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ULTRASONIC COMMUNICATION IN MICE: RELEVANCE IN NEUROSCIENCE

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TABLE OF CONTENT

Abstract
Mice emit ultrasonic vocalizations (USVs) to communicate each other in different social conditions: pups maternal separation, juvenile play, adults mating and social investigation. USVs can be acquired by means of specific tools and later analyzed on the base of both quantitative and qualitative parameters using manual or automated systems of calls classification. In recent years, the relevance of USVs has been consolidated as a valid tool for behavioral analysis of mice in both the context of ethological studies and in the field of studies of pathologies, especially those characterized by deficits in communication as neurodevelopmental disorders (NDDs) and autism spectrum disorders. Indeed, altered ultrasonic communication is found in several mouse models of NDDs and currently the evidence has emerged that the study of USVs can provide additional value to NDDs models. In addition, alterations in USV pattern are detected in mice also after pharmacological treatments in NDDs context.
This thesis covers the topics of USVs features in mice, contexts for USVs emission and factors that modulate their expression. A particular focus will be devoted to analysis of USVs in the context of NDDs murine models (e.g. Fmr1 knock-out mice, CB1 knock-out mice).

Sommario
I topi emettono le vocalizzazioni ultrasoniche per comunicare tra di loro in diversi contesti sociali: la separazione materna per i cuccioli, il gioco per i topi adolescenti, accoppiamento e interazione sociale per i topi adulti. Le vocalizzazioni ultrasoniche possono essere acquisite grazie a strumenti specifici e analizzate sulla base dei parametri quantitativi e qualitativi utilizzando sistemi manuali o automatizzati di classificazione. Negli ultimi anni, l'importanza delle vocalizzazioni ultrasoniche è stata consolidata come un valido strumento per l'analisi comportamentale dei topi sia nel contesto degli studi etologici che nell'ambito medico, specialmente per lo studio di patologie caratterizzate da deficit nella comunicazione come i disordini del neurosviluppo e in particolare i disturbi dello spettro autistico. Infatti, è stato dimostrato che vi è una comunicazione ultrasonica alterata in diversi modelli murini di disordini del neurosviluppo e attualmente è emersa l'evidenza che lo studio delle vocalizzazioni ultrasoniche può fornire un valore aggiuntivo ai modelli dei disturbi del neurosviluppo. Inoltre, alterazioni nello schema della comunicazione ultrasonica sono state rilevate nei topi anche dopo trattamenti farmacologici, sempre nel contesto dei disturbi del neurosviluppo.
Questa tesi comprende lo studio dei seguenti aspetti: le caratteristiche delle vocalizzazioni ultrasoniche dei topi, i contesti dove vengono emesse le vocalizzazioni e i fattori che ne modulano l'espressione. Un focus particolare sarà dedicato all'analisi delle vocalizzazioni ultrasoniche nel contesto dei modelli murini dei disturbi del neurosviluppo (es. i topi Fmr1 knock-out e i topi CB1 knock-out).

A) INTRODUCTION

1. Ultrasonic communication

Mouse communication occurs both in the audible and ultrasonic range of sound frequencies (Zippelius and Schleidt, 1956; Ehret and Bernecker, 1986). Mice predominantly communicate using the ultrasonic vocalizations (USVs) with a frequency from 30 to 110 kHz, above the limit of human hearing that is 20 kHz (Holy and Guo, 2005). Three types of USVs have been largely studied in laboratory mice: - isolation-induced USVs in pups, - interaction-induced USVs in juvenile mice and - interaction-induced USVs in adult mice (**Figure 1**).

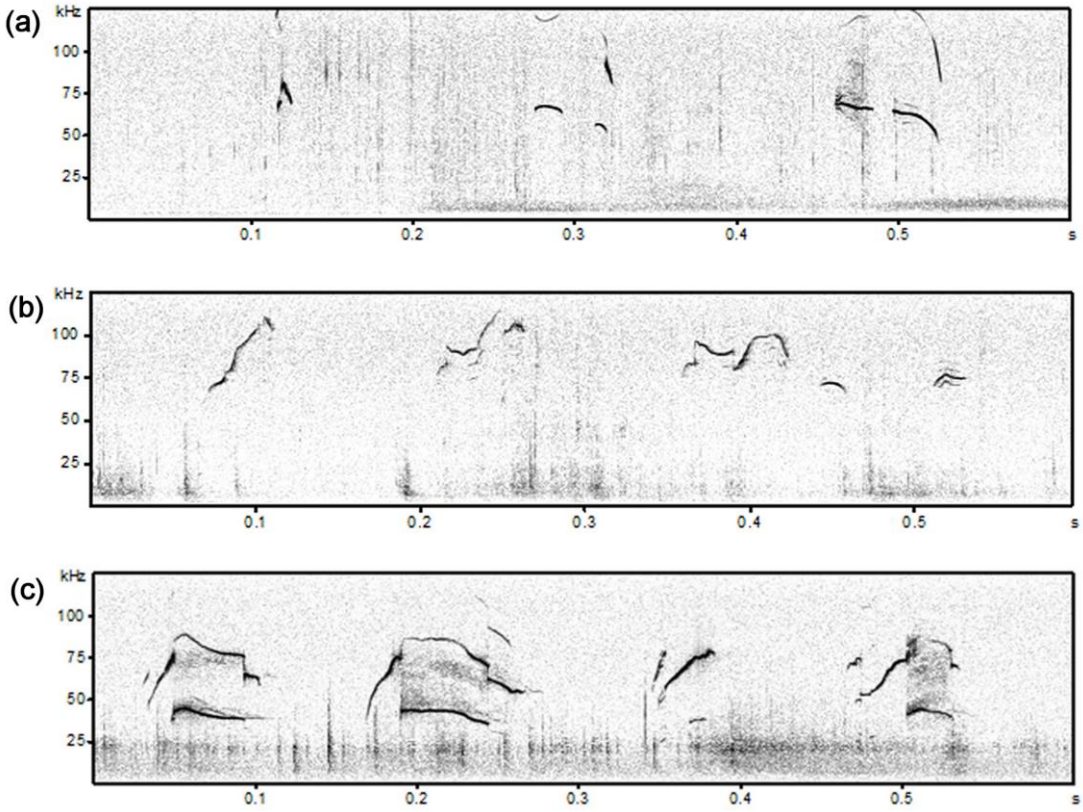

Figure. 1. Examples of isolation-induced USVs emitted by B6;129PF2 pups (a), male-female interaction-induced USVs produced by juveniles (b) and adult B6;129PF2 mice (c). In *y* axis is reported frequency expressed in kHz and in *x* axis time expressed in seconds (s).

Isolation-induced USVs in pups

For the first time, Zippelius and Schleidt described USVs emitted by pups during separation from the mother and the littermates and they referred to USVs as "whistles of loneliness" able to elicit mother retrieval (Zippelius and Schleidt, 1956). Pups use USVs to induce a caregiving behavior of the mother, as in the first post-natal days they are totally dependent upon her, because of their sensorial immaturity and inability to thermoregulate themselves. Pup USVs represent an early communicative behavior of the mother-pup dyad; indeed, USVs trigger maternal care and facilitate communication between mother and offspring (D'Amato et al., 2005; Hernandez-Miranda et al., 2017).

In addition, alterations in the pup USVs features can reveal modifications in emotional states of pups and therefore in arousal states of mother (D'Amato et al., 2005; Lahvis et al., 2011). Indeed, different types of calls are produced in response to

particular conditions such as the presence of a threatening stimulus like the odor of an unfamiliar, potentially infanticidal, adult male (Branchi et al., 1998). Moreover, experiments using mice with alleles linked to social bonding and separation distress, like oxytocin or mu-opioid receptor, demonstrated that isolation USVs are emitted in response to affective variations (Winslow et al., 2000; Moles et al., 2004a). In addition, it is also interesting the idea that emotional state and responsiveness of mother influence the emission of pup USVs. D'Amato and colleagues reported an increased number of isolation calls in pups born from mothers with a lower maternal responsiveness such as BALB/c females in comparison with C57BL/6 mothers (D'Amato et al., 2005).

Interaction-induced USVs in juvenile mice

The second type of USVs concerns vocalizations of juvenile mice during social interactions and it is correlated with social bonding and motivational level of mice (Panskepp et al., 2007; Peleh et al., 2019).

Interaction-induced USVs in adult mice

Adult mice produce USVs in different situations such as courtship, mating and social interaction. The most characterized adult mice USVs are those emitted during male-female interactions and/or in presence of female odor cues/urine. This type of calls is primarily attributed to males to attract females and has an important role in social/sexual behaviors (Egnor and Seagraves, 2016). Using devocalized males, no USVs were detected during male-female interactions and this supports the idea that are male mice that emitted vocalizations (Sugimoto et al., 2011). However, other authors do not exclude the possibility of USV production by the female mice because during interaction it is not easy to distinguish the animal that produces vocalizations. With the use of new technologies such as a microphone array system and sound source localization method, it is possible to localize and assign USVs to individual mice during a social context (Neunuebel et al., 2015; Heckman et al., 2017; Warren et al., 2018a). This permitted to demonstrate that also female mice vocalize during interaction with male mice, mainly during pursuit by males and when in close proximity with males (Neunuebel et al., 2015). Nevertheless, further researches are needed in this field.

USVs can be detected also during male-male and female-female social interactions. Different authors proposed some functions for these vocalizations such as to mediate competition over social status (Nyby et al., 1976; D'Amato, 1991; Zala et al., 2017) and to establish territorial dominance (Matsumoto and Okanoya, 2018) for male and to have affiliative purposes for female (Maggio and Withney, 1985; Moles et al., 2007; Zala et al., 2017). Usually, male mice emit USVs during male-male non-aggressive encounters and the number of their vocalizations is often low (Gourbal et al., 2004). On the contrary USVs rate produced by female mice in female-female interaction is comparable to the males in the male-female interaction with a similar acoustic structure (Hammerschmidt et al., 2012). USVs are emitted during the first minutes of social interaction by the resident female usually accompanied with high level of social investigation of the female intruder (Moles et al., 2007).

1.1 USVs NATURE

The nature of mice USVs is another interesting theme in this area of research that is still under investigation. Are vocalizations innate or learned? This is a debate yet ongoing. Supporting innate nature of vocalizations there is the fact that mouse pups

when born are deaf and their sense of hearing starts to develop after ten days of pups life (Ehret, 1975). Therefore, mice cannot be vocal learners during their infancy. Nevertheless, Holy and Guo suggested that USVs produced by adult male mice have features similar to songs of birds that are vocal learners (Holy and Guo, 2005). This has opened the debate on the idea that mice can be a model for vocal learning. Imitation of another species calls is essential to demonstrate vocal learning. Kikusui and colleagues demonstrated the incapacity of mice to imitate USVs of other strains during cross-fostering experiments (Kikusui et al., 2011). This is another evidence for innate nature of USVs. In addition, auditory experience during development is not necessary to produce normal vocalizations in mice (Hammerschmidt et al., 2012). Indeed, USVs of hearing and deaf mice were qualitatively similar (Mahrt et al., 2013). For these reasons, USVs seems to be innate and not learned. Other recent studies reported limited vocal plasticity in mice linked to the absence or limited presence of a forebrain pathway that it is very developed in vocal learners such as birds and humans (Arriaga and Jarvis, 2013). On the contrary, to demonstrate vocal learning in mice, several experiments focused on acoustic changes of USVs in different contexts were done. In particular, recent studies found evidences for learned nature of murine USVs due to modifications of USVs features during development (Grimsley et al., 2011), after isolation (Chabout et al., 2012) and in a competitive social condition (Arriaga et al., 2012). Therefore, the argument about the innate or learned nature of USVs is still totally open and under study.

1.2 USVs FEATURES

Regarding specific features that describe mice USV types, there are several temporal spectral components such as vocalization rate, frequency and duration that depend from different factors including age and genetic background of mice. In particular, the USV rate induced by maternal isolation follows a clear ontogenetic profile increasing during the first week of pup life, reaching a peak that depends from genetic strain of mice, and it decreases until the end of the second week of pup life (Elwood and Keeling, 1982). Then, the emission of USV from mouse pups is interrupted for a period that corresponds to the insurgence of social stimuli aging as trigger for USV. Later, vocalization rate increases again during social interactions especially between males and females (Warburton et al., 1989).

Furthermore, the vocal repertoire concerning different types of vocalizations (as the syllable vocabulary) is unvaried by age of mice. Indeed, both pups and adults are able to emit all types of USVs except noisy syllables (Grimsley et al., 2011). Nevertheless, the proportion of different types of USVs changes by age and also the other acoustic features, such as the duration and the frequency of USV types, decrease with mice advancing age (Grimsley et al., 2011; Heckman et al., 2016; Peleh et al., 2019). In particular, with the increase of age, pups emit calls with more complex features. This pattern continues in juvenile mice with a decreased number and duration of calls (Grimsley et al., 2011; Peleh et al., 2019). Pups produce many calls with a frequency above 100 kHz unlike adult mice that emit calls with a lower frequency (Grimsley et al., 2011). Other differences in calls features concern duration of intervals between syllables with a longer inter-syllable interval in younger than adult mice (Grimsley et al., 2011).

1.3 USVs CATEGORIES

All features of vocalizations described above together with others, such as amplitude and bandwidth (i.e., the range of frequencies that a signal spanned), can be detected

recording USVs with an ultrasound sensitive microphone (**Figure 2**) and can be quantitatively analyzed by specific software (e.g., SASLab by Avisoft Bioacoustics, Metris Sonotrack and Noldus UltraVox XT).

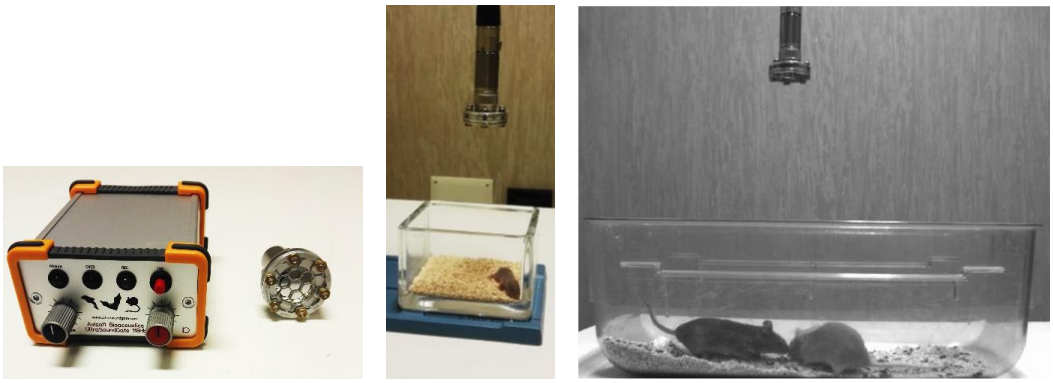

Figure 2. Mouse experimental settings to record USVs. Microphone (left panel) used to record calls during mother separation in pups (middle panel) and during adult male-female social interaction test (right panel).

Each vocalization can be also qualitatively classified into different categories based on several criteria. One of the most used classification for mice is described by Scattoni and colleagues including 10 categories, defined according to the internal frequency changes, duration and spectrographic shape, (Scattoni et al., 2008) such as:

1) Complex calls, that display one syllable containing two or more directional changes in pitch, each ≥6.25 kHz.

2) Harmonics calls, that display one main call, resembling the complex call, but with additional calls of different frequencies surrounding the main call.

3) Two-syllable calls, consisting of two components: a main call (flat or downward) with an additional punctuated component towards the end.

4) Upward-modulated calls, that exhibit a continuous increase in pitch that is ≥12.5 kHz, with a terminal dominant frequency at least 6.25 kHz more than the pitch at the beginning of the vocalization.

5) Downward-modulated calls, that exhibit a continuous decrease in pitch that is ≥12.5 kHz, with a terminal dominant frequency at least 6.25 kHz less than the pitch at the beginning of the vocalization.

6) Flat calls, that display a constant beginning and the ending of the pitch frequency remains constant (≤3 kHz of each other).

7) Chevron calls, resembling an "inverted-U", which is identified by a continuous increase in pitch ≥12.5 kHz followed by a decrease that is ≥6.25 kHz.

8) Short calls, that are punctuated and shorter than 5 ms.

9) Composite calls, that are formed by two harmonically independent components, emitted simultaneously.

10) Frequency steps, that are instantaneous frequency changes appearing as a vertically discontinuous ''step'' on a spectrogram, but with no interruption in time.

Examples of spectrograms of these 10 different calls typologies emitted by mice are

reported in **Figure 3**.

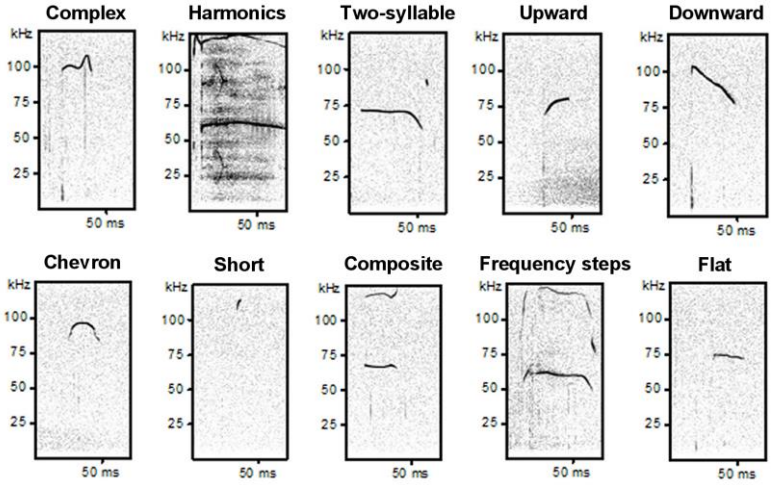

Figure 3. Examples of spectrograms of different USVs categories emitted by B6;129PF2 mice. In y axis is reported frequency expressed in kHz and in x axis is time interval expressed in milliseconds (ms).

In addition, also other qualitative classification methods exist in the literature (Holy and Guo, 2005; Gaub et al., 2016; Grimsley et al., 2016). To date the precise meaning of the different calls types is still unknown, and additional data is needed to better unravel this issue, allowing an essential step forward in the field of mouse behavioral neuroscience.

Finally, some automated systems can be used to deeply analyze USVs of rodents with standardized methods of machine learning (van Segbroeck et al., 2017; Coffey et al., 2019; Vogel et al., 2019). These tools permit to obtain a faster and objective identification of spectrographic features and also a standardization of USVs analysis in comparison of the manual classification of calls; however, to date the manual categorization of vocalizations still allows for the most highly detailed characterization. All standardized tools can automatically separate audio inputs into calls and background noise. They then use a classification algorithm to label the obtained vocalizations. For example, MUPET (van Segbroeck et al., 2017) and DeepSqueak (Coffey et al., 2019) systems execute unsupervised clustering with a great number of categories. MUPET can only cluster into a known a priori number of classes that must be determined by the user, whereas DeepSqueak can automatically discover the best-fitting number of clusters using statistical approaches. Furthermore, MUPET cannot apply its models to datasets other than those for which they were originally developed for, thus requiring separate models for each dataset. On the contrary, our research group in collaboration with Information Engineers of University of Brescia developed an *ad hoc* method for automatic USVs classification on the basis of USVs classification pattern published by Scattoni and colleagues (Scattoni et al., 2008). Briefly, we began our classification by using an audio track that had already been segmented into vocalizations and noise, with a dataset of 48699 labelled segments. We designed and employed two deep learning methods, a Convolutional Neural Network (CNN) and a Multilayer Perceptron (MP), as well as two machine learning techniques, i.e., Support Vector Machines (SVM) and Random Forests (RF), in order to achieve an automatic classification task. The dataset was manually created using Avisoft software, which includes capabilities for visualizing

and manually segmenting audio tracks in the form of spectrograms. The obtained USVs were then processed to extract some informative features required for training the learning algorithms. The results demonstrated that utilizing the entire time/frequency information of the spectrogram results in a better performance than taking into account only a subset of numerical features (Premoli et al., 2021). The findings seemed to be encouraging, and could offer a significant standard for future research in this field.

1.4 RELEVANCE OF USVs STUDY

Mice produce USVs to convey information related to positive or negative emotional states and to mediate social interactions. Communication is intensely linked to social behavior and for these reasons, USVs study has become a valid assay in behavioral readout and monitoring in this context (Granon et al., 2018).
For several decades, USVs have been extensively analyzed from an ethological point of view but in recent times the USVs study has acquired large importance in the field of psychiatric and neurological disorders, starting from those characterized by communication and social interaction deficits such as neurodevelopmental disorders (NDDs) and in particular autism spectrum disorders (ASD) (Moy and Nadler, 2008; Scattoni et al., 2009; Fischer and Hammerschmidt, 2011; Simola and Granon, 2019). Quantitative and qualitative alterations in USVs are reported in numerous mouse models of NDDs and ASD (Roy et al., 2012; Lai et al., 2014; Hodges et al., 2017; Premoli et al., 2019; Caruso et al., 2020).
It is very interesting to analyze USVs from a qualitative point of view because it permits to investigate the meaning and potential functions of vocalizations in order to use them as a valid tool in NDDs and ASD field. Regarding this, experiments were performed to assign specific USV calls to different behaviors during social interactions (Warren et al., 2018b). In addition, Sangiamo and colleagues demonstrated that distinct ultrasonic vocalizations are associated with different types of murine social behavior highlighting important role of communication in social contexts (Sangiamo et al., 2020).
Furthermore, since communication impairment is one of the core symptoms of ASD, altered calling patterns in mice can offer a useful assay for the diagnosis of ASD and to study the effects of pharmacological/behavioral interventions. Therefore, mouse ultrasonic communication analysis is a fundamental tool to investigate the mechanisms at the basis of these brain disorders and to test the efficacy of pharmacological treatments for these pathologies.

1.4.1 USVs and murine models of NDDs

NDDs are a heterogeneous group of neurobehavioral disorders with an early onset during development characterized by alterations in one or more domains of functioning such as social interactions, communication, cognition and motor behaviors (Homberg et al., 2016; Silverman and Ellegood, 2018; Mossa and Manzini, 2021). The Diagnostic and Statistical Manual version 5 (DSM-5) categorizes different NDDs such as:

- Intellectual Disorders: Intellectual Developmental Disorder, Global Developmental Delay, Unspecified Intellectual Disability;
- Communication Disorders: Language Disorder, Speech Sound Disorder, Childhood-Onset Fluency Disorder, Social Communication Disorder, Unspecified Communication Disorder;
- Autism Spectrum Disorder (ASD)

- Attention-Deficit/Hyperactivity Disorder
- Specific Learning Disorders: Impairment in reading and/or in written expression and/or in mathematics;
- Motor Disorders: Developmental Coordination Disorder, Stereotypic Movement Disorder;
- Tic Disorders: Tourette's Disorder, Persistent Motor or Vocal Tic Disorder, Provisional Tic Disorder, Other Specified Tic Disorder, Unspecified Tic Disorder;
- Other NDDs

Several genes have been associated with increased risk for NDDs, and rodent models are very useful for research in this field. Murine models with a high construct validity (conforming to the underlying disease rationale) and face validity (mimicking some of features of the disease) are able to reproduce molecular and behavioral modifications typical of human pathologies (Foxe et al., 2018; Vogel et al., 2019). Behavioral alterations in these models can offer markers for disease symptoms. For these reasons, different authors studied ultrasonic communication as a fundamental tool for early and adult behavioral phenotyping of NDDs and in particular of ASD mouse models (Caruso et al., 2020).
Below is reported the analysis of USVs in some models of NDDs and ASD models.

Fmr1 KO mice
Fragile X syndrome (FXS) is a NDD caused by the expansion of a CGG triplet in the X-linked fragile X mental retardation gene (FMR1) resulting in the absence of the FMR protein (FMRP) (Pieretti et al., 1991) that modulates mRNA trafficking, dendritic maturation and synaptic plasticity (Greenough et al., 2001). FXS is the most common inherited form of intellectual disability and monogenic cause of ASD (Rogers et al., 2001). Models of *Fmr1* KO mice were generated and they are very useful to study both FXS and autistic disorders. Indeed, *Fmr1* KO mice display many abnormalities reminiscent of the symptoms typical of FXS and ASD patients, including cognitive deficits, altered social interaction and communication, repetitive behaviors, seizures and also cortical cytoarchitecture deficits (Gaudissard et al., 2017; Hodges et al., 2017; Sarè et al., 2019). Regarding ultrasonic communication, several authors have studied USVs in *Fmr1* KO mice (**Table 1**) founding different results, mainly due to the use of different backgrounds of mice. Lai and colleagues (2014) investigated features of USV spectrograms emitted by *Fmr1* KO pups with FVB/NJ background, and they found an increased number of calls, especially frequency jump calls on postnatal day (PND) 7 in comparison with WT pups. In addition, a shift in USVs temporal distribution was found in *Fmr1* KO pups (Lai et al., 2014). Another analysis of ultrasonic communication has been performed by Reynolds and colleagues (2016), showing a reduction in number and duration of USVs in *Fmr1* KO with FVB/NJ background in comparison with WT pups. Furthermore, sex differences were found in calling pattern of this model (Reynolds et al., 2016). On the contrary, no quantitative differences in number or duration of USVs, but only a decreased proportion of downward calls, were detected in *Fmr1* KO mice with B6 background on PND 8 from Roy's study (Roy et al., 2012). Finally, in a study on *Fmr1* KO pups with B6 background it emerged that they emitted a similar USVs rate, but with longer duration than WT pups (Gaudissard et al., 2017). USVs analysis has revealed more calls emitted by adolescent than adult mice but without genotype differences (Gaudissard et al., 2017). Other papers described the

study of ultrasonic communication in *Fmr1* KO adult mice. For example, Rotschafer reported a reduced rate of USVs in *Fmr1* KO with FVB background compared to WT adult male during mating behavior (Rotschafer et al., 2012). Instead, no differences in USVs number but only a different proportion of calls typologies were recorded in another courtship paradigm including high level of upward syllables for KO with B6 background and harmonics calls for WT adult males (Belagodu et al., 2016). Lastly, the effect of exposition to female urine on USVs features was evaluated, resulting in higher frequency, lower amplitude and duration of vocalizations in *Fmr1* KO adult male with FVB background in comparison with control mice as well as a different USVs repertoire (Hodges et al., 2017).

MOUSE MODEL OF ASD	**USV FEATURES vs WT mice**		**REFERENCES**
***Fmr1* KO (FVB background)**	**Quantitative parameters**	**Qualitative parameters**	
pups	↑ number on PND 7	↑ frequency steps calls on PND 7	Lai et al., 2014
	↓ number and duration on PND 9-10, 13		Reynolds et al., 2016
adults	↓ duration and amplitude, ↑ peak frequency	↑ complex, chevron and flat; ↓ composite and frequency steps calls	Hodges et al., 2017
	↓ number		Rotschafer et al., 2012
***Fmr1* KO (B6 background)**			
pups	no differences in number but ↑ duration on PND 4, 6, 8, 10 and 12		Gaudissard et al., 2017
	no differences in number and duration on PND 8	↓ downward calls on PND 8	Roy et al., 2012
adolescents	no differences in number and duration		Gaudissard et al., 2017
adults	no differences in number and duration	↑ upward calls	Belagodu et al., 2016; Gaudissard et al., 2017

Table 1. Altered ultrasonic communication in *Fmr1* KO mice

BTBR mice

The BTBR T+tf/J (BTBR) mouse strain was bred for insulin resistance studies, diabetes-induced nephropathy and phenylketonuria (Clee et al., 2005) but recently, it has been associated also to ASD. Indeed, it is one of the most known idiopathic models of ASD because it displays abnormalities reminiscent of core symptoms

typical of ASD patients such as social deficit, impaired communication and repetitive stereotype behaviors as well as neuroanatomical abnormalities (Meyza and Blanchard, 2017; Faraji et al., 2018). Analyses of ultrasonic communication in this model (**Table 2**) have revealed a higher number of isolation-induced USVs with a longer duration than WT pups. BTBR pups emitted louder USVs with a lower frequency in comparison with WT pups on PND 6 and 8. From a qualitative point of view, specific alterations of calls typologies were found such as high level of harmonics, two-syllable and composite calls on PND 8 (Scattoni et al., 2008). This unusual calling pattern in BTBR pups suggests the translational value of pups USVs with human babies cry in ASD context (Scattoni et al., 2008; Esposito et al., 2017).
Communication alterations continue during adolescence and adulthood with a decreased USVs number in BTBR than control mice (Scattoni et al., 2011, 2013). Indeed, reduced USVs rate was detected in BTBR adult mice during male-male, female-female and male-female social interactions. In particular, BTBR males emitted fewer chevron, two-syllable and frequency steps in presence of other males. Females displayed a narrow repertoire of USVs that included upward and short calls when they interacted with other females with a reduction of complex, chevron and frequency steps calls. Instead, during male-female interactions, BTBR mice emitted fewer short and frequency steps calls than control mice (Scattoni et al., 2011). Furthermore, Yang and colleagues studied interaction-induced USVs, founding a distinct calling scheme of BTBR males after the removal of females (Yang et al., 2013). Finally, ultrasonic communication deficit was found in BTBR adult male mice also in presence of female mice urine (Wöhr et al., 2011a).

MOUSE MODEL OF ASD	USV FEATURES vs WT mice		REFERENCES
BTBR	**Quantitative parameters**	**Qualitative parameters**	
pups	↑ number, duration and peak amplitude; ↓ peak frequency on PND 6 and 8	↑ harmonics, two-syllable and composite calls on PND 8	Scattoni et al., 2008
adolescents	↓ number		Scattoni et al., 2013
adults	↓ number	different USVs repertoire on basis of different social interaction test	Scattoni et al., 2011; Wöhr et al., 2011a; Yang et al., 2013

Table 2. Altered ultrasonic communication in BTBR mice

Cntnap2 KO mice and Nlgn mutant mice

Mutations in genes coding for synaptic cell adhesion molecules, such as neuroligins, neurexins and contacting-associated proteins have been associated to NDDs and ASD (Penagarikano et al., 2012; El-kordi et al., 2013). Contactin associated protein-like 2 (*Cntnap2)* KO mice display a reduced ultrasonic communication (**Table 3**) as well as other ASD core behavioral features and neurophysiological alterations (Penagarikano et al., 2011; 2015, Mohrle et al., 2020). In particular, *Cntnap2* KO pups emit less USVs than WT pups when they are isolated from the mother

(Penagarikano et al., 2011). USVs production remains altered in this model also during adulthood in presence of female mice urine (Brunner et al., 2015). Also in another model of ASD, the neuroligins (*Nlgn*) mutant mouse, ultrasonic communication was analyzed. Reduced isolation-induced USVs were found in *Nlgn2* KO pups but without differences in other USVs parameters in comparison with their control pups (Wöhr et al., 2013). Conflicting results were detected in *Nlgn4* KO mice. Indeed, a reduction of USVs number was reported in female juvenile mice of this model (Ju et al., 2014) and during interaction of adult males with adult females (Jamain et al., 2008). On the contrary, Ey and colleagues did not find alterations in USVs pattern in pups and adults of this model (Ey et al., 2012).

MOUSE MODEL OF ASD	**USV FEATURES vs WT mice**		**REFERENCES**
***Cntnap2* KO**	**Quantitative parameters**	**Qualitative parameters**	
pups	↓ number on PND 3, 6 and 9		Peñagarikano et al., 2011
adults	↓ number		Brunner et al., 2015
***Nlgn2* KO**	**Quantitative parameters**	**Qualitative parameters**	
pups	↓ number and total calling time on PND 7		Wöhr et al., 2013
***Nlgn4* KO**	**Quantitative parameters**	**Qualitative parameters**	
pups	no differences in number and duration		Ey et al., 2012
adolescents	↓ number and duration and ↑ latency to first call		Ju et al., 2014
adults	↓ number		Jamain et al., 2008
	no differences in number and duration		Ey et al., 2012

Table 3. Altered ultrasonic communication in *Cntnap2* KO mice and *Nlgn* mutant mice

Shank models

SHANK proteins are a family of scaffolding proteins that connect the actin cytoskeleton of the dendritic spine with proteins of the postsynaptic membrane, promoting synapse formation and spine maturation (Monteiro and Feng, 2017). Several studies have reported a correlation between mutations in SHANK genes and autism (Sato et al, 2012; Bai et al, 2018). Three *Shank* mouse models for ASD have been generated and all of them have some characteristic abnormalities of this neurodevelopmental disorder (for Shank1: Sungur et al., 2016, 2017, 2018; for Shank2: Eltokhi et al, 2018; for Shank3: Wang et al., 2011; Balaan et al., 2019).

Shank1 KO

Four studies on ultrasonic communication analysis in this model have been conducted (Wöhr et al., 2011b; Wöhr, 2014; Sungur et al., 2016, 2017; **Table 4**). At first, Wöhr and colleagues (2011) reported that *Shank1* KO pups emitted less USV induced by maternal separation and spent less time calling in comparison with WT pups on PND 8. Furthermore, their USVs peak frequency was higher and frequency modulation was lower than those of WT pups. This calling pattern was present only in females and not in males pups (Wöhr et al., 2011b). Wöhr found two groups of USVs (e.g. the first between 50 and 80 kHz and the second between 80 and 100 kHz) present in both genotypes but with a different distribution. Indeed, the majority of WT mice USVs belonged to the first group, whereas *Shank1* KO mice USVs were distributed similarly between the two groups. Comparing the two genotypes, reduced number of KO miceUSVs was related to the first group of vocalizations (Wöhr, 2014). Reduced USVs in *Shank1* KO pups was confirmed also extending analysis of USVs to several days of pups development and evidenced the fact that ultrasonic communication deficits were more marked in presence of a social odor (Sungur et al., 2016). Regarding interaction induced USVs in juveniles, no genotype differences were detected (Sungur et al., 2017). At adulthood, the number of USVs produced by males during female urine exposition did not vary between genotypes. However, calling pattern of WT mice changed on the basis of their previous exposure to female but not in *Shank1* KO male mice (Wöhr et al., 2011b).

Shank2 KO

Shank2 KO models display alterations in ultrasonic communication (**Table 4**). Schmeisser and colleagues observed increased number of USVs in female, and not in male, *Shank2* KO pups with deletion of exon 7 on PND 4 and 10 (Schmeisser et al., 2012). Another study was conducted on this model but no differences between male and female mice were detected (Ey et al., 2013). In addition, *Shank2* KO pups lost their typical inverted U-shape developmental pattern with calls peak on PND 6, leading to a reduced number of calls in this day (Ey et al., 2013). During female-female social interaction test, ultrasonic vocalizations were recorded obtaining a reduced calls rate in *Shank2* KO adult female mice (Schmeisser et al., 2012; Ey et al., 2013). Longer latency to emit the first USV during male-female social interactions was found in *Shank2* KO in comparison with WT adult mice but no change in number of calls was noticed (Schmeisser et al., 2012; Ey et al., 2013). In other Shank2 KO model (with deletion of exon 6 and 7), Won and colleagues reported a reduced emission of USVs in adult male mice in presence of a female (Won et al., 2012).

Shank3 KO

Analysis of ultrasonic communication in *Shank3* KO pups (**Table 4**) did not reveal genotype effects on calling rate and duration of USVs (Yang et al., 2012; Balaan et al., 2019). On the contrary, adult *Shank3* KO females emitted fewer calls with a shorter duration than WT females during social interaction with females. Also their USVs frequency modulation was decreased in comparison with WT mice (Wang et al., 2011). Opposite results were obtained in *Shank3* KO male mice, including an increased USVs rate compared to WT mice in one study (Wang et al., 2011) and no genotype change in USV emission founded in another study (Yang et al., 2012). Finally, reduced USVs emission pattern with an increased latency to call was detected in adult heterozygous males during social interaction with females (Bozdagi et al., 2010).

MOUSE MODEL OF ASD	USV FEATURES vs WT mice		REFERENCES
***Shank1* KO**	**Quantitative parameters**	**Qualitative parameters**	
pups	↓ number and frequency modulation, ↑ peak frequency	different distribution of USVs in two groups (50-80 kHz and 80-100 kHz) on PND 8	Wöhr, 2014; Wöhr et al., 2011b; Sungur et al., 2016
adolescents	no differences in number		Sungur et al., 2017
adults	no differences in number		Wöhr et al., 2011b
***Shank2* KO (exon 7)**			
pups	↑ number in female on PND 4 and 10		Schmeisser et al., 2012
	↓ number on PND 6		Ey et al., 2013
adults	↓ number in female-female social interaction test; no differences in number in male-female interaction social test		Schmeisser et al., 2012; Ey et al., 2013
***Shank2* KO (exon 6-7)**			
adults	↓ number		Won et al., 2012
***Shank3* heterozygous**			
adults	↓ number and ↑ latency to call		Bozdagi et al., 2010
***Shank3* KO**			
pups	no differences in number and duration		Yang et al., 2012; Balaan et al., 2019
adults	↓ number, duration and frequency modulation in female-female social interaction test; ↑ number, ↓ duration and frequency modulation in male-female social interaction test		Wang et al., 2011
	no differences in number in presence of female urine		Yang et al., 2012

Table 4. Altered ultrasonic communication in *Shank* models

p50 KO mice

p50 KO mice have a deletion of the NFκB1 gene coding for the NF-κB p50 subunit. Nuclear factor-κB (NF-κB) pathway is involved in many processes such as neuron plasticity, neurogenesis, cell proliferation, apoptosis and immune system regulation (Kaltschmidt and Kaltschidt, 2009; Gutierrez and Davies, 2011). *p50* KO mice display immune responses defects, hyperactivity, reduced anxiety behavior and altered hippocampal neurogenesis associated with cognitive deficit in spatial short-term memory (Sha et al., 1995; Kassed and Herkenham, 2004; Denis Donini et al., 2008). Recently, they have been characterized as a model of NDDs because they have cortical structural abnormalities and some behavioral alterations typical of neurodevelopmental disorders, such as social interaction deficit and ultrasonic vocalizations impairment (Bonini et al., 2016; Mastinu et al., 2018; Premoli et al., 2019). Our research group analyzed ultrasonic communication in this model both in infancy and adulthood. We found that WT pups emitted an inverted U-shaped call emission pattern that followed a typical ontogenetic profile of USVs mouse pups, and it was exacerbated in *p50* KO pups. Furthermore, they emitted significantly more and longer USVs compared to WT pups. A detailed analysis displayed that WT pups emitted a quite homogenous repertoire of calls; instead, *p50* KO pups emitted a different repertoire of calls, which included higher number of two- syllable and frequency steps calls with a longer duration of these calls than WT pups (Premoli et al., 2019; **Table 5**). During male-female social interaction test, adult *p50* KO mice emitted decreased number of USVs than WT mice associated with a reduced social interaction, in particular reduced sniffing behaviors. We hypothesize that this reduced number of USVs found in adult *p50* KO mice can be linked to reduced communication and social interaction typical of adult human patients with NDDs. Interestingly, the same categories of calls altered in *p50* KO pups were reduced also in *p50* KO adult mice. Therefore, alterations of ultrasonic communication found in *p50* KO mice are not generalized but call-specific; this could have an important role in the context of specific call meaning. To date, the precise meaning of calls categories is still unknown but it is very interesting to note how the deletion of a subunit of a gene involved in several basic cellular processes (as NF-κB) results in a altered ultrasonic communication in a specific manner.

MOUSE MODEL OF ASD	**USV FEATURES vs WT mice**		**REFERENCES**
***p50* KO**	**Quantitative parameters**	**Qualitative parameters**	
pups	↑ number and duration	↑ two-syllable and frequency steps calls on PND 6	Premoli et al., 2019
adults	↓ number	↓ two-syllable and frequency steps calls	Premoli et al., 2019

Table 5. Altered ultrasonic communication in *p50* KO mice

1.4.2 USVs and environmental modulation

Several stimuli grouped as "environmental stimuli" can induce USVs modifications. Since mice emit USVs to communicate their emotional state or to coordinate social interaction (Simola and Granon, 2019), these environmental stimuli affect these fields. They can be split in two main categories: - social context, and - immunity.

USVs and social context

Social context can constitute a positive stimulus for mice emotional state, such as social enrichment, or negative, such as social stress or segregation. All these situations significantly affect mice vocalizations (**Table 6**).

In pups social odors play an important role in modulating USVs emission (Branchi et al., 1998). This is particularly relevant for ASD mouse models, as ASD is characterized by deficits in the processing of social context information (American Psychiatric Association, 2013). In the context of maternal separation, mouse pups emit fewer USVs when exposed to nest odor than to clean bedding (D'Amato and Cabib, 1987; Moles et al., 2004a; Wöhr, 2015). Typically, the presence of odors from mothers and littermates produce a calming response of the isolated mouse pup, and hence to a reduction in isolation-induced USVs emission (Moles et al., 2004a; Zanettini et al., 2010). Interestingly, in a mouse model of ASD, BTBR mice, it was observed that this social context produces a reduction in isolation-induced USVs, but, contrary to WT pups that emitted USV with shorter call durations and lower levels of frequency modulation, it had no effect on acoustic call features (Wöhr, 2015). Furthermore, the exposure to pheromones of an adult male induces a significant increase in pups USVs emission, as a result of anxiety related-behavior (Kessler et al., 2011). In another model of ASD, *Shank1* KO pups, this adverse social context (adult male odor) caused a significant reduction in USVs emission compared to WT pups, so exacerbating communication deficits in the ASD mice (Sungur et al., 2016).

As positive stimulus, early social enrichment, in particular housing pups until weaning with the mother and an additional female, resulted to decrease both number and duration of USVs emitted during maternal separation in PND 8 pups (Oddi et al., 2015). This effect was observed both in WT and in a murine model of ASD, *Fmr1* KO mice and suggests a weaker emotional response to maternal separation. Also our research group applied early social enrichment paradigm, founding no effect on USVs and social interaction in *p50* KO pups and adult mice. Indeed, social interaction and ultrasonic communication remained altered in this mouse model of NDDs, strengthening the fact that genetic background prevailed on environmental enrichment (Premoli et al., 2019). In another study, communal nesting was used as procedure to induce early social enrichment and it provoked decreased USVs emission rate in the social recognition test in adult female offspring (Gracceva et al., 2009).

Another important stimulus for pup vocalizations is early life stress. It was demonstrated that mice, both Balb/c and C57BL/6, emit significantly more isolation-induced USVs in the first PNDs after infant maternal separation (a common stressful stimulus) compared to standard-facility reared pups (Feifel et al., 2017). In addition, another model to induce early life stress in mice consists of limiting the amount of bedding and nesting material during the first postnatal weeks of pup life (Ivy et al., 2008; Rice et al., 2008; Schmidt et al., 2011; Molet et al., 2014). This paradigm has been demonstrated to alter maternal behavior and increase ultrasonic communication in offspring (Heun-Johnson and Levitt, 2016).

In adult mice, social context is a key factor for USVs emission and regulation. Mice emit the highest number of calls with the largest diversity of call types during social interaction. In particular, it has been proven that mice of both sexes emit vocalizations at a higher rate and higher frequencies during opposite-sex compared to same-sex interaction and that male mice emit USVs with higher amplitude in presence of a male mouse compared to a female mouse stimulus, thus suggesting an USVs modulation depending on the sex of potential receiver (Zala et al., 2017).

Furthermore, housing conditions (isolation or grouping), that act as a modulator factor for social motivation, significantly influence vocal behavior during social interaction (Chabout et al., 2012). Also Caruso and colleagues have demonstrated significant effects on UVSs after post-weaning isolation in adult mice during male-female interactions (Caruso et al., 2022). In addition, Burke and colleagues demonstrated that prior social experiences deeply influence USVs production (Burke et al., 2018). They found significant differences in USVs number and call types that isolated mice (CBA/CaJ strain, both sexes) produce across exposure conditions (following period of isolation or an exposure of a same- or opposite-sex mouse).
Also, a complex social environment such as the presence of additional male as listeners during male-female mice courtship, alters vocal behavior (Seagraves et al., 2016). In particular, Seagraves and colleagues demonstrated the existence of an "audience effect" in mice: male vocal behavior elicited by female odor is affected by the presence of a male audience, with changes in vocalizations count, acoustic structure and syllable complexity. This effect requests multiple cues, indeed a single sensory cue as odor or vocalizations, indicating the presence of a male audience, was not sufficient to elicit an effect; probably there is the need for an audience to be apparent. Moreover, a socially safe environment (in the experiment consisting in the three chamber apparatus, without physical threat) dramatically reduces communication between two adult mice competing for a natural reward, compared to a more uncertain environment (in the experiment consisting in two mice freely interacting in a large and novel cage) (Chabout et al., 2013). A relevant factor for male mice vocalization is also social status; indeed, Nyby and colleagues (1976) demonstrated that when interacting with a female, dominant males produce more USVs compared to subordinate males. Dominant males also emit wider vocal repertoire than mice avoiding social interactions (Sangiamo et al., 2020).
Finally, adult mice emit USVs also in non-social context, such as exploration of a novel environment and restrain stress, also if much less compared to social context, and only during exploration task social motivation has been demonstrated to significantly modulate vocal behavior (Chabout et al., 2012).
Stress is another key factor modulating USVs in adult mice. A commonly used behavioral paradigm to induce stress is the restrain (immobilization in a small container for few minutes), as it causes anxiety-like behavior and increase in corticosterone levels. Lefebvre and colleagues demonstrated that restrain induces low-frequency USVs ($\leq$ 60 kHz) in mice and these calls emission is increased by both a previous period of social isolation and the presence during restrain of a social congener (Lefebvre et al., 2020). Free exploration of a novel environment is also considered a stressful situation, as it induces anxiety. This behavioral task induced low-frequency USVs that resulted to be quantitatively decreased by a previous period of social isolation (Lefebvre et al., 2020).

USVs and Maternal Immune Activation

In the context of NDDs, an interesting hypothesis has recently emerged: aberrant immune activity during critical periods of neurodevelopment could participate in the generation of neurological dysfunction characteristic of several NDDs. This immune system dysregulation during pregnancy is also called "maternal immune activation" (MIA), and it has recently emerged as an important risk factor for several NDDs including ASD. Activation of the maternal immune system during pregnancy, by infection through common viruses or bacteria, has been linked to lifelong changes in brain function and behavior of offspring. A combination of genetic background,

autoimmune status and second hits during childhood and adolescence combines with the consequences of MIA to increase the likelihood of offspring developing NDDs or psychiatric disorders as adults (Bilbo and Schwarz, 2009; Boksa, 2010; Meyer et al., 2011; Harvey and Boksa, 2012; Estes and McAllister, 2016).

Several studies in MIA models founded a robust effect of immune system on USVs in mice. Indeed, stimulation of immune system of pregnant dams with bacterial or viral agents, causes significant changes in offspring vocalizations (Malkova et al., 2012; Carlezon et al., 2019; Jouda et al., 2019). Final effect of infection on USVs differs between different experiments due to the stimulus used, the dose, gestational time, offspring sex and frequency of injection. In brief, injection of poly I:C, a viral stimulus, in C57BL/6J female mice at 10.5, 12.5 and 14.5 gestational days caused a reduction of isolation-induced USVs in pups from PND 8 to 12, and qualitatively differences in comparison with USVs of pups born from saline-injected mothers (fewer harmonics, more complex and short syllables). Adult male offspring also presented altered vocalizations, with fewer USVs in response to social encounters (Malkova et al., 2012). Carlezon and colleagues (2019) studied both the effect of MIA (by poly I:C injection at gestational day 12.5) and the effect of early life immune system activation (EIA, by the injection of a bacterial agent named lipopolysaccharide, LPS, in the offspring at PND 9) on C57BL/6J mice communication. They found that MIA significantly reduced maternal separation-induced USVs compared to vehicle at PND 10, specifically in male pups, whereas EIA led to an increase in USVs in both males (at PND 14) and females (at PND 12); furthermore the two-hits (MIA+EIA) induced a significant increase in USVs compared to vehicle-treated pups in both female and male pups at PND 12, 14 and 16. Concerning USVs quality, they found that at PND 12, EIA and two-hits induced in both sexes an increase in calls under 75 hertz frequency compared to vehicle pups vocalizations (Carlezon et al., 2019). In another study, a single poly I:C injection at embryonic day 12.5 caused no significant changes in maternal isolation-induced USVs number at PND 8 in C57BL/6J strain pups, but only in the BTBR strain pups; instead, calls emitted at PND 10 resulted significantly increased in pups of both strains compared to control pups (Schwarzer et al., 2013). Also Choi and colleagues recorded increased isolation-induced USVs number in pups at PND 9 in the same MIA model, demonstrating a direct link with the pro-inflammatory interleukin 17 (Choi et al., 2016; Yim et al., 2017). In addition, an another model of ASD, *Cntnap2* mice, was prenatally exposed to LPS (0.3 mg/kg at embryonic day 7) and this provoked a significant decrease in maternal separation-induced vocalizations at PND 3. Furthermore, the MIA dependent effect on USVs resulted to be the strongest hit, when compared to other two hits: genotype (KO versus WT) and sex (male versus female) (Schaafsma et al., 2017). Also Fernandez de Cossio and colleagues founded altered ultrasonic communication in terms of decreased duration of calls in pups on PND 8 in pups treated with LPS (100 µg/kg) at embryonic day 15 (Fernandez de Cossio et al., 2017). Finally, our research group also used LPS as MIA inducing stimulus (0.3 mg/kg chronic administration during pregnancy) in B6;129PF2 mice, and this caused a significant increase in both number of isolation-induced ultrasonic calls in pups on PND 8 (Aria et al., 2020). Furthermore, in order to better understand the biological mechanisms underlying the inflammatory response induced by LPS in the offspring, we treated dams with meloxicam, a well-known nonsteroidal anti-inflammatory drug cyclooxygenase-2 preferential throughout pregnancy. Analysing USVs, it emerged that LPS-prenatally exposed pups of mother treated with meloxicam during pregnancy emitted significantly more USVs than LPS-pups. Also

their duration of calls was higher compared to vehicle- and to LPS-pups. So, we demonstrated that a chronic LPS injection during pregnancy causes communication alteration in pups, and that a maternal meloxicam treatment exacerbates this abnormal vocalization pattern (Aria et al., 2020).
Therefore, perturbation of the immune system during brain development, both in the pre-natal and the post-natal periods, causes dramatic and long-lasting effects on mice communication, that sometimes exacerbate the mice social behavior impairment.

Effect of environmental modulation on USVs	**USV FEATURES**	**STRAINS**	**REFERENCES**
SOCIAL CONTEXT			
pups	↓ number when exposed to adult male odor	*Shank1* KO	Sungur et al., 2016
	↓ number when exposed to nest odor vs clean bedding	WT, μ-opioid receptor KO, serotonin receptor 1A KO	D'Amato and Cabib, 1987; Moles et al., 2004a; Zanettini et al., 2010
	↓ number and duration when exposed to soiled vs clean bedding	WT and BTBR	Wöhr, 2015
	↑ number when exposed to pheromones of adult male	WT	Kessler et al., 2011
	↓ number when exposed to maternal enrichment	WT and *Fmr1* KO	Oddi et al., 2015
	no differences in number and duration when exposed to maternal enrichment	p50 KO	Premoli et al., 2019
	↑ number when separated from mother vs standard-facility reared	Balb/c and WT	Feifel et al., 2017
	↑ number when exposed to early life stress	WT and *Met* heterozygous	Heun-Johnson and Levitt, 2016
adults	sex effect on number, frequency and amplitude	WT	Zala et al., 2017
	↓ number when exposed to communal	WT	Gracceva et al., 2009

	nesting vs standard nesting		
	↓ number when grouped vs isolated	WT	Chabout et al., 2012
	↓ number when exposed to post-weaning isolation	WT	Caruso et al., 2022
	↓ number when isolated vs exposed to other mice	CBA/CaJ	Burke et al., 2018
	male audience effect on number and acoustic features	SWR/J	Seagraves et al., 2016
	↓ number in socially safe environment	WT and neuronal nicotinic acetylcholine receptor KO	Chabout et al., 2013
	↑ number when emitted by dominant male vs subordinate male mice	DBA/2J	Nyby et al., 1976
	↑ number of restrain-induced USVs by isolation and presence of a social congener; ↑ number of free moving-induced USVs by isolation	WT	Lefebvre et al., 2020
MIA CONTEXT	**MIA vs vehicle group**		
pups	↑ or ↓ number	LPS and Poly I:C models	Jouda et al., 2019
	↓ number and duration on PND 8, 10 and 12	Poly I:C model	Malkova et al., 2012
	↑ number on PND 8 in BTBR and ↑ number on PND 10 in BTBR and WT	Poly I:C model	Schwartzer et al., 2013
	↓ or ↑ number	Poly I:C model; LPS model	Carlezon et al., 2019
	↑ number	Poly I:C model	Choi et al., 2016; Yim et al., 2017
	↓ number	LPS model	Schaafsma et al., 2017
	↓ duration	LPS model	Fernandez de Cossio et al., 2017

	↑ number	LPS model	Aria et al., 2020
adults	↓ number	Poly I:C models	Malkova et al., 2012; Jouda et al., 2019

Table 6. Environmental modulation effect on ultrasonic communication

1.4.3 Pharmacological USVs modulation

Mouse vocalizations can be modulated by pharmacological interventions (**Table 7**). One example of the pharmacological treatment that resulted effective in USVs modulation is minocycline, a tetracycline antibiotic. Rotschafer and colleagues (2012) demonstrated that a 4-week minocycline treatment, from PND 0 to 28, was able to reverse vocalization deficits of *Fmr1* KO mice, in terms of calling rate, during mating.

Also Toledo and colleagues confirmed effects on ultrasonic communication of treatment with this antibiotic for 28 days starting at PND 30 in this ASD mouse models (Toledo et al., 2019). In addition, minocycline treatment rescues reduced mother call rate also in another NDDs model: oxytocin receptor-KO mice (Miyazaki et al., 2016).

In another study, the effect of several anxiolytics (as citalopram, escitalopram, R-citalopram, paroxetine, fluoxetine and venlafaxine) were tested on Carworth Farms Webster mouse pups of 7 days old and maternal-separation USVs were recorded; acute treatment (45 minutes before USVs recording) with all these drugs induced a reduction in USVs emission compared to vehicle treated pups (Fish et al., 2004). Veronesi and colleagues (2017) studied the effect of an anti-inflammatory drug as dypirone administered to dams during lactation; they found that it induced increase number of USVs in male pups, but not females, compared to vehicle pups, suggesting a greater vulnerability of male pups to anti-inflammatory treatment during lactation. An interesting toxicological study concerned the impact of dioxin exposure on USVs emission in infant mice born to dams treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on gestational day 12.5. Total USVs and mean call durations in infant mice exposed to 3 μg/kg dioxin were shorter than those in the control mice. In addition, the percentages of complicated call types (i.e., chevron and wave) in dioxin-exposed mice were decreased compared to control mice (Kimura and Tohyama, 2018).

Generally, antianxiety agents (such as benzodiazepines, serotoninergic agents, opioid agonists) resulted in a decrease in USVs while anxiogenic (beta-carboline) and oxytocinergic agents and opioid antagonist (naloxone, naltrexone) tended to increase USVs number during maternal isolation (D'Amato, 2021).

Effect of pharmacological/ behavioral modulation on USVs	USV FEATURES	STRAINS	REFERENCES
pups	minocycline treatment effect on number	WT and Oxytocin receptor KO	Miyazaki et al., 2016
	anxiolytics treatment effect on number	Carworth Farms Webster	Fish et al., 2004
	dypirone treatment effect on number	WT	Veronesi et al., 2017
	dioxin exposure effect on number, duration and call types	WT	Kimura and Tohyama, 2018
adults	minocycline treatment effect on number	WT and *Fmr1* KO	Rotschafer et al., 2011; Toledo et al., 2019

Table 7. Pharmacological/behavioral modulation effect on ultrasonic communication

B) RESULTS OF RESEARCH STUDIES

During my PhD course, I analyzed ultrasonic communication in two mouse models of NDDs: the CB1 null mutant and the Fmr1 KO.

Mice lacking the CB1 primary endocannabinoid receptor: CB1 null mutants, were used in our paper published on *Autism Research* to evaluated USVs and social interaction during development and at adulthood.

Fmr1 KO mice represent a mouse model of ASD and their ultrasonic communication was analyzed also in a modified environmental context as published in our paper on *Scientific Reports*.

Finally, our last paper published on *Frontiers in Behavioral Neuroscience* displays a detailed analysis of USVs emitted by adult male and female mice, underlining the impact of sex differences and repeated testing on features of calls.

2. First paper:
Communication and social interaction in the cannabinoid-type 1 receptor null mouse: Implications for autism spectrum disorder

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Journal: Autism Research

Clinical and preclinical findings have suggested a role of the endocannabinoid system (ECS) in the etiopathology of ASD. Previous mouse studies have investigated the role of ECS in several behavioral domains; however, none of them has performed an extensive assessment of social and communication behaviors, that is, the main core features of ASD. So, the aim of this study was to evaluate ultrasonic communication and social interaction in mice lacking the primary endocannabinoid receptor (CB1R): CB1 null mutants, homo ($CB1^{-/-}$) and heterozygous ($CB1^{+/-}$) for the mutation, during development and at adulthood, supporting the role of the ECS in ASD-relevant core domains.

2.1 INTRODUCTION

The endocannabinoid system (ECS) is a retrograde inhibitory signaling pathway composed by endocannabinoids, their receptors and the enzymes for their biosynthesis and degradation. N-arachidonoylethanolamine (AEA), also called anandamide, and 2-arachidonoyl glycerol (2-AG) are the most studied endocannabinoids, a family of fatty acid derivatives (Devane et al., 1992; Sugiura et al., 1995). AEA and 2-AG are synthesized on demand from membrane phospholipids and then rapidly released (Di Marzo and De Petrocellis, 2012). Several enzymes participate in the production of these endocannabinoids. N-acylpho-sphatidylethanolamine-selective phospholipase D (NAPE-PLD) is responsible for the hydrolysis of N-acylphosphatidylethanolamide (NAPE) and the synthesis of AEA (Magotti et al., 2015). Whereas, phospholipase C (PLC) and diacylglycerol lipase α (DAGLα) or β (DAGLβ) hydrolyse 2 arachidonic acid in 2-AG (Sugiura et al., 2002). Furthermore, there are other important enzymes that regulate the degradation of AEA and 2-AG, such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) respectively that produce arachidonic acid and ethanolamine or glycerol (Sugiura et al., 2018). Endocannabinoids interact with two G protein-coupled receptors: the type 1 cannabinoid receptor (CB1R) and the type 2 cannabinoid receptor (CB2R). The CB1R is localized in the brain and in peripheral tissues such as the intestine, liver, adipose tissue and immune cells (Di Marzo, 2018). Instead CB2R is distributed on spleen, tonsil, and immune cells and recently they have been found in glial and neuronal cells (Chen et al., 2017). In addition, AEA and 2-AG are involved in different (i.e., independent to the CBR1 and CBR2) pathways interacting with non-cannabinoid receptors including the transient receptor potential vanilloid 1 (TRPV1) channel, transient receptor potential ankyrin 1 (TRPA1), the peroxisome proliferator-activated receptor-gamma (PPARγ), nuclear receptor and the orphan G protein-coupled receptor (GPR55) (Di Marzo, 2018).

ECS is a highly promising candidate to study the etiopathology of ASD and to identify novel therapeutic targets (Carbone et al., 2021). ECS modulates neuronal functions, as demonstrated by the abundance of CB1 receptors in the brain (Mackie, 2005), and regulates synaptogenesis and neuronal interconnectivity during development (Berghuis et al., 2007; Mulder et al., 2008), i.e., all processes that are

known to be affected by ASD pathology (Pardo and Eberhart, 2007). In addition, brain expression levels of CNR1, the gene coding for CB1R, increase during the late embryonic stage and peak during postnatal development (Marsicano and Lutz, 1999). Also, alterations in the expression of CB1R and other ECS components, as well as in their functionality, have been described in ASD patients (Smith et al., 2017; Karhson et al., 2018; Aran et al., 2019) and in several ASD animal models (Zamberletti et al., 2017). Finally, recent clinical and preclinical studies supported the efficacy of pharmacological modulators of ECS as therapeutic approaches to autistic symptoms (Jung et al., 2012; Bar-Lev Schleider et al., 2019; Pretzsch et al., 2019). Interestingly, the idea that an increase in endocannabinoid tone, for example inhibiting the enzyme FAAH, could ameliorate symptoms of ASD has been hypothesized by different researchers and also investigated in ASD and FXS rodent models (Melancia et al., 2018; Servadio et al., 2016; Trezza et al., 2012, Wei et al., 2016).
Studies with null mice ($CB1^{-/-}$) have demonstrated that CB1R plays a key role in the regulation of several behavioral responses (Haller et al., 2004; Litvin et al., 2013; Shonesy et al., 2018), including ASD-relevant repetitive (Varvel and Lichtman, 2002; Terzian et al., 2011), and social behaviors (Haller et al., 2004; Haring et al., 2011; Litvin et al., 2013; Terzian et al., 2014). Mice lacking CB1R also have brain connectivity alterations, a neurological phenotype of ASD (Berghuis et al., 2007; Diaz-Alonso et al., 2012). However, to the best of our knowledge, no studies have been performed specifically on the role of CB1R and the ECS in modulating social communication, that is, one of the major domains altered in ASD. Only one USV study has been conducted in $CB1^{-/-}$ pups, but within a chronic stress paradigm (Fride et al., 2005). Also, most behavioral studies, including those investigating social interest and interaction (Haller et al., 2004; Haring et al., 2011; Litvin et al., 2013; Terzian et al., 2014), have so far focused exclusively on homozygous $CB1^{-/-}$ male mice. Hence, little is known about potential "dosage" and sex-dependent effects of the CB1 mutation on behavior, especially in relation to ASD-relevant phenotypes. Here we therefore examined ultrasonic communication in male and female CB1 null mutants, homo ($CB1^{-/-}$) or heterozygous ($CB1^{+/-}$) for the mutation, during development and at adulthood. Both quantitative and qualitative analyses of spectrographic measurements were performed in order to provide with an extensive characterization of USVs in CB1 null mutants and their WT littermates. To complete the assessment of ASD-relevant social phenotypes, social interest in the three-chamber test and social investigation towards a conspecific were also evaluated at adulthood. As confounding differences in anxiety (Vivian and Miczek, 1993; Fish et al., 2004; Veronesi et al., 2017; Simola and Granon, 2019) or sensori-motor abilities (Webber et al., 2013; Wada, 2017) may influence ultrasonic communication and social behavior, adult mice were also assessed in the elevated plus maze and auditory startle tests. The assessment of these behavioral confounders also contributed to the evaluation of the ASD-relevant phenotypes of the CB1 mouse line, since anxiety and enhanced auditory startle are recognized as additional ASD symptoms and are therefore often investigated in genetic mouse models of ASD (Crawley, 2007; Silverman and Crawley, 2014).

2.2 MATERIALS AND METHODS

2.2.1 Ethics approval

All experimental procedures were in accordance with the European Communities Council Directive 2010/63/EEC, and approved by local ethical committee ("Comité

d'Ethique pour l'experimentation animale de Bordeaux", CE 50) and the French Ministry ("Ministere de l'enseignement superieur de la recherché et de l'innovation").

2.2.2 Animals

All experiments were performed in homozygous CB1 null mutant ($CB1^{-/-}$) mice with a targeted deletion of CNR1 gene and their heterozygous ($CB1^{+/-}$) and wildtype ($CB1^{+/+}$) littermates. Mice were obtained from breeders on a C57BL/6N congenic background, generated as previously reported (Marsicano et al., 2002). $CB1^{+/-}$ virgin males and females were paired for breeding in a temperature- (21 ±1°C) and humidity- (40%) controlled animal facility (lights on at 07:00 am); approximately two weeks afterwards, pregnant females were individually housed and left undisturbed.

Three batches of mice were used, as described in detail in **Table 1**: one batch (36 males and 36 females) was tested for USVs during development between PND 4 and 10 (Experiment 1a); a subgroup of the same batch (24 males and 24 females) was tested again for USVs at adulthood (Experiment 1b). A second batch of adult mice (23 males and 26 females) underwent the tests of social interest in the three-chamber apparatus and of direct social interaction with an adult female. A third batch of mice (34 males and 27 females) was tested at adulthood in the elevated plus maze followed by the auditory startle test.

TABLE 1 Experimental plan of the study

Experiment	Testing age/cohort	*N* (male)	*N* (female)	Behavioral test	Behaviors analyzed
1a	PND 4, 6,8,10/ cohort 1	9 WT	13 WT	Maternal separation	Ultrasonic vocalizations
		14 HET	17 HET		
		13 KO	6 KO		
1b	Adult (3 m)/cohort 1	6 WT	8 WT	Direct social interaction with an intact adult NMRI female	Ultrasonic vocalizations
		11 HET	11 HET		
		7 KO	5 KO		
2	Adult (3 m)/cohort 2	8 WT	12 WT	Three-chamber-test, direct social interaction with an ovx adult NMRI female	Social interest, social investigation
		10 HET	8 HET		
		5 KO	6 KO		
3	Adult (3 m)/cohort 3	9 WT	7 WT	Elevated plus maze, auditory startle	Anxiety-like behavior, startle reactivity
		16 HET	9 HET		
		9 KO	11 KO		

Note: A subgroup of the cohort used for Experiment 1a was re-assessed in Experiment 1b, while separate cohorts of adult mice underwent Experiments 2 and 3.
Experiment 1a used male and female pups obtained from 11 litters, including all genotypes.
Abbreviations: Ovx, ovariectomized female; PND, postnatal day.

Overall, our sample size was in line with the guidelines for data analysis in animal studies. Nonetheless, we were partially limited in the sample size by the following factors; (a) litters including all three genotypes were preferentially selected in the study, (b) primiparous females had to be used for breeding in all experiments (as commonly done in studies including pup USVs, since previous maternal experience may affect USV's characteristics, e.g., Francia et al., 2006), and (c) breeding was based on heterozygous crossings as suggested by guidelines for mouse studies on behavioral genetics to control for maternal effects (Crusio, 1996; Crusio et al., 2009). Experiment 1a used male and female pups obtained from 11 litters, including as much as possible all three genotypes. The approach used here, including a high number of litters together with the use of WT and mutant littermates and testing all litter members without sampling, has been suggested as the most appropriate to protect from the risk of false positives related to litter effects, by past (Chiarotti et al., 1987; Zorrilla, 1997) and recent (Jimenez and Zylka, 2021) guidelines on mouse

neurodevelopmental studies. On PND 4 pups were marked after testing by paw tattoo, using a non-toxic odor-less tattoo ink (Ketchum permanent Tattoo Inks green paste, Ketchum MFG. Co.), as previously described (Wöhr et al., 2011b; Yang et al., 2012). This procedure is routinely used for pups' marking and identification, as it is associated with minimal stress and pain. On the same day tail samples were collected for DNA extraction and subsequent PCR assessment of the genotypes as previously described (Marsicano et al., 2002). Mice were weaned at 3 weeks of age (PND 21), housed in same-sex cages in groups of 3–5 mice/cage in polycarbonate standard cages (33 x 15 x 14 cm in size; Tecniplast). Mice were left undisturbed until Experiment 1b began, that is, at 3 months of age. Animals for Experiments 2 and 3 were bred and housed as described from Experiment 1, but they were left undisturbed until PND 21, when they were weaned, identified, and genotyped and they were all tested at 3 months of age.

Stimulus mice used for the adult assessment of USVs (Experiment 1b) and of social interest and investigation (Experiment 2) were adult (10 weeks of age) female NMRI mice (Janvier, Le Genest-Saint-Isle), as this strain is commonly employed in social studies (Moles et al., 2000; Moles et al., 2007) since (a) it is characterized by high levels of sociability, (b) is an albino strain, so it facilitates the behavioral analysis during social encounters with B6 mutants, and (c) it has been used in several social tests with mouse models of ASD (Pietropaolo et al., 2011a; Hébert et al., 2014; Pietropaolo et al., 2014; Oddi et al., 2015; Gaudissard et al., 2017; Gauducheau et al., 2017) and other neurodevelopmental and neurological disorders (Pietropaolo et al., 2011b; Pietropaolo et al., 2012; 2015), as well as in mouse social studies with CB1r antagonists (Pietropaolo et al., 2020). They were housed in groups of 3–4 per cage in the same conditions used for test subjects and left undisturbed for 2 weeks before being used in behavioral tests. We employed intact and ovariectomized NMRI stimulus females, for the adult assessment of USVs (Experiment 1b) and social interest/investigation (Experiment 2), respectively. This allowed us to be in line with most ASD-relevant previous studies assessing USVs in adult male and female mice, including a recent mouse study on USVs and pharmacological inhibition of CB1r (Pietropaolo et al., 2020). These USV experiments mostly employed intact stimulus females (Caruso et al., 2020), concomitantly assessing their estrous phase, as controversial findings have been reported on the impact of estrous phase on USVs (Nyby et al., 1979; Moles et al., 2007; Hanson and Hurley, 2012; Yang et al., 2015; Kim et al., 2016). Hence, we have also used intact female stimuli for USV adult assessment, taking into account their estrous phase as well as that of the female resident in the statistical analysis of the data. The choice of an ovariectomized female for the three-chamber and direct social interaction tests allowed us to directly compare our findings with those from a previous study with CB1 male mutants (Terzian et al., 2014), concomitantly avoiding the potential impact of estrous phase on social interest/ interaction and limiting the occurrence of sexual behaviors due to the longer duration of the testing session of direct social interaction. All animal cages were covered by a stainless metal wired lid, provided with sawdust and *ad libitum* food and water; they were provided with nesting material as environmental enrichment.

2.2.3 Behavioral testing

First, ultrasonic communication was evaluated on PND 4, 6, 8, and 10 in response to maternal separation (Experiment 1a), and again at adulthood in response to an adult female intruder (Experiment 1b). Second, adult social interest and investigation were

assessed respectively in the three-chamber and direct social interaction tests (Experiment 2). Finally, adult mice were tested for anxiety-like behavior in the elevated plus maze and for auditory startle response, that is, two behavioral confounding variables potentially acting on ultrasonic communication and social behavior (e.g., Simola and Granon, 2019; Webber et al., 2013). All behavioral procedures were based on experimental protocols used in our previous studies on genetic mouse models of ASD (Pietropaolo et al., 2011a; Hébert et al., 2014; Pietropaolo et al., 2014; Zhang et al., 2014; Oddi et al., 2015; Gaudissard et al., 2017; Gauducheau et al., 2017). Behavioral tests were performed in adult mice with a 48 h-interval between subsequent tests, and they were carried out by experimenters blind to animals' genotypes. Except for pups' assessment, male and female mice were tested on separate days, in order to avoid olfactory interference in the testing environment.

Experiment 1a: Assessment of isolation-induced USVs in pups

USVs of $CB1^{-/-}$, $CB1^{+/-}$, and $CB1^{+/+}$, littermates were repeatedly assessed on PND 4, 6, 8, and 10, during a 3-min daily session. Pups were taken individually from the nest in a random sequence and placed into a glass container (10 x 8 x 7 cm; open surface), containing clean bedding material (3 cm). USVs were captured by an UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics) placed 20 cm above the bedding. The microphone used is sensitive to frequencies of 15 to 180 kHz with a flat frequency response (± 6 dB) between 25 and 140 kHz. It was connected via an UltraSoundGate 116 USB audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were recorded with a sampling rate of 250 kHz in 16-bit format by Avisoft RECORDER (version 2.97; Avisoft Bioacoustics). At the end of the 3-min session, each pup was weighed and identified.

For acoustic analyses, recordings were transferred to Avisoft SASLab Pro (Version 5.20; Avisoft) and a Fast Fourier transformation was applied (512 FFT length, 100% frame, Hamming window, and 75% time window overlap). Call detection was provided by an automatic threshold-based algorithm and the accuracy of call detection by the software was verified manually by an experienced user. Based on previous studies (Wöhr et al., 2011b), the number of USVs was computed, as well as their mean duration, peak frequency and peak amplitude. In addition, call subtypes were determined by density plots depicting the distribution of total calls for each genotype at peak frequency versus peak amplitude, peak frequency versus duration, and peak amplitude versus duration, as described in details elsewhere (Wöhr, 2014; Mosienko et al., 2015).

Experiment 1b: Assessment of interaction-induced USVs in adults

$CB1^{-/-}$, $CB1^{+/-}$, and $CB1^{+/+}$ male and female littermates were then tested at adulthood in a 33 x 15 x 14 cm plastic cage with 3 cm of sawdust and a metal flat cover. Male experimental subjects were habituated to this apparatus for 15 min prior to testing, while female subjects were isolated in the testing cage for 72 h, in order to induce a status of resident in adult females and therefore promote the emission of USVs toward an adult female intruder (Moles et al., 2007). An unfamiliar stimulus female mouse was then introduced into the testing cage and left there for 3 min. Previous studies have shown that in these experimental settings USVs are mainly emitted by the male mouse in the male–female interaction (Whitney et al., 1973; Warburton et al., 1989; Wang et al., 2008), and by the female resident in the female–female interaction (Maggio and Whitney, 1985; Moles et al., 2007). The ultrasonic

microphone previously described was mounted 2 cm above the cover of the testing cage; subsequent scoring of USV parameters was performed following the same procedures described for experiment 1a.
The estrus phase of adult females was assessed by analysis of vaginal smears (Caligioni, 2009) performed on the testing day in both the experimental subjects and NMRI stimulus mice. The evaluation of $CB1^{-/-}$, $CB1^{+/-}$, and $CB1^{+/+}$ females was conducted after their testing, in order to minimize the potential stress effects of the manipulation necessary for determining the estrous phase. Stimulus NMRI females were approximately half in diestrus and half in estrus phases, and their assignment to social encounters was equally distributed between genotypes and sexes. The estrus phase of experimental female subjects included proestrus, estrus and diestrus, following a distribution that was balanced across genotypes ($CB1^{-/-}$ females included 2 in proestrus, 2 in estrus and 1 in diestrus; $CB1^{+/-}$ were 4 in proestrus, 4 in estrus and 3 in diestrus; and $CB1^{+/+}$ included 3 in proestrus, 2 in estrus and 3 in diestrus).

Experiment 2: Assessment of social interest and social investigation in adult mice
Mice of a second cohort were assessed first in the three-chamber test for social interest and 48 h later in the direct social interaction; both tests used an ovariectomized NMRI adult female as the social stimulus, since the estrous phase of the stimulus animal is known to affect social interest and investigation (Baudoin et al., 1991; Liu et al., 2010). The estrus phase of experimental female subjects was assessed as described in Experiment 1b, and no differences in the distribution of estrous phases were found between genotypes.

Three-chamber test for social interest
The three-chamber apparatus was made of transparent Plexiglas (Gauducheau et al., 2017). Each side compartment contained a perforated stimulus cage (8 x 8 x 15 cm) placed at a distance of 5.5 cm from the side walls.
Each experimental animal was placed in the middle of the central compartment and allowed to explore the whole apparatus for two trials of 5 min each (Pietropaolo et al., 2011a). On the first trial the stimulus cages were empty and the experimental mouse was left undisturbed to explore the apparatus and habituate to the testing environment. At the end of this trial, the experimental mouse was confined in the central compartment using two transparent Plexiglas magnetic doors for 40 s. On the second trial, a stimulus mouse was introduced in one of the stimulus cages, while a novel object (a glass red cylinder) was introduced in the other one. The position of the social stimulus and of the object was counterbalanced between genotypes. The time spent in each of the side compartments containing the stimulus cages was computed from the video files obtained from a camera placed above the center of the apparatus. An experimenter blind to stimulus position and animals' sex and genotypes performed the analysis using Observer XT (version 7, Noldus).

Direct social interaction with an adult female
Each experimental animal was confined in one of the side compartments of the three-chamber apparatus and an unfamiliar stimulus NMRI female was introduced and left for 10 min. Testing sessions were recorded by a camera placed on the side of the compartment and videos analyzed with Observer XT. One observer who was unaware of the genotype and sex of the animals scored the behavior of the test mice, quantifying the time spent performing affiliative behaviors (Pietropaolo et al., 2011a; Pietropaolo et al., 2014; Oddi et al., 2015; Gaudissard et al., 2017; Gauducheau et al.,

2017), that is, sniffing the head and the snout of the partner, its anogenital region, or any other part of the body; contact with partner through traversing the partner's body by crawling over/under from one side to the other or allogrooming. Nonsocial activities were also measured: rearing (standing on the hind limbs sometimes with the forelimbs against the walls of the cage); digging; self-grooming (the animal licks and mouths its own fur). The testing session was analyzed in 5-min bins, in order to assess social habituation, that is, the expected time-dependent decrease in affiliative behaviors that is known to occur after the initial minutes of a social encounter and is often altered in mouse models of ASD (Shah et al., 2013; Pietropaolo et al., 2014). Furthermore, the separate analysis of the first 5 min allowed a better comparison of our results with those obtained from other studies on social interaction/interest tests, since short testing session (typically between 3 and 5-min-duration) are most commonly employed in experiments on both male and female mice (e.g., Nadler et al., 2004; Moles et al., 2007; Ricceri et al., 2007).

Experiment 3: Assessment of potential confounding nonsocial behavioral alterations in adult mice

Mice of a third cohort were assessed first for anxiety-like behavior in the elevated plus maze and 48 h later in for auditory startle response. The estrus phase of female subjects was assessed as described in Experiment 1b at the end of each behavioral test, and no differences in the distribution of estrous phases were found between genotypes.

Elevated plus maze

The maze was placed 55 cm above floor level, in a quiet testing room with diffuse dim lighting (Pietropaolo and Crusio, 2009; Pietropaolo et al., 2011a). A digital camera was mounted above the maze, and images were transmitted to a PC running the Ethovision (Version 11, Noldus Technology) tracking system. To begin a trial, the mouse was gently placed in the central square with its head facing one of the open arms and allowed to explore freely for 5 min. We measured the percent time in open arms as ($time_{(open\ arms)}/time_{(open + closed\ arms)}$) x 100. Total distance moved was also assessed.

Auditory startle response

The whole-body startle response to low intensity auditory stimuli was measured using startle response boxes (SRLAB, San Diego Instruments), as described in details in Gaudissard et al., 2017. Briefly, mice were habituated to the boxes for 24 h prior to testing for 5 min to reduce stress. On the days of testing, mice were presented with pulses of 20-ms duration and varying intensity: +6, +12, +18, and + 24 dB over a white background noise at 66 dB (namely 72, 78, 84, and 90 dB). Startle reactivity was assessed by the scores obtained for the mean of trials for each stimulus level presented.

2.2.4 Statistical analysis

Data from experiment 1a were analyzed using a 3 x 2 x 4 parametric analysis of variance (ANOVA) with genotype and sex as between-subject factors, and day as within-subject factor. For all other experiments, data from males and females were analyzed separately using a one way ANOVA with only genotype as between-subject factor. These separate analyses were necessary as male and female mice had to be

tested (a) on different days to avoid odor interference in all experiments, and (b) using a different experimental protocol to allow USV detection in Experiment 1b.
Within-subject factors, that is, stimulus compartment, 5-min-bins, stimulus intensities, were added to the ANOVAs of the data of social interest, social interaction, and auditory startle. Post hoc comparisons using Fisher's PLSD test were performed when appropriate. To better conform to the assumptions of parametric ANOVA, a natural logarithmic transformation was applied to the startle reactivity scores (Experiment 3). Data from the density plots did not undergo statistical analysis, but were used to obtain a qualitative three-dimensional evaluation of USV data, as in previous studies (Wohr, 2014; Mosienko et al., 2015). All statistical analyses were performed using SPSS Statistics Version 25 and GraphPad Prism 8 (GraphPad Software).

2.3 RESULTS

Experiment 1a: Assessment of isolation-induced USVs in mouse pups

CB1 mutation affected the body weight of mouse pups and this effect was detected only in females, where it differed across postnatal days (interaction sex x genotype x day: $F_{(6,198)} = 3.58$, $p < 0.01$, and interaction genotype x day in females: $F_{(6,33)} = 3.596$, $p < 0.05$; **Figure 1**). On PND 4, both $CB1^{+/-}$ and $CB1^{-/-}$ females weighted less than their $CB1^{+/+}$ littermates, and this difference was still found on PND 10, but for $CB1^{-/-}$ pups only (post hoc, $p < 0.05$). No significant genotype difference in body weight gain emerged in male pups (genotype and interaction genotype x day, all n.s).

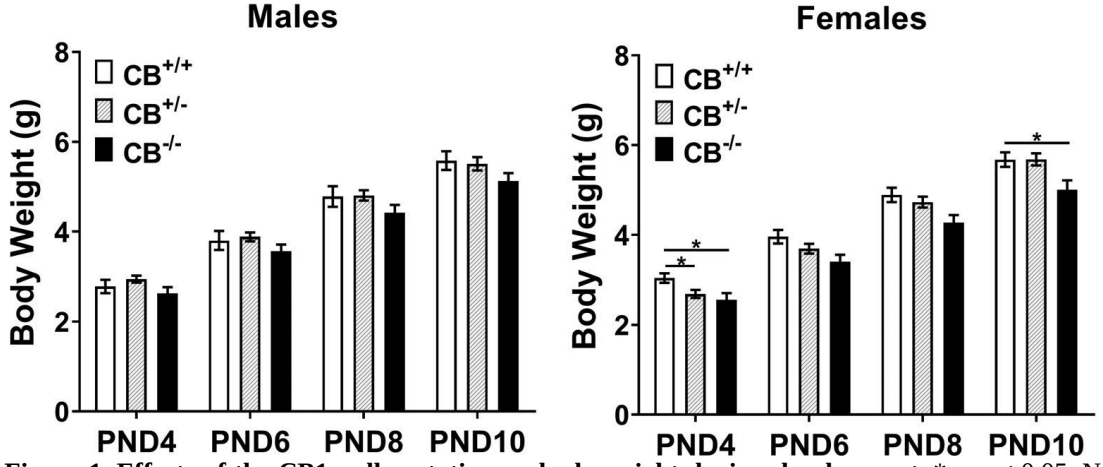

Figure 1. Effects of the CB1 null mutation on body weight during development. * = $p < 0.05$. N (males) = 9 ($CB1^{+/+}$), 14 ($CB1^{+/-}$), 13 ($CB1^{-/-}$). N (females) = 13 ($CB1^{+/+}$), 17 ($CB1^{+/-}$), 6 ($CB1^{-/-}$). Male and female pups were obtained from 11 litters, including all three genotypes ($CB1^{-/-}$, $CB1^{+/-}$, and $CB1^{+/+}$). Data are mean ±SEM

All USV parameters followed a developmental pattern, with changes across PNDs. As expected, the number of USVs emitted by pups of both sexes showed a peak occurring on PND 4 and 6 followed by a decrease on PND 8 and PND 10 (day effect: $F_{(3,198)} = 44.272$, $p < 0.0001$; **Figures 2a,b**). This pattern was altered in CB1 mutants, with slight differences between sexes (genotype x day: $F_{(6,198)} = 2.645$, $p < 0.05$; sex x genotype x day: $F_{(6,198)} = 2.309$, $p < 0.05$). The most prominent decrease was observed in males on PND 10, and it was less marked in $CB1^{-/-}$ littermates only (genotype x day in males $F_{(6,99)} = 2.674$, $p < 0.05$; post hoc: $p < 0.05$; **Figure 2a**), while in females it was observed on PND 8 and it was attenuated in both $CB1^{+/-}$ and

$CB1^{-/-}$ animals (genotype x day in females $F_{(6,99)} = 2.95$, $p < 0.05$; post hoc: $p < 0.05$; **Figure 2b).**

A similar pattern was detected also on USV duration, with a peak occurring on PND 4–6 and a reduction afterwards (day effect: $F_{(3,198)} = 19.13$, $p < 0.0001$; **Figures 2c, d**). This pattern was more marked in male than in female mice, with a more dramatic decrease in call duration on the last days in males (interaction sex x day: $F_{(3,198)} = 5.67$, $p < 0.01$), where it tended to be attenuated in $CB1^{-/-}$ mice (interaction genotype x day in males: $F_{(6,99)} = 2.75$, $p < 0.05$; post hoc: $p < 0.05$, **Figure 2c**; in females, genotype effects or its interactions: all n.s., **Figure 2d**).

The peak frequency of the calls increased on PND 8 and 10, and this pattern differed between sexes and genotypes (genotype x sex: $F_{(2,66)} = 3.07$, $p = 0.05$; **Figures 2e,f**). The highest increase in peak frequencies was observed in males on PND10 and this was markedly reduced in $CB1^{-/-}$ pups only (interaction genotype x day in males: $F_{(6,33)} = 10.463$, $p < 0.0001$; **Figure 2e**), while in females it was detected already on PND 8, and it was almost absent in both $CB1^{-/-}$ and $CB1^{+/-}$ pups (interaction genotype x day in females: $F_{(6,33)} = 4.989$, $p < 0.05$; **Figure 2f**).

The peak amplitude of USVs tended instead to decrease (softer calls) on PND8 and PND10 (day effect: $F_{(3,198)} = 155.959$, $p < 0.0001$; **Figures 2g,h**), with no differences between sexes and genotypes (all effects and interactions, n.s.).

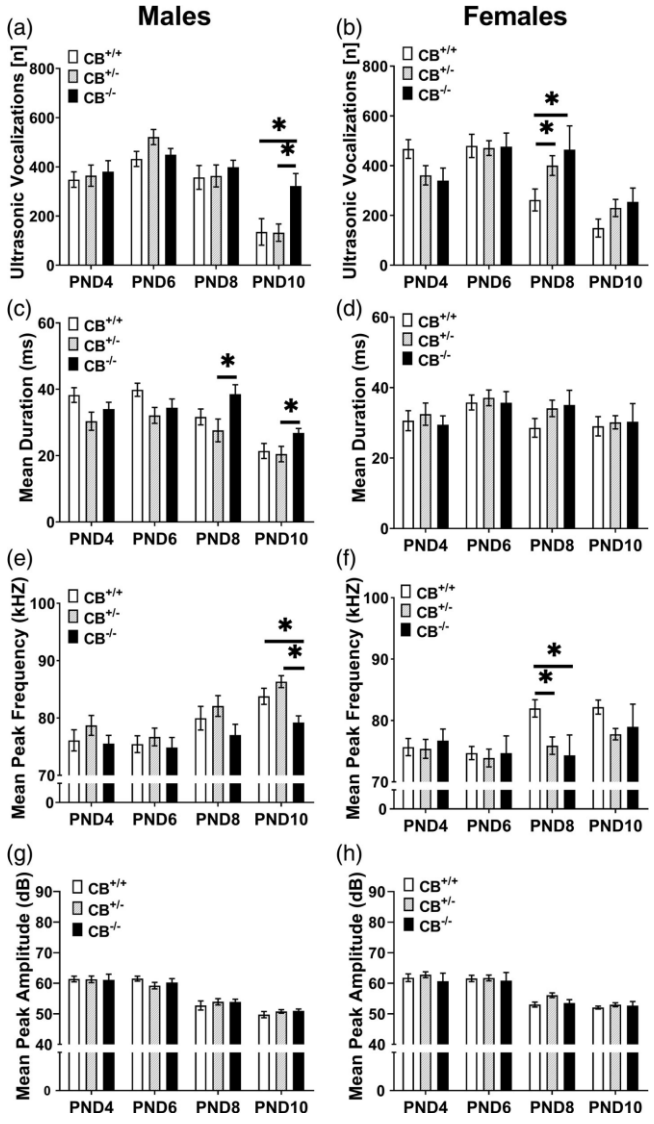

Figure 2. Effects of the CB1 null mutation on ultrasonic vocalization during development in mouse pups. * = $p < 0.05$. N (males) = 9 ($CB1^{+/+}$), 14 ($CB1^{+/-}$), 13 ($CB1^{-/-}$). N (females) = 13 ($CB1^{+/+}$), 17 ($CB1^{+/-}$), 6 ($CB1^{-/-}$). All pups were obtained from 11 litters, including all genotypes. Data are mean ± SEM

In a subsequent detailed analysis based on 28,756 calls emitted by $CB1^{+/+}$ pups, 44,724 calls by $CB1^{+/-}$ pups, and 31,452 calls by $CB1^{-/-}$ pups, different clusters of isolation-induced USVs were revealed by density plots (**Figure 3**). In $CB1^{+/+}$ mice a single cluster was identified on PND 4, most USVs being characterized by peak frequencies between 60 and 70 kHz. On PND 6, a second cluster between 80 and 100 kHz appeared, became more prominent on PND 8, and included most USVs as a single cluster on PND10. A similar pattern was observed in $CB1^{+/-}$ and $CB1^{-/-}$ pups except on PND10, when both mutants continued to produce the majority of their USVs in two distinguishable clusters. This effect was found in both male and female mice.

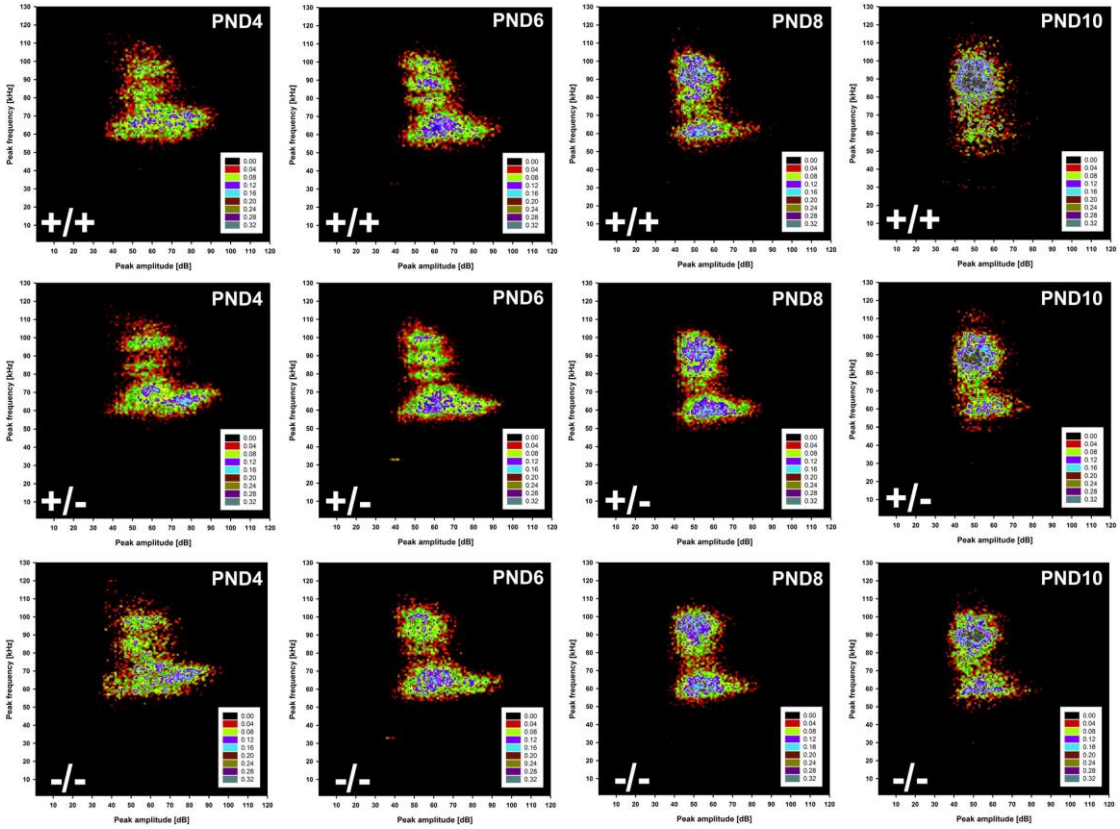

Figure 3. Density plots depicting the distribution of individual ultrasonic vocalizations by PND in mouse pups. Distribution of individual USVs depending on peak frequency in kHz and amplitude in dB in $CB1^{+/+}$, $CB1^{+/-}$, and $CB1^{-/-}$ littermates. Color coding reflects frequency in percentages. Pooled data for both sexes are represented, as no difference between males and females was detected.

Experiment 1b: Assessment of interaction-induced USVs in adult mice

The CB1 mutation affected the number of USVs produced at adulthood by both males and females (genotype effect, respectively: $F_{(2,21)} = 15.89$, 4.06, and $p < 0.05$; **Figures 4a,e**), with $CB1^{-/-}$ mice emitting less USVs than their $CB1^{+/+}$ and $CB1^{+/-}$ littermates (post hoc: $p < 0.05$). No differences in other parameters, including duration, peak frequency and peak amplitude were detected in either sex (all genotype effects, n.s.; **Figures 4b–d, f–h**).

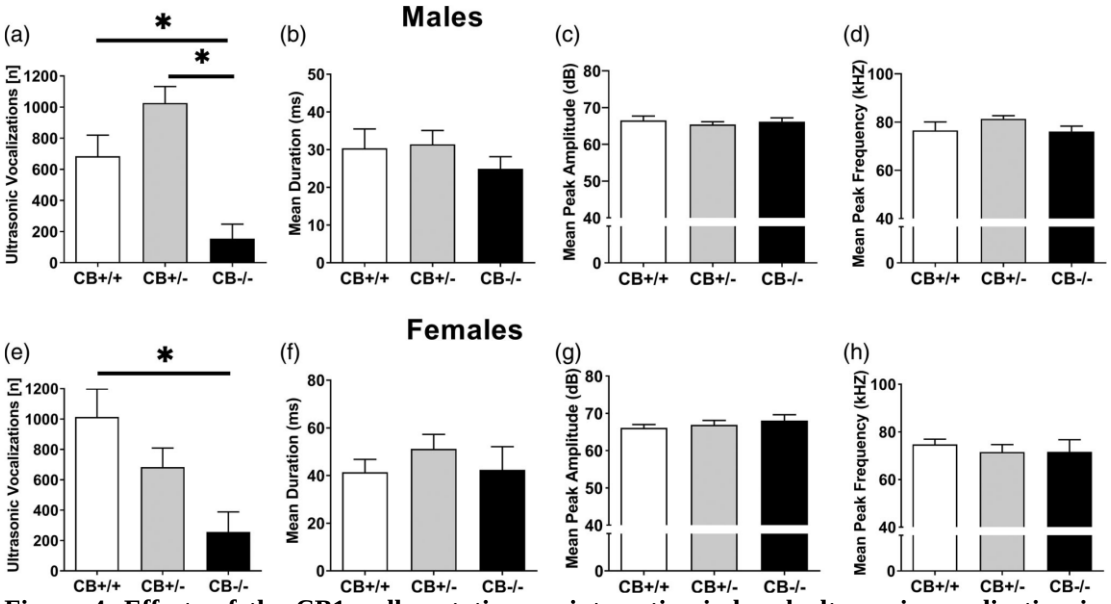

Figure 4. Effects of the CB1 null mutation on interaction-induced ultrasonic vocalization in adult mice. USVs were analyzed in male and female mice during a 3-min session of direct social interaction with an intact NMRI stimulus female. * = $p < 0.05$. N (males) = 6 ($CB1^{+/+}$), 11 ($CB1^{+/-}$), 7 ($CB1^{-/-}$). N (females) = 8 ($CB1^{+/+}$), 11 ($CB1^{+/-}$), 5 ($CB1^{-/-}$). Data are mean ± SEM

Stimulus NMRI females were approximately half in diestrus and half in estrus phases, and their assignment to social encounters was balanced between genotypes and sexes. In males, no significant main effect of the estrous phase of the stimulus females ($F_{(1,18)}$ = 1.24, 0.51, 0.03, 1.17, for number, mean duration, peak amplitude and peak frequency, all n.s.) or its interaction with genotype ($F_{(2,18)}$ = 0.09, 1.02, 0.79, 0.73, for number, mean duration, peak amplitude, and peak frequency, all n.s.) was detected on any USV parameter. In females, similar results on the impact of the estrous phase of the stimulus animals were obtained, with no main effect ($F_{(1,18)}$ = 0.03, 0.01, 0.62, 0.05, for number, mean duration, peak amplitude and peak frequency, all n.s.) or interaction with genotype ($F_{(2,18)}$ = 0.03, 1.13, 1.4, 0.83, for number, mean duration, peak amplitude and peak frequency, all n.s.). The estrus phase of experimental female subjects included proestrus, estrus, and diestrus, following a distribution that was mostly balanced across genotypes. The estrous phase of the experimental subjects did not induce any significant main effect ($F_{(2,15)}$ = 0.29, 0.94, 1.14, 1.12, for number, mean duration, peak amplitude and peak frequency, all n.s.) or interaction with genotype ($F_{(4,1)5}$ = 0.07, 0.69, 0.93, 1.89, for number, mean duration, peak amplitude and peak frequency, all n. s.) on all considered USV parameters.

As in Experiment 1a, a detailed analysis (**Figure 5**) was performed in males based on 4106 calls for $CB1^{+/+}$, 11,289 calls for $CB1^{+/-}$, 1082 calls for $CB1^{-/-}$ mice, and in females based on 9237 calls for $CB1^{+/+}$, 8600 calls for $CB1^{+/-}$, and 1283 calls for $CB1^{-/-}$ mice. In $CB1^{+/+}$ males the majority of calls were clustered between 70 and 85 kHz for peak frequency and 5 to 25 ms for the mean duration; while $CB1^{+/-}$ littermates exhibited a similar pattern, $CB1^{-/-}$ males showed substantially more variation in their calls in both mean peak frequency and duration, with the majority of calls occurring in clusters between 65 and 90 kHz and durations between 5 and 50 ms. In $CB^{+/+}$ females most USVs were distributed in two clusters, one between 70 and 75 kHz and another between 80 and 85 kHz, both with durations between 5 and 40 ms. These two clusters were less distinguishable in $CB^{+/-}$ females, and tended to

disappear in CB1$^{-/-}$ mice, emitting USVs with a wider variation in both mean peak frequency and duration.

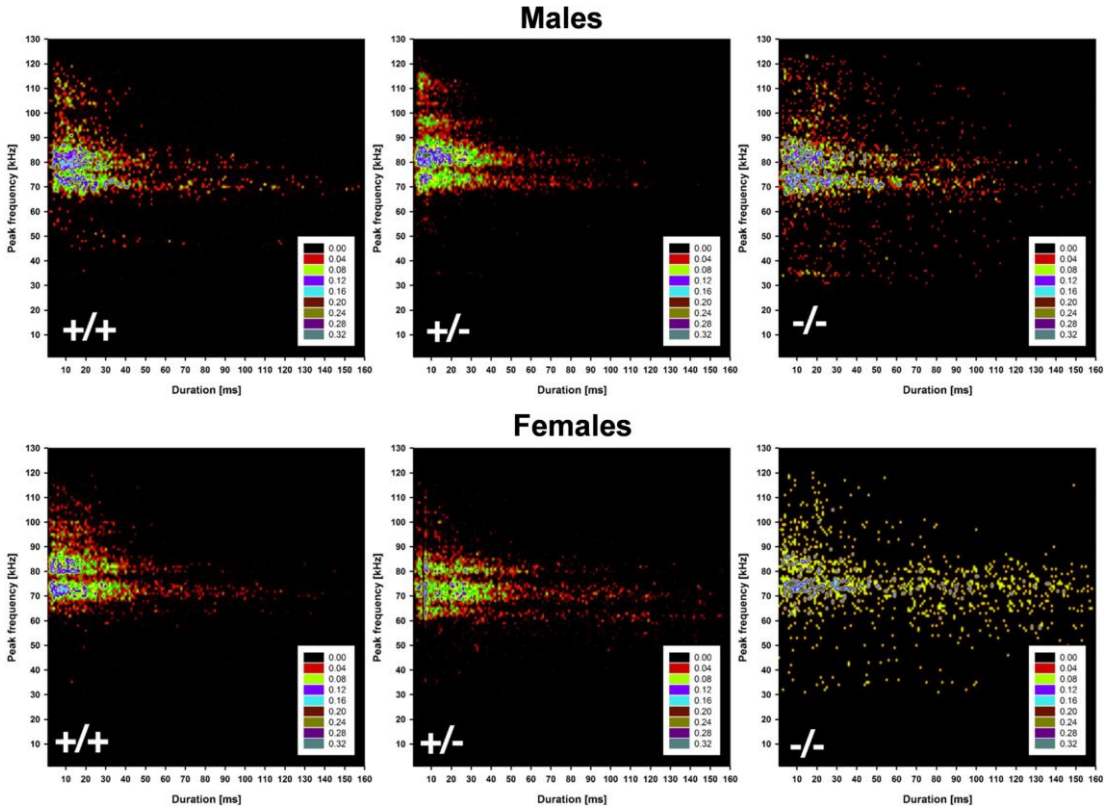

Figure 5. Distribution of individual ultrasonic vocalizations in adult males and females. Density plots depict the distribution of individual USV emitted during 3-min social interaction with an intact NMRI adult female, plotted by frequency in kHz and duration in ms. Color coding reflects frequency in percentages

Experiment 2: Assessment of social interest and social investigation in adult mice

One CB1$^{+/-}$ and a CB1$^{+/+}$ mouse, both females, were excluded respectively from the analysis of the data of the three chamber and direct social interaction tests because of problems in video recording. In the three-chamber test, CB1 mutation affected social interest in both sexes (interaction genotype x compartment: $F_{(2,20)} = 5.32$ and $F_{(2,22)} = 12.04$, $p < 0.05$, respectively in males and females; **Figures 6a,d**). CB1$^{+/+}$ and CB1$^{+/-}$ mice showed a clear preference for the compartment containing the social stimulus (compartment effect: $F_{(1,7)} = 46.91$ and $F_{(1,9)} = 10.97$, $p < 0.01$, respectively in CB1$^{+/+}$ and CB1$^{+/-}$ males, and $F_{(1,11)} = 43.66$ and $F_{(1,6)} = 16.73$, $p < 0.01$, respectively in CB1$^{+/+}$ and CB1$^{+/-}$ females), while social interest was absent in CB1$^{-/-}$ adult males and females (compartment effect: $F_{(1,4)} = 0.49$ and $F_{(1,5)} = 3.09$, n.s., respectively in male and female mutants).

In the direct social interaction test, all mice displayed social habituation, as demonstrated by the reduced time spent in affiliation from the first to the last 5-min of the testing session (effect of 5-min bins, $F_{(1,19)} = 28.19$, $F_{(1,22)} = 4.51$, $p < 0.05$, respectively, in males and females; **Figures 6b,e**). CB1 mutation reduced social investigation, as demonstrated by the reduced time spent in affiliation by CB1$^{-/-}$ males and females compared to their littermates (genotype effect, respectively: $F_{(2,19)} = 3.61$ and $F_{(2,22)} = 4.42$, $p < 0.05$; post hoc: $p < 0.05$; **Figures 6b,e**). In mice of both sexes, this effect was mainly due to a reduction in the time spent performing

anogenital sniffing (**Figures 6c,f**); in males, this reduction was observed in both $CB1^{+/-}$ and $CB1^{-/-}$ mice during the entire duration of the test (genotype effect: $F_{(2,19)} = 11.62$, $p < 0.001$; post hoc: $p < 0.05$; **Figure 6c**). In females, reduced anogenital sniffing was detected only in $CB1^{-/-}$ mice and during the first 5 min of the test (genotype effect: $F_{(2,22)} = 4.45$, $p < 0.05$; interaction genotype x 5-min bin: $F_{(2,22)} = 4.39$, $p < 0.05$; post hoc: $p < 0.05$; **Figure 6f**). No difference among experimental groups was found on nonsocial behaviors (data not shown).

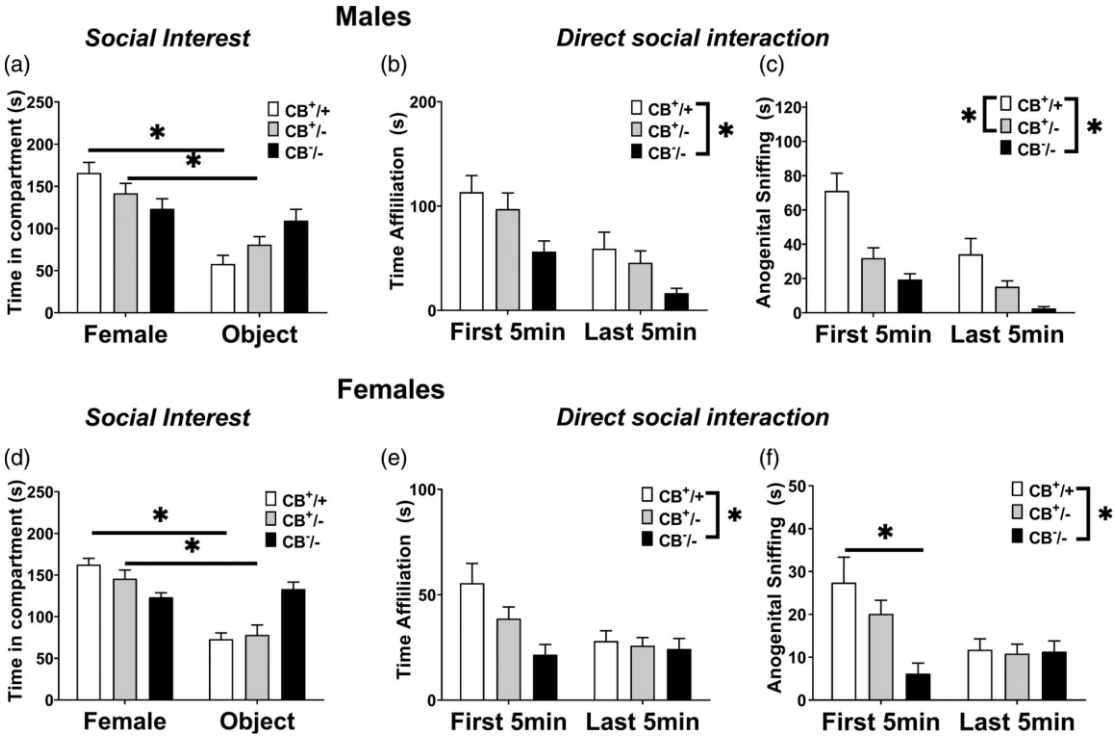

Figure 6. Effects of the CB1 null mutation on social interest and investigation in adult mice. Social interest (a, d) was assessed in the three-chamber test. Social investigation was evaluated during a 10-min session of direct social interaction (b,c,e,f), based on the time spent performing affiliative behaviors. The testing session was analyzed in 5-min bins in order to assess social habituation. * = $p < 0.05$ (in b, c, e, and F, it refers to the main effect of genotype on the entire 10-min testing session). N (males) for both tests = 8 ($CB1^{+/+}$),10 ($CB1^{+/-}$), 5 ($CB1^{-/-}$). N (females) for the three-chamber test = 12 ($CB1^{+/+}$), 7 ($CB1^{+/-}$), 6 ($CB1^{-/-}$). N (females) for the direct social interaction test = 11 ($CB1^{+/+}$), 8 ($CB1^{+/-}$), 6 ($CB1^{-/-}$). Data are mean ± SEM

Experiment 3: Adult assessment of potentially confounding nonsocial alterations

In the elevated plus maze, the CB1 mutation in males or females did not results in behavioral differences either in the percent time spent in the open arms (all genotype effects, n.s.; **Figures 7a,b**), or in the total distance traveled (data not shown). Similarly, no differences between genotypes were detected in the auditory startle response in both sexes, with only an overall expected effect of pulse intensity ($F_{(3,72)} = 12.529$, $F_{(3,93)} = 16.49$, $p < 0.0001$ in males and females, respectively; all genotype effects, n.s.; **Figures 7c,d**).

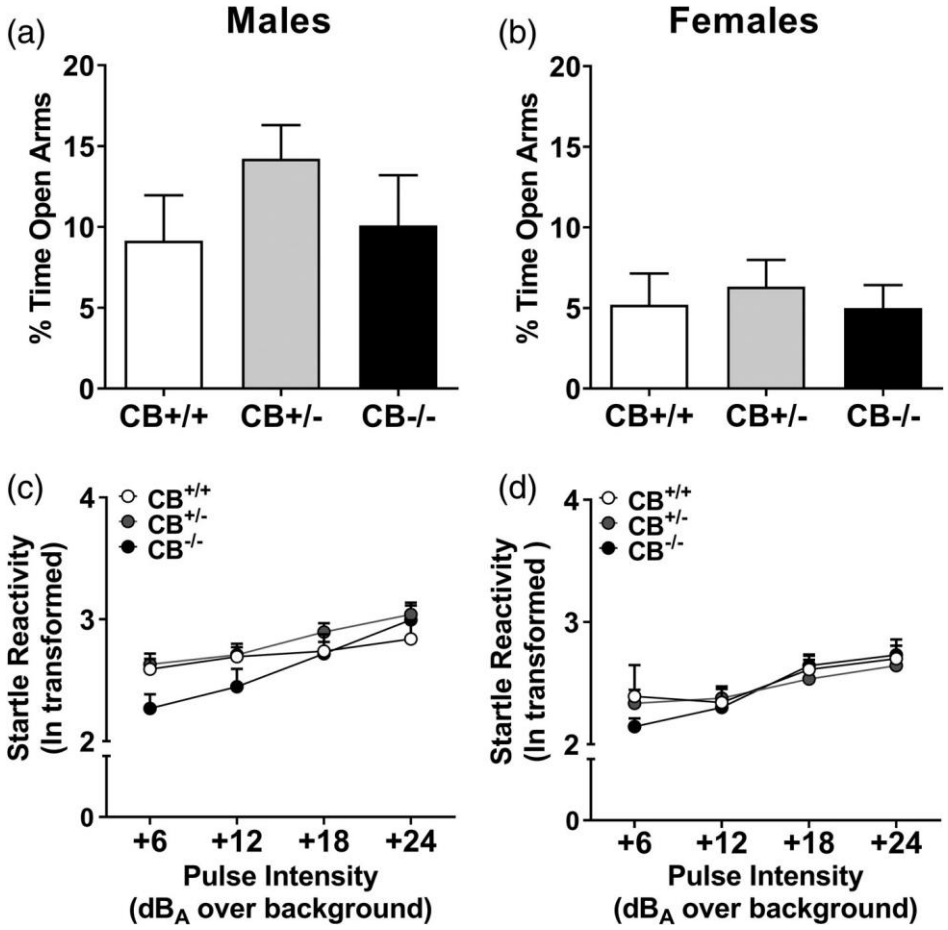

Figure 7. Effects of the CB1 null mutation on confounding nonsocial behaviors in adult mice. Anxiety-like behavior (a, b) in the elevated plus maze and auditory startle response (c, d) were assessed in adult mice. N (males) = 9 ($CB1^{+/+}$), 16 ($CB1^{+/-}$), 9 ($CB1^{-/-}$). N (females) = 7 ($CB1^{+/+}$), 9 ($CB1^{+/-}$), 11 ($CB1^{-/-}$). Data are mean ± SEM

2.4 DISCUSSION

Our data demonstrate that the CB1 mutation affects ultrasonic communication, both during development and at adulthood, as well as social interaction at adult age. These ASD-relevant behavioral alterations were observed in both male and female mice, and overall seemed more marked in $CB1^{-/-}$ than $CB1^{+/-}$ mutants. Importantly, the adult USV alterations were not confounded by differences in anxiety or sensori-motor abilities, as assessed by the elevated plus maze and auditory startle tests. These findings add to previous evidence showing that CB1 inhibition in mice induces repetitive and inflexible behaviors (Varvel and Lichtman, 2002; Gomes et al., 2011; Terzian et al., 2011; Pietropaolo et al., 2020), that is, another core ASD-like symptom. Hence, our results further support the potential role of the ECS in the etiopathology of ASD and therefore its relevance as a therapeutic target for autistic pathologies. Our data also support the validity of the CB1 null mouse line not only for preclinical studies on ASD, but also for studies on the neurobiological mechanisms underlying the general control of social behaviors and communication.

The present study performed for the first time a comprehensive analysis of ultrasonic communication in CB1 mutants during early postnatal development and at adulthood. During the postnatal period both male and female $CB1^{-/-}$ pups showed altered day-dependent patterns of expression of multiple USV parameters. These patterns included a developmental profile characterizing the number and mean duration of

calls produced by $CB1^{+/+}$ mouse pups, with a peak around PND 4–6, followed by a reduction on PNDs 8 and 10 (**Figures 2a–d**). We observed here a slight divergence from the typical inverted-U shaped developmental USV patterns described in mouse pup studies (e.g., Branchi et al., 2001; Sungur et al., 2016) that could be due to the specific genetic background of our CB1 mice, that is, the C57Bl6/N. B6/N mice differ from other B6 sub-strains in the number, peak amplitude and peak frequency of their USVs during infancy (Wöhr et al., 2008), while at adulthood show slightly higher anxiety levels and reduced auditory startle than B6/J, without differing in their social behaviors (Matsuo et al., 2010).

While $CB1^{-/-}$ pups demonstrated a similar peak in USV rate and duration, they did not show a comparable reduction on the following days. This finding suggests a delay in the development of communication abilities in $CB1^{-/-}$ pups, a finding supported by the analysis of other parameters of pups' USVs. Indeed, USV mean peak frequencies also followed a clear developmental pattern, increasing from PNDs 4 to 10 (**Figures 2e,f**), but this linear increase was markedly reduced in $CB1^{-/-}$ male and female pups. Furthermore, density plots revealed in $CB1^{+/+}$ pups the presence at PND 4 of a single cluster of lower mean peak frequency calls (50–70 kHz), associated at PND 6 and 8 with a second cluster of higher frequency calls (80–100 kHz), and disappearing on PND 10, when only the higher frequency cluster remained (**Figure 3**). In $CB1^{+/-}$ and $CB1^{-/-}$ pups both the high and low frequency clusters were instead still evident at PND 10; this finding resembles the pattern observed in the Shank1 mouse model of ASD (Sungur et al., 2016), and further supports a delay in the communication abilities of CB1 mutants.

The hypothesis of a general developmental delay in CB1 mutants is further supported by their reduced body weight gain during the first 10 postnatal days (**Figure 1**); this reduced body growth was previously described in mouse pups following not only genetic (Fride et al., 2005), but also pharmacological inhibition of CB1R (Fride et al., 2001; Schechter et al., 2012), and it has been linked to reduced suckling and milk intake (Fride et al., 2001; Schechter et al., 2012). Nonetheless, body weight differences cannot directly explain the alterations in USV emission rates of CB1 mutant pups, since they appear much earlier during postnatal development (i.e., at PND 4, while USV alterations were first detected at PND 8). Furthermore, reductions in body weight are thought to lead to reduced emission rates of USVs because of the decreased pulmonary-thoracic size (Scattoni et al., 2008), while here an increase in USV number was observed on the last postnatal days (**Figures 2a,b**). Interestingly, an increase in USV rate was also previously described on PNDs 11–13 in rats following systemic administration of the CB1 inverse agonist/antagonist rimonabant (McGregor et al., 1996). It is instead possible that an overall developmental delay in terms of reflexes and neuro-physiological development may be associated with the USV alterations found in CB1 mutant pups; future studies evaluating in depth developmental milestones (Branchi et al., 2004) are needed to investigate this issue that is, to our knowledge, still unknown in these mouse mutants.

Our findings on neonatal USVs point out to a developmental delay in $CB1^{-/-}$ pups, since the day-dependent reduction of USV rate is supposed to correspond to increased pup maturity and reduced dependence from the mother (Caruso et al., 2020). Furthermore, previous studies have shown that USV rate in rodent pups reflect their affective state (Zimmerberg et al., 1994; Branchi et al., 2001), with an increase corresponding to an adverse emotional state (Caruso et al., 2020). An increase in pups' USV rate has been described in several mouse models of ASD [reviewed in (Caruso et al., 2020)] and it has been often interpreted as a sign of

enhanced emotional distress; when accompanied by alterations in spectrographic parameters, such as mean duration or peak frequency, it has also been interpreted as a marker of altered communication abilities, within the context of mother-infant interactions. Interestingly, infants with ASD or at risk for developing ASD, when compared to typically developing infants, emit more cries with certain specific spectrographic characteristics (e.g., higher fundamental frequencies), that have been linked to more aversive conditions and negatively affect parental responses (Esposito and Venuti, 2010; Esposito et al., 2017). Other studies have interpreted the enhanced rate of USVs described in mouse models of ASD as similar to the excessive talking and repetitive speech often found in autistic patients (e.g., Wheeler et al., 2014; Gauducheau et al., 2017). Interestingly, an enhanced mean duration of USVs was also described in the Fmr1-KO mouse model for ASD, both in pups (Gaudissard et al., 2017) and juveniles (Gauducheau et al., 2017), thus suggesting that longer calls may represent a consistent ASD-like phenotype, at least in young mice.

An alternative explanation for USV alterations in CB1 pups may involve their altered response to stress, as USVs were assessed following a short maternal separation; several studies demonstrated an abnormal behavioral response of CB1 null mice to stressors in general (Miller et al., 2008; Busquets-Garcia et al., 2016), often accompanied by enhanced neuro-endocrine reactivity (Barna et al., 2004). Furthermore, previous data demonstrated specifically an altered ultrasonic response to acute stress in $CB1^{-/-}$ mouse pups (Fride et al., 2005), similar to that previously described in rats following pharmacological CB1 modulation (Varga et al., 2012; Varga et al., 2017; Myose et al., 2019). Moreover, the persistence of USV alterations into adulthood in our CB1 mutants strongly supports the presence of a genuine deficit in communication abilities in these mutants, which was indeed confirmed in the non-stressful context of direct social interaction with an adult female.

Male and female $CB1^{-/-}$ mice showed in fact again at adulthood USV alterations, including a reduction in the call rate (**Figures 4a–e**), and higher variations in the peak frequency and mean duration of the calls (i.e., reduced clustering), as revealed by the analysis of the density plots (**Figure 5**). The reduced USV rate is in line with what is observed in other ASD mouse models (Silverman et al., 2010), for example, the BTBR (McFarlane et al., 2008; Scattoni et al., 2011), Shank (Ey et al., 2011; Schmeisser et al., 2012) or Fmr1-KO mouse lines (Rotschafer et al., 2012), thus supporting the relevance of this quantitative USV alteration as an ASD-like phenotype.

Interestingly, the USV alterations of CB1 mutants were not accompanied by altered anxiety or reduced startle response, as found in other mouse models for ASD (Pietropaolo and Subashi, 2014; Yang et al., 2015). In fact here CB1 mutants did not differ from their WT littermates in the elevated plus maze and auditory startle tests (**Figure 7**), in line with previous studies showing that behaviors in these tests were not consistently and robustly affected by CB1 homozygous deletion (Haller et al., 2002; 2004; Marongiu et al., 2012). While the USV alterations observed in CB1 male and female mutants at adulthood were not linked to emotional or sensori-motor abnormalities, they were instead associated with deficits in social interest in the three-chamber test and in social investigation (**Figure 6**); interestingly, the genotype differences were more marked for anogenital sniffing, a behavior that has been shown to positively correlate with USVs rate in adult mice (Nyby, 1983; Moles et al., 2007). This finding, together with the presence of social and USV alterations in both sexes, suggests that CB1 mutation may affect social interactions and communication by acting on the general sociability of mice. It is possible that other confounding

factors may influence the genotype effects observed here on adult social behaviors, such as deficits in olfactory abilities, as well as general reduced explorative behavior and altered locomotor activity. Nonetheless, these behavioral confounders were ruled out by previous studies describing no abnormalities in olfactory discrimination tests (Hutch et al., 2015), and inconsistent alterations in object exploration and locomotion (e.g., Haller et al., 2002; Lafenetre et al., 2009; Dubreucq et al., 2010; Haring et al., 2011). Our data from the total distance moved in the elevated plus maze and nonsocial exploration in the direct social interaction test further support the lack of confounding differences in locomotion/exploration between WT and CB1 mutants.

The deficits showed in social interest and behavior in adult CB1 mutants are in agreement with previous reports on $CB1^{-/-}$ male mice (Haller et al., 2004; Haring et al., 2011; Litvin et al., 2013; Terzian et al., 2014), even with stimuli of different sex (Haller et al., 2004; Haring et al., 2011; Litvin et al., 2013; Terzian et al., 2014) and genetic background (Terzian et al., 2014), thus confirming these as a robust behavioral phenotype of CB1 null mice. Reduced social investigation and social interest in $CB1^{-/-}$ male mice was previously described toward an ovariectomised female in hormonally-induced estrous phase (Terzian et al., 2014), thus suggesting the limited impact of the estrous phase of the female stimulus on the social deficit of CB1 mutants. Also, similar deficits in USV rate and social investigation (again, especially on anogenital sniffing) were observed following pharmacological CB1 inhibition by rimonabant in male mice, in a dose-dependent manner (Pietropaolo et al., 2020). In agreement with our results, stimulating the endocannabinoid tone by the administration of anandamide hydrolysis inhibitor URB597 induced an increase in social investigation, as well as in the emission rate of USVs (although mostly those at 50 kHz) in adult rats (Manduca et al., 2014). To our knowledge, this is the first time that USV and social alterations are described also in $CB1^{+/-}$ mice, also including female subjects; here, females seem more sensitive to the early effects of CB1 mutation, since during development $CB1^{+/-}$ females (but not males) differed in body weight and USVs from WT littermates similarly to $CB1^{-/-}$. At adulthood, "a dosage" effect of CB1 mutation seemed evident in males and females, with one allele somehow protecting from the effects of CB1 deletion, and $CB1^{+/-}$ positioning between $CB1^{-/-}$ and $CB1^{+/+}$. Despite a traditional focus on the male sex, ASD-research is indeed increasingly interested in evaluating pathological behavioral phenotypes also in females subjects, as ASD female patients may have unique clinical presentations relative to their male counterparts, a factor that may have led to under diagnosis of ASD in the female sex (Loomes et al., 2017). Hence, the presence of communication and social phenotypes in CB1 mutant females add to the value of the CB1 null mouse to study ASD, an issue that is receiving increasing attention in preclinical research on this pathology (e.g., studies in female Fmr1-KO mice modeling ASD, (Gauducheau et al., 2017).

In conclusion, our data support the use of the CB1 null mouse in preclinical research on ASD. The lack of nonsocial alterations, that is, emotional or sensori-motor abnormalities, does not undermine the validity of CB1 mutants to study ASD, although they may be considered ASD-like phenotypes; first, because recapitulating the full ASD-like phenotypes is increasingly considered an unrealistic and unnecessary goal of mouse models (Crawley, 2004, 2007; Moy et al., 2006; Silverman et al., 2010), second because it allows to rule out important confounds potentially acting on social and communication behaviors. Thus, the CB1 null mouse may be instrumental in specifically investigating the neurobiology of social behaviors and communication, that is, the core ASD symptoms, without including

other nonsocial symptoms. This approach is particularly suitable to the CB1 null model, because of the availability of mutant CB1 mouse lines with region- and cell-specific deletions (Marsicano and Lutz, 1999; Bellocchio et al., 2010; Hebert-Chatelain et al., 2014; Busquets-Garcia et al., 2016; Oliveira da Cruz et al., 2016; Robin et al., 2018), allowing dissecting the behavioral role of CB1 according to its expression site (e.g., glutamatergic, gabaergic, dopaminergic neurons, in the whole cell or mitochondria only). Hence, future studies combining region-and cell-specific deletions of CB1 will be able to identify the structures and circuits responsible for the social and communication deficits, thus providing novel avenues for research on ASD.

3. Second paper:
Autistic-like behavioral effects of prenatal stress in juvenile Fmr1 mice: the relevance of sex differences and gene-environment interactions

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Journal: Scientific Reports

The aim of this study was to analyze the ultrasonic communication of a validated monogenic mouse model for ASD, Fmr1 mutant mice, compared to that of WT mice, also in a modified environmental context evaluating the relevance of early environmental stressors to interact with genetic factors to influence the appearance of ASD-like phenotypes.

3.1 INTRODUCTION

Fmr1 KO mouse line models Fragile X syndrome (FXS) that is a neurodevelopmental disorder characterized by multiple behavioral alterations, including mental retardation, hyperactivity, anxiety, cognitive and social deficits (Hagerman and Hagerman, 2002). Autistic symptoms, including altered social interaction and communication, are also often detected in FXS patients (Bailey et al., 1998; Hagerman, 2006): FXS is indeed considered as the most common monogenic cause of autism spectrum disorder (ASD). FXS is due to a mutation in the X-linked FMR1 human gene consisting in more than 200 CGG repetitions leading to the absence of FMRP protein (Pieretti et al., 1991) playing a major role in synaptic and neuronal functionality (Greenough et al., 2001). The lack of FMRP has been recapitulated by the Fmr1-KO mouse model of FXS together with several relevant behavioral alterations (Dutch-Belgian Fragile X Consortium, 1994).
Despite its clear and well-defined genetic origins, the FXS behavioral phenotype can be critically modulated by environmental factors, both in terms of its severity and of the timing of appearance. Environmental stimulation is for instance known to attenuate/delay the expression of behavioral alterations both in FXS patients and Fmr1-KO mice (Dawson et al., 2002; Oddi et al., 2015). Conversely, exposure to stressful life events may exacerbate the behavioral deficits of FXS patients (Hessl et al., 2001; Dyer-Friedman et al., 2002), especially when occurring during early life phases. Exposure to prenatal stress is a powerful tool to induce early adversity in a genetic mouse model and therefore to study the impact of gene-environment interactions in the expression of its behavioral phenotype. Surprisingly, to our knowledge, the behavioral effects of prenatal stress have never been investigated in

the Fmr1-KO mouse, or in other models of ASD (while they were demonstrated in genetic mouse models of neuropsychiatric and neurodegenerative disorders (Oliver et al., 2009; van den Hove et al., 2011; Sierksma et al., 2013).

Furthermore, prenatal stress is known to induce marked long-term behavioral alterations in wild-type rodents, including cognitive, emotional, motor and social abnormalities (reviewed in Weinstock, 2008; Sandi et al., 2015). These studies have pointed out in particular the relevance of the unpredictable chronic mild stress procedure, as the most suitable experimental approach to model early environmental adversity in laboratory rodents (Mineur et al., 2003, 2006; Wilner, 2005). This procedure, combining multiple stressors of different nature, has also the advantage to minimize habituation and exclude pain or nutritional effects (Imbe et al., 2006; Campos et al., 2013). In most existing preclinical studies (reviewed in Weinstock, 2008; Sandi et al., 2015) stress exposure was implemented during the last week of gestation of the dams, as this phase is a preferential target to induce long-term brain and behavioral modifications in the offspring, because of its high environmental and stress sensitivity (Misdrahi et al., 2005; Enayati et al., 2012).

The inclusion of mice of both sexes in the behavioral analysis of the offspring is considered of critical relevance for preclinical studies on prenatal stress exposure. Several sex differences have been indeed described in the behavioral response to stress in rodents; these include differences in the severity of stress effects, but also in their specificity to selected behavioral domains (Weinstock 2007; Sierksma et al., 2013; Sickmann et al., 2015). The inclusion of subjects of both sexes is also important for studying FXS, both in human and preclinical research. Although FXS is more common in boys than girls, increasing attention has been devoted to heterozygous females, as they are the ones producing the affected offspring (Nolin et al., 1996), and they represent the majority of FXS female patients, as homozygous *FMR1* mutations are extremely rare (Vafaeie et al., 2021). In humans, FXS female carriers present several behavioral symptoms, including hyperactivity (Wheeler et al., 2014) mild cognitive impairments (Loesch and Hay, 1988; Loesch et al., 2003) and autistic behaviors (Mazzocco et al., 1997). In mice, similar behavioral abnormalities were described in Fmr1 mutant females, especially at adulthood (as reviewed in Pietropaolo and Subashi, 2014).

Here we therefore evaluated whether exposure to unpredictable chronic mild stress during the last prenatal week could advance and/or exacerbate the juvenile behavioral phenotype of Fmr1-KO offspring of both sexes. To this end, Fmr1-KO male (hemizygous, -/Y) and female (heterozygous, +/−) mice, together with their WT littermates, underwent behavioral tests for exploration, spatial memory, social interaction and communication at the juvenile age of 7–8 weeks, i.e., when most of the FXS-like behavioral alterations are absent or mild. At this age, Fmr1-KO males do not show any remarkable behavioral phenotype in the considered domains (Pietropaolo and Subashi, 2014; Gaudissard et al., 2017), while mutant females displayed mild alterations in social interaction and communication (Gauducheau et al., 2017). This age partially overlaps with adolescence (3-8 weeks of age in mice), a critical phase for brain and behavioral development in rodents and humans and largely involved in several neuropsychiatric disorders (Spear, 2000). This phase has been also extensively studied for the expression of social behaviors in laboratory mice, with a special emphasis on the post-pubertal phase (i.e., approximately after the 5 weeks of age), since it is characterized by important changes in the patterns of intra-specific social interactions (Terranova et al., 1993). Late adolescence (7–9 weeks) is also of particular interest, since most behavioral abilities are already well

developed in mice; it is therefore suitable to multiple behavioral testing, performing the same cognitive, emotional, and social tests done in adult mice and hence facilitating comparisons with data from adult subjects.

3.2 MATERIALS AND METHODS

3.2.1 Ethics approval

All experimental procedures were in accordance with ARRIVE guidelines (https:// arriveguid elines. org), European Communities Council Directive 2010/63/EEC. Furthermore, there were approved by local ethical committee ("Comite d'Ethique pour l'experimentation animale de Bordeaux", CE 50) and the French Ministry ("Ministere de l'enseignement superieur de la recherche et de l'innovation").

3.2.2 Breeding and stress procedure

Twenty adult (12 ± 1 weeks-old) virgin Fmr1 heterozygous (+/−) females and 10 C57BL/6J adult wild type males [16 weeks-old; purchased from Janvier (Le Genest St Isle, France)] were used as breeders to generate the tested offspring.

Each half of the female breeders was assigned to one of the following groups in which they were kept during the last week of pregnancy: no-stress, i.e., kept undisturbed in their home-cage, or stress, i.e., exposed to the unpredictable stress procedure described below.

The time line of the study is illustrated in **Figure 1**.

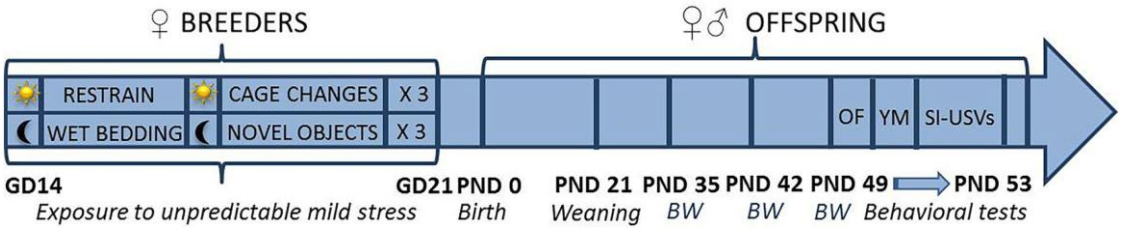


Figure 1. Schematic representation of the experimental design of the study and its timeline. Unpredictable mild stress was conducted during the last week of gestation. Behavioral tests were performed between 7 and 8 weeks of age, with 48hs interval between consecutive tests. GD = gestational day; PND = postnatal day; BW = body weight; OF = open field; YM = Y maze; SI = social interaction; USVs = ultrasonic vocalizations.

The stress procedure included the following 2-day sequence of events that was repeated three consecutive times during the last week of gestation:

- Day 1: 30 min of restrain stress (3 times each day during the light phase, with a 4 h-interval) in perforated conical tubes (3 cm in diameter, 11.5 cm long; Becton Dickinson Labware Europe, France), followed by overnight housing with wet bedding (50 ml of water were added to floor sawdust of the home cage at the beginning of the dark phase).
- Day 2: multiple sawdust and cage changes (3 times each day during the light phase, with a 4 h-interval), followed by overnight housing with novel objects (12 glass black beads, 1.5 cm in diameter were added in the home cage at the beginning of the dark phase).

Pregnant females were exposed to this sequence of events for 3 times during the last week before parturition: this procedure is based on previous studies (e.g. Pardon et al., 2000a; b; Negron et al., 2004; Misdrahi et al., 2005) and it is known to limit the habituation to stressful stimuli without using pain or nutritional manipulations. All breeders used for the study gave birth within 48hs after the last day of exposure to

stress procedure. They were left undisturbed until weaning of the pups. No alteration in the general health status of stressed breeders emerged at the end of the stress paradigm. The health measures were taken by the animal caretakers through the daily observation of the animals in their home cage in order to assess both behavioral and physical indicators of welfare (Burkholder et al., 2012). These included hunched posture, dull or sluggish movements, reduced locomotion/immobility, altered nest building and stereotypic behaviors, excessive grooming, absence of feces, rough hair coat, squinted eyes, skin abrasions/lesions (Burkholder et al., 2012).

3.2.3 Animals and housing procedures

At 3 weeks of age, all pups were weaned and housed in same-sex groups of 3–5 littermates in our animal facility (Oddi et al., 2015; Gaudissard et al., 2017). On the same day, tail samples were collected for DNA extraction and subsequent PCR assessment of the genotypes. Mice were then left undisturbed until the beginning of behavioral testing (i.e., at 7 weeks of age), except for the evaluation of body weight that was carried out once a week starting at 5 weeks of age (**Figure 1**). Only litters including males and females of both mutant (KO for males and HET for females) and wild-type (WT) genotypes were used for experiments, for a total of 14 litters. A total of 93 mice were subjected to behavioral testing: 45 males [25 WT and 20 KO (-/Y), n = 9–15 for stress condition) and 48 females [24 WT and 24 HET (+/−), n = 12 for stress condition].

Stimulus mice used for the direct social interaction test were adult (12 weeks of age) female NMRI mice, as this strain is commonly employed in social studies (Moles and D'Amato, 2000; Moles et al., 2007), especially those using the Fmr1-KO mouse model (Oddi et al., 2015; Gaudissard et al., 2017; Gauducheau et al., 2017). This strain is often chosen since it is characterized by high levels of sociability, and it facilitates the behavioral analysis during social encounters with B6 mutants because of its albino phenotype. NMRI mice were purchased at 10 weeks of age from Janvier (Le Genest-Saint-Isle, France), housed in groups of 3–4 per cage and left undisturbed for 2 weeks before being used in behavioral tests. The choice of the age of stimulus mice was based on previous studies with male mice (both adults and juveniles; e.g. (Whitney et al., 1973; Warburton et al., 1989; Wang et al., 2008; Lahvis et al., 2011; Castellucci et al., 2018; Caruso et al., 2020), and with females in the resident-intruder setting (Maggio and Whitney, 1985; D'Amato and Moles, 2001; Moles et al., 2007), all using adult stimulus females. Indeed, in these experimental contexts, adult stimulus females do not emit ultrasonic vocalizations that are instead mostly uttered by the experimental male (Whitney et al., 1973; Maggio and Whitney, 1985) or resident female (Maggio and Whitney, 1985; D'Amato and Moles, 2001), as demonstrated by alternately anesthetizing each pair member. For these reasons, we have previously used an adult female stimulus to assess ultrasonic communication in juvenile Fmr1 mice of both sexes (Gaudissard et al., 2017; Gauducheau et al., 2017). Indeed, juvenile females are known to produce a high number of USVs during same-age interactions (Panksepp et al., 2007), both with male and female experimental mice. During juvenile-juvenile interactions in mice, both pair members are indeed supposed to emit USVs, so that the USVs of each pair represent the only variable taken into consideration (e.g., Panksepp et al., 2007). This situation is easily detectable by spectrographic analysis, through the identification of "double calls", i.e., overlapping in their timing, but with different, non-harmonic, characteristics (e.g., different peak and mean frequency, modulation). Here the presence of these double calls was excluded by the additional inspection of all spectrograms.

3.2.4 Behavioral testing

Behavioral tests commenced at 7 weeks of age and were conducted as follows (see also **Figure 1**). On day 1, an open field test for locomotion and exploration was administered, followed on day 3 by a spontaneous alternation test in a Y-maze, and on day 5 by a direct social interaction test and the females' estrous cycle assessment. All behavioral tests were carried out during the light phase of the cycle (between 9 a.m. and 4 p.m.) by an experimenter who was blind to the group assignment of the subjects. All mice were habituated to the experimental room for at least 30 min before the beginning of each behavioral test.

Open field.

The open field consisted of a white plastic arena (42 × 26 × 15 cm) where the locomotion of each mouse was assessed during 10 min using automated tracking (Ethovision, Noldus, The Netherlands).

Y maze.

The apparatus for this test consisted of a plastic Y-maze composed by three arms similar in appearance and spaced at 120° from each other. Each arm was 42 cm long and 8 cm wide. The entire maze was enclosed by a wall 15 cm high and 0.5 cm thick. The Y maze test was employed to assess spontaneous alternation through a 5-min habituation trial, followed by a 2-min test trial (Gaudissard et al., 2017). During the first sample phase, access to the third novel arm was blocked by a door; mice were placed at the end of the start arm and allowed to freely explore the start and the other unblocked arm for 5 min before being returned to a waiting cage. After 2 min in the waiting cage, the test phase began: the door was removed; mice were placed at the end of the start arm and allowed to explore the entire maze for 2 min. Time spent in each arm during the habituation and testing phases was scored by automatic tracking and percent alternation rates during the test phase were derived as follows: 100 x (time in novel arm/time in all arms).

Social interaction and ultrasonic communication.

Male experimental subjects were habituated to the testing apparatus for 30 min prior to testing, while female subjects were isolated in the testing cage for 72hs, in order to induce a status of resident in experimental females and therefore promote the emission of USVs towards an adult female intruder (Moles et al., 2007). An unfamiliar stimulus female mouse (an adult NMRI female) was then introduced into the testing cage of either male or female subjects and left there for 3 min. Testing sessions were recorded by a camera placed on the side of the cage and videos analyzed with Observer XT (Noldus, The Netherlands). One observer who was unaware of the genotype and sex of the animals scored the behavior of the test mice, quantifying the time spent performing affiliative behaviors (Oddi et al., 2015; Gaudissard et al., 2017; Gauducheau et al., 2017), i.e., sniffing the head/body/anogenital region of the parter; contact with the partner through traversing the partner's body by crawling over/under from one side to the other or allogrooming. Nonsocial activities were also measured (Oddi et al., 2015; Gaudissard et al., 2017): rearing, digging and self-grooming. An ultrasonic microphone UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics, Berlin, Germany) was mounted 2 cm above the cover of the testing cage. Recordings were then analyzed through Avisoft SASLab Pro (Version 5.20; Avisoft, Berlin, Germany) to compute the number of USVs as well as their mean duration, peak frequency and peak

amplitude (Oddi et al., 2015; Gaudissard et al., 2017). In addition, density plots depicting the distribution of total calls for each genotype at peak frequency versus duration were obtained (Wohr, 2014; Mosienko et al., 2015). Call subtypes were also determined for a more detailed qualitative analysis; for this purpose, USVs were automatically classified using the Sonotrack Call Classification Software (version 1.4.7, Metris B.V., The Netherlands), using categories previously described in details elsewhere (Caruso et al., 2020).
The estrus phase of female mice was assessed by analysis of vaginal smears (Caligioni, 2009) performed on the testing day in both the experimental subjects and NMRI stimulus mice. The evaluation of Fmr1 WT and HET (+/−) females used as experimental subjects was conducted after their testing, in order to minimize the potential stress effects of the manipulation necessary for determining the estrous phase. Stimulus NMRI females were approximately half in diestrus and half in estrus phases, and their assignment to social encounters was equally distributed between experimental groups. The estrus phase of experimental female subjects included pro-estrus, estrus and diestrus, following a distribution that was balanced across genotypes and stress conditions.

3.2.5 Statistical analysis

All data were separately analyzed in males and females. This was due to sex differences in (i) the X-linked Fmr1-mutation (i.e., hemizygous in males, heterozygous in females), (ii) in some behavioral testing procedures (such as different duration of pre-testing isolation necessary for USV assessment), (iii) in most of the behavioral phenotypes measured here. The latter sex differences were further confirmed in our data set, through a preliminary ANOVA showing overall sex effects in basically all measured variables (data not shown).
Data from each sex were analyzed with a 2 x 2 ANOVA with genotype and stress as the between subject factors. Within-subject factors were included when appropriate (e.g., testing time for body weight). Alternation rates from the Y-maze test were instead analyzed for differences from the chance level (with a t-test), in line with previous studies (Vandesquille et al., 2013). Post-hoc comparisons were performed using Fisher's LSD test when a significant interaction was detected. Separate ANOVAs were also conducted when appropriate. Data from the density plots of ultrasonic calls did not undergo statistical analysis, but were used to obtain a qualitative three-dimensional evaluation of USV data (Wohr, 2014; Mosienko et al., 2015).
Analyses were conducted using the software Statview and SPSS and α was set at 0.05. Results are expressed as mean ± SEM throughout the text. The exact number of mice is indicated in the legend of each figure; differences may be due to technical reasons (e.g., loss of behavioral video recordings) or to the exclusion of outliers (using Grubbs' ESD test adapted for small sample size) or of non-vocalizing mice for USV assessment (these included a total of 4 males and one female).

3.3 RESULTS

Body weight

Body weight was assessed once a week between 5 and 7 weeks of age (**Figure 2**). In males, there was an expected body weight gain with time [testing time effect: $F_{(2,82)} = 982.57$, $p < 0.0001$; **Figure 2a**] and this was more marked in WT mice than KOs [interaction genotype x time: $F_{(2,82)} = 9.22$, $P < 0.001$]. Nonetheless, this was mainly due to the overall higher body weight of WT-stressed males, as demonstrated by

separate ANOVAs showing a significant effect of stress in WT mice only [$F_{(1,23)}$ = 4.2, p = 0.05; in KO: n.s.; **Figure 2b**]. A similar pattern was found in females, where body weight also increased over weeks as expected [time effect: $F_{(2,88)}$ = 768.32, p < 0.0001; **Figure 2c**], and this gain did not differ between genotype or stress conditions [all interactions with time, ns]. In females also, stress increased the overall body weight, but equally in both WT and HET mice [main stress effect: $F_{(1,44)}$ = 9.17, p < 0.01; **Figure 2d**].

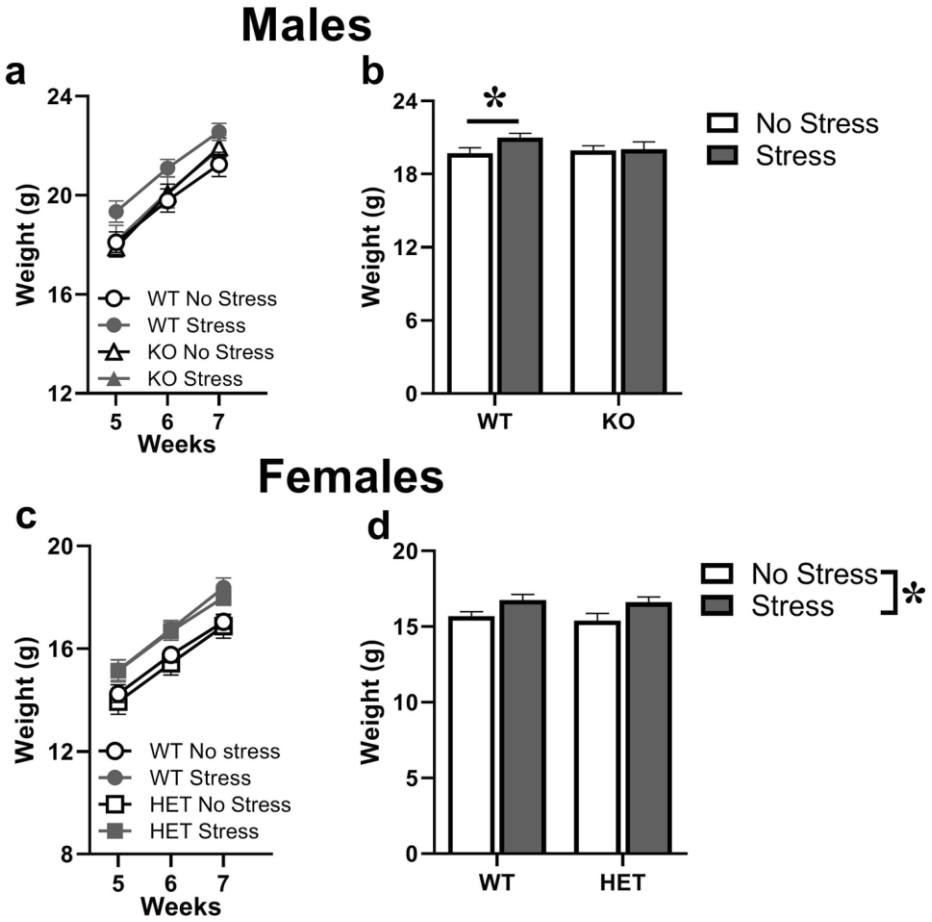


Figure 2. Effects of prenatal stress in juvenile mice on body weight. Body weight was assessed during the last two weeks before behavioral testing. Time course illustrates the expected weight gain in males and females (**a**–**c**), while overall group differences are shown by the mean weight values averaged across time-points in each sex (**b**–**d**). *p < 0.05. N for males: 15 WT-no stress, 10 WT-stress, 9 KO-no stress, 11 KO-stress; N for females: 12 in all groups. KO refers to -/Y in males, HET to +/− in females. Data are expressed as mean ± SEM.

Open field

In males, there was no difference among experimental groups in locomotor activity in the open field [genotype, stress effects and their interaction: all n.s.; **Figure 3a**]. In females, a tendency to a decrease in locomotor activity following stress was observed in mice of both genotypes [stress effect: $F_{(1,44)}$ = 3.87, p = 0.06; **Figure 3b**].

Y-maze

All male and female mice equally explored the maze arms during the habituation phase, and no differences among experimental groups were detected (data not shown). During the test phase, all males displayed spontaneous alternation, except stressed KO mice that showed a performance not significantly different from the chance level: (t = 2.16, ns; in other groups, all ts > 4, p < 0.01; **Figure 3c**). In females, none of the

four experimental groups showed significant levels of spontaneous alternation (t-tests: all ns; **Figure 3d**), suggesting that this cognitive ability is not sufficiently expressed in *Fmr1* WT and HET female mice at this juvenile age.

Social interaction

In males, WT stressed mice showed higher levels of affiliative behaviors towards the WT female stimulus [interaction genotype x stress: $F_{(1,38)} = 4.47$, $p < 0.05$; post-hoc: WT-no stress versus WT-stressed, $p < 0.05$; **Figure 3e**]. In females, HET mice showed enhanced levels of affiliation towards the WT female intruder compared to their WT littermates, but this genotype difference disappeared following stress, since stress tended to increase affiliative levels in WT mice [interaction genotype x stress: $F_{(1,44)} = 4.19$, $p < 0.05$; post-hoc: WT-no stress versus HET-no stress, $p < 0.05$; WT-no stress versus WT-stressed, $p = 0.06$; **Figure 3f**].

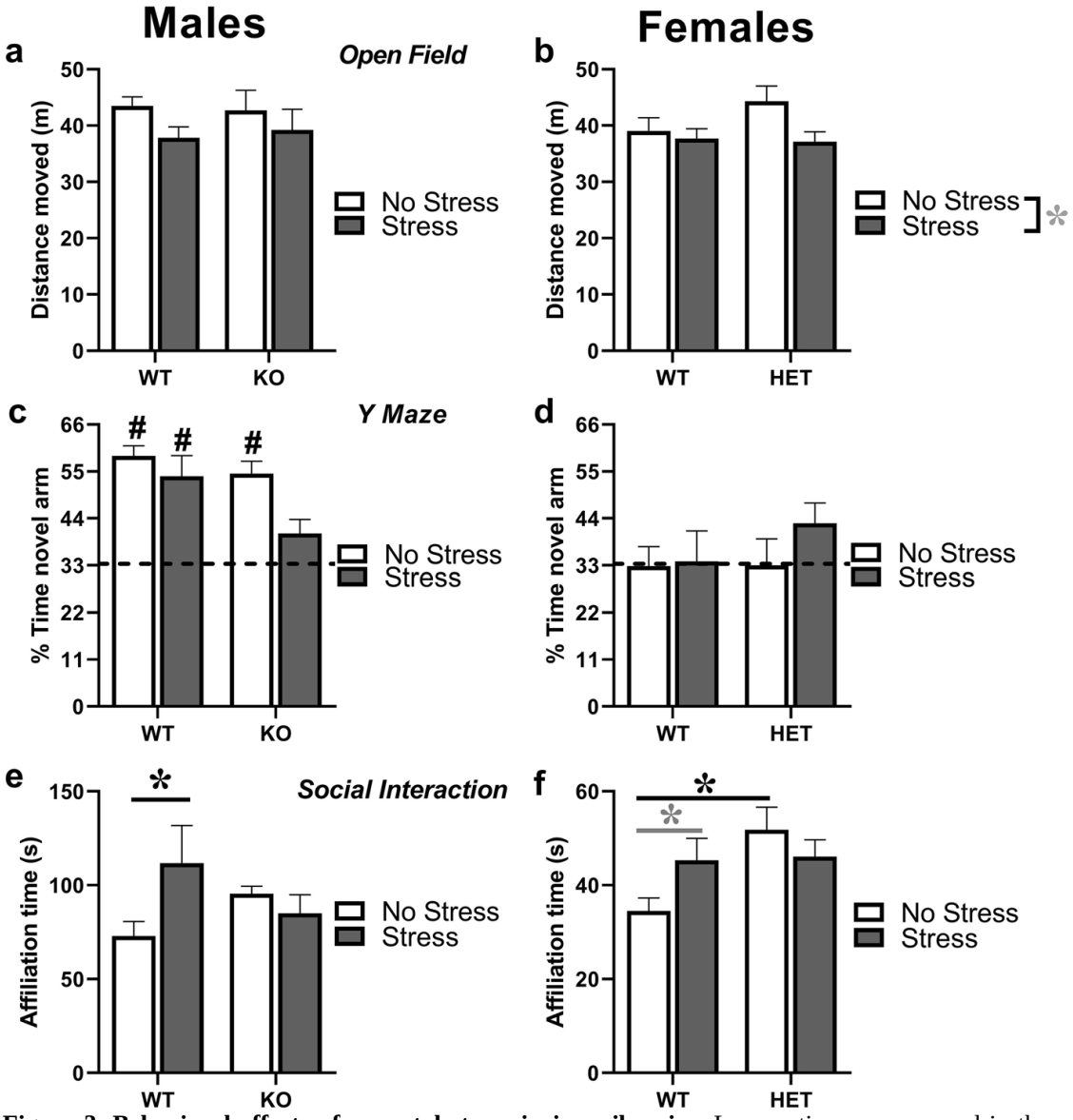
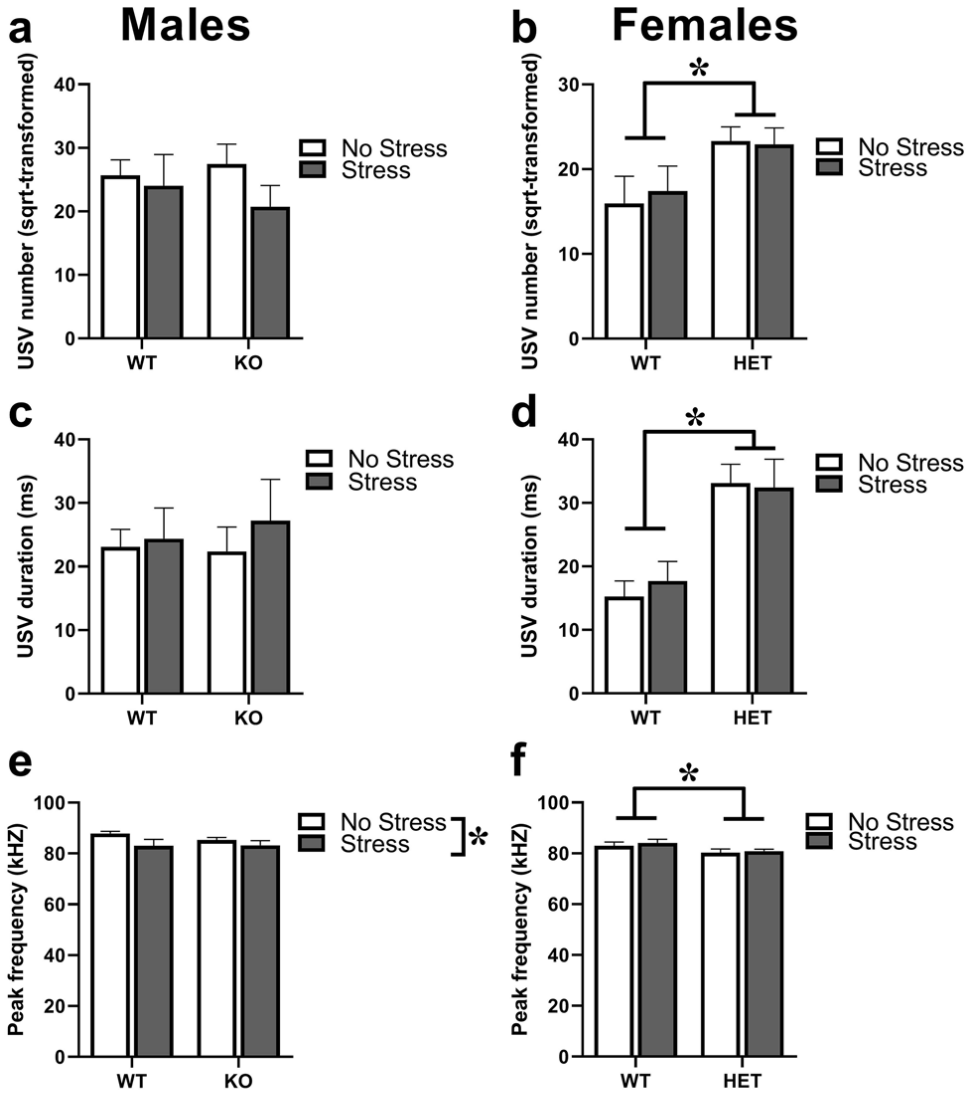


Figure 3. Behavioral effects of prenatal stress in juvenile mice. Locomotion was assessed in the open field test (**a,b**), while spontaneous alternation was evaluated in the Y maze (**c,d**). Social interaction was measured during a 3-min encounter with an adult NMRI WT female (**e,f**). *$p < 0.05$; * in grey $p = 0.06$; #$p < 0.05$ versus chance level (indicated by dotted line). N for males: 14 (**a,e**) or 13 (**c**) WT-no stress, 9 (**a,c,e**) WT-stress, 8 (**a,e**) or 9 (**c**) KO-no stress, 11 (**a,e**) or 10 (**c**) KO-stress; N for

females: 12 in all groups (**b,d,f**). KO refers to -/Y in males, HET to +/− in females. Data are expressed as mean ± SEM.

Ultrasonic vocalizations

In males, the number of USVs and their mean duration did not differ among experimental groups [genotype, stress effects and their interaction: all ns; **Figure 4a,c**]. Stress decreased the mean peak frequency in mice of both genotypes [$F_{(1,32)}$ = 4.50, $p < 0.05$; **Figure 4e**] and contribute to the emergence of a significant genotype difference in the mean peak amplitude, due to the highest values of WT-stressed mice [interaction genotype x stress: $F_{(1,30)} = 5.22$, $p < 0.05$; post-hoc: WT-no stress versus WT-stressed, $p < 0.05$; **Figure 4g**]. In females, HET mice emitted more and longer USVs compared to WT animals, and this effect was not altered by stress exposure [genotype effect on number (sqrt-transformed) and mean duration, respectively: $F_{(1,43)}$ = 6.65, 23.42, $p < 0.05$ and 0.0001 (**Figure 4b,d**); all other effect and interactions: ns]. USVs produced by HET females were also characterized by a significant lower mean peak frequency [genotype effect: $F_{(1,43)} = 5.38$, $p < 0.05$; **Figure 4f**] and by lower peak amplitude, but only under no stress conditions [genotype x stress interaction: $F_{(1,41)} = 4.81$, $p < 0.05$; post-hoc WT-no stress versus HET-no stress, $p < 0.05$; **Figure 4h**].



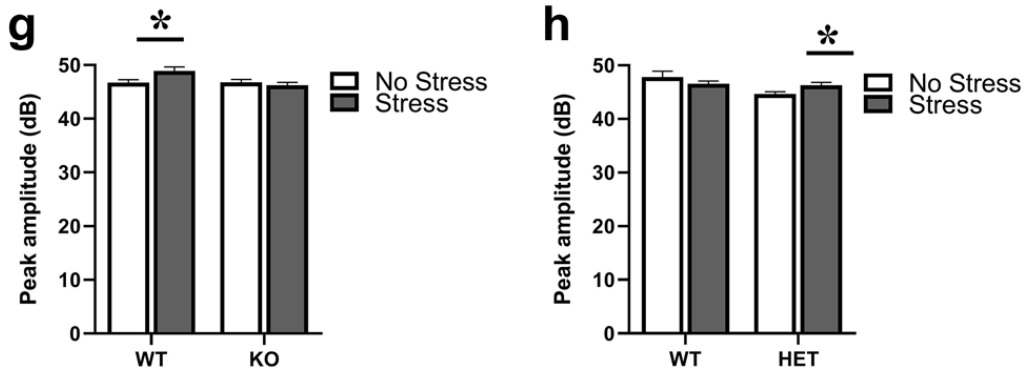


Figure 4. Effects of prenatal stress on ultrasonic communication in juvenile mice. USVs were assessed during the direct social interaction test with an adult NMRI WT female in Fmr1 mice of both sexes. The following parameters were measured through spectrographic analysis of the calls: total number (**a**,**b**), mean duration (**c**,**d**), mean peak frequency (**e**,**f**) and amplitude (**g**,**h**). The number of the calls was subjected to square-root (sqrt) transformation in order to meet the normality assumptions of parametric ANOVA. * $p < 0.05$. N for males: 10 (**a**,**c**,**e**,**g**) WT-no stress, 8 (**a**,**c**,**e**) and 7 (**g**) WT-stress, 8 (**a**,**c**,**e**) and 11 (**g**) KO-no stress, 10 (**a**,**c**,**e**,**g**) KO-stress; N for females: 11 (**b**,**d**,**f**,**h**) WT-no stress, 12 (**b**,**d**,**f**,**h**) WT-stress, 12 (**b**,**d**,**f**) and 11 (**h**) KO-no stress, 12 (**b**,**d**,**f**) and 11 (**h**) KO-stress. KO refers to -/Y in males, HET to +/− in females. Data are expressed as mean ± SEM.

The inspection of the density plots (**Figure 5**) extended the results previously obtained from the quantitative analyses of the ultrasonic spectrograms. In both males and females, stress tended to increase the occurrence of unusual long USVs (mean duration > 60 ms, **Figure 5**) an effect that appeared especially marked in mutant mice. In KO/HET-stressed mice there was an increased variability in the duration of the calls, an effect that was particularly dramatic in females (**Figure 5** lower panel).

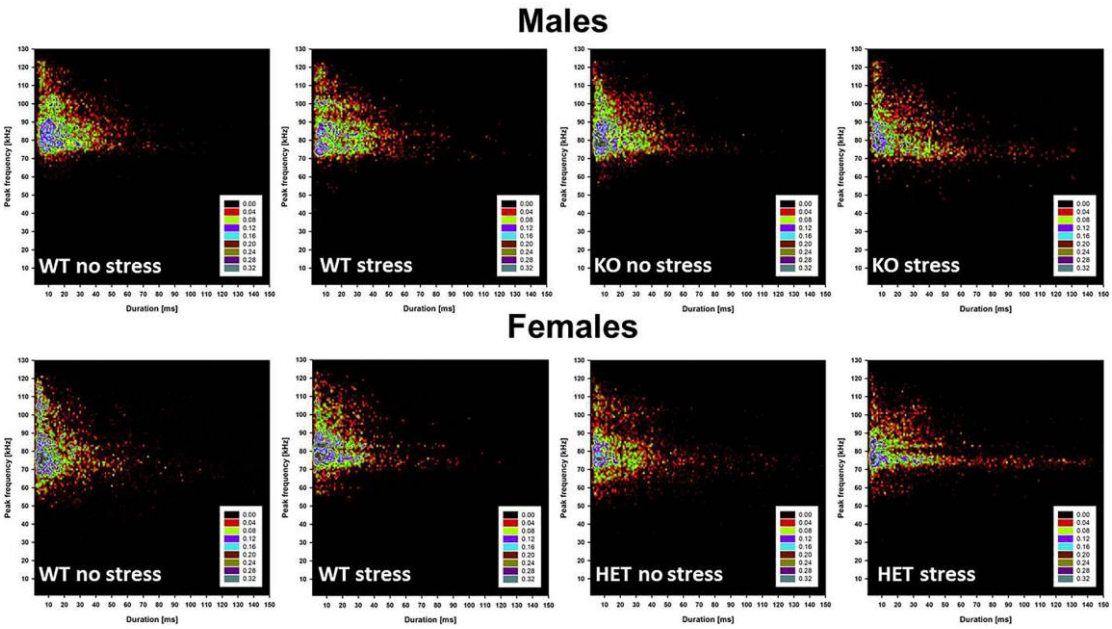


Figure 5. Density plots of individual ultrasonic calls. Density plots depict the distribution of individual USV emitted during 3-min social interaction with a NMRI adult stimulus female, plotted by frequency in kHz and duration in ms. Color coding reflects frequency in percentages.

The analysis of call subtypes (Caruso et al., 2020) revealed no major difference in the composition of the calls emitted by males [genotype, stress effects and their

interaction, all n.s.; **Figure 6**). In contrast, a clear genotype difference emerged in female mice, irrespectively of their stress conditions (**Figure 6**). Female Fmr1-HETs emitted less simple calls, i.e., based on one or two components [genotype effects, respectively: $F_{(1,42)}$ = 18.06 and 14.59, $p < 0.001$], and more complex calls, i.e., containing 3, 4, 5 or more components, than their WT littermates [genotype effects, respectively: $F_{(1,42)}$ = 57.21, 58.48, 35.16 and 26.26, $p < 0.0001$]. This is in line with the results of the density plots, since complex calls typically correspond to longer USVs.

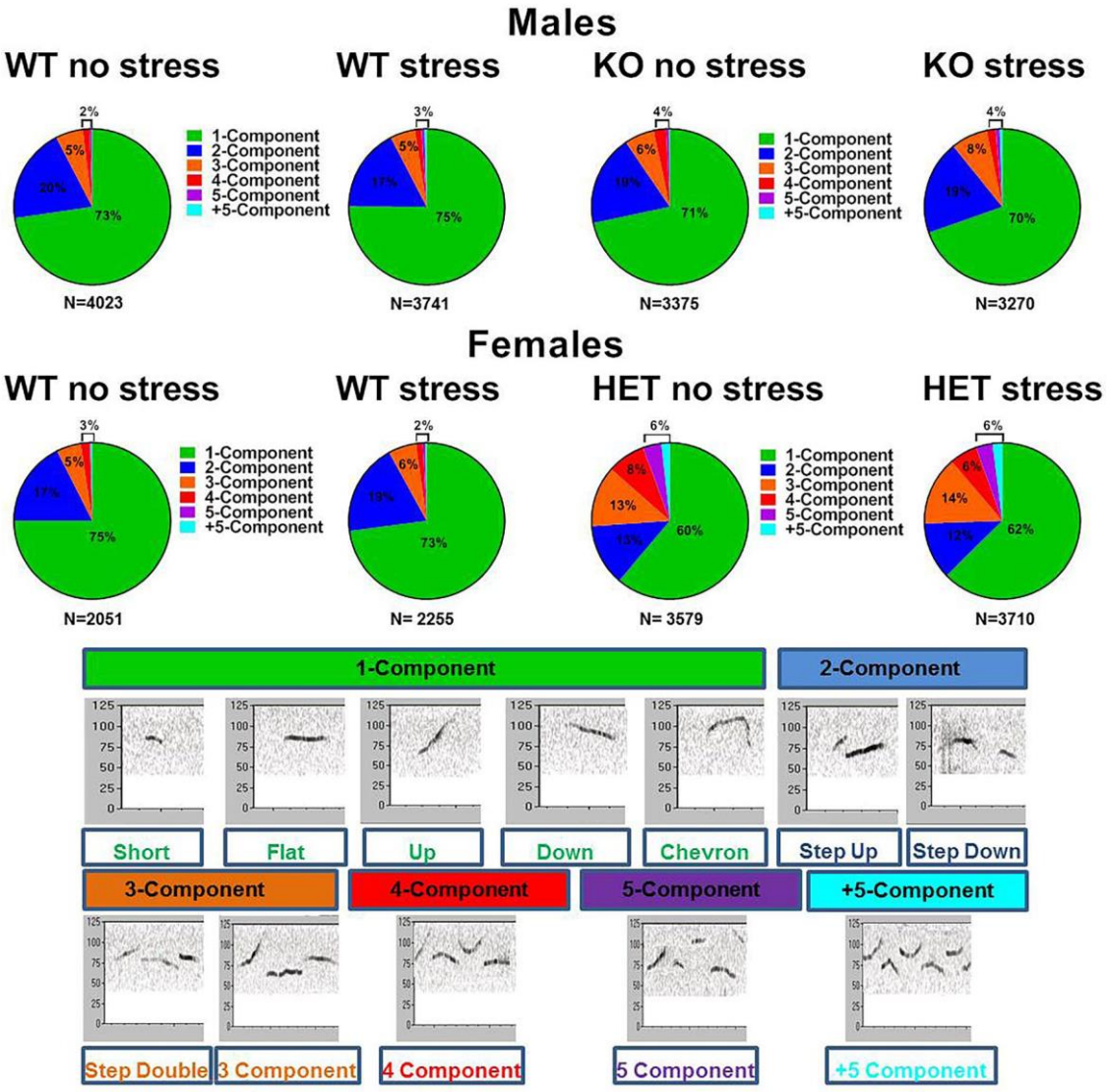


Figure 6. Composition of ultrasonic call types. Pie charts illustrate the different call types automatically classified by Sonotrack software. Call categories are expressed as percentages over the total number of USVs (N) for each experimental group.

Variables measured	♂		♀	
	KO genotype effect	Stress effect	HET genotype effect	Stress effect
Body weight (Fig. 2)	–	↑**only in WT**	–	↑ in WT and HET
Locomotion (Fig. 3a,b)	–	–	–	↓in WT and HET
Spontaneous alternation (Fig. 3c,d)	–	↓***only in KO***	–	–
Social interaction (Fig. 3e,f)	–	↑**only in WT**	↑ **in no stress**	↑ **only in WT**
Ultrasonic communication (Figs. 4, 5 and 6)	–	↓peak frequency in WT and KO ↑ **peak amplitude only in WT**	↑call number and duration, ↓peak frequency, ↑complex calls, ↓ simple calls	– – ↑ **peak amplitude only in HET**

Table 1. Summary of the results. All gene-environment interactions are marked in bold; italics bold refers to interactions inducing the emergence of a novel KO/HET phenotype (i.e., different from WT) under stressed conditions.

3.4 DISCUSSION

Our findings highlighted the impact of several gene-environment interactions on the behavioral phenotype of juvenile Fmr1 mutant mice that varies according to the sex of the animals. Overall, prenatal exposure to stress was able to induce several effects that were mostly dependent on sex differences and the considered behavioral domain. Our hypothesis, i.e., that stress exposure may advance/exacerbate the emergence of the behavioral alterations of Fmr1-KO mice was only partially confirmed, i.e., in the cognitive domain of spontaneous alternation and in male mice (**Table 1**).

As expected from previous reports (Pietropaolo and Subashi, 2014), our results confirmed that the behavioral phenotype of our juvenile Fmr1-KO mutants was almost undistinguishable from their WT littermates. This is the reason why we chose this testing age as it provided the optimal baseline conditions to evaluate a potential exacerbating/anticipating impact of prenatal stress avoiding floor or ceiling effects. In male KO mice, no alteration emerged in any of the considered behavioral domains under no stress conditions, supporting the view that FXS- and ASD-like behavioral abnormalities, such as hyperactivity, cognitive deficits and social alterations, appear only at adulthood (Pietropaolo and Subashi, 2014). In females, an hyper-social phenotype was the only one detected in our juvenile mutants, including enhanced affiliation levels, increased number of ultrasonic calls and their duration (with qualitative alterations). Once again, these results were in agreement with our previous reports: interestingly, these communicative and social abnormalities were observed only at the juvenile age as they disappeared in adult mutant females (Gauducheau et al., 2017). These hypersocial phenotypes may seem surprising in view of the ASD-like alterations shown by FXS patients, consisting mostly of social avoidance and reduced social interest. Nonetheless, the more abundant and longer USVs emitted by mutant juvenile females could be also interpreted as autistic-like phenotypes, since several studies have described excessive talking and repetitive speech as major autistic communicative alterations in FXS patients (see for example Wheeler et al., 2014). Furthermore, our analysis of the ultrasonic call types revealed a different composition of the USV repertoire of Fmr1-HET females (**Figure 6**), with a prevalence of complex multi-component calls compared to WT littermates. Although little is still known about the social meaning of different call types (Caruso et al., 2020), it is possible that Fmr1-HET females may emit more and longer USVs, but with less appropriate or adaptive communicative properties. The increased levels of affiliations could also be interpreted as an inappropriate social attitude since they are directed toward an intruder, i.e., a potential threat for the resident female. This testing context was indeed necessary to allow the detection of USVs in female mice (Moles et al., 2007). It is therefore still possible that a different social phenotype may appear in a different testing context, e.g., in a neutral environment; indeed, when Fmr1 mutant juvenile females were assessed for their social interest in the three compartments test no sign of increased sociability was observed (Gauducheau et al., 2017).

On this basis of genotype differences, prenatal exposure to stress was able to induce the appearance of a cognitive deficit in the spontaneous alternation Y maze test, although only in males. KO stressed male mice were indeed the only experimental group displaying a performance similar to the chance level (**Figure 3c**; **Table 1**). In

females, stress instead seemed to eliminate the hyper-social phenotype of mutant mice (**Figure 3e**) without affecting their ultrasonic communication profile (**Figure 4**). Nonetheless, these effects in females were actually due to a selective effect of stress in WT mice, rendering the WT phenotype similar to that of mutants. Hence, our data suggest that exposure to prenatal stress does not dramatically advance the appearance of pathological behavioral phenotypes in male and female mutants, juvenile stressed KO/HET mice being mostly comparable to their WT littermates, as in no-stress conditions. Indeed, with the exception of the Y maze effect in males, no selective effect of stress on mutant behavioral phenotypes was detected (**Table 1**). Our findings may therefore suggest a higher sensitivity of the cognitive domain to the effects of stress in the male sex, in line with clinical data describing a positive correlation between stress levels and cognitive deficits in FXS boys (Scherr et al., 2016). Nonetheless, additional memory tests other than the Y maze for spontaneous alteration would be useful to fully confirm the selective efficacy of prenatal stress in the cognitive domain, an issue that could be specifically addressed in future studies combining spatial and non-spatial memory tests.

Interestingly, stress did interact with genotype on several behavioral measures, but mostly by inducing its effects in WT mice only. This may suggest a reduced sensitivity of Fmr1-KO/HET mice to stress that could be interpreted as a deficit in the adaptive response to stressors, as already proposed by Qin and colleagues (2011). Previous studies have indeed described a reduced behavioral and endocrine sensitivity of adult Fmr1-KO mice (though only males were investigated) to the post-natal exposure to chronic stressors (Qin et al., 2011; Scherr et al., 2016). Here the genotype-specific effects of stress were characterized by clear sex differences: in males, stress enhanced body weight (**Figure 2a**), affiliative behaviors (**Figure 3e**) and peak amplitude (**Figure 4g**) in WT only, while it reduced peak frequency in both genotypes (**Figure 4e**). In females, stress enhanced affiliative behaviors in WT only (**Figure 3f**), while it enhanced body weight (**Figure 2b**) and reduced locomotion in both WT and mutant mice (**Figure 3b**). Furthermore, in female HETs stress increased USV peak frequency (**Figure 4f**). Overall, not the magnitude, but the behavioral specificity of the effects of stress differed between sexes, in line with most of the previous reports (Mueler et al., 2007; Advani et al., 2009; Schwendener et al., 2009; Hodes et al., 2015; Meng et al., 2020).

The promoting effects of stress on social interaction were observed in WT mice of both sexes and may be explained by multiple hypotheses. One possible explanation lies in the prosocial effects of increased oxytocin, since this has been described in hypothalamic and limbic brain regions following exposure to a variety of stressors (Takayanagi and Onaka, 2021). A second possible interpretation may consider the increased social interaction of WT stressed mice as a reflection of a the altered excitatory/inhibitory (E/I) imbalance induced by stress especially in brain circuits involving the prefrontal cortex (Sandi and Haller, 2015; Marchisella et al., 2021), known to critically control social behaviors in rodents (Gangopadhyay et al., 2021; Nakai et al., 2021). Our findings suggest that these potential changes in oxytonergic or E/I systems are in any case induced by stress only in WT mice, perhaps because of a reduced functionality of these adaptive mechanisms of stress response in our Fmr1 mutant animals.

Despite the overall agreement of the behavioral effects described in WT mice by our findings, an important difference between our and others' studies on prenatal stress should be underlined, that is, the genotype of our breeders exposed to prenatal stress. The dams exposed in our study to prenatal stress are indeed heterozygous Fmr1

mutant females and not WT as in previous similar studies: it is therefore possible that the sensitivity to stress of our female breeders may be different (as previously demonstrated for Fmr1-KO males with adult postnatal stress (Qin et al., 2011; Lemaire-Mayo et al., 2017)) and result in specific sex-dependent effects on the offspring behaviors. Studies comparing the behavioral and endocrine response to stress of Fmr1 mutant and WT dams should be performed in the future in order to clarify this issue; also, it would be interesting to evaluate the maternal behavior of stressed and no-stress dams to investigate whether the effects of stress on the Fmr1 offspring behavior could be mediated by alterations in the maternal care received. Similarly to other manipulations of the early environment (e.g., early enrichment (Branchi et al., 2009)), prenatal stress may induce its effects on the offspring both at the prenatal level, i.e., directly affecting pups' embryonic development, and during the early post-natal phase, i.e., interfering with normal mother–pup interactions and altering maternal behaviors (Moles et al., 2004b). In conclusion, our findings demonstrate for the first time the impact of prenatal stress on the juvenile FXS and ASD-like behavioral phenotype of Fmr1 mice, underlying the relevance of including sex differences and assessing multiple behavioral domains in mouse studies on FXS and ASD. These data therefore highlight the importance of complex gene-environment interactions in the etiopathology of neurodevelopmental disorders, also for a syndrome of clear genetic origins, such as FXS. The early timing of the stress exposure used here may be of critical relevance, since previous studies using post-natal chronic stress paradigms in the same mouse model showed less varied and marked effects on FXS-like neurobehavioral phenotypes (Qin et al., 2011; Lemaire-Mayo et al., 2017). Our results also focused on the juvenile age, which is critical for the early detection of behavioral abnormalities and their early therapeutic rescuing; this research focus could be extended in future studies by investigating the effects of prenatal stress on a longer term, for instance on the behavioral phenotype of Fmr1 mice at the adult age, i.e., when the behavioral alterations of mutants are more marked and well-established.

Summary of our results of USVs analysis in Fmr1 mutant mice

In basal condition:

- Fmr1-KO male mice displayed an altered ultrasonic communication in infancy with longer USVs than those emitted by WT pups. These alterations disappeared at adolescence (Gaudissard et al., 2017; Petroni et al., 2022) and adulthood (Gaudissard et al., 2017);
- No differences were observed in Fmr1 heterozygous female pups (Gauducheau et al., 2017), while at adolescence mutant females showed an increase in USVs number and duration (Gauducheau et al., 2017; Petroni et al., 2022). These alterations disappeared at adulthood, except for the increase in USVs duration that remained also later (Gauducheau et al., 2017). At qualitative level, mutant females emitted less simple calls than wild-type, showing a preference of long and complex calls (Petroni et al., 2022).

In stress conditions:

- Fmr1-KO adolescent male emitted calls with a reduced peak frequency (Petroni et al., 2022);
- Fmr1 heterozygous adolescent female produced calls with increased peak amplitude (Petroni et al., 2022).

4. Third paper:
Ultrasonic Vocalizations in Adult C57BL/6J Mice: The Role of Sex Differences and Repeated Testing

Authors: **Premoli M**, Petroni V, Bulthuis R, Bonini SA, Pietropaolo S.
Journal: Frontiers in Behavioral Neuroscience

Recent studies have developed several tools to analyze adult mouse USVs, especially in males, because of the increasing relevance of adult communication for behavioral phenotyping of mouse models of ASD. Little attention has been instead devoted to adult female USVs and impact of sex differences on USVs features. Most of the studies have also focused on a single testing session, often without concomitant assessment of other social behaviors (e.g., sniffing), so little is still known about the link between USVs and other aspects of social interaction and their stability/variations across multiple encounters. So, the aim of this study was to analyze USVs emitted by adult C57BL/6J (B6) male and female mice during 3 repeated encounters with an unfamiliar female, with equal or different pre- testing isolation periods between sexes. In addition, this study allowed for us to evaluate sex differences in several USV's characteristics and social behaviors.

4.1 INTRODUCTION

A large part of study on adult ultrasonic communication in mice have focused on USVs emitted by males, since these are more commonly employed than those by females for behavioral phenotyping of animal models of ASD and avoid the well-known impact of the estrous cycle on females' USVs (Moles et al., 2007). USVs produced by adult mice during male-female dyadic interactions are also the most extensively characterized, and they allow for easier identification of the emitting animal, since it has been demonstrated that in this context USVs are mostly produced by male mice to attract female mice (Sugimoto et al., 2011; Hammerschmidt et al., 2012b; Egnor and Seagraves, 2016). Nonetheless, USVs can also be emitted by adult females: recent studies have described that adult receptive female mice produced USVs either in the presence of male urines enriched with pheromones or in groups of four mixed-sex individuals (Neunuebel et al., 2015; Demir et al., 2020). However, in these studies, the female mice were assessed under experimental conditions maximizing the expression of their sexual interest, including being tested in a receptive estrous state, during the dark phase, and for long sessions (more than 30 min). Even under these conditions, the largest proportion of USVs registered during group interactions was produced by males (Neunuebel et al., 2015). Instead, female mice show their most prominent vocalizing abilities during adult female-female dyadic interactions, and in these cases USVs are used to establish affiliative relationships (Moles and D'Amato, 2000; Moles et al., 2007; Zala et al., 2017). In particular, Moles and D'Amato (2000), Moles et al. (2007) studied USVs of adult female mice in a resident-intruder setting, i.e., the resident being isolated in the testing cage 3 days before assessing the USVs with a female intruder. They demonstrated that in these experimental settings most of the USVs are uttered by the resident and suggested that the calls can facilitate proximity with the intruder and reduce its potential aggressiveness. Female USVs in this context can be also used as an index of sociability and social memory, since (i) a strong positive correlation was found between the number of calls and the time spent by the resident female mouse sniffing the intruder, and (ii) a marked decline was observed in the number of USVs

emitted by a resident female mouse when exposed multiple consecutive times to the same female intruder. USVs are mostly emitted during close contacts and approach behaviors in female-female interactions and male-female encounters (Ferhat et al., 2015, 2016). Nonetheless, the precise link between multiple USV characteristics and other social behaviors is still not fully understood in adult female mice, as in male mice.

Several studies have tried to analyze differences in USVs between male and female mice (Hammerschmidt et al., 2012b; von Merten et al., 2014; Zala et al., 2017; Matsumoto and Okanoya, 2018; de Chaumont et al., 2021), also with novel technical approaches such as deep learning networks (Ivanenko et al., 2020), yielding to the emergence of a variety of either quantitative or qualitative differences (or both) without, so far, a univocal pattern. Divergences among these studies mainly arise from differences in testing procedures, e.g., sex of the stimulus animal and pre-testing isolation conditions. The sex of the "receiver" is known to critically modulate several characteristics of USVs of the "emitter" in mice of both sexes (Zala et al., 2017); for instance, quantitative sex differences in USVs were described between female-female and male-female interactions (von Merten et al., 2014), while both quantitative and qualitative differences were observed in same-sex interactions (de Chaumont et al., 2021). Also, most of the studies on female USVs have applied relatively long periods (more than 24 h) of pre-testing isolation in order to induce a resident status in the subject and assure the identification of the emitter (Moles et al., 2007; Hammerschmidt et al., 2012b). In contrast, pre-testing isolation is not commonly applied in USV studies on adult male mice, as this manipulation is not necessary to induce their USV production. In general, isolation is known to alter USV emission in adult mice (Lefebvre et al., 2020; Zhao et al., 2021) and to modulate the correlation between USVs and other social behaviors (Chabout et al., 2012). Finally, most of the studies on sex differences in USVs have employed single testing sessions or multiple repeated sessions but with the same social stimulus in order to assess habituation (Moles et al., 2007). Hence, it is not clear whether sex differences in ultrasonic communication may be dependent on testing experience or are a stable trait across multiple social encounters with an unfamiliar stimulus. Here, we performed an extensive quantitative and qualitative characterization of sex differences in USVs emitted by adult C57BL/6J (B6) mice. To this end, we compared the USVs uttered either by an adult male or female toward the same type of stimulus, i.e., an adult CD1 female. The female mice were isolated for 3 days before testing in order to acquire the status of resident, i.e., becoming the major emitter of USVs during interaction with a female intruder. Their USVs were compared with those of males that were either isolated for the same duration before testing (study 1) or only habituated to the testing cage for 10 min before tests (study 2). These two studies allow us to evaluate sex differences respectively (1) in the same resident-intruder settings, thus controlling for isolation effects and (2) using the most common (and practically more suitable) experimental settings for USV assessment used in previous studies with ASD mouse models, i.e., in the resident-intruder context for females and without pre-testing long isolation in males (Pietropaolo et al., 2011, 2014; Hebert et al., 2014; Oddi et al., 2015; Gaudissard et al., 2017; Gauducheau et al., 2017; Fyke et al., 2021). In both studies, three subsequent social tests with a novel intruder were performed with an interval of 7–10 days in order to evaluate the potential stability of sex differences and their dependency on previous testing experience without confounding effects of social memory. For each encounter, social affiliative behaviors were also assessed in order to evaluate their potential link with USV

changes. Estrous cycle phases were assessed for experimental female subjects and female stimuli before each social encounter to control for potential hormonal modulation of social interaction and communication (Moles et al., 2007; Hanson and Hurley, 2012; Egnor and Seagraves, 2016; Kim et al., 2016). We chose B6 mice as the experimental subjects of our study because of the well-known relevance of this strain for behavioral neuroscience due to its large use for engineering genetically modified mouse lines. Stimulus females for all social tests were instead chosen from the CD1 strain because of its common use in social studies (Moles and D'Amato, 2000; Moles et al., 2007), especially those using genetic mouse models of ASD (Hebert et al., 2014; Pietropaolo et al., 2014; Oddi et al., 2015; Gaudissard et al., 2017; Gauducheau et al., 2017; Lemaire-Mayo et al., 2017; Fyke et al., 2021). This strain is preferentially employed in social interaction tests, since it is characterized by high levels of sociability and it facilitates behavioral analysis during social encounters with B6 animals because of its albino phenotype.

4.2 MATERIALS AND METHODS

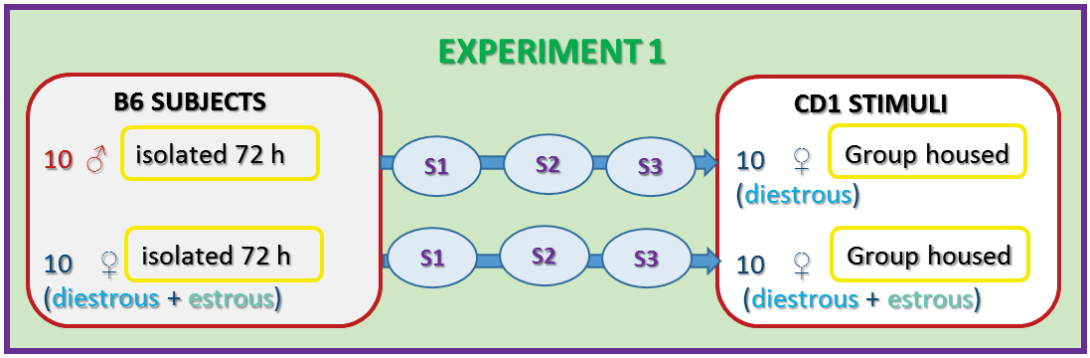
4.2.1 Ethics approval

All the experimental procedures were performed in accordance with the European Communities Council Directive 2010/63/EEC and approved by the Local Ethical Committee ("Comité d'Ethique pour l'experimentation animale de Bordeaux", CE 50) and the French Ministry ("Ministere de l'enseignement superieur de la recherché et de l'innovation").

4.2.2 Animals and Housing Conditions

Forty adult male and female C57BL/6J mice (10–12 weeks old, n = 10 per sex in each experiment), used as experimental subjects, and forty adult CD1 females, used as social stimuli, were purchased from Janvier (Le Genest St Isle, France). Upon arrival at the animal facility at Bordeaux University they were all housed in same-sex and same–strain groups of 5 individuals in standard polycarbonate cages and provided with sawdust bedding enriched with cotton nestlets. Food chow and water were provided *ad libitum*. The animals were maintained in a temperature- (22◦C) and humidity- (55%) controlled vivarium under a 12:12 h light–dark cycle (lights on at 7 a.m.). The mice were left undisturbed for 2 weeks upon their arrival before the behavioral tests began.

As illustrated in **Supplementary Figure 1**, two separate groups of mice were used for the two experiments of the study, each consisting of 20 B6 experimental subjects (10 male mice and 10 female mice) and 20 CD1 stimulus females.



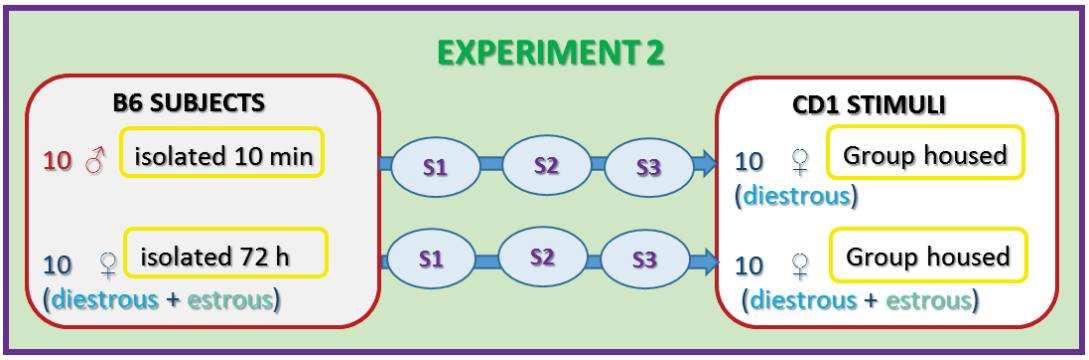


Figure S1. Schematic representation of the experimental procedures. B6 female and male mice encountered CD1 stimulus females during 3 testing sessions of 3 min each (S1-S3). Experiment 1 investigated sex differences in social behaviors and USVs in the same resident-intruder conditions, i.e., after 72 h isolation. Experiment 2 intended to assess sex differences using procedures commonly used to assess USVs in mouse models of ASD, i.e., in resident-intruder settings for females and with minimal isolation in the testing cage for males (i.e., 10 min habituation).

The CD1 female mice were all naïve to social experience with B6 mice at the time of the first testing session; each female stimulus was employed for a total of 3 times for each experiment but only once for each testing session. B6 mice of either sex encountered a novel female at each session. Separate batches of CD1 female mice were employed for male-female and female-female interactions in each experiment, so that a CD1 stimulus encountered either B6 female or male mice during the 3 sessions. *In experiment 1*, both male and female B6 subjects were single-caged for the same time (72 h) in the test cage before each testing session to assess social behaviors and USVs: this allowed for us to assess sex differences in the same resident-intruder settings and pretesting social isolation conditions. *In experiment 2*, the male mice were subjected to 10 min of isolation in the test cage, and their social behavior was compared with that of female mice exposed to 72-h pretesting isolation: this comparison served to evaluate sex differences under conditions that are commonly employed to assess male and female USVs in ASD mouse studies (Hebert et al., 2014; Pietropaolo et al., 2014; Oddi et al., 2015; Gaudissard et al., 2017; Gauducheau et al., 2017; Lemaire-Mayo et al., 2017; Fyke et al., 2021) and that are also more suitable for male behavioral assessment. USVs can in fact be also evaluated in male-female interactions without inducing a resident state in the male (e.g., Hebert et al., 2014; Oddi et al., 2015; Gaudissard et al., 2017; Lemaire-Mayo et al., 2017; Fyke et al., 2021), thus avoiding applying a social isolation period of at least 72 h that could interfere with several other behaviors. In contrast, female mouse USVs are most commonly assessed in a resident-intruder setting, at least in female-female dyadic interactions. In both experiments, after each testing session, the experimental mice were re-housed in groups with the same cagemates. The CD1 stimulus mice were kept under grouped conditions during the entire duration of the study.

4.2.3 Behavioral Testing

Behavioral testing was carried out during the light phase of the cycle. Social behavior and ultrasonic communication were assessed in a 33 × 15 × 14 cm plastic cage with 3 cm of sawdust and a metal flat cover during 3 testing sessions of 3 min each and with an interval of 7–10 days. In experiments 1 and 2, the female B6 subjects were isolated in the testing cage for 72 h prior to testing in order to induce the status of

resident in the adult female mice and therefore promote the emission of ultrasonic vocalizations toward an adult female intruder (Moles et al., 2007). The male B6 subjects were isolated either for 72 h (experiment 1) or for 10 min (experiment 2) in the testing cage before each social encounter. In all the experiments, an unfamiliar adult female CD1 stimulus was then introduced into the testing cage of either male or female subjects and left there for 3 min. Previous studies alternately anesthetizing each pair member have shown that in these experimental settings adult stimulus females do not emit USVs that are instead mostly uttered by the experimental male (Whitney et al., 1973; Maggio and Whitney, 1985) or the resident female (Maggio and Whitney, 1985; D'Amato and Moles, 2001). The lack of concomitant emission of USVs by the two interacting animals was indeed confirmed here by additional inspection of spectrograms, excluding the presence of "double calls", i.e., overlapping in their timing, but with different, non-harmonic characteristics (e.g., different peak and mean frequency, modulation). After each testing session of both experiments, the experimental and stimulus mice were returned to their home cages and kept with their original cagemates until the subsequent testing session.

The testing sessions were recorded with a camera placed on the side of the cage, and videos were analyzed with Observer XT (Noldus, The Netherlands). One observer who was unaware of the sex and experimental assignment of the animals scored the behavior of the test B6 mice only, quantifying the time spent performing the following behaviors (Pietropaolo et al., 2011, 2014; Oddi et al., 2015; Gaudissard et al., 2017; Gauducheau et al., 2017): - affiliative behaviors: nose/anogenital/body sniffing and contact with the partner by crawling over/under from one side to the other or allogrooming (grooming the stimulus mouse) - nonsocial activities: rearing, exploring the cage (locomotion and wall rearing), digging, grid-climbing and self-grooming.

An ultrasonic microphone, UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics, Berlin, Germany), was mounted 2 cm above the cover of the testing cage; it was connected via an UltraSoundGate 116 USB audio device (Avisoft Bioacoustics) to a personal computer, with which acoustic data were recorded with a sampling rate of 250 kHz in 16-bit format with Avisoft Recorder (version 2.97; Avisoft Bioacoustics). The recordings were then transferred to Avisoft SASLab Pro (Version 5.20; Avisoft, Berlin, Germany) and Fast Fourier transformation was applied (512 FFT length, 100% frame, Hamming window, and 75% time window overlap). Spectrograms were generated with Avisoft at a frequency resolution of 488 Hz and a time resolution of 0.512 ms. Signals below 30 kHz were cut to reduce background noise to 0 dB (Premoli et al., 2019). For USV detection, an interactive function with section labels was used. This tool permits to define manually USV borders by inserting section labels, and it is useful when automatic threshold-based USV separation may not work satisfactorily because of ambient noise or because of poorly structured vocalizations (manual guide of Avisoft Bioacoustics). Number, mean duration, peak frequency, and peak amplitude were calculated for each vocalization together with the calling time of the mice based on previous studies (Wöhr et al., 2011b). Call subtypes were also determined for a more detailed qualitative analysis. For this purpose, USVs were automatically classified with the Sonotrack Call Classification (version 1.4.7, Metris B.V., The Netherlands) software, using the categories described in detail in **Figure 1**, based on previous literature on mouse USVs (Caruso et al., 2020). To deal with background noise and artifacts in the ultrasound recordings, the Sonotrack Call Classification software applies various filters to remove unwanted signals such as white noise and artificial sound sources. In

addition, a logic filter is used that further processes the recorded signal by removing sounds that are too short or appear at many frequencies at the same time. The logic filter also reduces echo that is found in the recording and merges spectral elements that are interrupted by a very short time and a frequency gap.

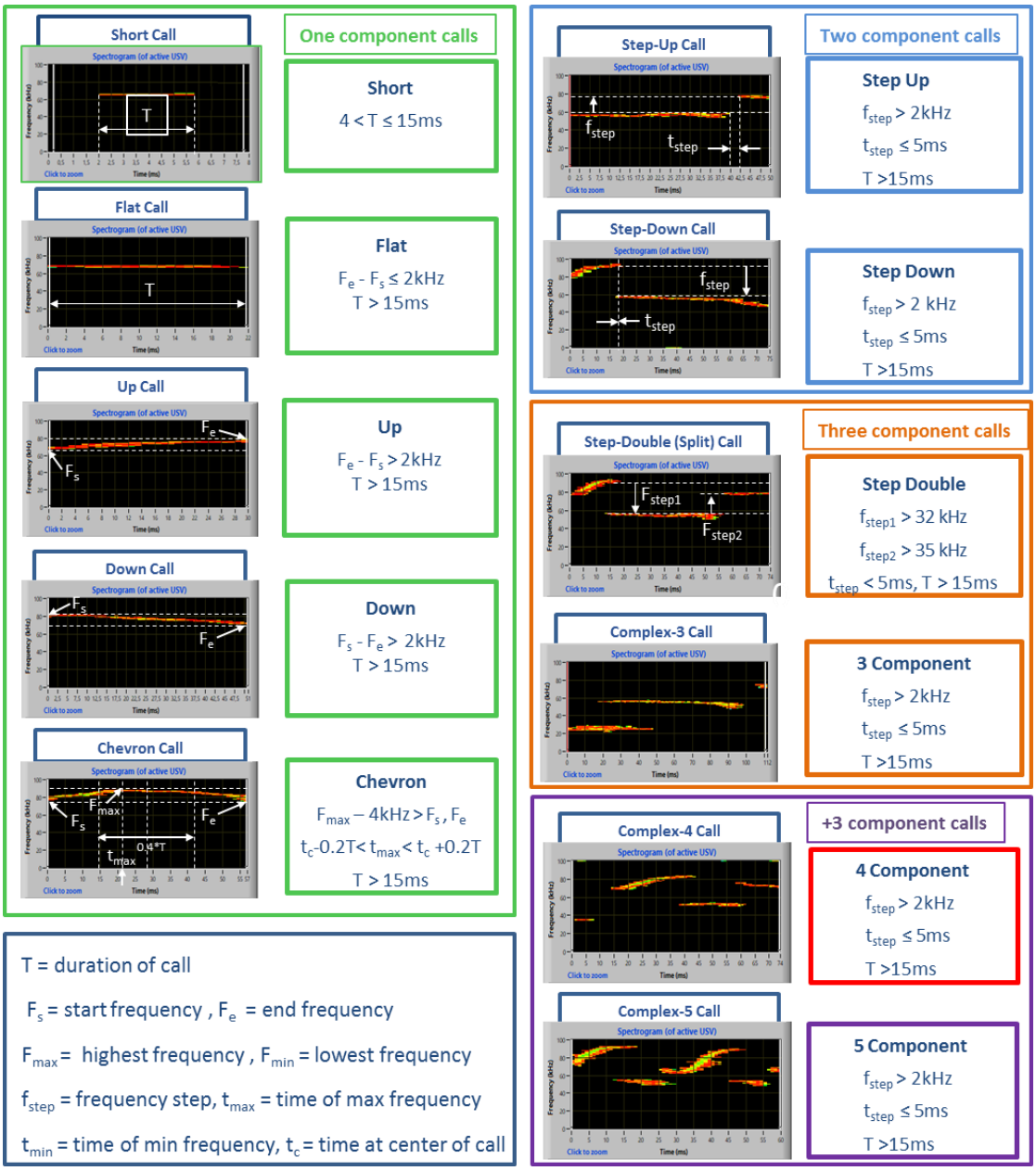


Figure 1. Examples of ultrasonic call types used to classify USVs in the study. The call types were automatically classified using the software Sonotrack and based on the parameters described above. Definitions of the call types were mutually exclusive. Overlap of components was removed when more than 70 % to prevent wrong call durations. Short gaps between components in both frequency (≤6 kHz) and time (≤5 ms) were interpolated (gaps can be caused by changes in microphone sensitivity or direction of vocalization). Complex "3 component" and "+3 component" calls were summed up into a "complex tot" category.

On each testing day, the vaginal estrous phases of both testing and stimulus female mice were assessed from the analysis of their vaginal smears (Caligioni, 2009). In both experiments, all stimulus females used for male-female interactions were in

non-receptive diestrous phase in order to minimize mounting attempts and other sexual behaviors that could confound the evaluation of sex differences in USVs and social behaviors. The stimulus females for female-female interactions were either in diestrous (non-receptive) or estrous (receptive) phase, and their assignment was counterbalanced depending on the estrous phase of the experimental subjects (i.e., approximately half of the intruders in the estrous phase encountered a resident in estrous and the other half was assigned to a resident in diestrous; the same design was applied to the intruders in diestrous). The estrous phases of the female residents and intruders for each testing session of female-female interactions are illustrated in **Table 1**.

Experiment	Estrous phase	Session 1		Session 2		Session 3	
		estrous	diestrous	estrous	diestrous	estrous	diestrous
1	resident	5*	5	4*	6	4*	6
1	intruder	5	5*	4*	6	5*	5
2	resident	4	6	7	3	7	3
2	intruder	5	5	6	4	5	5

Table 1. Estrous phase of the resident B6 female mice and the CD1 stimulus female intruders for each testing session. *One female B6 was excluded from the analysis of social behaviors because she was a statistical outlier on the time spent in affiliative behaviors (based on Grubbs' ESD test).

4.2.4 Statistical Analyses

Normality of data distribution was confirmed by Shapiro-Wilks test for each sex and testing session and for each variable of interest. Behavioral data from each experiment were separately analyzed by ANOVA with sex as the between-subject factor and testing session as the within-subject factor. Furthermore, behavioral data from the female mice in each testing session were subjected to an additional ANOVA with the estrous phase (estrous or diestrous) of the experimental B6 female and the estrous phase (e.g., **Supplementary Figures 2, 3**) of the stimulus CD1 female as the between subject-factors. The analysis of the data from male mice did not include the estrous phase of the stimulus, since all CD1 females selected for testing males were in diestrous phase. A further ANOVA with experiment as the additional between-subject factor was performed on the data from female mice only in order to quantify the replicability of the female phenotype (under the same testing conditions) across the two experiments. Post-hoc comparisons (Fisher's LSD test) and separate ANOVAs were performed when appropriate. All the analyses were conducted using the software Statview and SPSS, and α was set at 0.05. The data were inspected for exclusion of outliers (by Grubbs' ESD test adapted for small sample size). Outlier values were excluded only from a specific dataset (e.g., body sniffing time on session 1), except for the analysis of repeated measures, when values for all the 3 sessions had to be excluded for the affected variable. The results are expressed as mean ± SEM throughout the text. Individual data of social behaviors and USV parameters are also provided for all the animals in **Supplementary**

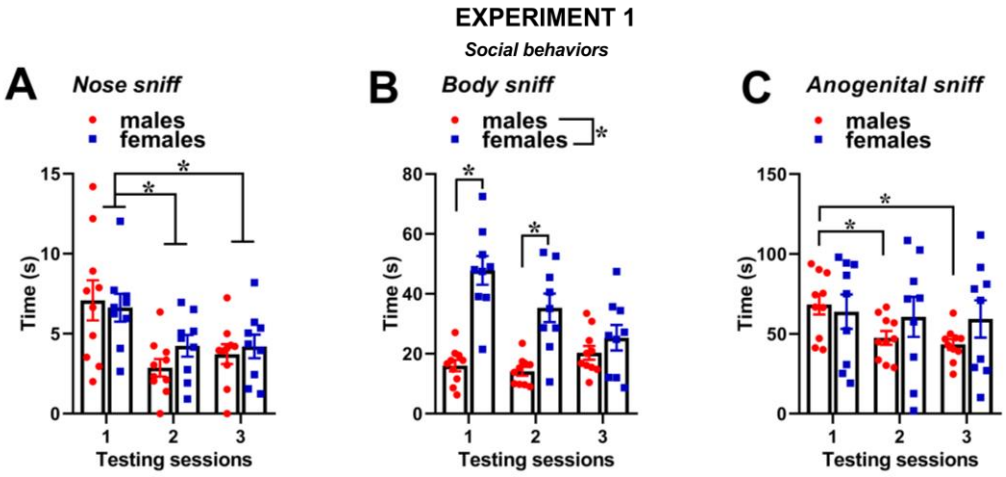
Figures 4, 5; in addition, the individual composition of call types is illustrated in **Supplementary Figures 6, 7** for half (i.e., 5 over 10) of individuals for each sex emitting the higher rates of USVs in each experiment.

4.3 RESULTS

Experiment 1: Same Pre-Testing Isolation Time in Both Sexes

Sex Differences: Social Interaction and USVs

Social behaviors were overall more markedly expressed in the female mice than in the male mice and tended to decrease with testing sessions; furthermore, sex differences depended on specific type of considered social behavior (**Figures 2A–E**). The time spent performing nose sniffing was overall similar in mice of both sexes and tended to decrease with repeated testing sessions [sex effect and its interaction, n.s., session effect: $F_{(2,34)} = 10.18$, $p < 0.001$, **Figure 2A**]. The female mice displayed more body sniffing than the male mice [sex effect: $F_{(1,17)} = 22.31$, $p < 0.001$, **Figure 2B**], and this effect was mostly observed during the first 2 sessions, since on the 3rd encounter body sniffing decreased in the female mice but increased in the male mice [interaction sex × session: $F_{(2,34)} = 18.59$, $p < 0.0001$, **Figure 2B**; post-hoc: $p < 0.05$]. The time spent performing anogenital sniffing did not differ overall between sexes, but it decreased with testing sessions in the male mice only [overall interaction sex × session: $F_{(2,34)} = 2.78$, $p < 0.07$, session effect on the male mice: $F_{(2,18)} = 14.68$, $p < 0.01$; on the female mice: ns, **Figure 2C**]. The female mice showed a tendency to display more affiliative behaviors than the male mice and explored significantly less the testing cage than males [sex effect, respectively: $F_{(1,17)} = 3.22, 6.43$, $p = 0.09$ and $p < 0.05$; **Figures 2D,E**]. In mice of both sexes, the levels of affiliative behaviors tended to decrease with testing sessions while those of cage exploration increased [session effect, respectively: $F_{(2,34)} = 25.01$ and 10.26, $p < 0.001$; **Figures 2D,E**].



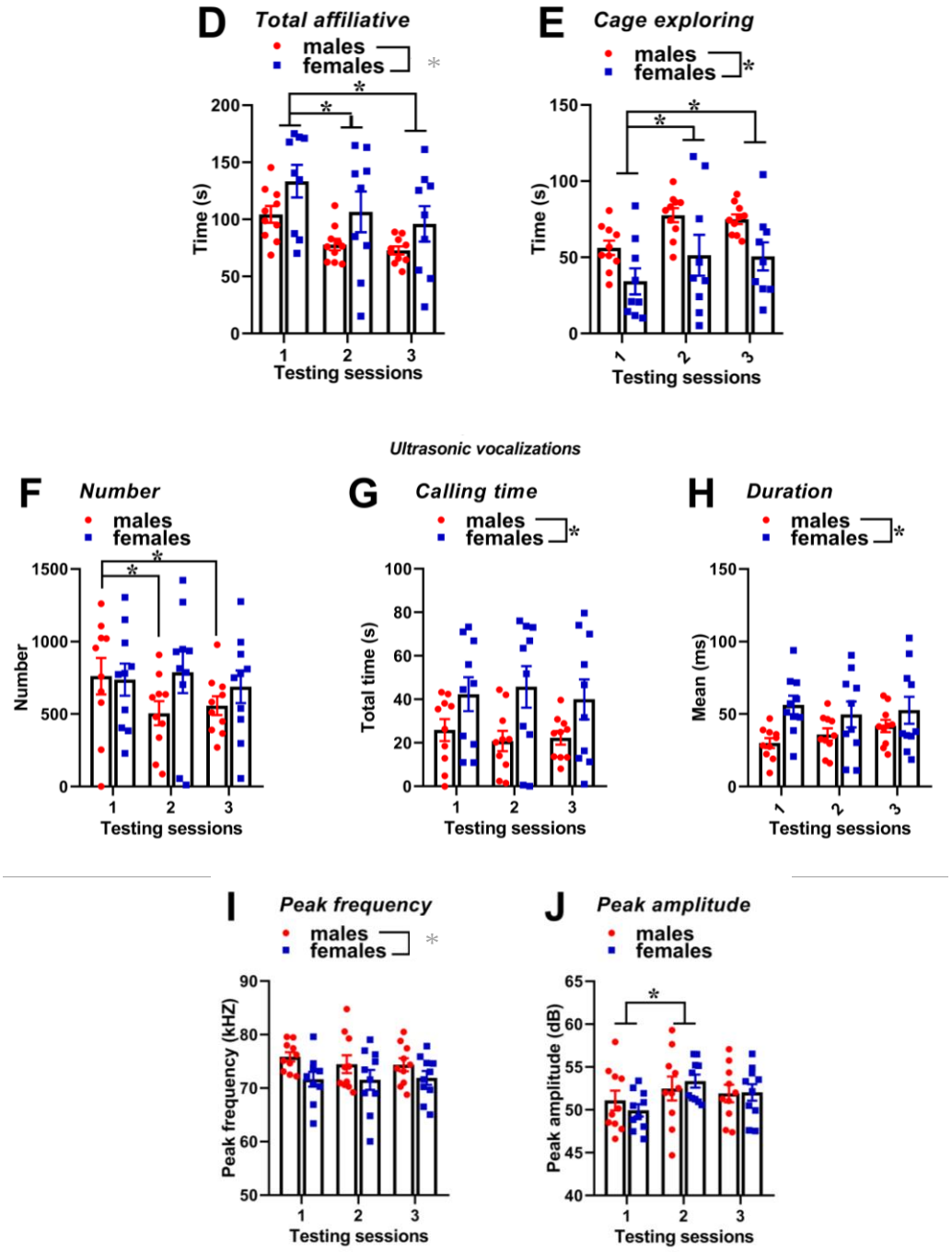
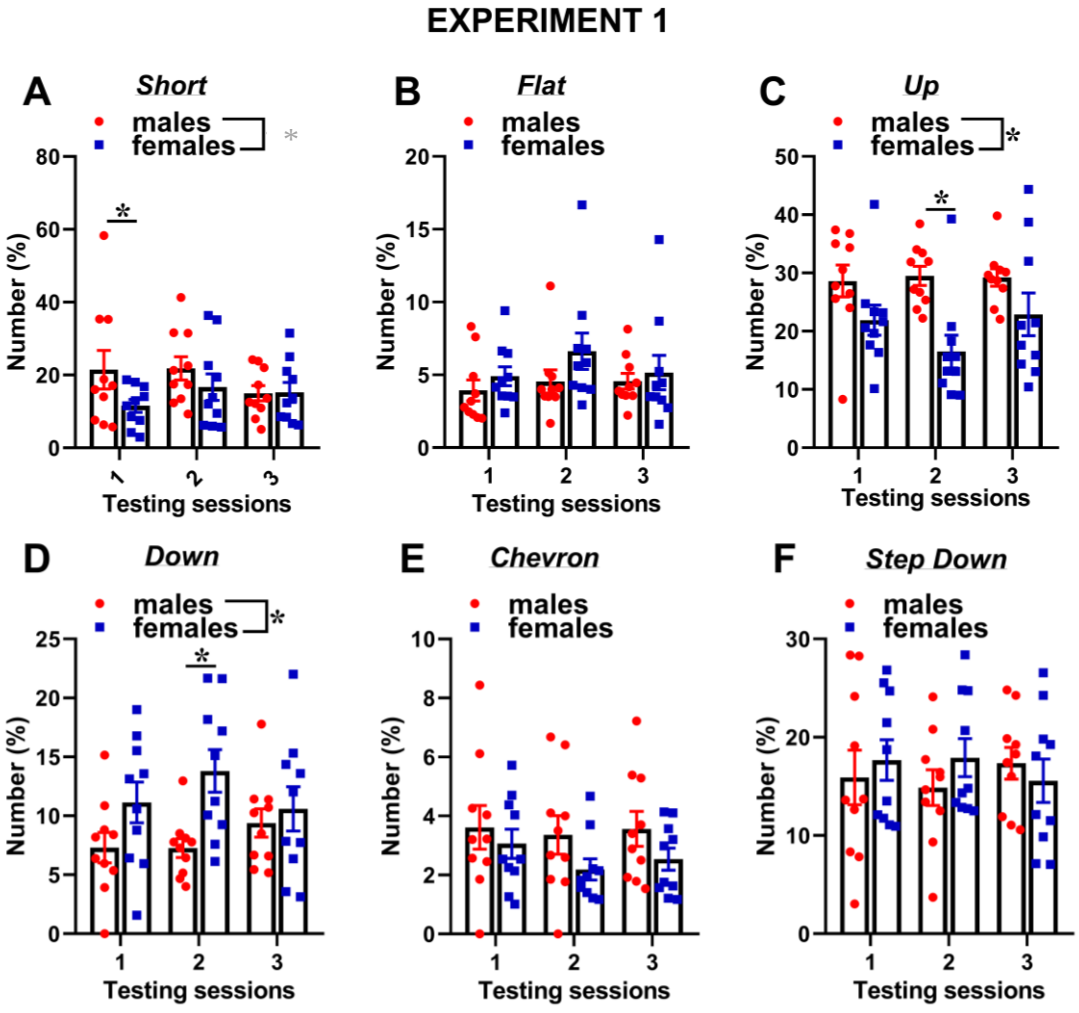


Figure 2. Sex differences in social behaviors and USVs in experiment 1. All behaviors were scored in male and female experimental B6 subjects (**A-E**) towards a stimulus CD1 female during 3 testing sessions of 3 min each. Data are mean ±SEM. N=10 for males and 9 for females (one mouse was excluded since it was a statistical outlier in affiliative time). For USVs (F-J): N=10 per sex. *p<0.05. * refers to a non-significant tendency (0.05<p≤0.09).

Several characteristics of USVs differed between the two sexes (**Figures 2F–J**). Although the number of USVs emitted was not significantly different (**Figure 2F**), the total calling time and the mean duration were higher in the female mice [sex effect, respectively: $F_{(1,18)}$ = 4.85 and 4.44, p < 0.05; **Figures 2G,H**], while the peak frequency tended to be lower than in the male mice [sex effect: $F_{(1,18)}$ = 3.62, p =

0.07; **Figure 2I**]. All the USV parameters did not significantly change across the testing sessions, with the exception of peak amplitude that increased from the first to the second session in mice of both sexes [session effect: $F_{(2,36)} = 4.27$, $p < 0.05$; **Figure 2J**]. Although the interaction sex × session did not reach statistical significance, it was evident that the number of USVs decreased across the testing sessions in the male mice only [separate ANOVAs on the male mice: $F_{(2,18)} = 9.59$, $p < 0.01$; in the female mice:, ns; **Figure 2F**].

The types of ultrasonic calls differed between sexes, and this pattern of results seemed more evident on the first 2 testing sessions (**Figures 3, 4**). The female mice tended to emit less "short" calls on the first testing session than the male mice [interaction sex × session: $F_{(2,36)} = 2.73$, $p = 0.08$, sex effect on session 1: $F_{(1,18)} = 3.17$, $p = 0.09$; **Figure 3A**]. Especially during the second session, the female mice also produced overall less "up" calls [sex effect: $F_{(1,18)} = 7.27$, $p < 0.05$; interaction sex × session: $F_{(2,36)} = 3.05$, $p = 0.06$, sex effect on session 2: $F_{(1,18)} = 16.01$, $p < 0.001$; **Figure 3C**] and more "down" calls [sex effect: $F_{(1,18)} = 4.91$, $p < 0.05$; interaction sex × session: $F_{(2,36)} = 3.2$, $p = 0.05$, sex effect on session 2: $F_{(1,18)} = 10.93$, $p < 0.01$; **Figure 3D**]. The female mice also emitted more "step double and more "complex" calls with 3 or more components [sex effect, respectively: $F_{(1,18)} = 11.44$ and 7.3, $p < 0.05$; **Figures 3H,I**].



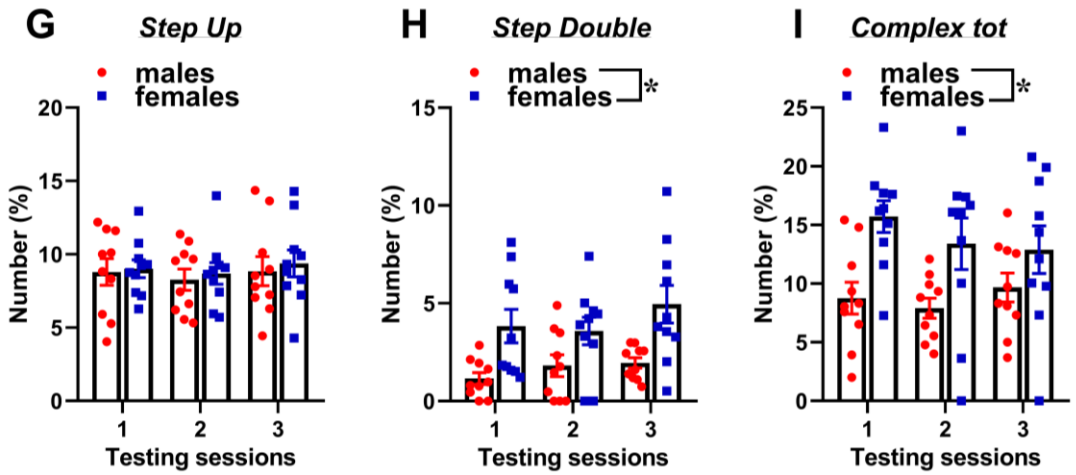


Figure 3. Sex differences in ultrasonic call types in experiment 1. (A–I) The different call types were automatically classified as detailed in Figure 1. Complex tot = complex 3 + complex 4+ complex 5. Data are expressed as percentages over the total number of USVs for each sex and session. Data are mean ± SEM. N = 10 for each sex. *p < 0.05. * Refers to a nonsignificant tendency (0.05 < p ≤ 0.09). Sex differences are reported as * in each graph legend when a significant main effect of sex was detected in the absence of any interaction with testing session.

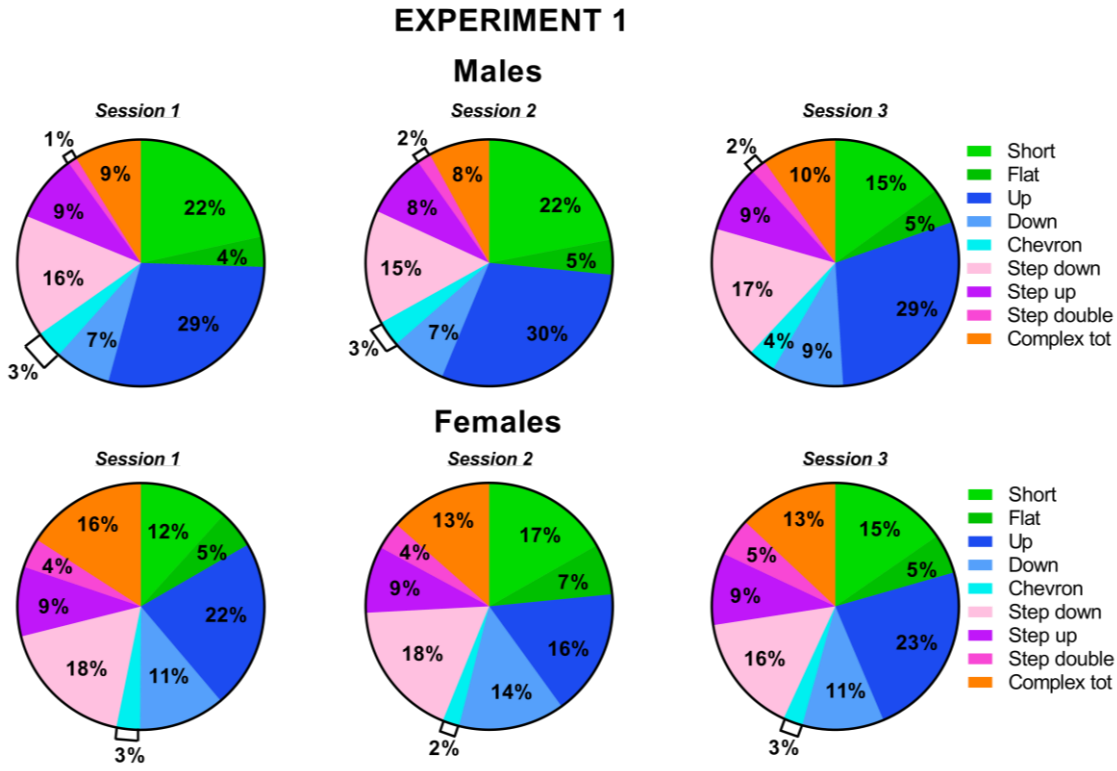


Figure 4. Pie charts depicting sex differences in ultrasonic call types in experiment 1. Distribution of call categories in each sex and testing session. Data are expressed as percentages over the total number of USVs for each sex and session. Data are mean ± SEM. N = 10 for each sex. Complex tot = complex 3 + complex 4 + complex 5 (refer also to Figure 1).

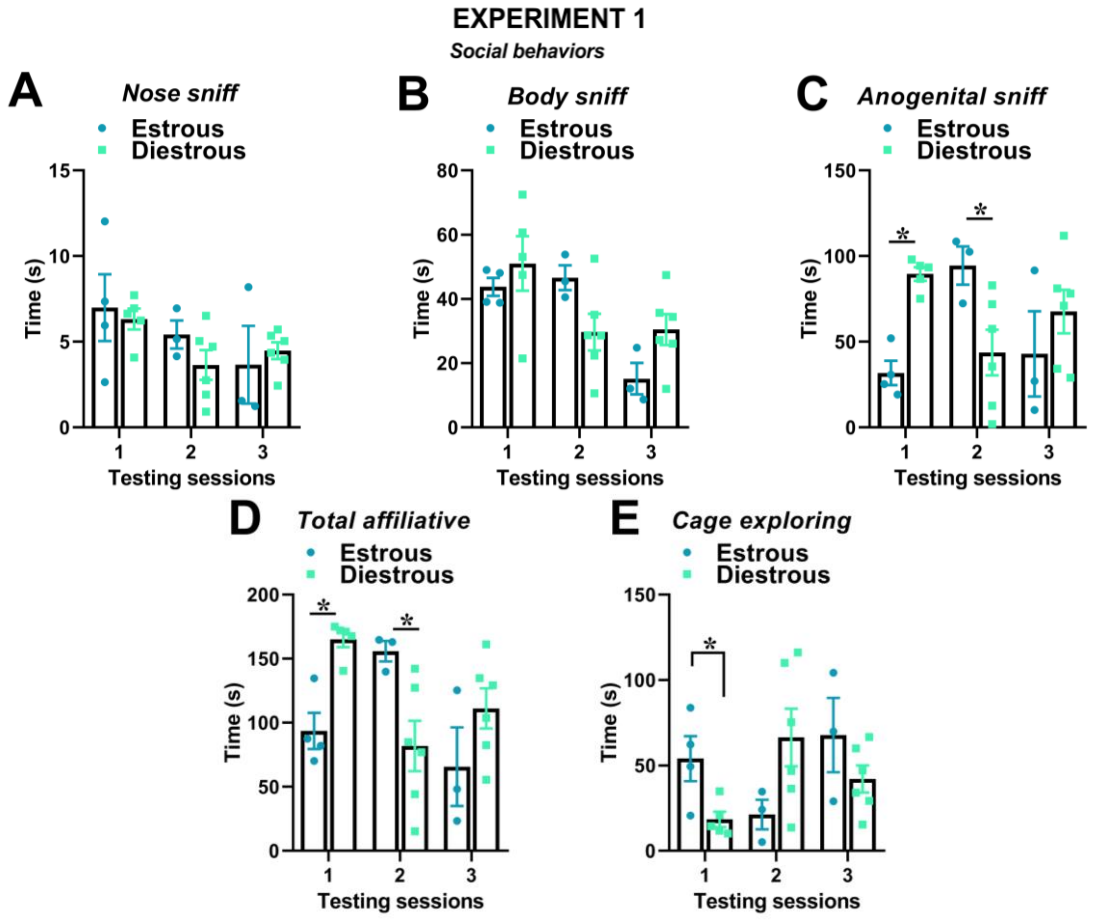
The Effects of Estrous Phase: Social Interaction and USVs in Female Mice

While the male B6 mice were all tested with a female CD1stimulus in diestrous phase, the female B6 mice were tested on each session with a female CD1 either in estrous or diestrous phase, with a balanced assignment across sessions (refer also to

Table 1). The estrous phase of the stimulus females did not affect the social behaviors in any of the testing sessions, neither any of the USVs' characteristics (stimulus' estrous phase effect for each testing session: all ns).

The estrous phase of the experimental B6 female mice affected their social behaviors on the first 2 testing sessions (**Figures 5A–E**): the female mice in estrous phase displayed less anogenital sniffing on session 1 and more on session 2 than those in diestrous [estrous phase effect on sessions 1 and 2, respectively: $F_{(1,7)} = 55.3$ and 5.96, $p < 0.001$ and <0.05; **Figure 5C**], and the same pattern was observed for total affiliative behaviors [estrous phase effect on sessions 1 and 2, respectively: $F_{(1,7)} = 24.97$ and 6.39, $p < 0.01$ and <0.05; **Figure 5D**]. In parallel, cage exploration was more evident in the estrous than in the diestrous female mice in session 1 [estrous phase effect on session 1: $F_{(1,7)} = 7.89$, $p < 0.05$; **Figure 5E**] and tended to decrease afterward.

The estrous phase of the experimental B6 female mice also affected certain characteristics of the USVs they emitted, especially in the first testing session (**Figures 5F–J**). The female mice in estrous phase emitted less USVs in session 1 than those in diestrous phase [estrous phase effect on session 1: $F_{(1,8)} = 16.74$, $p < 0.05$; **Figure 5F**]. The female mice in estrous phase also spent less time calling in session 1 compared to those in diestrous phase, while an increase was observed in session 2 [estrous phase effect on s1 and s2, respectively: $F_{(1,8)} = 12.50$ and 5.99 $p < 0.05$; **Figure 5G**]. The peak frequency of the USVs emitted by the female mice in estrous phase was also higher, although this effect was again detectable only in session 1 [estrous phase effect on session 1: $F_{(1,8)} = 5.35$, $p < 0.05$; **Figure 5I**]. No difference was found in the distribution of call types between estrous and diestrous experimental female subjects (**Supplementary Figure 2**).



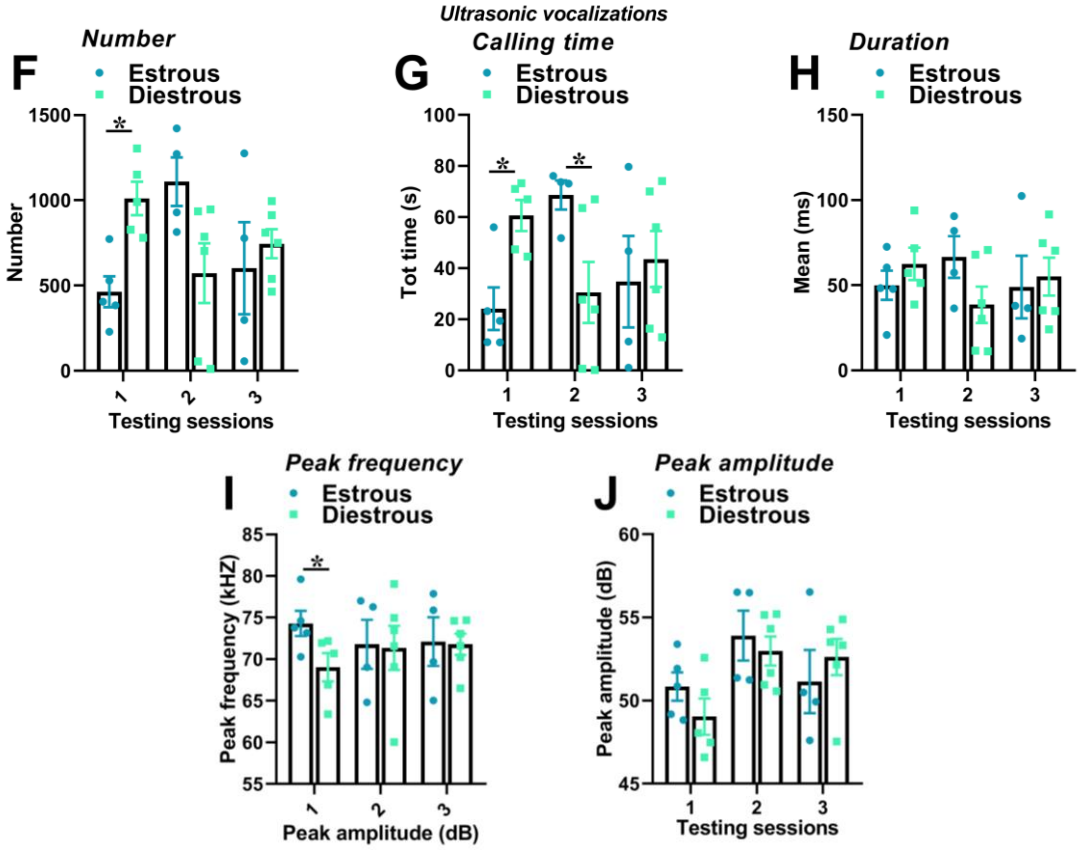


Figure 5. Effects of the estrous phase of the resident female mice on social behaviors and USVs in experiment 1. The impact of the estrous phase of the experimental female B6 subjects was investigated on social behaviors **(A–E)** and USVs' characteristics **(F–J)**. For the exact number of mice in each estrous phase, refer to Table 1. Data are mean ±SEM. Total N = 10 before exclusion of statistical outliers by Grubb's test for small samples.*p < 0.05.

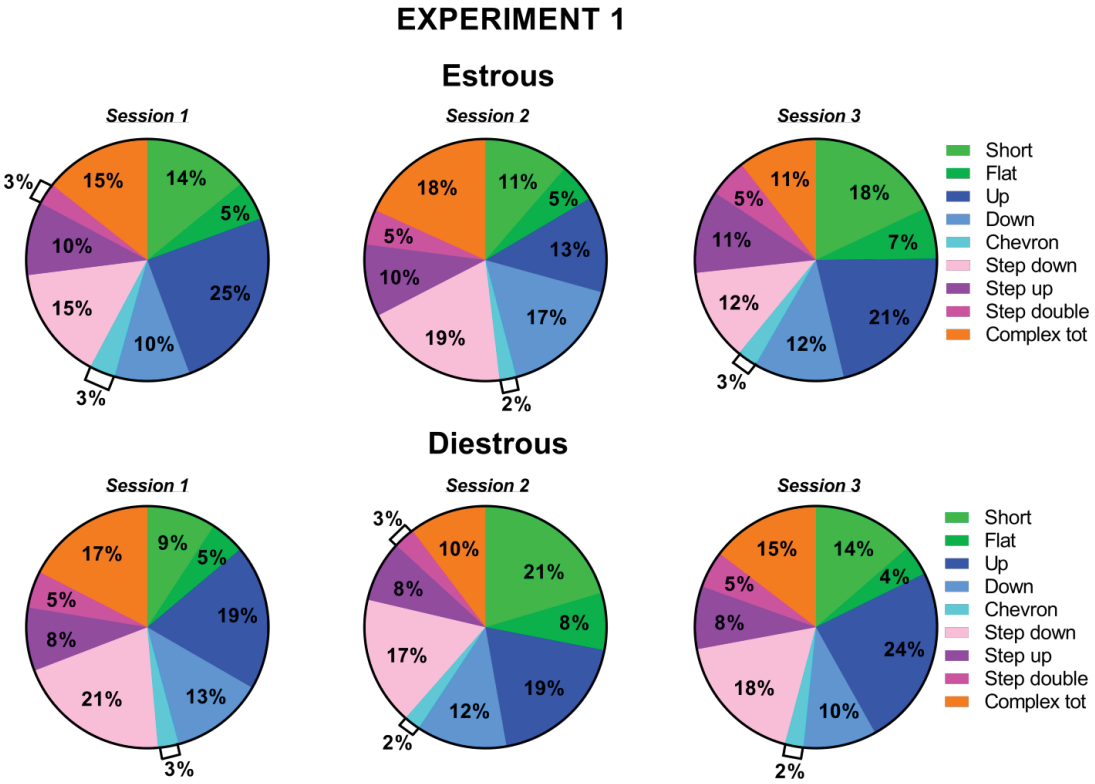


Fig. S2. The effects of the estrous phase of the resident female on USVs types in experiment

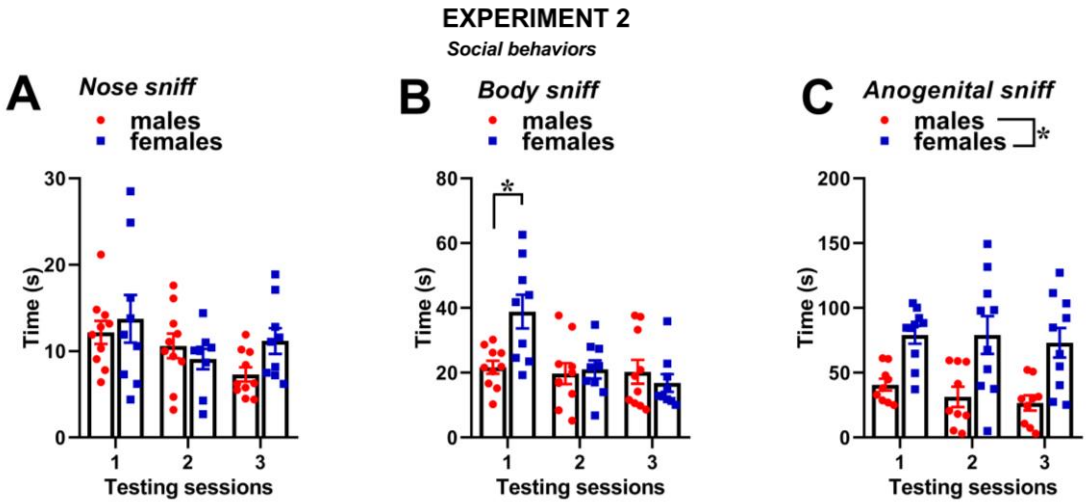
1. The distribution of call categories for each testing session and estrous phase is illustrated through pie-charts. Data are expressed as mean percentages over the total number of USVs for each sex and estrous phase. A total of 10 females were included in the dataset; their distribution across estrous phases is detailed in Table 1.

Experiment 2: Different Pre-Testing Isolation Time in Male and Female Mice

Sex Differences: Social Interaction and USVs

Similar to experiment 1, social behaviors were overall more expressed by the female mice than the male one and tended to decrease with testing sessions; furthermore, sex differences depended on specific type of behavior (**Figures 6A–E**). The female mice displayed more body sniffing than the male mice, especially in the first testing session [interaction sex × session: $F_{(2,34)} = 5.34$, $p < 0.01$; sex effect on session 1: $F_{(1,17)} = 10.36$, $p < 0.01$; **Figure 6B**]. The female mice were also overall engaged in more anogenital sniffing than the male mice [sex effect: $F_{(1,17)} = 16.72$, $p < 0.01$, **Figure 6C**], an effect that was stable across the sessions. The female mice display more affiliative behaviors and explored significantly less the testing cage than the male mice [sex effect, respectively: $F_{(1,17)} = 9.61$ and $F_{(1,18)} = 20.51$, $p < 0.01$; **Figures 6D,E**]. In mice of both sexes, the levels of affiliative behaviors tended to decrease with testing sessions, while those of cage exploration increased [session effect, respectively: $F_{(2,34)} = 6.55$ and $F_{(2,36)} = 10.98$, $p < 0.01$; **Figures 6D,E**].

Several characteristics of USVs differed between the two sexes (**Figures 6F–J**), in a highly similar manner to what was observed in experiment 1. Although the number of USVs emitted was not significantly different (**Figure 6F**), the total calling time and the mean duration were higher in the female mice [sex effect, respectively: $F_{(1,16)} = 6.6$ and 5.32, $p < 0.05$; **Figures 6G,H**]. All the USV parameters did not significantly change across the testing sessions (session effect and its interaction with sex: all ns).



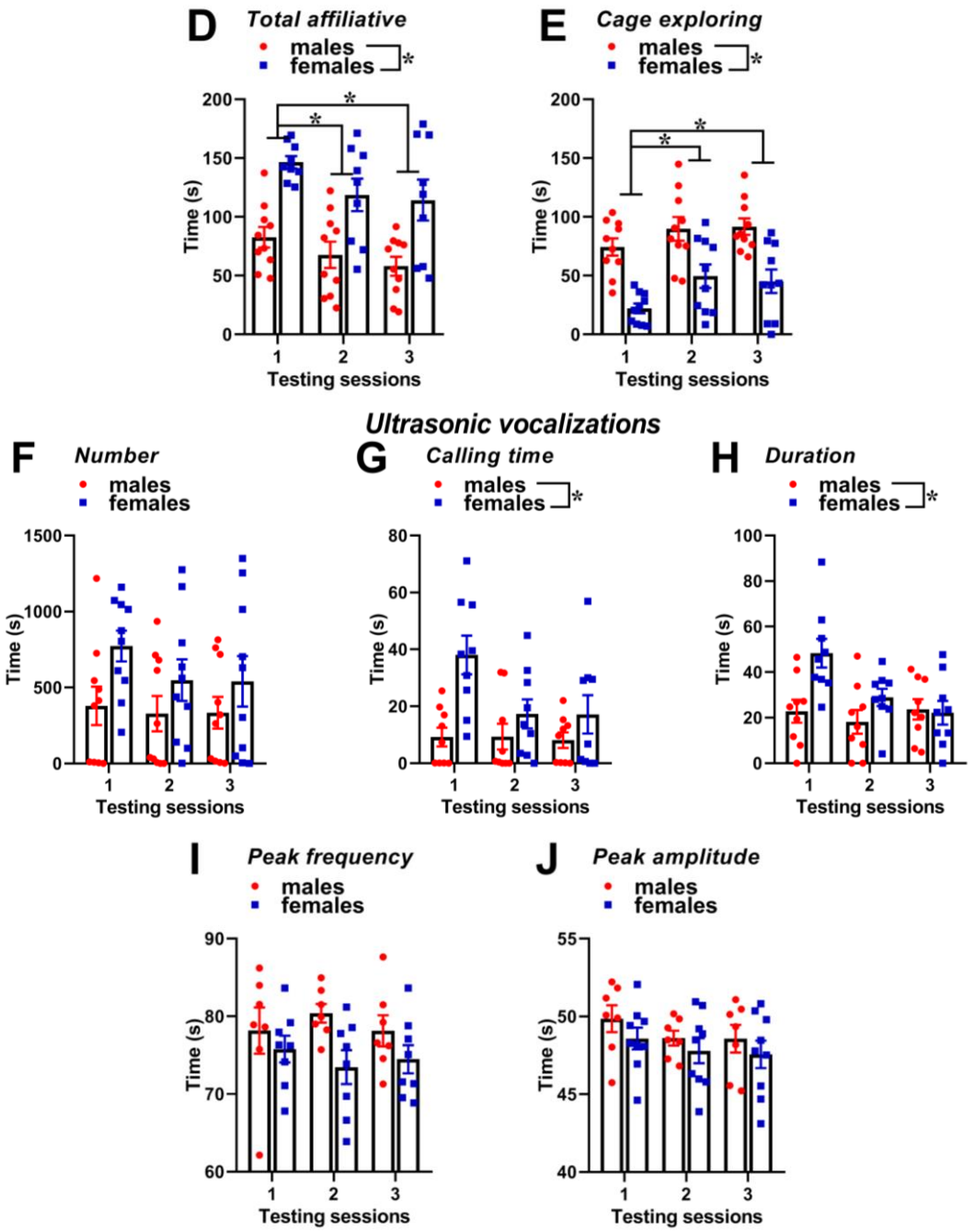


Figure 6. Sex differences in social behaviors and USVs in experiment 2. (A–E) Social behaviors and **(F–J)** USVs of the male and female experimental B6 subjects were assessed by analysis of the video recordings (Observer, Noldus) and spectrograms (Avisoft SASLab Pro), respectively. As in experiment 1, social behavior and USVs were analyzed during 3 testing sessions of 3min each using an adult female CD1 as stimulus. Data are mean ± SEM. N = 10 before exclusion of statistical outliers by Grubb's test for small samples.*$p < 0.05$. Sex differences are reported as * in each graph legend when a significant main effect of sex was detected in the absence of any interaction with testing session.

The classification of the call types revealed several sex differences that were mostly independent of the testing sessions (**Figures 7**, **8**). The female mice tended to emit less "short" calls than the male mice [sex effect: $F_{(1,18)} = 3.53$, $p = 0.08$; **Figure 7A**]. The female mice also produced more "down" calls [sex effect: $F_{(1,18)} = 9.15$, $p < 0.01$;

Figure 7D], more "step double" [sex effect: $F_{(1,18)} = 4.12$, $p = 0.06$ **Figure 7H**], and more complex calls [sex effect: $F_{(1,18)} = 7.3$, $p < 0.05$; **Figure 7I**].

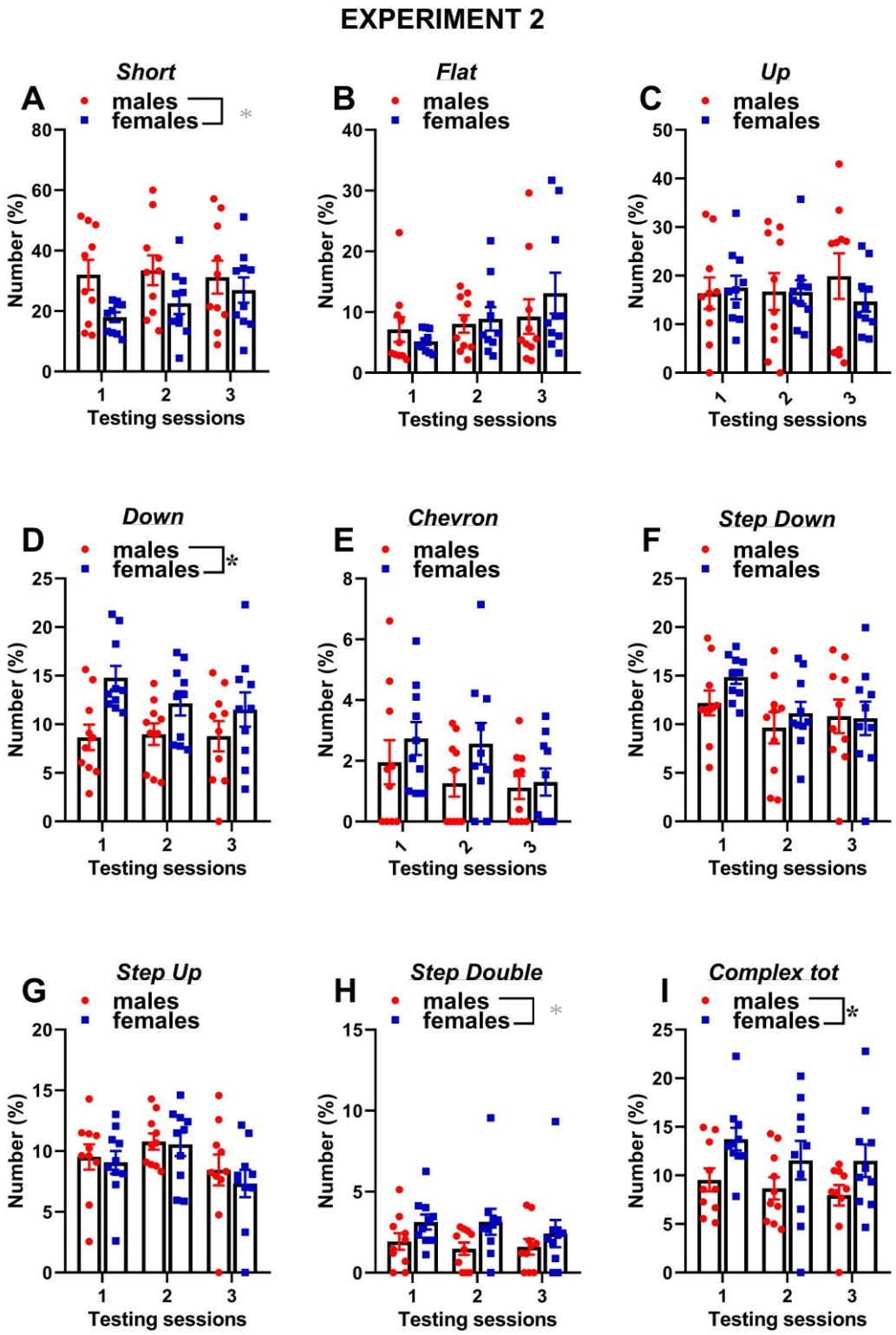


Figure 7. Sex differences in ultrasonic call types in experiment 2. (A-I) The different call types were automatically classified (refer to Figure 1 for a detailed description). Complex tot = complex 3 + complex 4 + complex 5. Data are expressed as percentages over the total number of USVs for each sex and session. Data are mean ± SEM. N = 10 for each sex. *$p < 0.05$.* Refers to a nonsignificant tendency ($0.05 < p \leq 0.09$). Sex differences are reported as * in each graph legend when a significant main effect of sex was detected in the absence of any interaction with testing session.

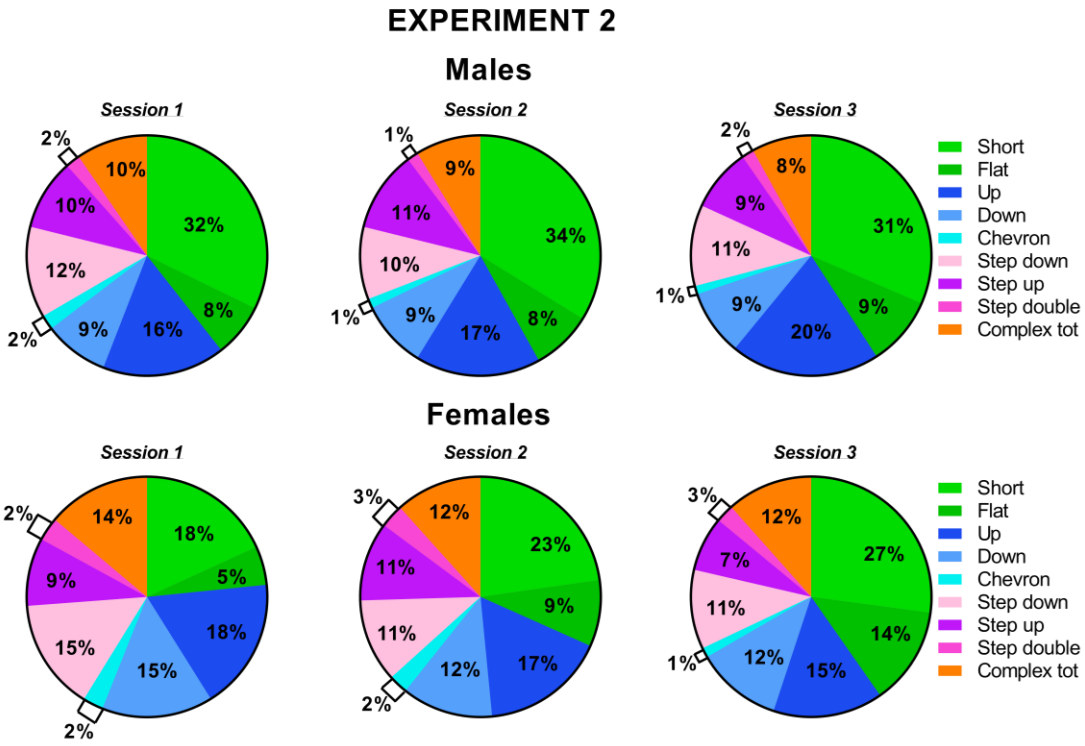


Figure 8. Pie charts depicting sex differences in ultrasonic call types in experiment 2. Distribution of call categories in each sex and testing session. Data are expressed as percentages over the total number of USVs for each sex and session. Data are mean ± SEM. N = 10 for each sex. Complex tot = complex 3 + complex 4+ complex 5 (refer also to Figure 1).

The Effects of Estrous Phase: Social Interaction and USVs in Female Mice

As observed in experiment 1, the estrous phase of the female stimulus (intruder) did not affect the social behaviors in any of the testing sessions or any of the USV characteristics (stimulus' estrous phase effect for each testing session: all ns).

In contrast to what was observed in experiment 1, the estrous phase of the female resident did not modulate any of its social behaviors in any testing session (effects of estrous phase on all sessions: ns; **Figures 9A–E**). Furthermore, no significant effect of the resident's estrous phase was found on any of the USV characteristics (effects of estrous phase on all sessions: ns; **Figures 9F–J**) or on their composition based on call types (**Supplementary Figure 3**).

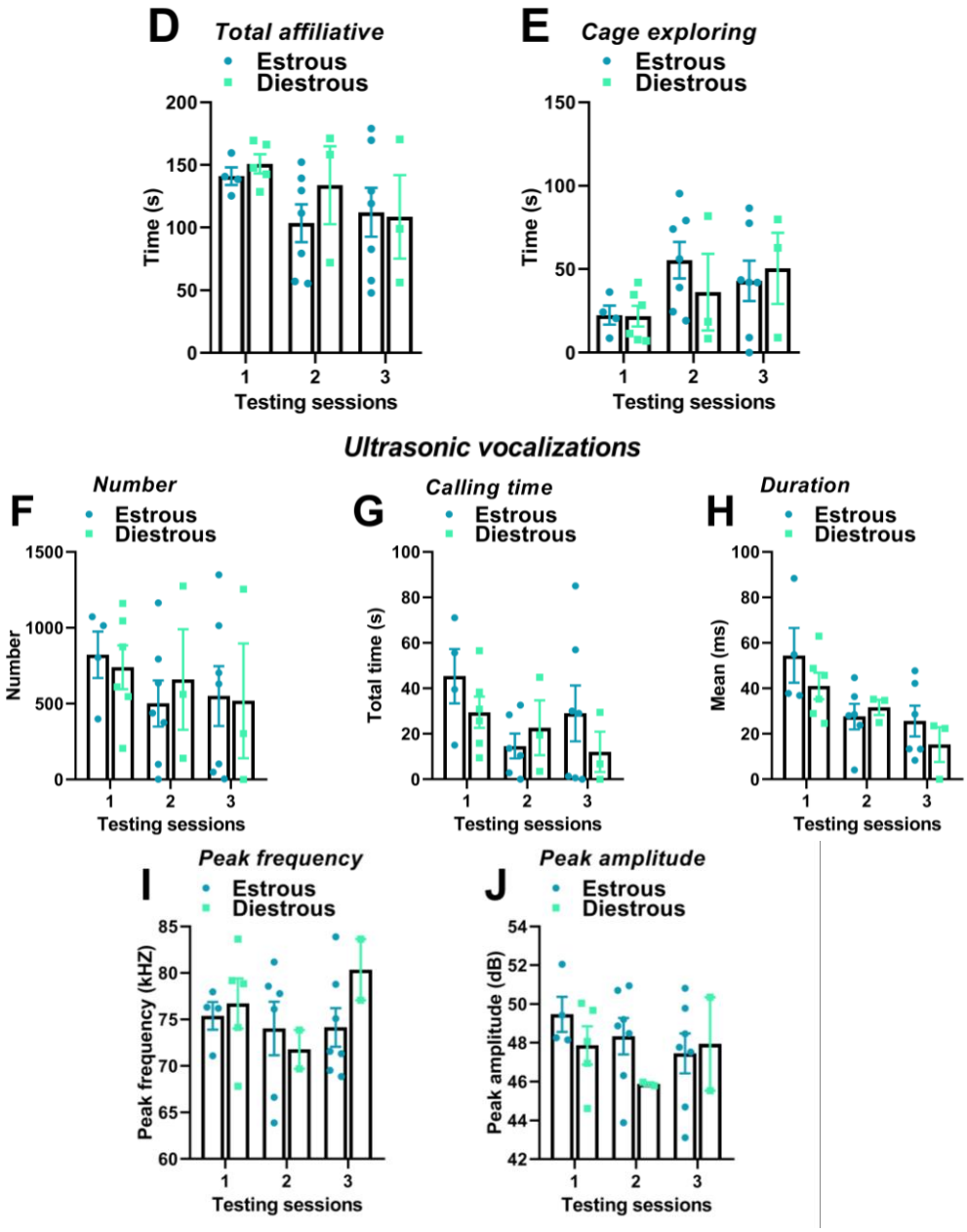


Figure 9. Effects of the estrous phase of the resident female mice on social behaviors and USVs in experiment 2. The effects of the estrous phase of the experimental female B6 subjects were investigated on **(A–E)** social behaviors and **(F–J)** multiple USV characteristics. A detailed distribution of the estrous phase in the female residents and intruders is provided in Table 1. Data are mean ± SEM. N = 10 before exclusion of statistical outliers by Grubb's test for small samples.

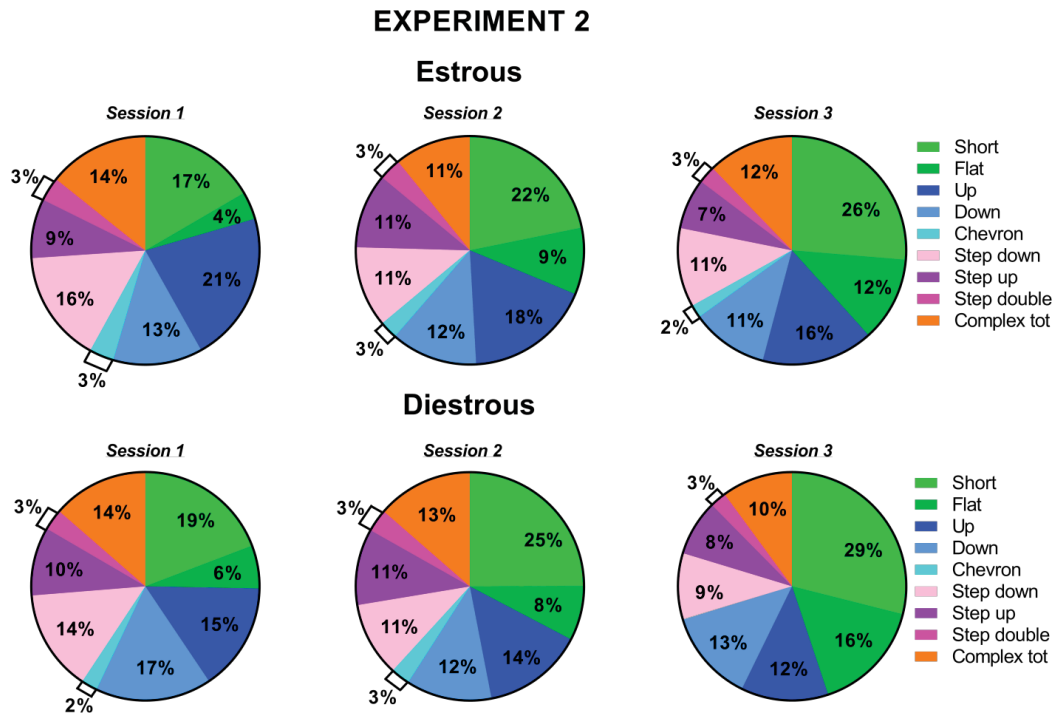


Fig. S3. The effects of the estrous phase of the resident female on social behaviors and USVs in experiment 2. Pie-charts illustrate the percentages of each call type calculated over the total number of USVs for each estrous phase and session (mean values). A total of 10 female B6 resident subjects was included in the dataset (for the precise n of each estrous phase, see Table 1). Complex tot=complex3+ complex4+complex5 (see also Fig.1).

<u>Comparison Between Experiments 1 and 2 in Female Mice: Social Interaction and USVs</u>

The female mice in both experiments were tested under the same experimental conditions. Since the female subjects belonged to two independent cohorts, in order to evaluate the replicability of female phenotypes, we analyzed the female dataset by an additional ANOVA with experiment as the between-subject factor and testing session as the within-subject variable.

Concerning social behaviors, the results on session-dependent changes were similar between the experiments, as shown by lack of the interaction experiment × session (all effects, n.s.). The levels of affiliative behaviors tended to decrease with the testing sessions while those of cage exploration increased [session effect, respectively: $F_{(2,32)}$ = 10.31 and $F_{(2,34)}$ =10.47, p<0.001; **Figures 2, 6**]. Independently of the experiments, all the USV parameters did not significantly change across the testing sessions (**Figures 2, 6**), and the expression of the different call types (**Figures 3, 4, 7, 8;** session effect and its interaction with experiment: all ns). Significant interaction experiment × session was only found in peak amplitude that tended to increase from the first to the second session but only in the first experiment [$F_{(2,34)}$ = 5.5, p < 0.05; **Figure 2J**].

The effects of the estrous phase of the resident female mice were instead statistically different between the two experiments, both on social behaviors and USVs parameters, as expected because of the presence of estrous phase effects in the first but not in the second experiment. A significant interaction experiment × estrous phase was found in the time spent in anogenital sniffing, affiliative behaviors, and cage exploration (**Figures 5, 9**) in session 1 [$F_{(1,15)}$ = 8.62, 9.28, 5.27; p < 0.05] and in affiliative time in session 2 [$F_{(1,15)}$ = 6.14; p < 0.05]. Concerning the USV

parameters (**Figures 5, 9**), a significant interaction experiment × estrous phase was found for session 1 in the number of USVs and calling time [$F_{(1,16)} = 6.13, 10.2$; $p < 0.05$].

4.4 DISCUSSION

Our findings provide convincing evidence for sex differences in ultrasonic communication and social interaction in the C57BL/6J mouse strain. In the context of male-female vs. female-female interactions, differences in ultrasonic communication between sexes were mainly qualitative, while those in social behaviors were both quantitative and qualitative. Sex differences were highly similar between the two experiments, i.e., their detection was not substantially affected by differences in pre-testing isolation of the male mice. Nonetheless, subtle differences in the social and ultrasonic profiles of the male mice emerged between the two experiments, suggesting an impact of pre-testing social isolation on the male mice. Sex differences were mostly stable across the three testing sessions with an unfamiliar intruder, although an overall tendency to social habituation occurred in both experiments and sexes.

The estrous cycle of the resident female mice altered the social behaviors and ultrasonic communication of the female mice, although these effects were significantly detected only in the first experiment. Here, both quantitative and qualitative differences were indeed observed between receptive and nonreceptive female residents, while the estrous phase of the intruder did not modulate any of the considered behavioral parameters.

Sex Differences and Isolation Effects (Comparison Between Experiments 1 and 2)

Sex differences in social behaviors and ultrasonic communication were overall highly comparable between the two experiments, with the female mice displaying more affiliative behaviors and less cage exploration than the male ones while emitting longer USVs and with less simple one-component calls (e.g., “short” and “up”) but more complex calls (e.g., “step double” and “total complex”). Nonetheless, subtle additional sex differences were found only in experiment 1, including a male-specific decrease with testing sessions in anogenital sniffing and USV number, as well as an overall higher peak frequency of male USVs.

Hence, our data suggest that the experimental settings used in our experiment 1, may be the most suitable to detect both major and minor sex differences in social and ultrasonic behaviors. Since the female mice were tested under exactly the same experimental conditions in experiment 2, it is natural to infer that the differences emerging between our two experiments are due to corresponding differences in the behaviors of the male mice that were exposed to pre-testing isolation only for experiment 1. The behavior of the female mice was indeed highly comparable in our two experiments, as confirmed by the statistical comparison of the two female datasets, supporting the replicability of female social and ultrasonic behavioral profiles across the repeated testing sessions. Male behaviors appeared instead slightly different between the two experiments, although we could not conduct a statistical quantification of these differences because of the confounding effects of independent testing on social isolation.

Nonetheless, the visual comparison of **Figure 2** with **Figure 6** and **Figure 3** with **Figure 7** clearly shows that the male mice in experiment 1, i.e., with longer pre-testing isolation, displayed higher levels of anogenital sniffing, more USVs, and more one-component ‘up” calls, suggesting a higher expression of these behaviors in

territorial, i.e., isolated, male mice. The hypothesis of a territoriality effect of social isolation is further supported by the predominance of the differences between the two experiments during the first testing session, since this effect may be attenuated by repeated experience of social encounters with an intruder. Furthermore, it should be noted that a resident-intruder setting was employed only in experiment 1, while experiment 2 used a basically neutral testing environment as a consequence of the short pre-testing isolation (10min). The reason for this experiment design was intrinsically related to the major aim of our study, which was not to specifically investigate the effects of social isolation on ultrasonic communication in male and female mice, as previously conducted by others (e.g., Zhao et al., 2021; this also explains the lack of an additional female group with minimal pre-testing isolation in our design). Instead, our goal was to evaluate sex differences either under the exact same experimental conditions for male and female mice (i.e., in the resident-intruder paradigm) or under the experimental conditions most suitable and commonly used in research studies on USVs in ASD mouse models (i.e., in a resident-intruder setting for female mice and with a short habituation to the testing environment in the case of male subjects).

Interestingly, when the effects of 72-h social isolation were previously assessed, only subtle changes were described in male B6 mice (Zhao et al., 2021). These discrepancies may be due to the longer duration of the testing session used in the previous study (i.e., 30 vs. 3 min in ours): Zhao et al. indeed described no effects of isolation on the number of USVs emitted by a male mouse toward a female intruder when the entire session was considered, but they detected a significant increase in isolated vs. grouped male mice when only the first 5 min of the session was analyzed, and they described a higher first latency to USV emission in the isolated male mice (with an average value of ~3min, i.e., the duration of our testing session). Differences in the estrous cycle of the female intruder could also contribute to the discrepant outcomes of ours and Zhao's study on social non-vocal behaviors of isolated male mice: the authors reported a tendency, although not significant ($p = 0.08$) to an overall increase in the time spent in social interaction in isolated male mice compared to grouped ones that was accompanied by an increase in the occurrence of mounting behavior. The higher engagement in mounting of their isolated male mice could have attenuated the isolation effects on affiliative behaviors that we instead found in our study. We did not detect mounting in our tested male mice, and this is not surprising considering our short testing duration and the non-receptive estrous state of our female intruders (the estrous cycle was not assessed in Zhao's study). In conclusion, our results from the male mice and their comparison with previous findings suggest that 3 days of isolation of the male mice increases social affiliation and promotes USV emission during the initial phases of the social encounter with a female mouse. Nonetheless, the duration of the testing session and the estrous cycle of the intruders may critically influence the social effects of isolation, an issue that deserves to be specifically investigated in future studies.

Independently of the experiments, our findings suggest that the major sex differences affecting mouse ultrasonic communication are of qualitative nature (duration and call composition) rather than quantitative. While the presence of longer USVS in the female mice was in line with previous studies (e.g., von Merten et al., 2014), the lack of sex difference in the number of USVs that we found here is in disagreement with a previous study reporting that female mice emitted more USVs than male mice toward a female intruder (Hammerschmidt et al., 2012b). This discrepancy may be due to the different substrain used in this study, since B6/N and B6/J are known to

have markedly different ultrasonic profiles. Indeed, in B6/J mice, another study described no difference in the number of USVs (Matsumoto and Okanoya, 2018) but a reduced number of short calls and prevalence of complex calls in female mice.
As short calls, together with simple calls in general, have been detected especially under territorial conditions, e.g., in male-male interactions (Matsumoto and Okanoya, 2018) and following long-term male isolation (Chabout et al., 2012), it is possible that male mice preferentially communicate using short calls. In contrast, complex calling bouts may be useful in maintaining the group structure necessary for female mice and promote interactions and cooperation (Matsumoto and Okanoya, 2018), in agreement with previous studies showing that this type of call is more attractive for female mice (Chabout et al., 2015). It is indeed increasingly accepted that ultrasonic calls from female mice facilitate proximity between animals in order to help residents to acquire relevant social information on intruders and promote group relationships (Moles et al., 2007).
Since the strain of the mice involved in the social encounter may play a role in their ultrasonic profile and potential related sex differences, it is important to underscore that our study employed different strains for the test subjects (B6) and the stimulus mice (CD1). Although mouse USVs are often analyzed during interactions within the same strain, our experimental setting is not unusual, as it has been employed in previous studies assessing the emission of USVs by resident female mice (Maggio and Whitney, 1985) and male mice (Sugimoto et al., 2011) during dyadic interactions. One study in particular (Sugimoto et al., 2011) demonstrated a lack of USVs emitted by the female stimulus (derived from the CD1 strain) also when the "devocalized" male was of a different background (B6), i.e., under conditions highly similar to ours. Furthermore, using stimuli of a different strain to induce emission of USVs by tested subjects is a typical procedure of several studies on urine-elicited USVs (Nyby et al., 1979, 1983; Nyby, 2010). The choice of the CD1 strain as stimulus enhances the applicability of our present data to the research field of neurodevelopmental disorders. Indeed, several studies with mouse models of Autism and Fragile X syndrome obtained from the B6 background have performed social and ultrasonic testing (in both male and female mice) through interactions with female CD1 stimuli (Hebert et al., 2014; Pietropaolo et al., 2014; Oddi et al., 2015; Gaudissard et al., 2017; Gauducheau et al., 2017; Lemaire-Mayo et al., 2017; Fyke et al., 2021).

Estrous cycle effects on social behaviors and USVs (experiments 1 and 2)
In both experiments, the estrous cycle of the intruders did not alter either the social behaviors or any parameter of ultrasonic communication. The estrous cycle of the residents instead modulated both behavioral domains, and these effects were more marked in experiment 1 (**Figure 5**) the female mice in estrous phase exhibited less affiliative behaviors in session 1 than those in diestrous phase, but this tendency inverted its direction in session 2 to return to the initial situation in session 3. These effects were mainly due to differences in anogenital sniffing. The effects of estrous cycle on social behavior followed those observed on USVs, since the number of USVs was also initially and finally lower in the estrous than in the diestrous female mice with a shift in session 2. USVs seemed more affected by the estrous cycle in session 1 when their number, call time, and peak frequency were all different between estrous and diestrous residents. The overall reduced social investigation and number of USVs of the estrous female mice are in agreement with previous reports (Moles et al., 2007) and fits with the reduced social interest of receptive female mice in a conspecific of the same sex. It has been suggested that oxytocin mediation of

social processes is likely to play a role in the effects of the estrous phase, since it is known to be regulated by ovarian circulation (Choleris et al., 2003). Nonetheless, we failed to replicate the significant effects of the estrous cycle in experiment 2 despite the experimental testing conditions of the female mice being unchanged. It should be noted that the pattern of results of experiment 2 was still in line with what observed in experiment 1 (**Figure 9**), although the composition of the estrous vs. diestrous female in each session was less balanced than in experiment 1 (see **Table 1**). It is possible that the lower number of female mice, especially in the diestrous phase (3 in some testing sessions), may have limited the emergence of significant differences. In none of the experiments any effect of the estrous cycle was detected on the call types (**Supplementary Figures 2, 3**).

Effects of testing experience at the group and individual levels (experiments 1 and 2)

We observed several group differences in our study, both in social non-vocal and vocal behaviors. It is intriguing to question whether the group differences could be confirmed at the individual level; to this end, the visual evaluation of individual plots (**Supplementary Figures 4, 5**) supports interesting considerations.

Concerning social non-vocal behaviors, most of the female individuals showed the expected reduction with testing sessions in the time spent performing body sniffing in both experiments (**Supplementary Figures 4B, 5B**), while a decrease in anogenital sniffing was confirmed in the male mice only in experiment 1 (**Supplementary Figure 4C**). In mice of both sexes in both experiments, the levels of affiliative behaviors tended to decrease with testing sessions while those of cage exploration increased (**Supplementary Figures 4D,E, 5D,E**).

Concerning individual trends in USV-related parameters, in experiment 1, we confirmed that all the USV parameters did not significantly change across the testing sessions, with the exception of peak amplitude that increased from the first to the second session in mice of both sexes (**Supplementary Figure 4J**) and the number of USVs that decreased across the testing sessions in the male mice only (**Supplementary Figure 4F**). None of the session differences appeared at the individual level in experiment 2 (**Supplementary Figures 5F–J**), when the male mice were not isolated before testing (confirming the lack of differences observed at the group level).

Concerning the types of ultrasonic calls, it is important to underscore that the stability of call compositions in each sex across the testing sessions was confirmed at the individual level (**Supplementary Figures 6, 7**) when we selected the individuals with the highest total calling rates (half of each sex for each experiment). The overall higher proportion of short calls in males mice and the lower proportion of complex calls (“step double” and “complex tot”) were also evident in this subset of individuals.

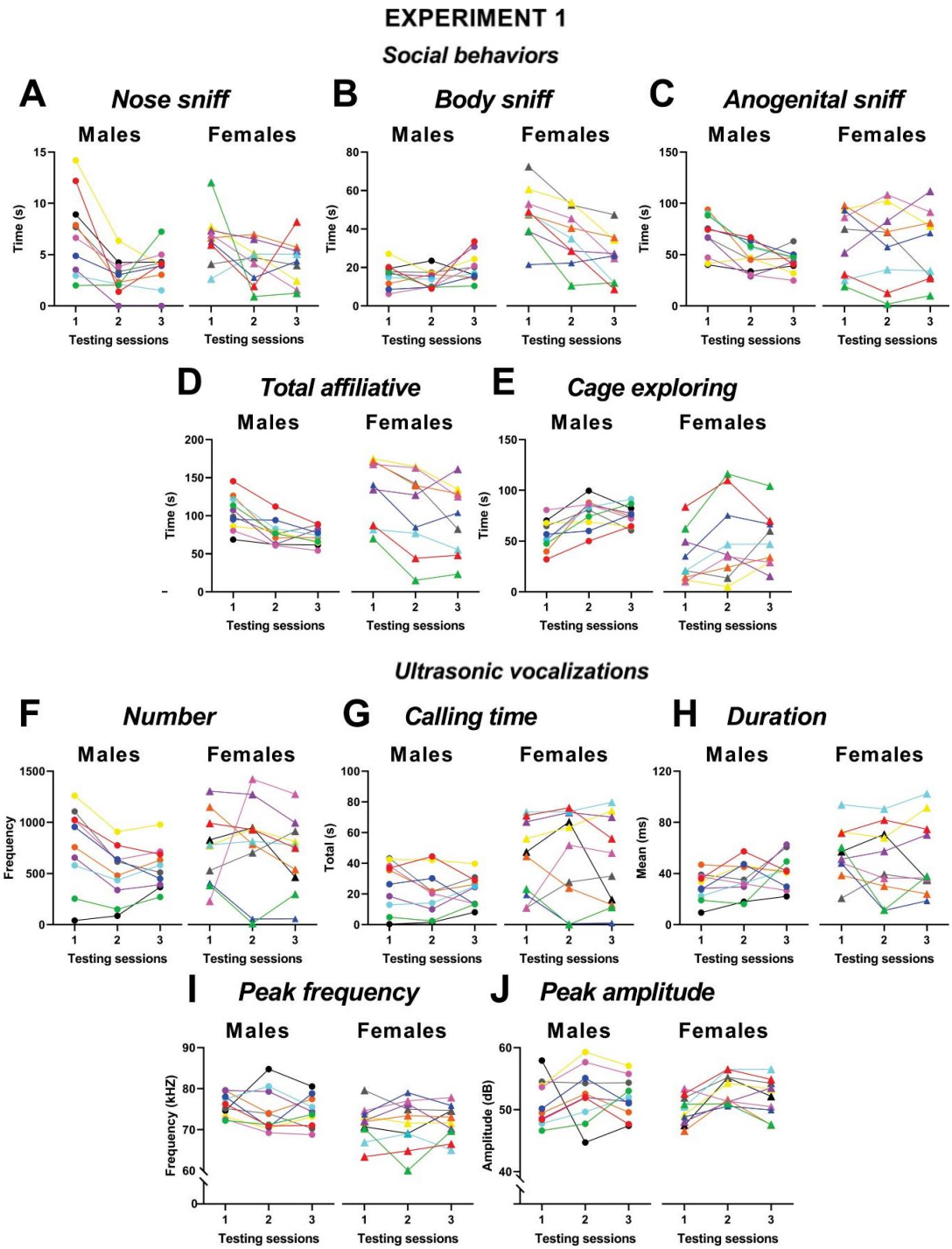


Figure S4. Individual line plots of social behaviors and ultrasonic communication in experiment 1.

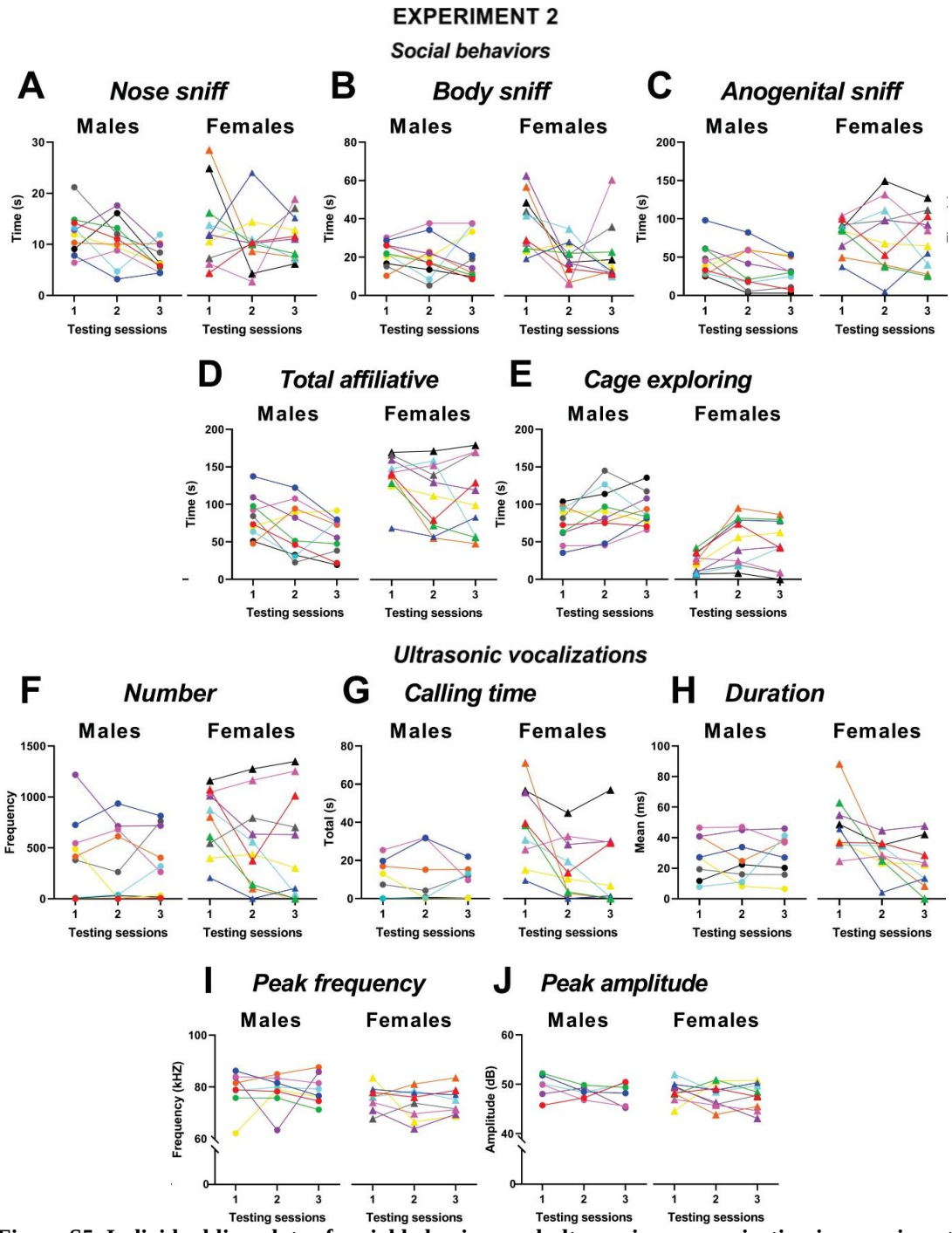


Figure S5. Individual line plots of social behaviors and ultrasonic communication in experiment 2.

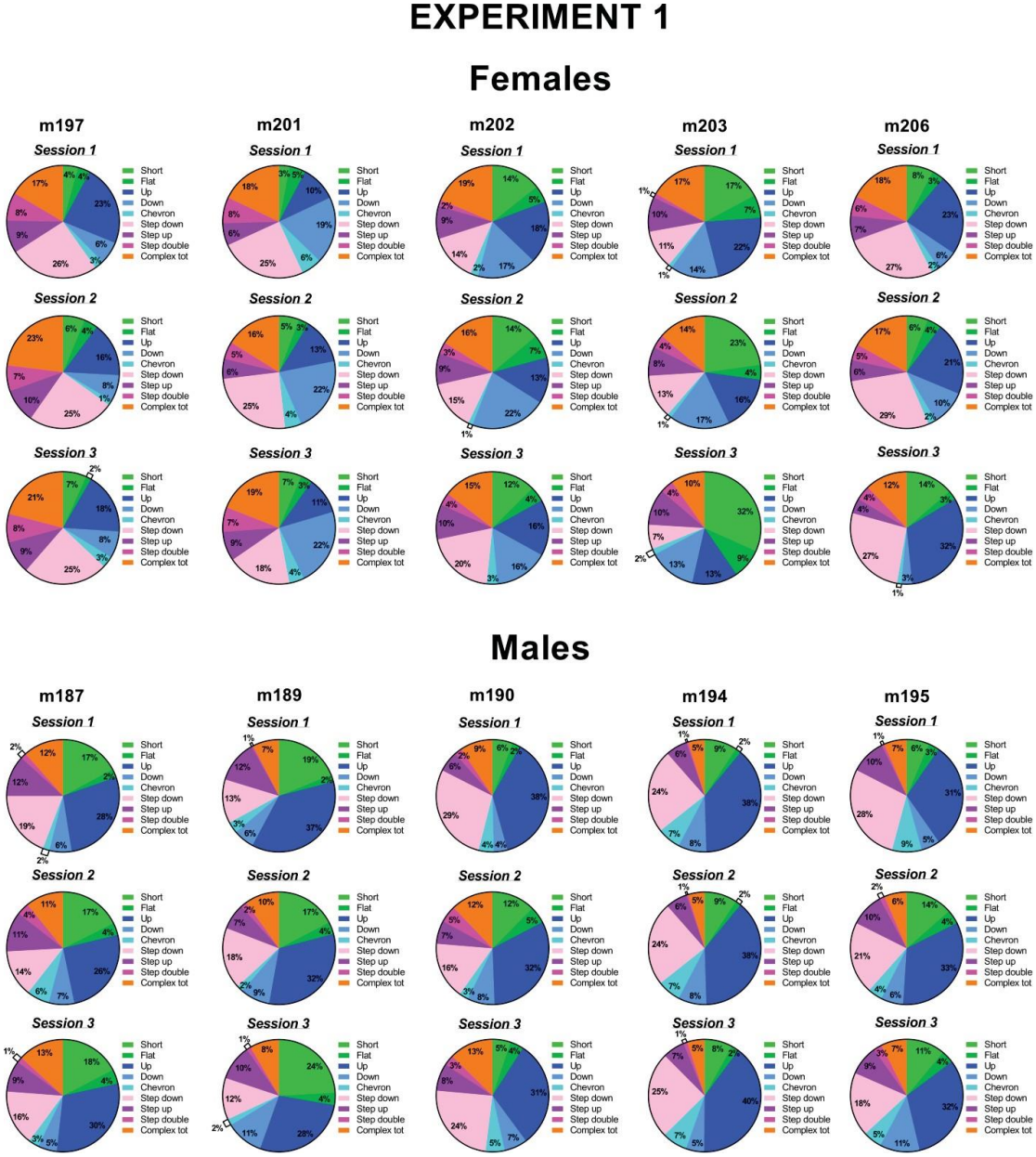


Figure S6. Stability of call type composition across testing sessions in high emitting individuals from experiment 1. In order to evaluate whether the stability of call composition across testing sessions was confirmed at the individual level, we selected 5 individuals for each sex with the highest total number of USVs. Pie-charts illustrate the percentages of each call type calculated over the total number of USVs for each testing session. m= mouse.

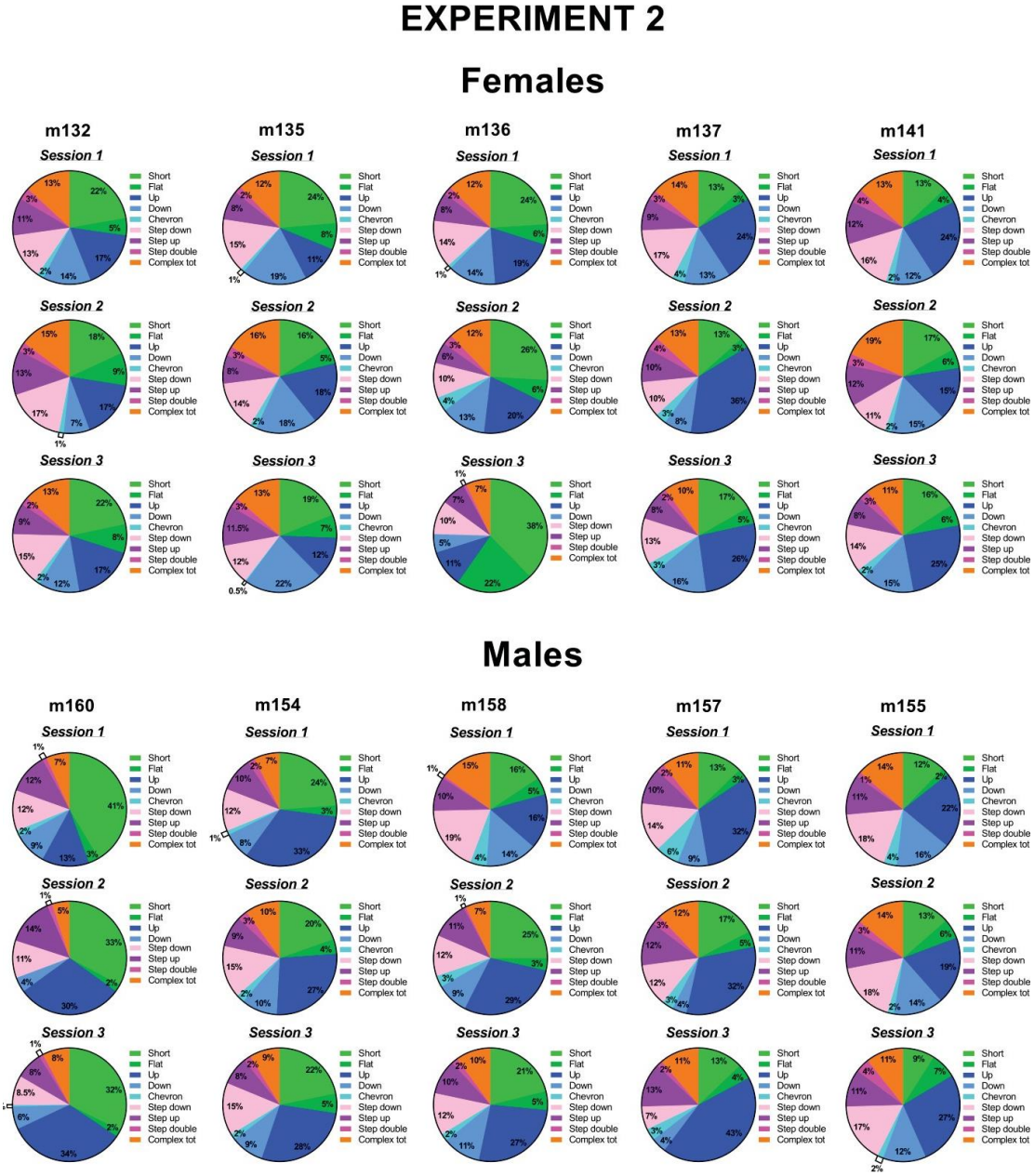


Figure S7. Stability of call type composition across testing sessions in high emitting individuals from experiment 1. Individual patterns of changes in call composition across testing sessions were evaluated in 5 individuals for each sex emitting the highest total number of USVs. Pie charts illustrate the percentages of each call type calculated over the total number of USVs for each session. m= mouse.

In conclusion, our findings provide novel evidence for marked sex differences in ultrasonic communication that are mirrored by differences in other social behaviors of adult B6 mice. The replication of the sex differences with and without pre-testing isolation in the male mice suggests their strong consistency and should be taken into account for designing future studies using male and female adult mice. This could be especially important for studies on genetic mouse models of ASD or other pathologies involving communication deficits where pretesting isolation may induce undesirable confounding effects more marked than in their wild-type littermates (Pietropaolo et al., 2008). Furthermore, our results demonstrate that the sex differences observed in ultrasonic communication and social behaviors are not

limited to the first testing session and represent a stable trait that seems independent of the novelty of the social stimulus. Finally, these data clearly show the importance of an extensive qualitative analysis of ultrasonic communication in adult mice, since this approach contributes to unravel the more complex structure of female vs. male calls.

D) CONCLUSION AND FUTURE PERSPECTIVES

This thesis investigated different factors, genetic and environmental, that affect ultrasonic communication in mice. A particular interest is devoted to USVs analysis in the field of neurodevelopmental disorders (NDDs), especially autism spectrum disorders (ASD), at both early phases of development and adulthood. Indeed, several alterations are found in USVs emitted by CB1 null mutants and Fmr1 KO mice, that are models of NDDs and ASD.

These results underlie the relevance of USVs analysis in neuroscience context. In last years, USVs of pups and adults are very well-characterized in many mice strains and have been a fundamental tool for preclinical research in neuroscience. expecially to investigate those brain disorders characterized by communication deficits as NDDs and ASD. USVs also represent a non-invasive tool to acquire data about the health status of the animals and effects of treatments in rodents models of brain pathologies.

Several commercial instruments and softwares have been developed to record and perform the spectrographic analysis of USVs. Mice emitted ultrasonic calls with different shapes and features that can potentialy deliver different messages to the receiver. The meaning of these different types of USVs is still under investigation and it is one of the main challenge for the future. The USV playback studies can be helpful to analyze the consequences of ultrasonic acoustic signal on animal behavior. This will allow to better understand the role of different typologies of calls and the meaning of the message trasmitted, at least in positive or negative terms. Further research is needed in this context. As soon as this key topic will be unravelled, the study of ultrasonic communication will become even more important in neuroscience field and also the translational value of USVs will dramatically increase.

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