our lab currently uses Kaleidoscope Pro, in which users can use cluster analyses to recognize a predetermined number of call types. This allows us to accurately sort and classify various calls, significantly accelerating the analysis of USVs. We have successfully classified over 70,000 USV calls with an accuracy rate exceeding 80%, which we can then manually correct to reach near 100% accuracy. We are able to share these training data with interested researchers upon request.

In conclusion, our meta-analyses reveal notable sex differences in USVs, especially in response to MIA. However, these differences vary depending on developmental stage and species. Importantly, none of the analyses showed differences across all USV call parameters, indicating a need for researchers to expand their focus beyond call number to include at minimum call duration and mean call duration. We also recommend incorporating an analysis of USV call types, as the existing studies, while few at this point, have identified sex-specific differences in call types [34, 45]. Additionally, we highlight that visual inspection via the "spot method" [86] is a highly accurate method for determining sex in common laboratory strains (e.g., C57Bl/6 mice and Long Evans rats) and advocate for its use to encourage the inclusion of sex in neonatal research.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13293-025-00685-9.

Additional file 1.

Author contributions

A.M.R., S.S. & A.S.-G. conceived the researched question. A.M.R., S.S. & L.F.F. conducted the review of manuscripts and data collection, T.A. & D.A.P. conducted the statistics. T.A., S.S. & L.F.F. prepared figures and tables. All authors contributed to the interpretation and write up of the manuscript.

Funding

This research was supported by a Canadian Institutes of Health Research (CIHR) project grant (495842) and Natural Sciences and Engineering Research Council (NSERC) Discovery Grant (RGPIN-2019-04999) to ASG.

Data availability

Data is provided within the manuscript or supplementary files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Department of Psychology, Memorial University of Newfoundland and Labrador, St. John's NL, Canada. [2]Cognitive and Behavioural Ecology Program, Memorial University of Newfoundland and Labrador, St. John's NL, Canada. [3]Department of Psychology and Counseling, Immaculata University, Immaculata, PA, USA. [4]Department of Anthropology, Pennsylvania State University, University Park, PA, USA.

Received: 16 October 2024 Accepted: 13 January 2025
Published online: 25 January 2025

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