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4/15/2010

Quantitative Approach to Analyzing Acoustic Communication Behavior in the House Mouse

by

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Abstract

Quantitative Approach to Analyzing Acoustic Communication Behavior in the House Mouse

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A key question in audition concerns abilities and mechanisms underlying how organisms recognize, detect and discriminate communication vocalizations. The mouse has become a useful tool for investigating this, both at the behavioral and neural levels. One particular communication context in the mouse has proven particularly valuable for this: lost mouse pups call for help to their mother using ultrasonic vocalizations known as isolation calls. Upon hearing these, mothers search out and retrieve the pups. Previous behavioral research has demonstrated that 1) mothers preferentially approach sounds with similar frequency, bandwidth and duration to pup isolation calls, and 2) mothers show equal approach between any two sounds that are pup-like in these acoustic dimensions. However, whether this truly implies that mothers cannot discriminate between natural calls of different pups is the subject of the current work. We examined whether aspects of a mother's locomotor behavior may indicate both the detection as well as discrimination of pup calls, when those calls are taken from a conspecific versus a foreign mouse strain. We tested c57bl/6J-strain mouse mothers in a two-alternative choice maze, using automated video tracking of the animal to assess their behavior, playing back both c57bl/6J (conspecific) and CBA/CaJ vocalizations; another group of mothers presented with silence served as controls for call detection. Our results suggest that the amount of time and distance travelled per approach best indicated the mother's detection and discrimination abilities. This study provides the first evidence that mouse mothers can display behaviors suggestive of discrimination between two natural types of isolation calls.

Quantitative Approach to Analyzing Acoustic Communication Behavior in the House Mouse

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Introduction

Animals communicate by sending signals to other animals, with intent to modify the receiver's behavior. The animals can send signals via chemical, visual, tactile, or auditory modalities for social purposes such as warning predators, attracting mates, or calling others for help. Many animals have utilized their auditory processing to detect and recognize the acoustic signals from other animals. More specifically, the animal uses its auditory system to detect the incoming sound, distinguish the acoustic signal from environmental noise, and recognize the call as a relevant signal before behaviorally reacting (G. Ehret, 2005). There is growing interest in animal acoustic communication as a likely evolutionary origin of human speech, and there have even been attempts to use acoustically communicating animals to model human speech disorders and social impairments (Fitch, 2000; McFarlane et al., 2008).

Despite the interest, there is still much to learn about both the underlying neurobiology and psychology of communication sound processing, particularly within the central auditory system. Researchers have made some progress in studying this in animals (Günter Ehret & Romand, 1996; Hauser, 1996). For example, by studying and manipulating the animals' genes, they have found genetic factors that contribute to auditory processing (Johnson, Erway, Cook, Willott, & Zheng, 1997; Kurt, Groszer, Fisher, & Ehret, 2009). For instance, a single gene was found to be responsible for age-related hearing loss in some mice (Johnson et al., 1997). They have also performed behavioral studies using operant conditioning and trained animals that respond to sounds (Wada, 1999). However, studying audition alone is not enough to characterize the extent of call perception in animal communication.

Experiments using animals trained via operant conditioning are not suited for a behavioral study of acoustic communication, because the animals are far-removed from a social setting. In an operant conditioning chamber, the animal is usually taught to associate tones with rewards or punishments, rather than perceiving natural sounds as a broadcast of another individual. Their conditioned behavior typically lies outside of the animal's natural ethogram (repertoire of an animal's intrinsic behavior), and does not resemble its natural response to hearing social sounds. Finally, the operant conditioning experiment does not replicate the environment in which animals behave socially. Hence, a behavioral study of acoustic communication demands an approach that resembles more natural conditions.

To study animal acoustic communication from a behavioral perspective, an ethology experiment (the study of animal behavior) as described below is more appropriate. Such experiments reconstruct a natural environment in which an untrained animal behaves freely. The researcher varies the environmental conditions to elicit and observe the subsequent behavior of the animal. To study how an animal in a social setting behaves as it hears calls of another animal, the environmental context and the sound stimuli must closely resemble the natural experience. No extraneous experimental conditioning of the animal is necessary.

However, ethological experiments in a laboratory setting are not without their challenges; the animal must be given the appropriate stimuli in order to evoke the intended behavior. The laboratory environment must be free of any distractions that can interfere with the behavior. The animal's natural behavior can be more subtle, uncontrolled, and unpredictable compared to the trained activity of conditioned animals.

Ethological experiments are also difficult to conduct because the measurements rely heavily on human observers. The experiment demands the observers' attention over extended periods of time. A human observer's measurements are susceptible to subjectivity, observer bias, imprecision, and inaccuracy. These problems can be ameliorated by requiring additional observers scoring behavior independently. Even then, observers can measure only obvious behavioral activities that are visible. Because ethological experiments are difficult to design and conduct, many questions remain unanswered regarding animal behavior in response to communicative sounds.

Despite the difficulties of studying acoustic communication, one animal is rising to the challenge: the house mouse. The house mouse has an auditory system capable of plasticity and association of sounds with memories (Weinberger, 2004). As a mammal, they have an auditory cortex and neural circuitry similar to humans, so that in principle mice can be used to study the mechanisms and disorders of human hearing (Avraham, 2003). Despite their small size, their nervous system can be used for *in vivo* electrophysiological experiments, such as recording the neural activity of an awake mouse during sound exposure (Galindo-Leon, Lin, & Liu, 2009). The mouse genome has been fully sequenced, and genetically modified strains can be bred to study the genes involved in auditory perception and communication (Liu, 2006). Finally, mice communicate using vocalizations and respond with stereotyped behavior, making the house mouse useful for behavioral experiments (Haack, Markl, & Ehret, 1983).

The house mouse is a social animal that vocally communicates during courtship and during infancy. In a typical litter, the mother gives birth to about 4-10 infant mice (mouse "pups") in a nest. Because these pups are blind, deaf, barely capable of locomotion, and are in danger of hypothermia, they require close maternal care during the first two weeks after birth (G. Ehret, 2005). The pups have a diverse repertoire of vocalizations that alert the mother, such as vocalizations for nursing, pain, or hypothermia (Haack et al., 1983). Each type of call varies in frequency, duration, and harmonics.

If a pup becomes lost outside its nest or becomes hypothermic, it vocalizes at a single-harmonic, ultrasonic call of about 60 kHz in frequency (Liu, Miller, Merzenich, & Schreiner, 2003). Known as an "isolation call", this vocalization attracts the mother and motivates her to retrieve the lost pup back to its nest. Because lost pups readily emit isolation calls and the mother consistently retrieves them back to the nest, the isolation call/retrieval is a convenient form of communication for behavioral researchers to exploit. Interestingly, isolation calls can vary across different strains of mice, possibly due to some genetic determinant that modifies call characteristics (Hahn, Hewitt, Adams, & Tully, 1987; Wöhr et al., 2008).

Some of the earlier studies of call perception used a two-alternative forced choice test to observe how mother mice discriminated between two different ultrasonic sounds, emitted from opposing loudspeakers (G. Ehret & Haack, 1982). The researchers played models of isolation calls that varied in duration and frequency range, while counting how many times the mothers approached each of the speakers. The mothers behaved as if sounds within certain ranges of frequency, duration and bandwidth belonged to a discrete category of attractive stimuli (G. Ehret, 1992; G. Ehret & Haack, 1981, 1982). If given a choice between a sound within versus outside this category ("inter-category" comparison), the mothers approached the former significantly more often. However, mothers did not seem to discriminate between two sounds that were either both within or both outside this category (“intra-category” comparisons) (G. Ehret & Haack, 1981). The researchers characterized this as an auditory form of categorical perception, since the acoustic boundary between pup-like and non pup-like categories was relatively sharp.

Recognizing a pup-like category of ultrasounds appears to be a useful way to perceive these calls and attract a mother to the distressed pup. However, if the calls are truly categorically perceived by mothers, then the acoustic variation between natural calls within this category would not be discriminated. This might mean that a mother would not be able to distinguish a call of her own pup from that of a foreign pup if they have systematically different call characteristics, as noted above. Such behavior may not be adaptive, thereby motivating the question of whether more subtle aspects of the mother’s behavior (aside from the number of approaches) might still differentiate between two pup-like sounds.

Quantifying subtle behavioral measures can be difficult, even for overt behaviors like locomotor responses. Fortunately, modern technology can analyze the mouse behavior at a finer detail while facilitating ethological observations at the same time. With steady advances in processing power, a personal computer can now automatically track animal movement and conduct simple ethological observations without assistance of a human observer. Some commercial software such as TopScan™ by Clever Sys Inc. or Ethovision™ by Noldus Information Technology can process video recordings of rodents from a birds-eye-view—either pre-recorded or live feed—and track the location of the animal on a frame-by-frame basis. This continuous tracking of the animal can quantify the speed and the distance the animal traveled—a measure that cannot be quantified so easily by a human observer. Some software can also track the orientation of the mouse and the position of its nose, providing additional resolution to the behavioral measurement.

By automating the observation one can now collect a wealth of behavioral measurements on locomotion during two-alternative choice experiments, possibly uncovering subtle features of the behavior that may indicate a finer ability to discriminate ultrasound calls in mice. The studies described here test this by playing back to mothers two different types of isolation calls—one from a conspecific strain, the other from a foreign strain. Whereas the earlier behavioral studies imply that no difference will be found in the number of approaches the mothers make towards each, our automated analysis will examine whether other aspects of the locomotor behavior show clearer signs of discrimination. We hypothesize that the mother will behaviorally show detection of the sounds, and also exhibit behavior indicating a discrimination between conspecific and foreign isolation calls.

MATERIALS AND METHODS

The Emory University Institutional Animal Care and Use Committee approved all procedures. For recording pup vocalizations and for behavioral experiments, c57bl/6J inbred mouse mothers with pups were used. All animals were housed in an acrylic cage with filtered lid under reverse-light cycle (lights on from 18:00-08:00) and had access to food and water *ad libitum.* Mothers between 12-16 weeks were used for recording/habituation.

-Recording

Isolation calls were individually recorded from approximately 10 pups at postpartum day 7 (p7), inside an anechoic chamber. Pup isolation calls were elicited by removing a single pup from its nest and placing it in an empty cage with clean bedding, within the anechoic chamber. Pup vocalization was recorded for ten minutes from a microphone suspended above the cage. Sound files containing pup recordings were high-pass filtered to attenuate low frequency noise. Eleven samples of 620 millisecond recordings (hereafter termed “snips”) were selected. Each snip contained one or more complete isolation calls with good signal-to-noise ratio and with no extraneous sound (i.e. low frequency calls, noise from animal movement). Each snip was amplified so that the maximum amplitude would be at 80dB SPL at 12cm away from the speakers. Similar procedures were used to obtain eleven snips of CBA/CaJ isolation calls, although we obtained these recordings from an earlier experiment.

Using the software: Brainware™ as a sound playback device, the eleven c57bl/6J snips and eleven CBA/CaJ snips were set to play back continuously for 10 minutes. The software would cycle through the eleven snips in a random order, for twelve cycles. The sound files from the two strains were set to play back simultaneously from two different speakers.

-Habituation & Experiment

As stated in the introduction, two-alternative choice maze was used (Figure 1). In this maze, an open-top center cage (30.5cm x 19.5cm x 21cm) is connected to two side cages (29.5cm x 18.5cm x 13cm) via hallways (36cm x 9cm x 15cm). A speaker (10cm x

1.5cm x 10cm: Infinity® EMIT High Energy Speaker), is placed 1cm away from each side cage. On the wall opposite from the speaker is a hole through which a mouse can exit (W=3.5cm, H=2.5cm). Except for the wall with an exit hole, the other three walls of each side cage have 36 small holes for sound to pass through (6 holes x 6 holes, diameter of the holes =3mm, holes 1cm apart). Within the center cage are removable metal gates (W=7.5cm, H=10cm) that block exits (W=3.5cm, H=2.5cm) leading out to the hallways. The "wingspan" of the maze is 120cm, and the distance from the center cage hole to the speaker was 64cm. The entire maze is enclosed within an anechoic chamber to minimize environmental noise.

Experiments were carried out on twenty-five c57bl/6J litters over two days, all between 12-16 weeks old at the time of experiment. All mothers used in the experiment (n=25) were rearing between five and nine pups. When the pups matured to p6 ("habituation day"), the mother, the pups, and some nesting material were gently hand-placed into the center cage of the two-alternative choice maze. The mother was then given 15 minutes to habituate and explore the maze. The metal gates were left open so the mother could discover the right and left hallways/cages. The observer watched the mother's behavior through a live video camera feed while outside the anechoic chamber. The observer also listened for any ultrasonic vocalizations from the pup, using a remote bat detector suspended over the center cage. The speakers were not used to play back sounds on this day.

After the 15 minutes of habituation, four pups were removed from the center cage and evenly scattered within the two side cages for the mother to retrieve back to the nest. For litters with only five pups, only two were scattered. A bat detector left in the anechoic chamber confirmed that the pups were vocalizing. The mother was given 10 minutes to retrieve the pup. If the mother did not retrieve any pups, the remaining pups were scattered within the center cage, and the mother was given additional 10 minutes to retrieve. The number of pups retrieved and the time it took to retrieve all pups were recorded. Mothers who refused to retrieve any pups after the 20 minutes were disqualified from the experiment. The mothers and the pups were gently placed back into their home cage, and the maze was cleaned using a 5% bleach solution. The center cage was left soiled with dirty bedding so that the mother will recognize the maze on the following day.

On the following day ("experiment day"; pups at p7), the mothers were confined within the center cage by two closed gates for the first five minutes. This period was for the mother to habituate to the maze again. After five minutes, a single pup was removed from the nest and kept a heating pad outside of the anechoic chamber. Immediately afterwards, the gates were removed, granting the mother access to side cages.

As soon as the gates were open, the pre-recorded isolation calls were played back to mothers from the "Playback group" (n=10). The isolation calls of conspecific and foreign strain were played back from each speaker in the side cages. In a single trial, each speaker played back the snips in a random order, but consistently played the calls of a particular strain. The other mothers (n=15) were not exposed to sound playback and served as the "Control group." The mother's activity in the maze was tracked and recorded for 10 minutes. Automated video tracking was performed by *TopScanNI*™, a software produced by Clever Sys Inc.

The software works by first constructing a time-averaged "background" image of the maze; by averaging together multiple frames of the video, the mother disappears and the static image of the maze remains (Figure 2b). Next, the background is subtracted from the live image on the camera or the current frame in a recorded video (Figure 2a), and the computer identifies the difference between the two images as the mother's immediate location (Figure 2c). In addition, the experimenter defines "zones", or the location of the center cage, right cage, right hall, left cage, and left hall within the maze. The computer calculates the mother's coordinates and decides which zone she is currently occupying. Since the experiment is 10 minutes long and the video is recorded at 30 frames per second, the mother's position is tracked about 18,000 times in an experiment.

TopScanNI™ outputs the tracking result in a text format. Custom-written MATLAB script imports the tracking result to calculate the animal's distance traveled, time spent, speed, and number of entries within each zone.

RESULTS

-Natural calls of c57bl/6J and CBA/CaJ pups

Since these calls are recordings of live pups rather than computer-generated models, it was impossible to make them equivalent in all acoustic parameters. However, in order to make the recorded sounds comparable, recordings were made on the same postnatal day, and the snips were amplified to match the peak intensity and checked for extraneous noises and non-ultrasonic calls.

Each snip contained 1-4 calls. The CBA/CaJ snips had an average of 2.0 calls per snip. The c57bl/6J snips had an average of 2.5 calls per snip. The peak amplitude of each snip for calls from both strain were set to 80dB SPL. Other call characteristics are as outlined in Table 1. The mean frequencies of both strains were about 70-75 kHz, higher than the typically reported frequency of about 60 kHz. The c57bl/6J had shorter, softer calls with wider frequency range. The spectrogram of the calls supported these quantitative findings; generally, CBA/CaJ call spectrograms were relatively flat (little change in frequency) and the calls strongly contrasted against the background noise (Figure 7). The c57bl/6J calls were fainter and had very large "sweeps" of frequency changes (Figure 8).

These differences reflect the innate acoustic properties that distinguish CBA/CaJ calls from c57bl/6J calls. It may be possible for mothers to discriminate between conspecific calls and foreign-strain calls, based on these acoustic differences.

-Overall behavior

On habituation day, the mother typically took about 2-5 minutes before discovering both openings in the main cage that lead out to side cages. The mother sporadically dug into the bedding or reared up against the wall. The mother typically roamed between left and right cages. When tested, most mothers made the effort to retrieve; one more mother was habituated but was not included as part of the Playback group when she showed no signs of retrieving. When the metal gates were raised on experiment day, the mother entered one of the side cages within 1 minute, regardless of whether the calls were played. The mothers sporadically dug into the bedding and reared against the wall. Again, the mother typically roamed between left and right cages.

Across both days, all mothers tested were actively explorative within the apparatus and had visited each zone at least once. In general, the mother was continuously on the move, occasionally stopping to pivot around, dig, or rearing against the cage wall. The mothers occasionally sniffed at the pups but did not interact much with them. None of the mothers stopped to nurse the pups during habituation or experiment. None of the pups moved appreciably far from the nest. The litter almost never produced ultrasonic vocalizations during habituation or experiment. Blinded observation and partial scoring of the videos suggest that the rate of digging and rearing activities were not significantly different between the Playback group and the Control group. Without knowledge about the audio playback, the observer could not distinguish from the video whether the mother was from the Playback group or the Control group.

-*Quantitative Analysis*

Using the tracking data of the mothers' position outputted by TopScan™, we quantified different aspects of the mothers' behavior, such as *distance traveled*, *time spent* in a zone, and *number of entries* into the zone. We were particularly interested in the mothers' behavior within the side cages, in proximity of the speakers.

Each animal made multiple entries into each side cages. The number of entries into each side cages during a trial was counted as *number of entries*. During each entry, the mother traveled a certain distance, and spends a certain span of time exploring the cage. We define the representative sample of one of these entries by finding the median length of time a mother spent in an entry ("*time spent per entry*"), or the median distance a mother traveled in an entry ("*distance traveled per entry*"). We took the median rather than an average, because the median value would less likely be skewed by outliers.

As a different measure, we also took the total distance a mother traveled in each side cages, and the total time the mother spent in each side cage. These values were designated "*total distance traveled*" and "*total time spent*" respectively.

- *Detection*

To determine whether the Playback group mothers detected the calls, we compared their behavior on experiment day to Control group mothers who only heard silence. Data from both side cages were combined because sound detection was expected to be reflected similarly in both.

We found that the Playback group made significantly fewer entries into the side cages compared to the Control group, within the 10 minutes of observation (Figure 3a; Mann-Whitney U-test, $p=0.036$). We interpret that this is because the Playback group traveled a significantly greater distance per entry (Figure 2b; Mann-Whitney U-test, $p<0.01$) and spent a significantly longer time per entry (Figure 2c; Mann-Whitney U-test, $p<0.01$) in the side cages compared to the Control group. Consequently, there was no significant difference in the *total distance traveled* and the *total time spent* between these two groups (Mann-Whitney U-test, $p>0.05$).

Another way to visualize the data is to plot each animal on a grid based on their *distance traveled per entry* and *time spent per entry* (Figure 4). While there is some overlap, the Playback mothers generally occupy a different region of the graph than the

Control mothers. Hence, the Playback group clearly behaved quite differently from the Control group.

-Discrimination

The Playback group was exposed to calls from two different strains (CBA/CaJ isolation calls and c57bl/6J isolation calls). Half of the animals were exposed to CBA/CaJ vocalizations from the left side cage and c57bl/6J vocalizations from the right side cage, whereas the other animals were exposed to the reverse. We compared the Playback mothers' behavior in the CBA/CaJ cage with the behavior in the c57bl/6J cage, regardless of which side the sound was playing from.

Since discrimination involved paired results (each mother's behavior in the CBA/CaJ cage versus the c57bl/6J cage), the Wilcoxon Signed Rank test was used rather than the Mann-Whitney U-test. The Playback mothers showed a clear trend of spending greater time and traveling a greater distance near the CBA/CaJ cage, but these trends fell short of reaching statistical significance ($p = 0.08$ in both cases, Figures 5 & 6). Interestingly, one mother (the first we tested) behaved very differently from the rest by showing an extreme preference for the c57bl/6J cage. Without her, the p-values for both behavioral measures would have reached statistical significance ($p<0.01$). Since this animal was an outlier creating variability in our estimates, we performed a power analysis to try to estimate how many total animals we might need to achieve a significant effect. Assuming that the observed mean distance traveled per entry and time spent per entry are representative of any mother's behavior, this power analysis predicts the need for 22-24 mothers to reach the $p < 0.05$ level of statistical significance ($1-\beta = 0.75$).

Discussion

We concluded that the *distance traveled per entry* and the *time spent per entry* are the measures of locomotion that are most effective for indicating differential responsiveness to ultrasounds versus silence (detection), and conspecific versus foreign strain calls (discrimination). These data provide the first evidence to our knowledge that mouse locomotion can exhibit discrimination between two sounds falling within the pup-like category. Since a computer and the tracking hardware/software were absolutely necessary for measuring the distance an animal travels and the time an animal spends in the side cage, this study also illustrates the benefits of automated animal tracking.

-Mother's behavior

For the detection task, the mothers exposed to the sound spent more time per entry in the side cages, and traveled greater distance per entry. We hypothesize that the mothers expressed this behavior because the continuing playback of behaviorally relevant calls kept the mothers in the side cages, possibly to search for their missing pups. Incidentally, this likely meant that the Playback mothers would make fewer entries than the Control mothers into the side cages during the fixed 10 minutes of observation, as we observed.

Surprisingly, the mothers behaved as if they preferred CBA/CaJ vocalizations over c57bl/6J vocalizations, as shown by their tendency to travel a greater distance and spend more time within the CBA/CaJ cage. This finding was puzzling, since it seems maladaptive for the mothers to be attracted to a foreign strain vocalization over the conspecific vocalization immediately after their own pup has disappeared. Nevertheless, there are two possible explanations for this behavior. Perhaps the different acoustic features of the CBA/CaJ calls that we mentioned earlier made the CBA/CaJ vocalizations sound more urgent or distressed, making the stimulus more attractive for the mothers. In ethological terminology they may be more representative of "super stimuli" for pup calls, or stimuli with exaggerated features that are particularly effective at eliciting a behavior (Carew, 2000; Tinbergen, 1951). The other possibility is that the CBA/CaJ vocalizations are simply novel to the mother, and she was more attracted to novel stimulus. Our next goal would be to identify what acoustic feature made the CBA/CaJ call so attractive by observing the mothers' attraction for artificial calls with more modifiable parameters.

Though there are many differences in the how the experiments were designed compared to Ehret's two alternative forced choice test, our behavioral measure: *number of entries* is arguably similar to the *number of approaches* they measured. The stimuli we used were isolation calls from two different mouse strains, which should be intra-category sounds within the pup call category. It is not surprising then, that we found no significant difference in the number of approaches toward one sound over another. On the other hand, other behavioral measures suggest that mothers are capable of discriminating and showing a preference between two types of isolation calls. Our results do not necessarily discredit Ehret's study that suggests mothers can behave as if they categorically perceive ultrasonic sounds. However, our result is an instance where a mother shows discrimination between two supposed intra-category sounds.

However, we also have reasons to suspect that the mothers' *number of entries* into the side cage was due to random exploration, irrespective of the sound played back. The intended design of this experiment was for the mother to hear sounds from both sides of the maze while in the center cage, and then make a decided approach to one side. However, the sounds were attenuated by the time they reached the center cage, and it is possible that the sounds only became sufficiently detectable after she had entered a side cage. This possibility could explain why the mothers showed no significant difference in the number of entries in the CBA/CaJ cage versus the number of entries in the c57bl/6J cage. Upon entering the side cages, the mother may have perceived the calls to be attractive, but the CBA/CaJ calls more so than the c57bl/6J calls. The effect of the call, then, may be to keep the mothers in the side cages for a longer duration once they enter; this idea would also explain the detection result. We are in the process of testing the animals' ultrasonic hearing thresholds and the actual level of sound attenuation at the center cage of the maze. If the sound is indeed too attenuated, then this explanation becomes an increasingly likely possibility. To determine if the mothers would have shown a difference in *number of entries* had they heard the sound, additional experiments are necessary with amplified calls.

We have yet to learn what acoustic feature made the CBA/CaJ vocalizations more attractive to the mothers. One study has classified the isolation calls by their acoustic properties into five different subtypes, and found that certain subtypes of isolation calls are emitted more readily depending on what environmental stressors elicited the call (Branchi, Santucci, Vitale, & Alleva, 1998). Even if the variations in pup sounds can be informative about the environmental stressors, it has not been shown whether the mothers respond any differently to particular pup calls emitted under different circumstances. There remains a possibility that the mothers might prefer one sound over another based on its acoustic properties. If the mother displays discrimination between two subtypes of isolation calls, the behavioral difference is predicted to be even more subtle than our findings or Ehret's.

It is also not known whether the mother can identify individuals or strains based on their isolation calls. Mouse mothers have been shown to discriminate between their own pups and foreign pups, but the mother is likely discriminating using olfactory cues if given a choice between two live pups (Ostermeyer and Elwood, 1983). The ability to discriminate between individuals based on ultrasonic vocalization has been shown in other mammals such as bats (Yovel et al., 2009). Our discovery of the mothers' preference for CBA/CaJ pup calls over c57bl/6J pup calls may be the very first evidence that mice can discriminate between strains. However, additional experiments will be necessary before we can conclusively demonstrate this possibility.

-*Conducting behavioral experiments*

Some analyses for detection or discrimination showed a clear trend, but did not attain statistical significance due to variations in the mothers' behavior. Most notably in the discrimination task, the distance traveled per entry and time spent per entry did not reach statistical significance but showed a very strong trend. When we looked into the data more closely, we noticed that one mother acted as an extreme outlier. In addition to increasing the sample size, we believe that the behavioral variability can be reduced by improving upon the methods we used.

The main challenge of this experiment was the process of streamlining the procedure, so that the mother's response to the call would be robustly expressed. The quality of the behavioral data depends heavily on how well the mothers respond to the isolation call. Although retrieval is an instinctual behavior readily seen in the mice, the mother can easily be distracted by fear, stress, noise, novelty of the maze environment, foreign smell, and the presence of the experimenter. For the mother to retrieve most effectively (or respond to isolation calls with the intent to retrieve), the environmental context should prime her for maternal behavior, the stimulus should trigger her maternal behavior, and distractions should be minimized. Below, we have identified a list of useful techniques to optimize future behavioral experiments, based on our experiences in this study and subsequent behavioral tests.

-*Minimizing distractions*

As was done in our study, the maze should be located in a quiet, open room with minimal clutter. The experiment should be conducted under dim red lighting, outside the visible spectrum of the mother. The floor should be stable with no nearby sources of mechanical vibration. The experimenter should check that no electrical appliances are appreciably emitting ultrasonic sounds.

The mice are very sensitive to smell, and it is important to keep the room and maze clean and ventilated. Since the mice will undoubtedly react to the scent of any previous mice that occupied the maze, the apparatus should be thoroughly cleaned after each experiment. While we have been using 5% bleach solution to clean the cages, other reports suggest diluted acetic acid or ethanol as a better cleaning solution. In addition, the center cage should be well-soiled with dirty bedding from the home cage, to mimic the smell of the home cage environment.

The mother should be allowed to explore the maze before an experimental trial, so that the maze is not a novel environment to the animal is being tested. The pups and some nesting material should be transferred over to the maze, as is done on the experimental trial. Transferring the entire litter to the maze is stressful for the mother, but by the experiment day the mother would have already experienced being handled. Whereas the habituation time in our experiment is 15 minutes, other researchers have spent up to 6 hours (G. Ehret & Haack, 1982). During habituation, the experimenter should leave the room and avoid interacting with the mice unless necessary.

Each trial should be conducted at a consistent time of the day, and conforming to the mother's circadian rhythm. To limit visual distractions, the experiment should be conducted under red light, outside the visible spectrum of the mother. Occasionally, the mice show a tendency to jump out of the maze. It has been reported by another lab that painting the surrounding floor black will discourage this (Dr. Joe Manns, personal communication). If a mouse jumps out during habituation or experiment, it should not be included as a data.

In earlier trials not included in this study, the openings leading out of the center cage and the side cages were much smaller, so that a mother carrying a pup had difficulty passing through the hole. Unable to pass through the hole while carrying the pup, some of the mothers seemed to lose the motivation to (although some mothers learned to drop the pup in front of the hole, walk through, and then turn around to pick up the pup). The hole should thus be of sufficient diameter to let the mother carrying a pup easily pass through (at least 3.5cm x 2.5cm for c57bl/6J-size females).

-Retrieval task

It is not unusual for a fraction of the mothers to be poor retrievers. To confirm that the mother is capable of retrieving, we scatter some of the pups to side cages following habituation. Whereas most mothers will retrieve the pups within 10 minutes, others only retrieve some of the pups or will not retrieve at all. We found that some mothers that did not retrieve after 10 minutes could be motivated by scattering the remaining pups in the center cage. Mothers that still do not retrieve should be excluded. We also found that scattering too many pups decreased the rate of retrieval. For litters with 6 or less pups, only 2 pups should be scattered. For 7 or more pups in a litter, up to four pups can be scattered. The pups should be scattered so that number of pups in any one region does not exceed the number of pups in the nest (i.e. one side cage should not contain more pups than the center cage).

-Sound stimuli

In our experiment, we played back isolation calls of 7-day-old c57bl/6J pups and 7-day-old CBA/CaJ pups. Earlier findings show that pups at p7 vocalize most frequently, at least among the CBA/CaJ strain (Liu et al., 2003). As far as we are aware, there is no evidence that a mother discriminates between pup vocalizations of her own litter and calls from foreign pups of the same strain. Thus, we saw no need to necessarily play back the vocalizations from the mother's own pups.

As stated in the Results, it is impossible to make the two sets of recorded sounds be acoustically equivalent, but some of the differences can be controlled by recording the pups on the same postpartum day, in a similar recording environment. Although we standardized the maximum amplitude of the two stimuli to make them comparable, there are other ways to make the sounds comparable (e.g. standardizing the mean amplitude). The recorded snips should have good signal-to-noise ratio. Computer-generated artificial calls have the advantage of giving the researchers the power to control and modify the sound, if the calls for playback do not need to be naturally derived.

In our experiments, we have been exposing the mother to continuous call playback. However, in a separate experiment that exposed the mice to continuous playback, the animal seemed to adapt to the stimulus rapidly and the response to the behavior was extinguished (Shepard & Liu, 2010). The rapid habituation may be because there is no mouse at the sound source, and the animal no longer responds socially to the call. One potential solution, then, will be to turn off the sound when the mother closes in near the sound source so that she is less likely to realize that the source of the sound is not within the apparatus.

Although it is tempting to play back the sounds simultaneously so neither the conspecific nor the foreign strain vocalization is played back first, simultaneous playback can potentially interfere with the mother’s perception of the sound. Due to auditory masking (a perceptual phenomenon when a sound makes inaudible a second, slightly delayed sound of similar frequency), the mother might not hear one of the sounds if both are played back at the same time within the audible range of the mother. The sounds should be played back asynchronously instead, either by alternating the playback or by randomly varying the onset of the sounds.

-*Symmetry*

For obvious reasons, the two-alternative choice maze should be bilaterally or radially symmetrical. Individual mothers typically behave asymmetrically between right and left side of the maze even in the absence of sound. Although asymmetry within an individual is usually not a problem when averaged across mothers, sometimes the mothers significantly prefer one side regardless of the sound playback. Since the mice may develop bias based on visual, olfactory, and auditory asymmetry, it is essential to minimize the asymmetry in the room. Furthermore, to demonstrate that the mother is discriminating between two sounds rather than behaving on any bias, it is necessary to switch the sound stimuli between left and right speakers.

One inadvertent source for spatial bias may have to do with how animals are handled within the cage. If the mother needs to be captured at the end of the habituation to be returned to the home cage, she should be captured in the center cage. If the mother is captured in one of the side cages during habituation day, she may develop place-conditioned fear and avoid that side of the maze on experiment day.

-*When to conduct control experiments*

If separate control trials need to be conducted, these trials should be done interleaved with experimental trials that are also ongoing. The mouse colony, experimenter's procedure, and testing conditions could imperceptibly change over time, affecting sequentially collected data.

-*Strain choice*

We originally intended to perform this experiment using both c57bl/6J and CBA/CaJ mothers, and compare their behaviors in response to the isolation calls. CBA/CaJ is in fact a more favorable strain to use for auditory studies because they do not develop a late-onset hearing loss that the c57bl/6J animals are prone to at an older age. Unfortunately, the CBA/CaJ mothers frequently leapt out of the maze or froze in one location, and we could not motivate them to respond to the calls. Because the CBA/CaJ mothers became too anxious to show a visible response to the sounds, we did not perform any further experiments using this strain. The c57bl/6J mothers from our experiment were tested before they developed age-dependent hearing loss, and the animals' ability to hear has been confirmed using Auditory Brainstem Response recording.

Table 1- Call characteristics, derived from the sound snips collected for sound playback

		Duration (ms)	Mean Frequency (kHz)	Frequency Range (kHz)*	Mean Amplitude (relative scale)
c57bl/6J	mean	40.36	75.62	26.94	13.59
(n=11)	std dev	19.55	6.52	7.10	1.75
CBA/CaJ	mean	53.59	72.88	11.10	23.64
(n=11)	std dev	13.03	8.14	8.18	6.91
	p value	**<0.05**	>0.05	**<0.0001**	**<0.001**

* -Frequency Range = Maximum frequency – Minimum Frequency

Duration is the length of the call, *Mean Frequency* is the average frequency of the call, *Frequency Range* is the maximum frequency minus the minimum frequency, *Mean Amplitude* is the average intensity of the call, and *Background Amplitude* is the noise level. P-value calculated using Mann-Whitney U-test.

1a

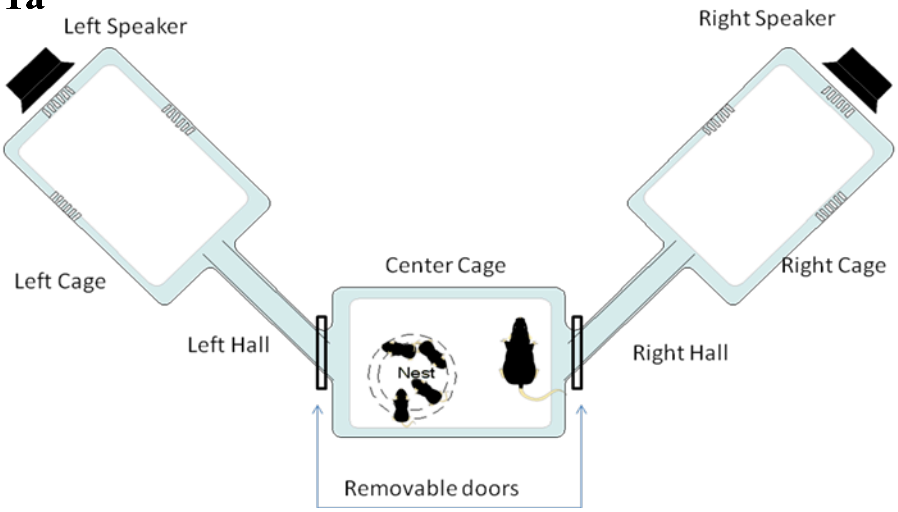
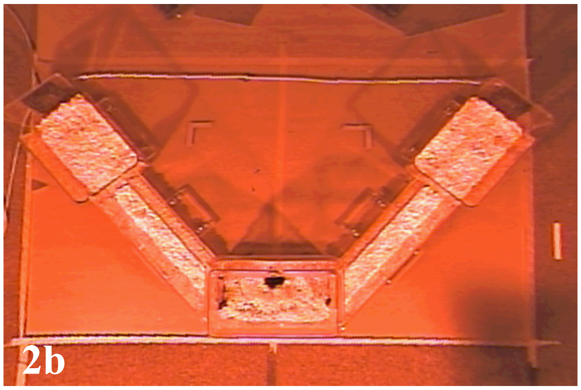


Figure 1a.
Schematic of a two-alternative choice maze. The center cage (30.5cm x 19.5cm x 21cm) is connected to the right and left side cages (29.5cm x 18.5cm x 13cm) via hallways (36cm x 9cm x 15cm). There is a speaker (10cm x 2cm x 10cm) adjacent to each side cage. There are removable gates (W=7.5cm, H=10cm) that block the holes (W=3.5cm, H=2.5cm) leading out of the cage.

Figure 1b.
Snapshot of the maze captured by the video camera inside the anechoic chamber. Both the mother and the pups are within the center cage. The bar and the box shows the scale of the picture in centimeters and pixels, respectively.



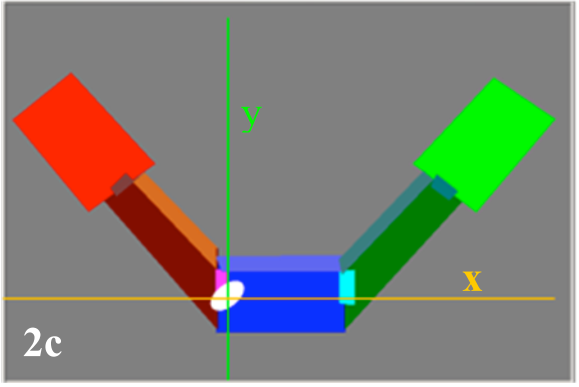
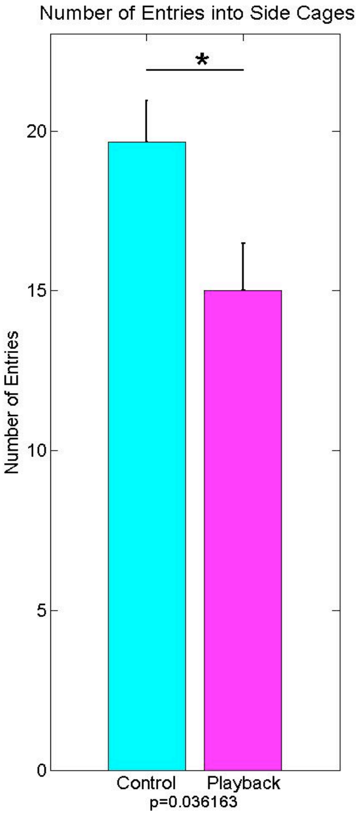


Figure 2a.
Same as figure 1 (lower). Mother is in the center cage of the two-alternative choice maze.

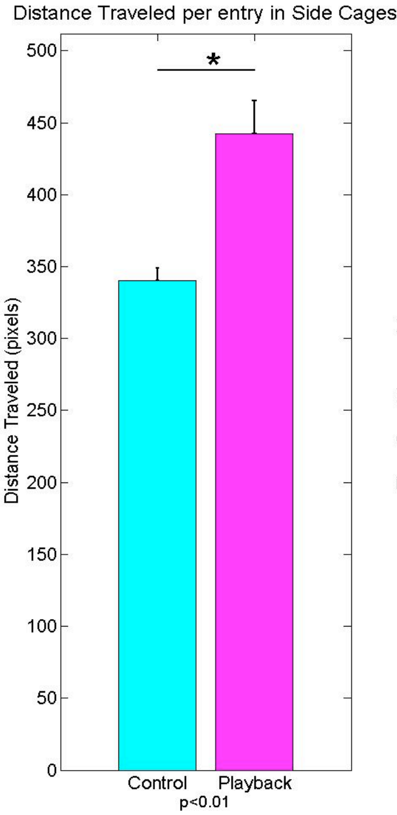
Figure 2b.
Time-averaged Picture of the maze. Note that the mother disappears because she is constantly moving. The pups remain in the background because they do not move.

Figure 2c.
Subtracting Figure 2b from Figure 2a, TopScanNI finds the location and the coordinate of the mother. TopScan also decides which zone the mother is in (blue; center cage). The mother's coordinate will be calculated this way, 30 times a second, throughout the entirety of the trial.

3a



3b



3c

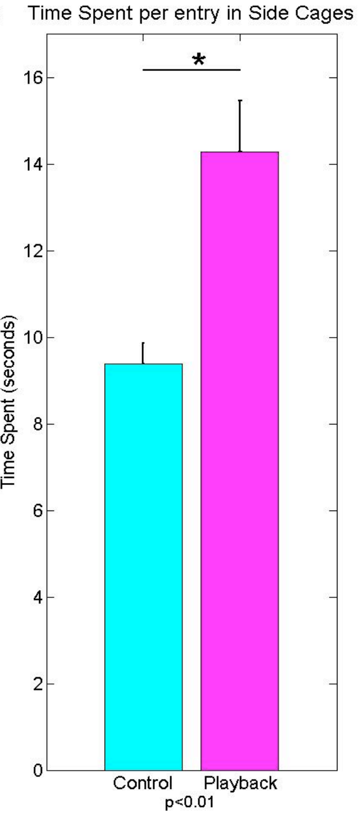


Figure 3a.

Number of entries into the side cages (left cage entry + right cage entry) by Control mothers (n=15) and Playback mothers (n=10) on experiment day. The Control mothers enter the side cages more often than Playback mothers. The difference is significant via Mann-Whitney U-test, p=0.036. Error bars indicate standard error.

Figure 3b.

Playback mothers' (n=10) and Control mothers' (n=15) *distance traveled per entry* on experiment day. The Playback mothers travel a significantly greater distance. p<0.01 via Mann-Whitney U-test. Error bars indicate standard error.

Figure 3c.

Playback mothers' (n=10) and Control mothers' (n=15) *time spent per entry*. The Playback mothers spend greater time in the side cages. p<0.01 via Mann-Whitney U-test.

4

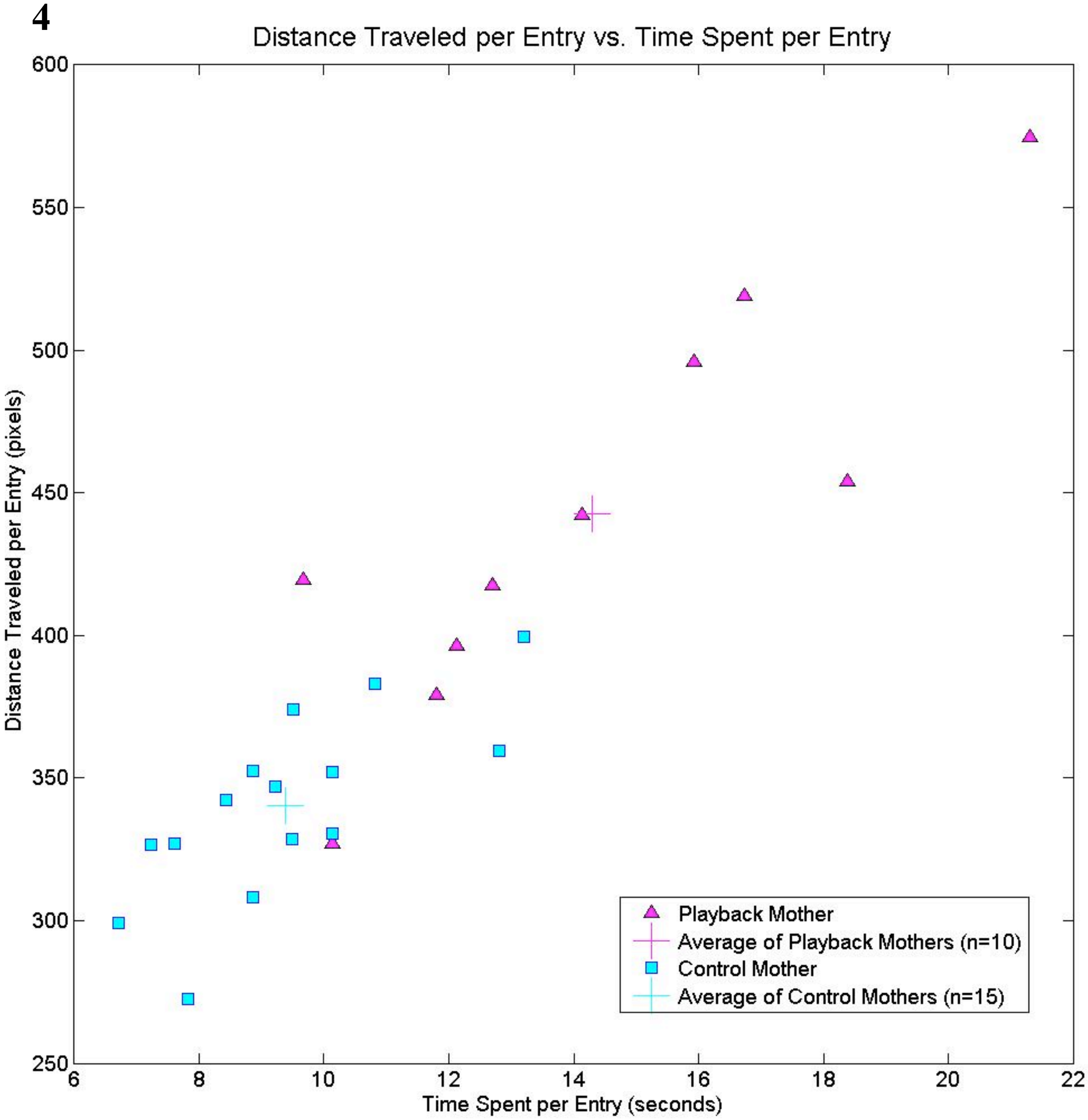
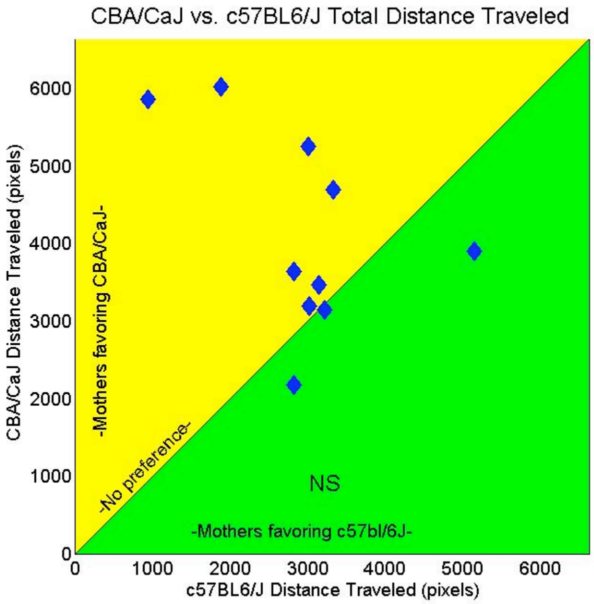


Figure 4
Scatter plot comparing the two mother groups on experiment day; the mothers' time spent per entry and their distance traveled per entry are denoted by x and y axis, respectively. The mothers exposed playback ("Playback mother", magenta triangle, n=10) spends greater time and travels a longer distance within the side cages than the Control mothers ("Control mother", cyan square, n=15).

5a



5b

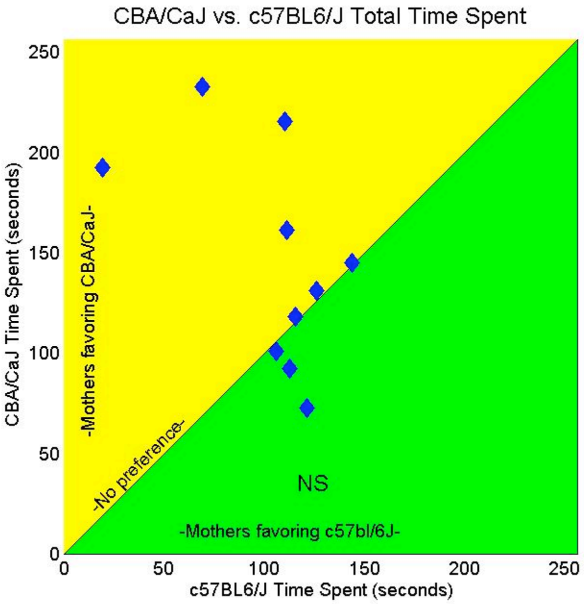


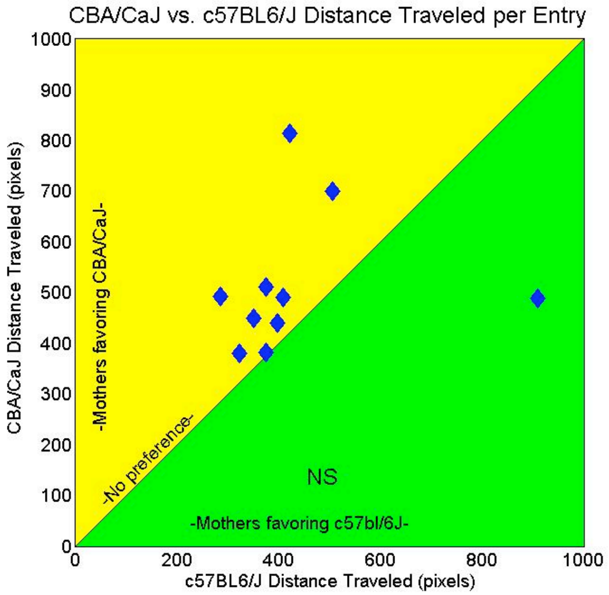
Figure 5a.

Scatter plot of mothers from the Playback group, and their *total distance travelled* while in the CBA/CaJ cage versus c57bl/6J cage. Each point represents a mother's *total distance traveled* in the two side cages. Mothers that traveled a greater distance in one cage over the other tend to lie near one of the axes (y axis = CBA/CaJ preference, x axis = c57bl/6J preference). Animals that lie near the dotted line at the center are showing no preference. According to this measure, the mothers show no significant preference for one sound over another (Wilcoxon Signed Rank test, p=0.105).

Figure 5b.

Scatter plot of mothers' *total time spent* while in the CBA/CaJ cage versus c57bl/6J cage. Each point represents a mother's *total time spent* in the two side cages. Mothers seemed to spend more time in the CBA/CaJ cage than in the c57bl/6J cage (most points lie closer to the y axis, which represents the CBA/CaJ *total time spent* in cage). This did not reach significance under a paired statistical test (Wilcoxon Signed Rank test, p=0.232).

6a



6b

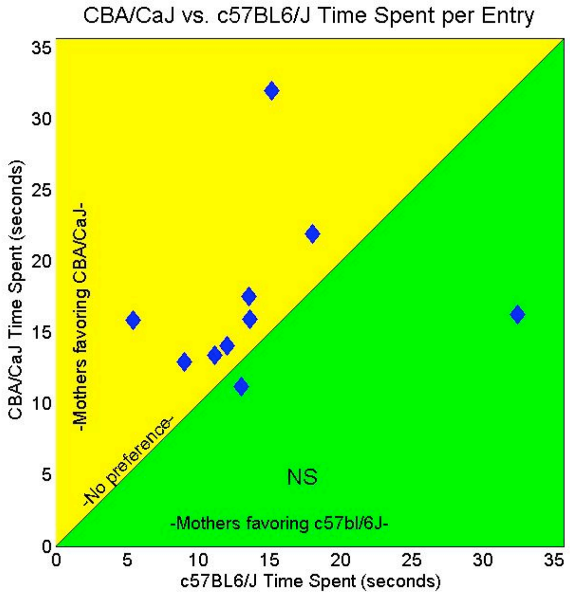


Figure 6a.
Scatter plot of mothers' *distance traveled per entry*, within the CBA/CaJ cage versus c57bl/6J cage. Mothers seemed to travel greater *distance traveled per entry* within the CBA/CaJ cage than in the c57bl/6J cage (most points closer to the y axis, representing the *distance traveled per entry* within CBA/CaJ cage). Despite the trend, this did not reach significance under a paired statistical test (Wilcoxon Signed Rank test, p=0.084).

Figure 6b.
Scatter plot of individual mother's *time spent per entry*, within the CBA/CaJ cage versus c57bl/6J cage. Mothers seemed to spend more time per entry in the CBA/CaJ cage than in the c57bl/6J cage (most points closer to the y axis, representing *time spent per entry* in CBA/CaJ cage). Despite the trend, this did not reach significance under a paired statistical test (Wilcoxon Signed Rank test, p=0.080).

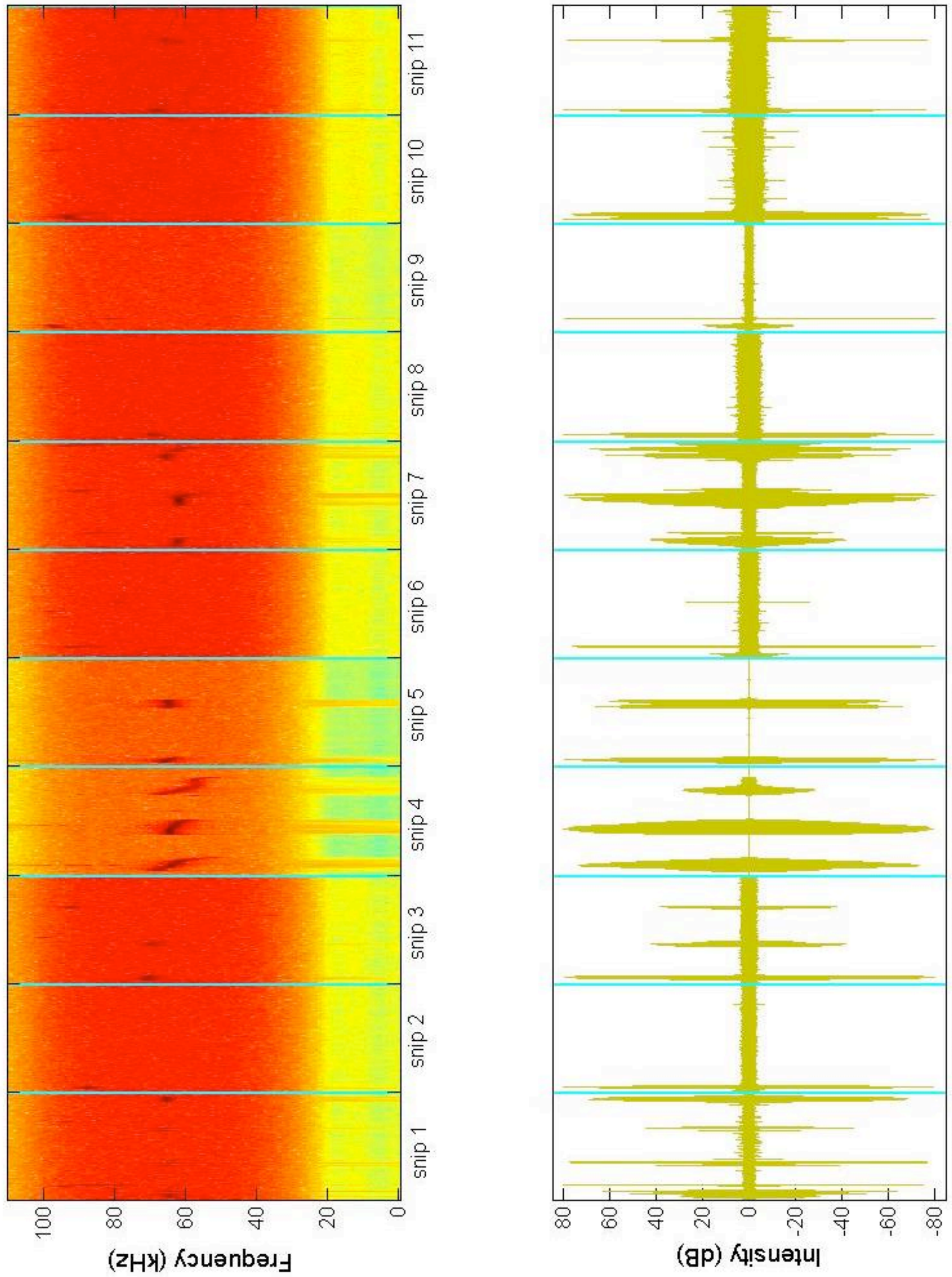


Figure 7
The spectrogram (left) and the waveform (right) of the snips, collected from CBA/CaJ pups at p7. Each snip is separated by a cyan line.

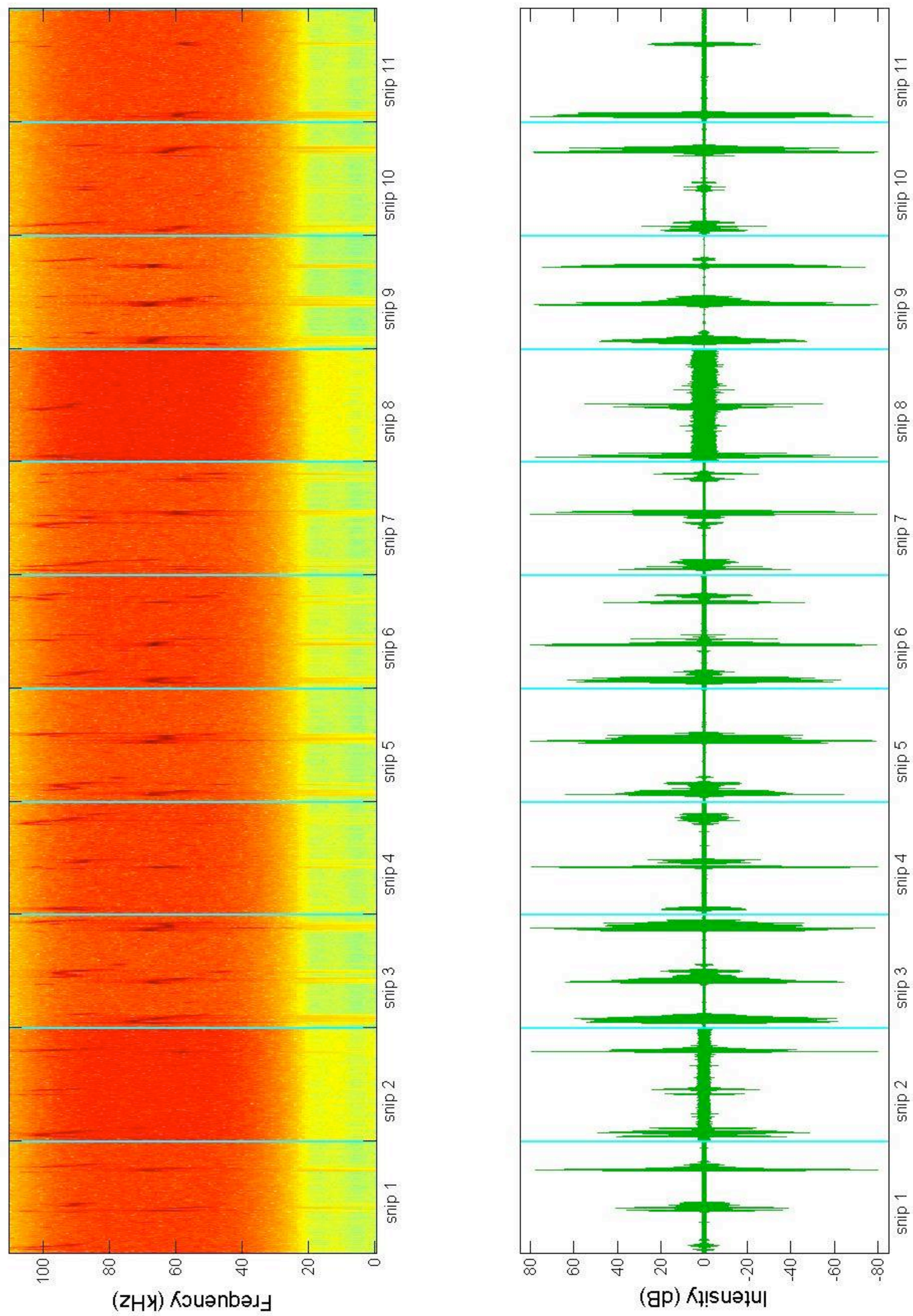


Figure 8
The spectrogram (left) and the waveform (right) of the snips, collected from c57bl/6J pups at p7. Each snip is separated by a cyan line.

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