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Danial Arslan April 13, 2020

Modality Specific Contributions to Multimodal Social Recognition in Pair-Bonded Prairie Voles

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Social encounters between animals often involve multiple sensory modalities with sensory cues containing information about the individual or about its affective state. A receiver's responses are, therefore, contingent on both individually and contextually specific cues. Identifying the social information conveyed by different modes of sensory cues would help to understand how those cues are used in social communication to recognize conspecifics and/or their affect. One potential sensory channel is olfaction, with previous studies showing that rodents rely heavily on the pheromones in urine to identify potential mates and intruders. Another is audition, where ultrasonic vocalizations (USVs) are known to be an important form of communication in mice and rats – both between adults and their pups as well as with each other. However, neither mice nor rats are known to have robust, long-term recognition of conspecifics, motivating exploration of these issues in other experimentally tractable rodent species with stronger prosocial interactions such as prairie voles. Here, we use adult prairie voles to analyze the role of audition and olfaction in social communication. These socially monogamous mammals form a life-long selective pair bond and collectively display complex prosocial behaviors, such as nest building. Therefore, we hypothesize that prairie voles not only use olfactory cues to, presumably, recognize their partners but also vocalize in the ultrasonic range to communicate with conspecifics. We first examine whether males recognize the odors of their partners and later investigate how they may display that recognition in both their investigative and vocal responses. We then look at whether emitted vocalizations might be used by receiving females to recognize their male partners. Hence, this work lays the groundwork for studying the neural bases of learning to recognize the social-sensory cues of a partner.

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Introduction

Many animals, including humans, live in complex social societies, where communication and the display of social behaviors is crucial for reproductive and survival fitness (Hauser, 1996). Living in such social structures requires animals to be able to participate in a broad range of social interactions that are incredibly intricate in nature, but where decisions may need to be made in a matter of seconds. Communication during these interactions requires the instant processing of cues from multiple, if not all, sensory modalities, which then shapes response. Despite the importance of these interactions, we have yet to fully delineate how the brain processes and integrates this variety of sensory information in order to modulate behavioral responses. To make progress we must start by better outlining the contributions of individual sensory modalities to social communication. We designed this study to trace putative sensory channels involved in communicating and recognizing others and their affective states. In particular, responses in social encounters are often contingent on both the perception of individually-specific cues, which are used to identify the other conspecifics involved, and the perception of context-specific cues that guide how one responds. Hence, the goal of this study was to understand which sensory modalities might be involved in the recognition of individuals and which may be involved in understanding the social environment or 'context' one is in. We did this by studying adult encounters in prairie voles.

Prairie voles (*Microtus ochrogaster*) are known for their socially monogamous nature that is, their ability to form life-long pair bonds. After forming a pair bond, male and female voles often

build a nest together where they dwell and care for each other and their offspring while exhibiting aggression towards any intruders (Carter & Getz, 1995; Getz et al., 1981). Prairie voles are among some of the few mammalian species to show empathetic responses towards others. They consolidate their stressed pair-bonded mates. (Burkett et al., 2016). To express such affiliative behaviors selectively, a pair-bonded vole has to be able to recognize its partner from others in order to respond to its needs appropriately. Hence, prairie voles provide a robust platform for investigating the processing of social signals to recognize conspecifics and their affective states (Ma et al., 2014).

Our work holds significant implications, since there is a gap in knowledge when it comes to understanding how prairie voles use social cues in communication and partner recognition. These mammals have garnered recent interest in behavioral neuroscience, since their monogamous nature provides an interesting avenue to study oxytocin mediated mesolimbic pathways which have been hypothesized to play a role in social memory formation and in attaching valence to social interactions (Bosch & Young, 2017). These pathways have also been implicated in disease pathologies such as schizophrenia, where their impairment can account for negative symptoms such as anhedonia and asociality (Amadei et al., 2017; Hammock & Young, 2006). Such symptoms have also been attributed to impediments in auditory processing and in the plasticity of the primary auditory cortex (A1) (Hames et. al., 2016; Martins & Froemke, 2015). Hence, there is a need to understand the processing of sensory cues and how they can eventually lead to the development of social memories which in turn can modulate

affect and subsequent behavioral responses. Our hope is that this work will lay the groundwork for studying the neural bases of social learning which will subsequently provide greater insights into how impairments in those mechanisms can result in social pathologies.

Work done in voles and other rodents, such as mice, point to the role of olfaction in communication. Mice secrete major urinary proteins (MUPs) in urine and differences in MUP signatures can be learned through social interactions (Roberts et al., 2018). Other groups have discovered that the major histocompatibility complex (MHC) can be involved in social signaling as well as adaptive immune responses. MHCs can impart unique body odors which do not change based on environmental variations (Ruff et al., 2012). In mice, MHCs can signal relatedness, genetic compatibility, and individuality. MHCs can modulate mate preferences and facilitate corporative behaviors across kin (Ruff et al., 2012; Kwak et al., 2008). Volatile odor profiles, from the 10^9 MHC phenotypes in mice, also invoke neurons in the main olfactory bulb differentially, highlighting their potential use in social recognition (Schaefer et al., 2001; Singh, 2001). Like mice, prairie voles seem to rely on chemosignals to identify counterparts which is exemplified by the fact that olfactory bulbectomies decrease the expression of sexual, parental, and other social behaviors in voles (Kirkpatrick et al., 1994). However, even if odors play an important role in prairie vole communication, any changes in body scent occur over a large timeframe. Thus, other sensory modalities must be involved in conjunction to olfaction for quick and effective social communication.

Rodents also utilize auditory signals to communicate, vocalizing in the ultrasonic range in response to conspecific odors. The calls are known as ultrasonic vocalizations (USVs). In species such as mice and rats, calls are emitted by adults selectively in relation to a range of behaviors, including exploration of a new environment, and encounters with another adult conspecific (Sangiamo et al., 2020; Hanson & Hurley, 2012, Holy & Guo, 2005, Knutson et al., 2002). USVs have been shown to hold a communicative salience, with calls emitted by distressed pups used by maternal mice to locate their young (Ehret, 2005; Liu et al., 2003). Acoustic features of calls such as duration and peak median frequency can provide a system to understand the affective state of an organism (Lahvis, 2011). Mice seem to emit specific types of calls in conjunction with certain social behaviors. For instance, in a social group setting, male mice emit calls which increase in pitch while expressing non-dominant behaviors, such as avoiding other mice by fleeing. Conversely, they emit calls that decrease in pitch while expressing dominant behaviors such as aggression towards other males and when courting females (Sangiamo et al., 2020). The coexpression of certain behaviors with a specific repertoire of vocalizations suggest that these calls can convey information on the vocalizing mouse's action, thus also communicating the affective state of the caller (Sangiamo et al., 2020).

Like mice, prairie voles are also thought to be "vocal creatures" (Stewart et al., 2015; Campi et al., 2010; Campi et al., 2007). In fact, unlike mice prairie voles have a disproportionately large auditory cortex, relative to other primary sensory cortices for rodents of their body size (Campi et al., 2010; Campi et al., 2007; Blake, 2002; Lepri et al., 1988). This could be explained due to

the parenting style of prairie voles which, as opposed to other rodents, involves high-contact and interaction with pups. Consequently, the enhanced sensory environment voles experience at infancy can appreciate the development and size of several cortical fields, including areas involved in sensory perception. (Bottom et al., 2020).

As a result, there is a possibility that prairie voles may be heavily reliant on both olfactory and auditory cues in order to communicate in various social scenarios. However, the degree to which olfaction and vocalizations can contribute to multimodal communication and recognition is unknown. Here, we seek to answer three behavioral questions to understand how social communication occurs in prairie voles. We first ask whether olfactory cues alone could be used by pair-bonded prairie voles to recognize their mates, hypothesizing that cues in this modality might in-fact be individually specific, perhaps due to the presence of peptides such as MUPs and MHCs that might be involved in generating distinct body odor profiles. We, therefore, performed a two-alternative choice odor test in a three-chambered cage, presenting a male subject with soiled bedding from its female partner or a female stranger. Next, we anticipated that auditory production in prairie voles may either convey information specific to the individual and thus be used in conspecific identification or these calls may outline the caller's affect and behavior. Hence, we presented males, in separate recording sessions, with urine from their female partner, a stranger female, a stranger male, or a water control. We also repeated this experiment with a separate cohort of males, this time using anogenital scent of conspecifics to elicit USVs instead. Lastly, we looked at whether vocalizations emitted by males

in contexts where they smell the scent of either their partner or stranger are used by a female receiver for social recognition. This was done via a two-choice playback experiment, where previously recorded USVs from male voles were played back to see if females showed preferences towards USVs from a specific conspecific over the other, indicating either recognition of their partner or the vocalization context (males smelling their partners' scents). Our results dissect the functional significance of olfactory and auditory cues in prairie vole adult interactions, thus providing a basis for investigating the neural underpinnings involved during communication in these socially monogamous mammals.

Methods

Ethics

The procedures mentioned were all performed in accordance with the National Institute of Health guidelines and were approved by Emory University's Institutional Animal Care and Use Committee prior to the experiment. Furthermore, all experiments were carried out in the light cycle period so as not to disrupt the diurnal circadian rhythm of any of the animals.

Animals

28 male and 27 female prairie voles (n=55) were used in total for all the behavioral experiments performed in this study. They were obtained from a colony that originally comprised of field-captured voles in Illinois (Getz & Hofmann, 1986; Getz et al., 1981). They were weaned on day 21 and socially housed with siblings, in same-sex groups of two or three until testing (see testing timeline below). All animals were subjected to a 10:14 hour light/dark cycle, in 20^{o}C, with *ad libitum* access to standard chow and water. All voles used for these experiments were kept under the age of 1.5 years and were no younger than 2 months old.

Ovariectomies and Priming

Females for all experiments, with the exception of Experiment 5, were ovariectomized bilaterally under isoflurane anesthesia prior to testing in order to normalize their hormonal states during behavioral experimentation (Amadei et al., 2017). Prior to each surgery, a Meloxicam injection (2mg/kg) was administered as an analgesic. Post-surgery, the animal was placed on a hot bed for recovery. This was followed by daily observation for a week after the

animal had been placed back into its home cage. Three days before starting cohabitation, all ovariectomized female subjects were primed with a solution of 1 - 2µg of estradiol benzoate (17-β-Estradiol-3-Benzoate) in 1 mL sesame oil (Amadei et al., 2017) by subcutaneous injection, once daily, for three days. This protocol helps to maximize a female's 'sociosexual interest' for male voles during cohabitation (Amadei et al., 2017; Young et al., 2010). In Experiment 5, the five female voles used did not undergo any ovariectomy and were intact during behavioral testing.

Cohabitation

A male and female vole (ovariectomized for Experiments 1-4 and intact for Experiment 5) were placed together in a clean, ventilated Plexiglass cage 26x18x19 inches, which was filled with Bed-o-cobbs Laboratory Animal Bedding and paper bedding to encourage nest building. The cohabitation period lasted 7 days with the same environmental conditions and same food and water availability as when the animals had been housed with siblings. This period of cohousing allowed for the facilitation of the pair bond between the female and male. After the cohabitation phase, both voles were housed individually in separate clean cages, to enhance social motivation before beginning behavioral testing. Partners for cohabitation were matched by age (approximately 70 days of each other) and by weight (within 5 gm of each other).

Experiments

At the point of behavioral testing, all animals went through the same periods of cohabitation and social isolation. Thus, all stimulus animals had the same degree of sexual experience, and

equivalent to that of the testing animal. Stimulus animals were defined as those whose odor or calls would be used in the following behavioral paradigms. Meanwhile, testing animals were defined as those prairie voles which actively participated in the behavioral tasks, perceiving the sensory cues from stimulus animals and responding accordingly. A separate cohort of animals was used in each of the experimental tests, which are described in detail below.

Experiment 1. Two-Alternative Choice Odor Paradigm

Seven males and seven females were used for this test. The females in this study were ovariectomized prior to cohabitation and used as "stimulus animals," whereas the males were subjects in the testing paradigm. As aforementioned, all pairs went through a weeklong cohabitation followed by 24-72 hours of social isolation where each animal was placed in a cage alone. During social isolation, which lasted at least 24 hours prior to the behavioral test, the soiled paper bedding from a female's cage was collected for presentation. The bedding would have the vole's scent, and we placed it in a petri dish with 30 perforations at its top and 30 at its bottom in order to enable adequate odor circulation. The goal was to present these petri dishes in the behavioral apparatus so that the perforations would allow the odor from the bedding to diffuse out into the behavioral chamber so that it would be detectable by the subject male. After collecting the bedding, these petri dishes were sealed immediately using a plastic film (Parafilm tape) to ensure airtightness. The dishes were stored in a freezer at -20^{o}C and thawed two hours before the behavioral assay. Five minutes before the start of the assay, we removed the parafilm tape.

In this assay, we utilized a three-chamber cage, wherein males were placed in the central, neutral chamber and allowed to roam freely for 30 minutes. In one of the side chambers, a dish containing the bedding from the female the male had cohabitated with (partner) was secured vertically on the wall so that it was at least 5 inches above the ground. On the other side chamber, we placed a dish containing the bedding of a female the male had not interacted with (stranger). The decision of which side contained which bedding was decided using a random number generator.

To familiarize the subject to the apparatus a day before testing, the male was allowed to investigate the three chambers without the presence of any bedding in the dishes. On the testing day, the male was once again placed in the neutral chamber (center chamber) of this cage and was allowed to roam for 5 minutes to habituate. Then, the two petri dishes were inserted on either side of the cage and the male was allowed to roam freely across the 3 chambers, while we recorded video from the top down at a rate of 30 frames per second. We then used these recordings for a comparative analysis of the time spent on each side of the cage and the total time spent investigating the bedding containing either conspecific's scent. Investigation was defined as periods of time when the male vole was within 5 inches of the petri dish and oriented towards it, or was on its rear feet and was actively sniffing or scratching the dish. These recordings were scored using Observer XT10 (Noldus Information Technology Inc., Leesburg, VA) by a scorer who was blinded to experimental conditions.

We used a separate cohort of three males and three females in a control experiment. Here, the testing males were placed in the center of the cage as before. However, in these trials the

dishes placed on both of the side chambers contained bedding from the same female conspecific i.e. the female partner. Once again, behavior was recorded for 30 minutes.

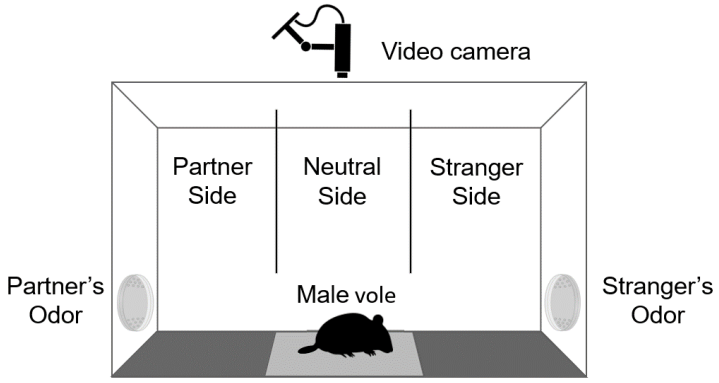


Figure 1. Odor Exposure Assay Setup. The male vole was placed in the center and allowed to roam in the three-chambered cage for 30 minutes.

Experiment 2. Urine-Elicited USV Recording Paradigm

Seven males and six females were used for this portion of the study. Six animal pairings were created, each consisting of one male and female. Each pairing initially underwent cohabitation and single housing as outlined above. In this paradigm, male subjects were presented with urine from conspecifics to elicit vocalizations which were recorded and analyzed. The females from each pairing, in addition to one male who was not paired were used as stimulus animals i.e. urine was extracted from these animals to elicit vocalizations from the six testing males who were cohabitated. Two of the groups had to be excluded from the study. In one of the groups the female had shown aggression towards the male and so cohabitation had to be terminated.

In the second group no urine could be collected from the female partner vole (urine collection method limitations are discussed later).

After cohabitation, the five females and the one stimulus male vole were euthanized via CO_2 asphyxiation. A ventral incision was made along the sagittal plane, to expose the vole's bladder. On successful location of the bladder, a syringe was used to penetrate the walls of the bladder and to access the urine stored inside, which was then removed. Hence, fresh samples of urine were obtained, which were stored in 0.2 mL Eppendorf tubes. These were placed in a freezer at -20^0C and stored for approximately 48 hours until needed for testing (McIntosh et al., 1984). At this point, the frozen urine samples were removed and thawed in a crushed ice bath for 2 hours until the samples liquified. Then, 10 µL of urine was added to 90 µL of distilled water to create a 10% dilution by volume of urine. Out of the 100 µL solution created, 50 µL was transferred, via a micropipette, onto a 2x2 cm gauze, which was placed in a petri dish with 60x 1 mm diameter perforations. The dish was sealed using parafilm, while making sure not to cover the perforations, and was used for testing within 10 minutes after the urine had been added to the cloth (Hoffman et al., 2009).

We created four experimental conditions and each testing male subject participated in all four conditions. The first condition involved recording vocalizations emitted when the male subject was presented with the urine odor of a female stranger vole. A stranger was defined as a vole that the male vole had not undergone cohabitation with, and who was not a sibling of the male subject. In the second condition, males were presented with their female partner's. In the third

condition, male subjects were presented with the odor of a non-sibling, male conspecific. In the fourth condition, males were placed in a cage with a sample of water as a negative control.

Twenty-four hours prior to vocalization recording, a male subject vole was placed in the middle of a Plexiglass chamber with one inch layer of paper cellulose bedding for 30 minutes for habituation. This was done since it has been shown that new environments can induce vocalizations in rodents due to anxiety, and so, a habituation phase was included in an effort to reduce such calls (Portfors & Perkel, 2014). On the day of testing, the vole was once again moved to a clean Plexiglass chamber with paper bedding. The vole habituated for 10 minutes, after which it underwent four 10-minute trials. During each trial, we placed into the cage a petri-dish containing a cotton gauze soaked in female stranger urine, female partner urine, male urine or 50 µL of water. Each of these trials was done in a random order, as determined by a random outcome generator software, and a 5-minute no-odor break was inserted between trials. During the entire test, the vole was allowed to roam freely in the chamber, and a Avisoft microphone was used to capture ultrasonic vocalizations as outlined in further detail the next section. The test was also recorded via a video camera to monitor behavior as aforementioned.

After testing, the male vole was placed back into its cage. where it was singly housed. Each subject received fresh paper bedding in the testing chamber, and we cleaned the Plexiglass testing chamber with soap and water and dried it before reused with another male subject.

All tests were performed in a room temperature-controlled room with a constant light intensity. These had to be controlled, since it has been shown that ambient temperature and

light intensity can affect vocalizations in rodents by inducing anxiety (Borta et al., 2006, Jelen et al., 2003).

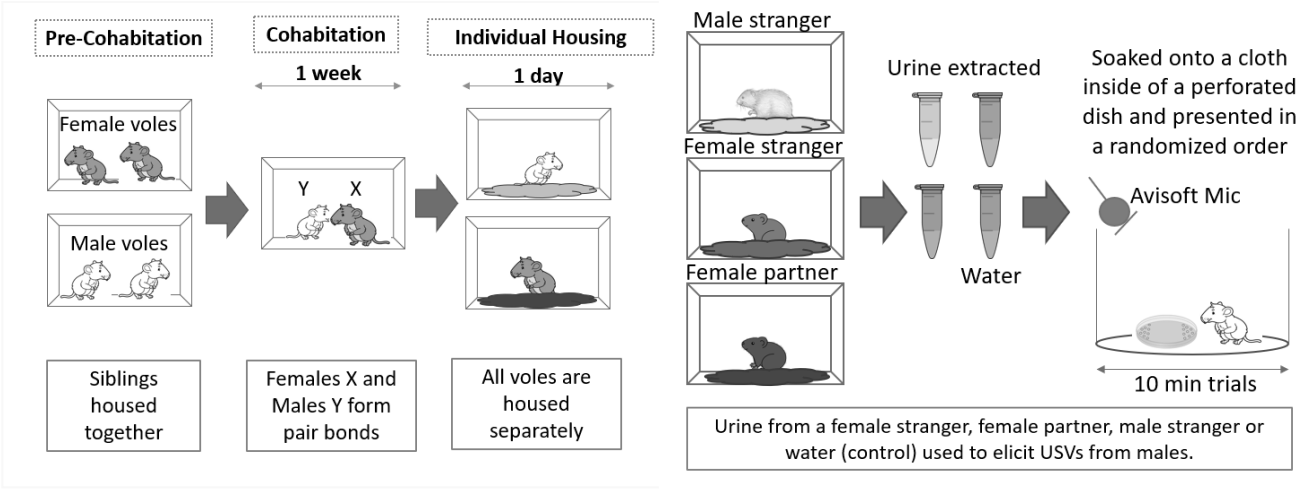


Figure 2. Schematic of the general experimental procedure for the urine-elicited USV recording paradigm.

Experiment 3. Anogenital Scent-elicited USV Recording Paradigm

A set of four males and four females were used for this part of the study. Four pairs were created, and each pair initially underwent cohabitation and single housing as outlined above. In this paradigm, males were presented with the anogenital scent from conspecifics, and we recorded and analyzed the resultant vocalizations in response to the different scents.

The experimental paradigm remained the same as that in Experiment 2, with the only difference being that we presented anogenital scent as a stimulus. Using a clean 2x2'' cotton gauze, the anogenital region of the stimulus voles was swabbed and placed in a petri dish. The collection occurred no later than 10 minutes before their presentation in the assay to reduce

the chance of evaporation. The experimental groups remained the same, i.e. anogenital scents were taken from each of the subject males, a female partner, a female stranger to the male, and a non-sibling male. (Ferkin et al., 1997; Ferkin & Johnston, 1995). Moreover, for our negative control, the 2x2'' gauze was soaked in 50 µL of water. The dishes were presented in randomized 10-minute trials in a singular chamber to the testing male who was habituated to the cage 30 minutes prior to first scent presentation, with a 5-minute break between trials. A microphone was used to record any vocalizations emitted, which were then analyzed as described in the next section.

Experiment 4. Two-Choice USV Playback Paradigm

Five females and five males were cohabitated in pairs for a week. All female subjects were ovariectomized. There were a total of five cohabitation pairs (A1-A5). Three of the males were from one litter 'Y1', and the other two were from another litter 'Y2'. On the other hand, the females were from three different litters 'X1', 'X2', and 'X3.' The pairs were set up so that if the males selected to form Pair A1 and Pair A2 respectively were from the same litter, Y1 for example, then the females selected for Pair A1 and Pair A2 were not littermates. In other words, in this case, if the female for Pair A1 was from litter X1, then the female for Pair A2 was from either litter X2 or X3 but not from X1. The reason behind this method of selection was to ensure that if two different pairs had sibling males, then they could not have sibling females and vice versa.

After seven days of cohabitation, the male and female in each cohabitation pair were housed in isolation. On day 8, the female vole for each pair was swabbed using a 2x2'' cotton gauze to extract anogenital scent. This was placed in the same petri dishes used in the previous assays. The dish was taped shut via parafilm while ensuring that the holes remained open. This procedure was performed not more than 10 minutes before the actual exposure of the dish to the male vole to elicit vocalizations. Meanwhile, the male vole for each pair was placed in an audio recording chamber to record vocalizations.

The male vole was exposed to three different scents and a control (a petri dish with a clean, dry cloth). One of the scents was that of the female partner. So, the A1 male, for example, was presented with the anogenital scent of the A1 female it had cohabitated with. The second scent was of a female that had been partnered with the male's sibling. Thus, given the aforementioned scenario, if Pair A1 and Pair A2 both had males taken from litter Y1, then the A2 female's anogenital scent was presented to the A1 male. Since the A2 female was not a littermate of the A1 female, and had been cohabitated with A2, who was male A1's sibling, she had no direct contact with male A1 and was therefore a complete stranger to it. Finally, the third scent was of another female stranger but who had been cohabitated with a male who was not related to Male A1 in any way (i.e. was from a different litter, Y2). The order in which these exposures occurred was randomly assigned via an online number generator.

The male was allowed to initially habituate to the empty cage for ten minutes after which the scents were introduced, with each scent constituting a new session. Each session lasted for 10 minutes, during which we recorded the male vocalizations in response to the olfactory stimuli

presented. Only two sessions were done at a time for each male, to maximize the vole's interest in the new stimuli at the beginning of each session in order to get the most vocalizations. There was an interlude of 10 minutes between the two sessions done in succession. The other two sessions were done at least an hour after the first set of sessions to prevent loss of interest in the male subject and to prevent a dampening in the number of calls emitted in the later sessions since continuous elicitation of calls could potentially lead to fatigue. An Avisoft-UltraSoundGate CM16/CMPA sensitive condenser microphone sensitive to frequencies from 2-200kHz was used to capture vocalizations, which were extracted, assessed and bundled together to form audio files for playback as described in the section below.

On day 9, after two days of social isolation for the females, a three-chamber cage was set up containing two Avisoft Ultrasound Dynamic Speakers Vifa (one in the left chamber and one in the right chamber). The two emitters were faced towards the center chamber and were used to play back two different audio files created from the above recordings. The doorways between the center chamber and the left/right chambers were closed using a translucent cover with holes for the subject female vole to nose poke and listen to the sounds emitted from the speakers, placed just behind the covers. A video camera provided a top down view of the cage. The female vole was placed in the middle chamber of the cage and 10-minute playback trials were conducted. Four different trials, randomized using a number generator, were conducted for each female vole:

1) The USVs of the female's male partner were played from one side of the cage, and the USVs of a non-affiliated stranger male were played back from the other side.

2) The USVs of a sibling of the male the female subject had cohabitated with was played on one side, and the USVs of a non-affiliated stranger male (non-sibling of the male the subject female had cohabitated with) were played from the other side.

3) The USVs of the partner male were played from one side, and the background file from the other. A background file was created for each male's USV recordings which contained the natural sound recorded by the microphone when the male was presented with dry cloth. Any calls detected in the recording were removed when the background noise file was created, however, the audio file contained other noises including sounds due to when the male played with the dish containing the gauze, scratched the walls of the cage, or moved in the cage.

4) The USVs of a stranger male were played from one side, and the background file from the other. The background file contained noise with no USVs.

The sides from which the calls were played back was also randomized beforehand and blinded to the experimenter. The generation of the audio files used for playback is explained in detail in a later section. Each trial was 10 minutes in duration. The female of each pair first underwent a 10-minute habituation in the new cage before testing. As in the male case, only two trials were done in succession, after which the vole was returned to her cage. Later in the day, the female was once again placed in the assay chamber for the other 2 trials. There was a 10-minute interlude between trials where no sounds were played back from the speakers. We recorded video at 40 fps for each trial, and analyzed the behavior using Observer X10. We marked the investigation/interest time for each type of USV (i.e. for each side of the cage). This investigation time was defined spatially, based on times during which the female vole was

within 3 inches of the translucent barrier (the center chamber the vole was placed in was approximately 9 inches in length) or was nose poking at the holes in the barrier, or was scratching at the barrier. We accumulated the investigation times for each side separately to determine which side the female vole showed preference towards, based on the sounds being played back from each side. This set-up was also blinded such that the scorer was unaware of which USVs were played back from which side of the cage.

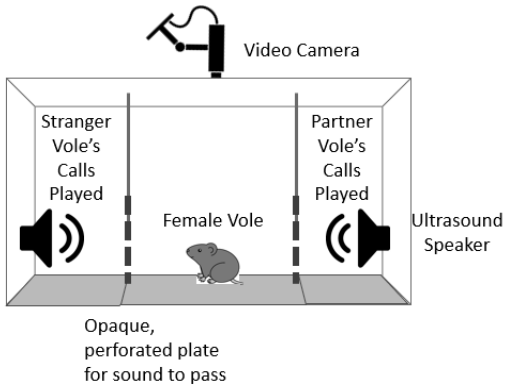


Figure 3. USV playback assay setup. The female vole was allowed to move freely within the center chamber which was closed off by perforated barriers behind which ultrasound emitters were placed which played back calls from two different conspecifics. In this example, a stranger male's calls in response to the scent of the testing female are played from the left and the calls from the partner of the testing female are played from the right, while the behavior of the female is tracked using the video camera.

Experiment 5. Replication Study – Two-Choice USV Playback Paradigm

A separate cohort of five males and five females were utilized in this experiment. For this replication experiment, the females were *not* ovariectomized. Furthermore, it turned out that two of the five cohabitation pairs (two males and two females) involved siblings and so the data

from both these pairs had to be discounted. The experiment otherwise replicated the methods in Experiment 4, with the one exception that we added a fifth playback trial for each female subject vole. The additional trial added was one where from one side, a speaker played back the USVs of the female subject's male partner who was calling back to her anogenital scent, whereas from the other speaker the USVs being played back were from a non-sibling, stranger male calling to its own partner. Hence, this tested whether the female subject could differentiate partner-directed USVs emitted by her partner versus a stranger.

Recording Vocalizations

All testing utilized the Avisoft-UltraSoundGate system to record audio at a rate of 300 kHz. The system was connected to a computer, where the acoustic data could be seen as a spectrogram in real time, using 1024 points of FFT-length. The system generated a 32-bit audio file . The peak amplitude knob on the sound gate system was set at the maximum possible setting on the recording system. A ¼" microphone by Avisoft (Model: CM16/CMPA) was attached, using a clamp stand and positioned roughly 20-25 cm above the testing chamber and angled to face the cage in a way that it covered the whole area of the experimental chamber. The microphone was sensitive to frequencies from 15 – 200 kHz (Mun et al., 2015).

Analyzing Vocalizations using MATLAB

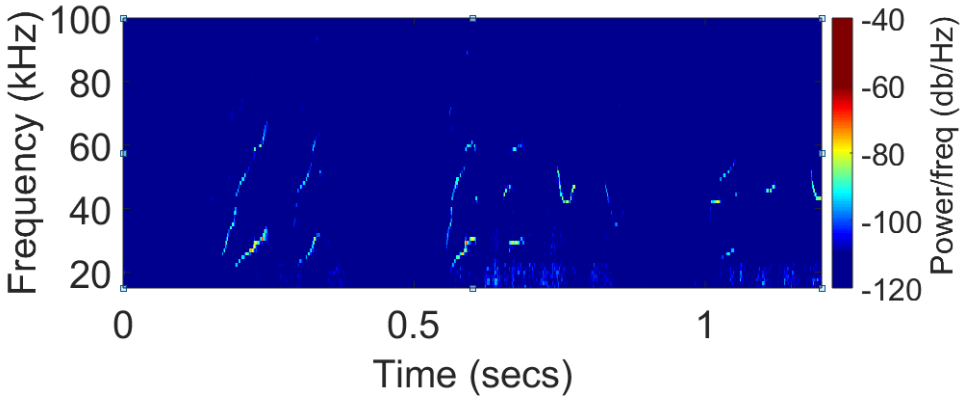
Initial audio recordings were analyzed in MATLAB (version 2018b, Mathworks), with potential USVs isolated from denoised files. This included filtering recordings via a high-pass 15 kHz corner, eight-order Butterworth filter, and spectrally denoising the audio, scaling it to RMS (root mean square) amplitude (Dunlap et al., 2013, Liu et al., 2003). The audio was then passed through a call detector algorithm, where calls with a duration less than 5 ms were rejected. The threshold was set at 5 ms since most signals identified below 3 ms were in fact artifacts, mainly spikes of noise which had been picked up due to the subject's movement in the cage or because the subject was scratching some surface or gnawing at the parafilm used to secure the dish. These spikes can be seen in Fig. 6A. The resultant identified calls were stored in a library table, which was the end output of the algorithm. These calls were then visualized as spectrograms and assessed for validity by looking at their peak frequency contours (Fig. 4). Furthermore, the portions of the audio recorded corresponding to the timepoints of the calls were also played back at a slower rate, to assess for audible whistles, which would confirm the presence of ultrasonic whistles emitted by the voles. This reduced the likelihood that the algorithm would mistake artificial noise for vocalizations. These methods were utilized for Experiments 2, 3, 4 and 5.

In Experiments 2 and 3, the number of calls per trial was calculated (Liu et al., 2003). Hence, the number of USVs emitted per experiment condition was calculated and compared across conditions. Meanwhile, in experiments 4 and 5, the resultant library tables were assessed to

find portions of bouts from which we could form USV playback files, as described in the next section.

A major limitation of experiment 2 was the difficulty of effectively attenuating sound and performing the experiments in the absence of an anechoic chamber. As a result, the audio recorded did have a low signal to noise ratio and a lot of the noise was not able to be completely removed during the data analysis on MATLAB (Fig. 4B). While significant improvements were made in reducing experimental sources of noise in Experiment 3, such as changing the bedding in the cage to a softer paper bedding to reduce noise due to animal movement, there was still noise picked up which overlapped with the USVs we were trying to study (Fig. 4C). So, certain parameters had to be included into the analysis such as rejecting any whistle detected less than 3 ms. Any single call detected which lasted for more than 300 ms was also rejected for this reason. Furthermore, the calls had to lie within 10kHz to 140kHz and anything recorded outside this range was also labelled as noise and excluded.

A



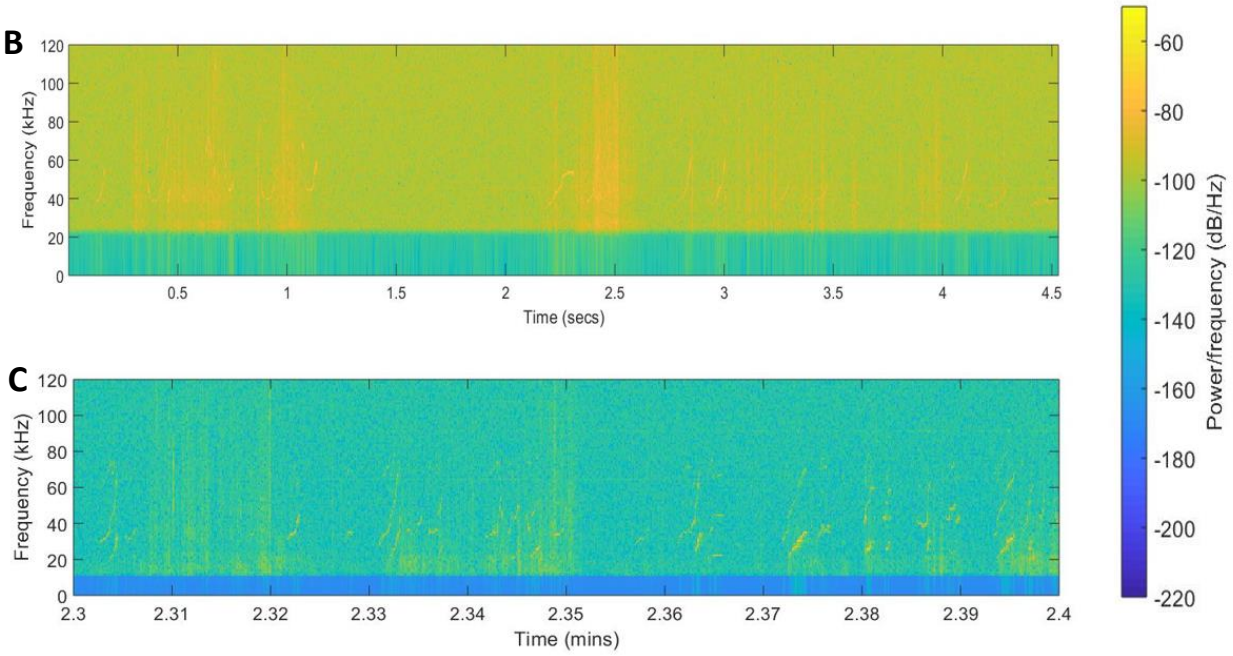


Figure 4. Spectrogram of a snippet of an audio file containing several male prairie vole USVs. **(A)** Spectrograms were used to confirm the presence of calls as are seen here. **(B)** Spectrogram of a specific portion of an audio file taken from Experiment 2. Broad-band noise was captured in the audio recordings in Experiment 2 which overlapped with the calls, making it hard to analyze them accurately. **(C)** Spectrogram of a snippet of an audio recording from Experiment 3. Here, the noise captured was reduced but still present and it still interfered with our ability to reliably focus on call statistics.

Generating Audio Files for USV Playback

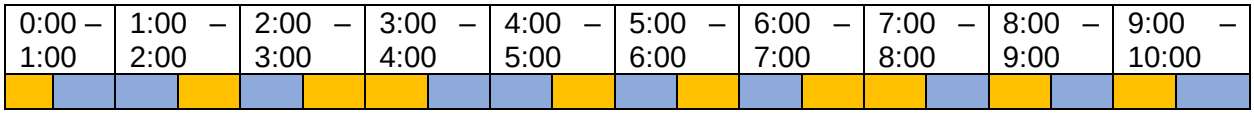
The audio recordings were analyzed using MATLAB for bouts of calls using spectrograms to visualize the audio data. These bouts were identified and the number of calls in each bout was manually counted. The files were subsequently edited, such that there were 30 second bouts interspaced with 30 or 60 seconds of 'intrinsic noise' from the same file (i.e. a region where no USVs are seen). This was done by first noting the time start and end time points of each bout, and then either splicing a longer USV bout so that it was 30 seconds long, or adjoining one bout (shorter than 30 seconds) with another bout period from the original file. Once, these 30

second bouts were arranged, a period of the audio recording with no USVs was placed between the bout periods (Fig. 5). A consideration while creating these new 30 second periods of bouts was ensuring that each bout contained around 20-40 calls. However, the total number of calls in a 10-minute playback file generated was approximately equalized across USV files at 300±10 calls. The audio was stored in mp3 format for playback.

A schematic of the two types of playback file combinations created is shown in Fig. 5. Note that for each playback trial, one side utilized a type A file and the other utilized a type B. So, for example, if the female's male partner USVs was created as a Type A file then the stranger calls played from the other side would be constructed as a Type B file. This allocation was randomly decided while creating these playback files.

A final 'background noise' file was constructed from the audio recordings from males who were presented with the control condition i.e. where the dish contained a dry gauze. Here, the bouts were periods of intense activity (on the part of the male as he gnawed at the Parafilm or scratched at the plate) and so the clicks and scratching noise from these behaviors was instead isolated for the 'bout periods'. The 'intrinsic noise' was once again portions of the raw recording file that were relatively quiet with just the microphone's intrinsic noise. It was ensured there were no USVs in these background files at all.

Audio File Type A – Played back by one of the speakers



Audio File Type B – Played back by the other speaker

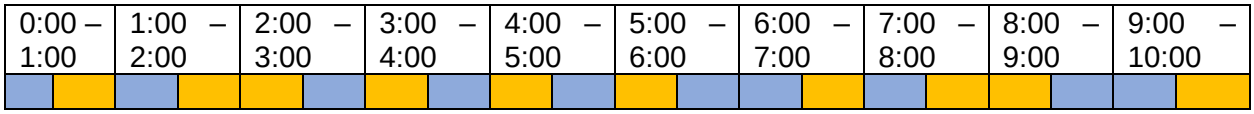


Figure 5. Schematic of the two types of audio files used for playback trials. The files were divided in 30 second intervals where the orange periods were bouts containing 20-40 USVs and the blue periods were intervals with intrinsic microphone noise containing no USVs. Both type A and B structures were utilized so that during the assay, the female vole had time to orient herself to calls from either ultrasound emitters followed by 30 second intervals when calls from both speakers would be played back simultaneously and 30 second intervals when no calls were played back from either speaker.

Analyzing Vocalizations Using DeepSqueak

DeepSqueak, an open-access USV detection analysis package for MATLAB, was used as an alternate software to analyse USVs to circumnavigate the problems encountered with the existing technique of using MATLAB . Our methods were closely adapted from Neumaier and colleagues, who have introduced the use of DeepSqueak in extracting calls (Neumaier et al., 2019).

The audio files were processed through a regional convolutional neural network architecture. This isolated particular snippets or spectrogram images that could be potential USVs. These snippets were then run through a classification network, which was a mouse-specific network that determined whether the frequency contours in these snippets represented any calls. The

secondary, classification network was made more robust, i.e. in its ability to reduce false positives, via manual labeling of individual detections as ‘noise’ or ‘USV.’ This was done by a trained scorer looking at the spectrograms corresponding to the regions of interest for the sole purpose of teaching the network. The sound recordings were processed by each network after first being split into segments of 6 s with 0.5 s overlaps. The frequency range was set at 18-100 kHz. A post-hoc denoising network was run afterwards on each potential call detected to improve the noise to power ratio. The output from these processes was saved onto a detection file which contained call statistics such as the minimum, maximum, and mean frequency, duration, slope, sinuosity, and power and confidence intervals for each USV identified – all calculated using the spectrotemporal properties of the contours of the USVs in the sonograms generated (Fig. 6B.). At this point, the tonality of all the potential calls was also found using the geometric mean of the power spectrum divided by the arithmetic mean and subtracted from 1 (Neumaier et al., 2019). Neumaier et. al. found that the tonality was vital in ascertaining whether the audio signals picked up by the algorithm were in fact calls or noise and found that using a tonality cutoff of 0.3 could best exclude certain false positives without missing USVs. Therefore, a contour threshold of 0.3 was established to cull calls (Neumaier et al., 2019).

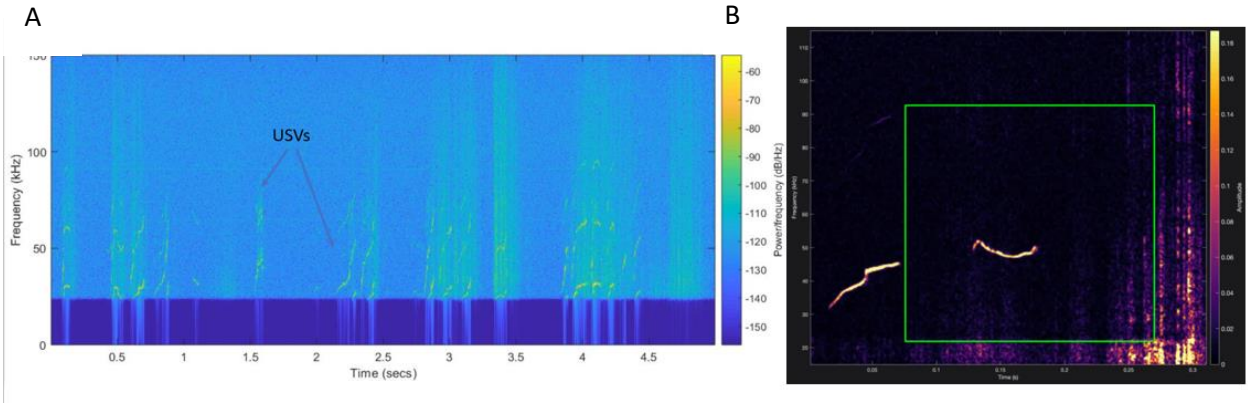


Figure 6. Spectrogram showing snippet of the audio file containing USVs with background and internal microphone noise. (A) Spectrogram after denoising via the initial method in MATLAB. The calls overlap with noise making it hard to study call statistics. **(B)** Spectrogram indicating a snippet of the audio file containing USVS after post hoc denoising in DeepSqueak. With a high signal to noise ratio, these calls were more easily identified by the call detector network and their statistics can now be studied more accurately.

An important step in our analysis was also to then randomly sample the detections from each file generated by the algorithm and view their spectrograms to confirm for the presence of contours that could confirm the existence of a USV while also playing back the audio file at the appropriate, corresponding segment at a slower speed to make that confirmation. We then looked at the frequencies and duration of the calls as a whole to make context-based comparisons.

Statistical Analysis

With alpha at 0.05, a difference with a p-value greater than 0.05 was considered due to chance and one below 0.05 was considered significant. A one way analysis of variance (ANOVA) followed by multi-comparison testing was performed to analyze the differences in time spent

and investigation times in Experiment 1 for one side over the other. Furthermore, the same method was also employed for studying multiple mean number of calls across experimental groups in Experiments 2, 3, and 5. For Experiment 4 and 5, a paired, two-tailed t-test was performed to see if there were differences in mean investigation times for the two different types of calls played back to female voles.

Results

1. Investigative Behaviors of Male Voles in Response to Female Odors

In the first odor exposure experiments, the total amount of time spent by the male vole investigating the chamber containing the stranger female's bedding, known as the stranger side, was calculated. Similarly, the time spent on the partner side was equated. The cumulative time spent on each side was averaged across voles for 10, 15 and 30 minutes following the start of the exposure. The average portion of total time spent on the stranger or partner sides did not vary significantly from one another at the 10 minute mark, presumably due to exploration in the initial portion of the assay (Fig. 7A; one-way ANOVA, df = 1, F = 1.12, p = 0.367). However, significant differences were seen by the 30 minute mark (Fig. 7A; one-way ANOVA, df = 1, F = 5.58, p = 0.023). Interestingly though, by this time male voles were spending a significantly longer time on average on the stranger side as compared to the partner side.

We saw similar results when looking at investigation times (see Methods). The average portion of time spent by the voles investigating the sealed, perforated dishes containing either the stranger bedding or the bedding of the partner at the 10 minute mark was similar. (Fig. 7A; one-way ANOVA, df = 1, F = 1.15, p = 0.239). However, at the 30 minute mark, the differences between the two portions became significant, with males spending longer time investigating the dish containing the stranger's bedding. (Fig. 7A; one-way ANOVA, df = 1, F = 4.89, p = 0.036).

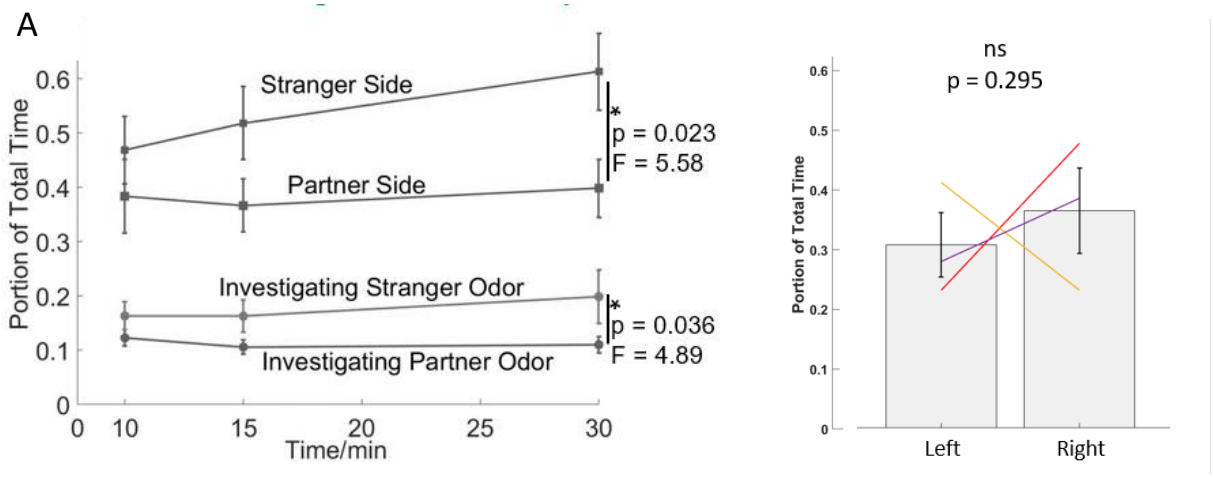


Figure 7. Male voles recognize partner bedding but prefer stranger bedding. (A) A temporal analysis of the average proportion of time spent by each male vole investigating partner or stranger odors or spending time on a specific side of the cage out of the total duration of the assay. Data collected from 7 males, and the error bars (here and in subsequent figures) represent ±one standard error. **(B)** Control study. Mean proportion of total time spent on the two side chambers of the 3 chamber cage, with both sides containing dishes with bedding from the same conspecific. The error bars represent ± one standard error.

In a control experiment with a separate cohort of three male-female pairs, the left and right chambers had dishes containing bedding from the *same* conspecific i.e. the partner of the male. In this case, the male voles did not show significant preferences for a particular side as seen in Fig. 7B. (One-way ANOVA, df = 1, F = 1.1 , p = 0.295).

2. Male Vocalizations in Response to Olfactory Cues

In Experiment 2, where urine was used to elicit vocalizations, male voles produced more calls in response to a urine sample of any conspecific over the water control (Fig. 8). This general trend was seen across all 4 male subjects, though there was high variability across subjects, resulting

in large standard errors across the four treatments. For instance, the number of calls elicited in response to the male urine varied between 177 to 712 among different subjects. As a consequence of this variability, significant differences were not observed between number of calls emitted across the different types of urine samples.

In Experiment 3, where anogenital scent was used to elicit vocalizations, male voles produce significantly more USVs for a female stranger's anogenital scent than for partner's (Fig. 8; one-way ANOVA; df = 1, F = 6.76, p = 0.041). The mean number of calls per male did not differ between stranger females and stranger males (Fig. 8; one-way ANOVA; df = 1, F = 1.668, p = 0.244). Meanwhile, once again the mean number of calls for all groups where a conspecific's scent had been presented was significantly higher than in the control condition. Interstingly, the number of calls produced in Experiment 2, where urine was used was almost three-fold higher than the number of calls seen in response to anogenital scent exposure.

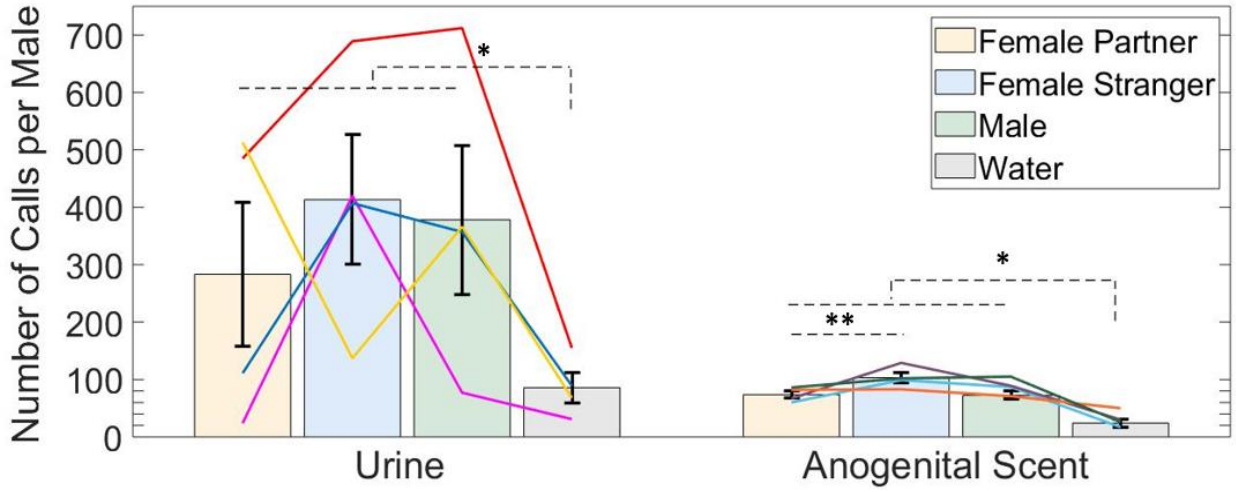


Figure 8. Average number of USVs emitted by each male vole when exposed to conspecific odor in the form of urine or anogenital scent or water (control) for 10 minutes. Each colored trace represents calls recorded from one male.

Besides differing in the number of calls produced under each context, we also investigated whether the acoustic properties of the calls differed. We generated contour plots of the distribution of USV frequency and duration across the four different contexts (Fig. 9). There was a group of calls seen in the male treatment group which ranged between 60-80kHz in frequency which was not seen in any other group. Meanwhile, while the contour maps of the calls on exposure to the female partner urine and female stranger urine groups looked similar, they seemed dissimilar to those from the male treatment and those from the control group (Fig. 9). Further delving into the acoustic features of the calls, the call durations mostly ranged from 20 to 60ms, with the median duration lying around 30 ms, however, calls could last anywhere from 10ms to 100ms. It was also noted that calls elicited from the male urine, the female stranger urine or the female partner urine did not significantly differ from each other on the basis of call durations. The calls were also significantly shorter when males were exposed to distilled water as compared to those elicited on the presentation of a urine sample from any female conspecific. Comparisons using other calls statistics did not show significance, but further data may be able to overcome noisiness in the distributions and result in meaningful comparisons between the calls emitted under the different experimental conditions.

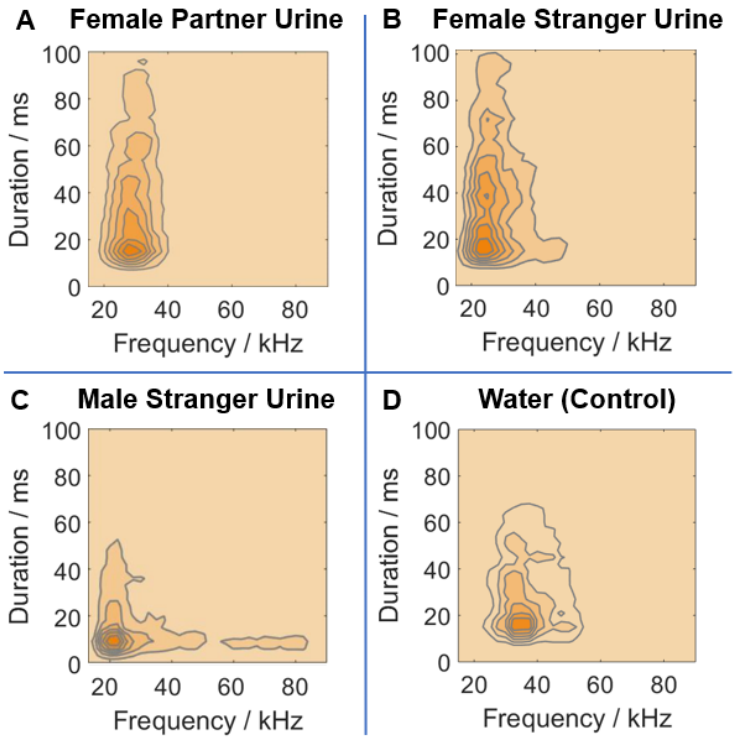


Figure 9. Male vole vocalizations show differences in acoustic features based on social context. Contour plot of the USVs recorded when the male vole was exposed to: **(A)** female partner urine **(B)** female stranger urine **(C)** water (control) **(D)** male urine. The duration of the calls and the median peak frequency of the calls is shown. The calls are aggregated from trials done in four males.

3. Male Vocalizations in Response to Olfactory Cues

To look at female responses to male vocalizations, we utilized a two-choice USV playback paradigm as shown in Fig. 3. Here in Experiment 4, the total time the female spent investigating the calls from the two speaker was assessed in the 10-minute period of the assay. The calls played back were emitted by males when they were previously exposed to the anogenital scent of the subject female, as done in Experiment 3, and so, the males were calling in response to

female odors. We saw that female subjects, on average, spent more time investigating calls from the stranger male as compared to their partner (Fig. 10A; two-tailed paired t-test, df = 4, p = 0.0118).

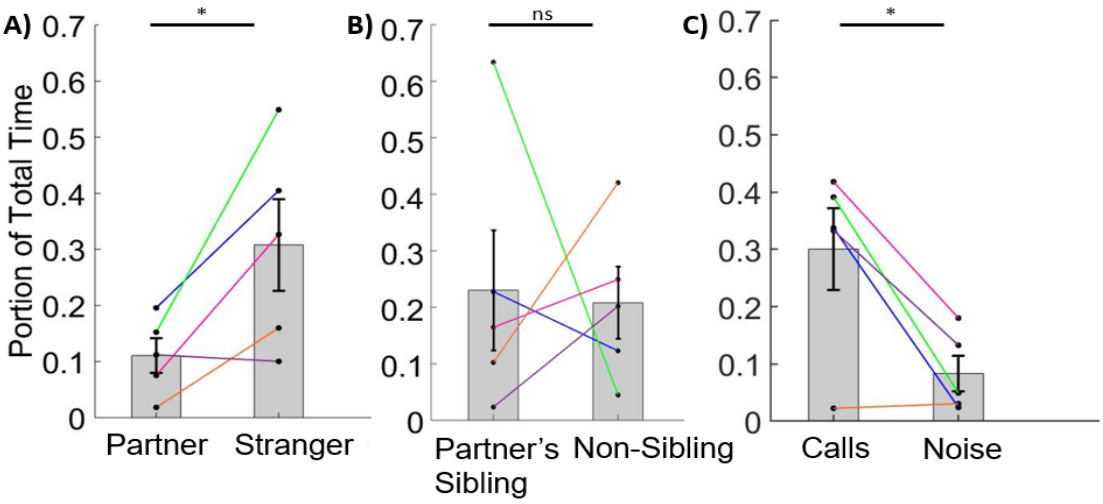


Figure 10. Female voles can discriminate between different vocalizations and between calls and noise. (A) Average portion of time spent by a female vole investigating the calls from her male partner and those from a male stranger. **(B)** Average portion of time spent by each female vole investigating the calls from a stranger who was the partner's brother or those from a stranger who was not the partner's sibling. **(C)** Average portion of time spent by each female vole investigating male USVs or a background noise that was devoid of any calls. The error bars represent ±one standard error. Each color trace represents data taken from females from one of the five cohabitation pairs.

At this point, we do not know whether the USVs from individuals of different litters would be systematically different from those of the same litter. We hypothesized that that USVs emitted by brothers from the same litter could be similar, so that a female would show recognition for a stranger sibling of its partner, when compared with a stranger from another litter. So, we performed an experiment where both the speakers played back calls from strangers from different litters. On one side, the sound was from a stranger was a sibling of the male who had

been partnered with the female, while on the other side, calls were played from a male who was not the sibling of the female subject's partner. In this case, the female voles did not show any preferences for either types of calls (Fig. 10B; two-tailed paired t-test, df = 4, p = 0.8934). Finally, a control experiment was done to see whether females could distinguish the USVs from other broad-spectrum, non-USV noise present in the recordings. In this case, the females spent significantly longer investigating the side where the USVs of male conspecifics emanated as compared to the background noise side (Fig 10C; two-tailed paired t-test, df = 4, p = 0.0245).

Finally, in our Experiment 5 replication study, we used four males and four intact females (after discarding the results of one male-female pair due to the fact that they were overlooked as littermates). In this experiment, the females were intact (not ovariectomized), unlike the case in Experiment 4. Nevertheless, as in that case, female subjects here spent more time investigating calls from strangers as compared to those from their partners (Fig. 11A; two-tailed paired t-test, df = 2, p = 0.0373,). Meanwhile, as in Experiment 4, here the female subjects again seemed to show no consistent, systematic attraction to the stranger calls from the partner's sibling or from a stranger of another litter (Fig. 11B; two-tailed paired t-test, df = 2, p = 0.8408). Also, the control experiment had the same results as before, where females preferred the speaker playing back USVs over the one only playing back noise (Fig. 11C; two-tailed paired t-test, df = 2, p = 0.0283).

In this experiment, we wanted to determine what the females were recognizing in the calls. Simply put, we wanted to see whether the calls could be used to identify individuals, or whether the calls were context-dependent such that the females were not recognizing the

individuals who were producing the calls but rather the context that they signified. Hence, in this trial, one speaker played back the USVs of each female subject's own male partner, while the other played back the USVs of a stranger male calling back to its own partner (i.e. not the current female subject). The idea here was that the context under which the calls were produced was the same i.e. the male was vocalizing for his partner. If, the female could show an overall preference for the calls as seen in the previous experiment then this could indicate that the calls were perceived as coming from different individuals, however, if no overall preference was seen then we would conclude that the calls were in fact perceived as similar and that they signified context. We found that, on average, females treated the calls from both conspecifics equally, spending an equal amount of their time investigating the calls coming from both sides of the chamber (Fig. 11D; two-tailed paired t-test, $df = 2$, $p = 0.5689$).

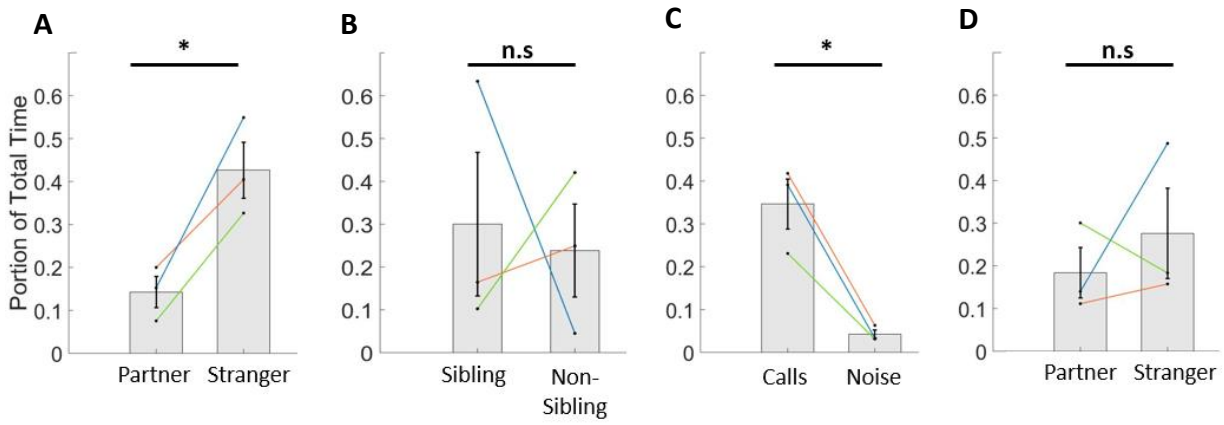


Figure 11. Intact female voles can discriminate between different vocalizations and between calls and noise. (A) Average portion of time spent by a female vole investigating the calls from her male partner and those from a male stranger. In this case, both the partner and stranger males were presented with the anogenital scent of the female who was later placed in the testing cage, and therefore, they were calling to her specifically. **(B)** Average portion of time spent by each female vole investigating the calls from a stranger who was the partner's brother or those from a stranger who was not the partner's sibling. **(C)** Average portion of time spent by each female vole investigating male USVs or a background noise that was devoid of any calls. **(D)** Average portion of time spent by each female investigating calls from their partner vs calls from a stranger male, however, in this case the calls were recorded under the same context, i.e. males called in response to their respective female partners. The error bars represent ±one standard error. Each color trace represents data taken from females from one of the three cohabitation pairs.

Discussion

Our study looked at how prairie voles utilized social-sensory cues from different modalities, specifically looking at whether males could recognize olfactory cues from their female counterparts and whether females could recognize the auditory cues received from males. In summary, we found that male voles can recognize their own partner's odors (bedding, urine or anogenital scent), but when given a choice between odors, spend more time with a stranger's odors. Furthermore, in the specific case of anogenital scents, males will also vocalize more when presented with the odor of a stranger female, although it also vocalizes somewhat differently (in both quantity and acoustic characteristics) in response to its partner's and another male stranger's scent. Finally, after equalizing the quantity of USVs played back in a two-choice test, female subjects show a preference for USVs emitted by a stranger vocalizing to her own anogenital scent, compared to the USVs emitted by her own partner to her scent. Our preliminary follow-up study suggests that this may reflect context-specific acoustic differences in the USVs emitted toward partner versus stranger scents, rather than the female's ability to recognize the specific USV "voice" of one's own partner. We will now explore each experiment and investigate the reasons behind the trends we observed.

First, we found that male prairie voles are more interested in stranger odors when they are presented side by side with partner odors. This preference could be due to the novelty of these cues. In other words, since males had been cohabitated with their partners for a week, they were familiar with the odors associated with their mates. However, the stranger female's odor

was new, since the stranger had not interacted with the male previously. This propensity towards 'social novelty' has been observed in studies involving mice with an absence of this behavior linked to autism related impediments (Pearson et al., 2010; Moy et al., 2007; Moy et al., 2004). Thus, this novelty might have instigated interest in the male subject vole. It is also important to note that the duration of this assay was only 30 minutes long, and over time we could speculate that the male vole might even return to the side of the cage which had the partner's scent, spending the rest of his time there, as is usually seen in partner preference tests. Another possibility is mate guarding. That is, a strong pair bond, usually involving mating with cohabitation, can lead males to display aggression towards strangers. This selective aggression has been shown to persist for weeks after a pair-bond is formed and it is displayed not only towards conspecific males but also toward socially receptive females. (Gobrogge & Wong, 2011; Wang et al., 1997; Carter et al., 1995). In either case, to display curiosity towards novel cues or to express mate-guarding would require an ability for males to differentiate between odor cues and to use them in identifying conspecifics and this is shown by our first test. In other words, our results from the first experiment suggest that prairie voles have individually distinctive odors, which can be distinguished by conspecifics.

Our second and third sets of experiments focused on the vocalizations that males produced in response to the olfactory cues of other prairie voles. Here, we saw that males produced more vocalizations in response to a female stranger's anogenital scent over that of a partner. This once again indicated a preference for strangers. While more USVs were produced by males in

response to the stranger female compared to their partner, the difference between the number of calls produced for the stranger female and that for the male stranger was not significant. This could suggest that males vocalize more when aroused. Previous studies in other small rodents have demonstrated that vocalizations are positively correlated with arousal and thus such a finding is not surprising. (Bell, 1974). Consequently, male voles were more aroused after sensing the odor cues of a novel individual. The arousal itself may be due to curiosity or might even be due to feelings of aggression against the stranger counterpart (Gobrogge & Wong, 2011; Wang et al., 1997).

Furthermore, we found that males produced more calls when presented with urine as compared to anogenital scent, which may not be surprising given that urine contains a higher concentration of pheromones which are used by rodents to identify other conspecifics. Anogenital scent, on the other hand, usually contains any body odor, urine, and residual fecal matter absorbed into the gauze, used to swab the region, thereby containing a lower concentration of pheromones (Ferkin et al., 1995).

The acoustic analysis of the calls suggests differences in the types of calls emitted in different conditions, as seen in Fig. 9. Most USVs having a median peak frequency between 20 kHz to 40 kHz. This made sense, given the fact that previous anatomical work has shown that the middle ear of a prairie vole is receptive to frequencies that lie in this very same range (Stewart et al.,

2015). Further analysis of call bandwidths will help delineate whether USVs by prairie voles do in fact vary over a narrow range of frequencies that are used to indicate emotional affect and state (Stewart et al., 2015). Given that, Fig. 9 shows that the USVs emitted by males in the presence of female urine were different than the ones emitted when presented with male urine, with the median peak frequency being slightly lower when the vole was exposed to a female scent over the male one or water. This could indicate lower frequency vocalizations being utilized to indicate positive valence or anticipation of copulation whereas higher frequencies could indicate a sense of distress, aggression or danger. Interestingly, this is the opposite to what is seen in rats where 22-kHz calls are considered threat signals and can induce aversion, whereas 50-kHz can induce approach behaviors (Wöhr & Schwarting, 2007).

Our preliminary analysis suggests that there are systematic differences in USVs emitted in the different contexts. DeepSqueak or other more detailed methods will be needed to ascertain what specific differences there are beyond the duration and some median frequency differences. Additionally, investigations illustrate that auditory cortical pyramidal neurons in mice can develop sensitivities to combinations of certain acoustic features, which makes the frequency, amplitude and duration of these calls behaviorally relevant (Shepard et al., 2015). However, further experimentation will need to be done to solidify such conclusions.

Taken together, males produced more vocalizations for the scent of conspecifics versus neutral odors (water). This suggests that males respond to arousing olfactory cues by vocalizing, perhaps to communicate their affective state. The contour plots only further hint that the vocalizations might convey context-specific information, which may be then received by females. Hence, male voles produce acoustically distinctive USVs based on which conspecific's scent they are exposed to. Audition plays an important role in social communication in voles and the USVs produced are likely to have social salience (Campi et al., 2007; Blake, 2002; Lepri et al., 1988). Here, these USVs are seen to be produced differentially by males in response to olfactory cues alone.

One confounding factor that emerged in our olfactory studies was the high variability in urine-elicited USVs due to the limitations in our urine collection methods, which would yield an unpredictable amount of urine from the animal. While at times sufficient urine was successfully collected from the animal's bladder, there were a few instances when enough urine could not be collected simply because the bladder of the animal was empty when the subject was euthanized. In fact, one group had to also be excluded from behavioural testing because there was no urine obtained from the female vole in that group during urine extraction. As a result, we switched to anogenital scent to successfully elicit vocalizations from males.

Finally, our last set of experiments looked at the reception of these vocalizations and the interest they might induce in females, if at all. We noticed that female voles spent significantly longer investigating stranger vocalizations. This was similar to what we saw with males preferring to approach the stranger's females bedding. As in that case, this could be due to novelty. Females, unaccustomed to the vocalizations from a stranger, might be drawn to their calls over their partner's for the same reasons males were attracted to stranger female odors.

A key question here concerns how to interpret the preference shown by female subjects for the USVs emitted by the stranger. One possibility is that male USVs contain certain features which make them specific to an individual which females use to recognize their mates. Another possibility is that the two sides differed not only because the partner was vocalizing from one side and a stranger was vocalizing from the other, but also because of whose scent elicited those males' vocalizations. In other words, the fact that the partner male perceived the subject as his partner and vocalized as such, as compared to the stranger male who perceived the subject female as a stranger as well and vocalized accordingly, might explain the differences in the overall investigation time females subjects exhibited for each audio signal. Thus, context could also explain the difference.

To determine which factors may play the stronger role, we ran a key control trial in experiment (Experiment 5) to investigate this growing suspicion that these vocalizations might be context

dependent. Here, the male partner's calls to the female in the chamber were played from one side of the cage, whereas a stranger male called to *its own* partner from the other side of the cage. Since no systematic preferences were seen here, this could further support the argument that acoustic communication in prairie voles is context-dependent, i.e. the information gained from vocalizations is about specific affective state of the animal producing the calls, which can then be used to infer the sociosexual condition at hand. With that information, one could then modulate their social behaviors accordingly.

Furthermore, since we did not know whether USVs from different litters would be systematically different, we performed a playback test with the USVs of two strangers - one a sibling of the female subject's male partner, and the other a male from a separate litter. Previous studies have speculated that call traits by animals from the same litter are more similar in nature, as compared to calls between males from different litters (Thornton et al., 2005). One interpretation of the lack of overall preference we observed in the sibling trial compared to the preference seen in the partner vs stranger trial is that the female did not perceive the sibling USVs to be similar to those of its partner. Instead, perhaps both stranger males perceived the female subject's scent as novel, vocalized as such, since an average preference across subjects was not seen for one or the other. If calls were more individually specific, and more similar across siblings due to genetic factors, female subjects would have mistaken the sibling's calls as familiar, thereby showing a preference for the non-sibling (Thornton et al., 2005). Yet, this was not the case.

Several of our results give some confidence that our findings are robust. First, an important positive control was to demonstrate that our methods could reveal an expected preference for calls over background sounds. Second, we ran two cohorts of females in our USV preference assay – one with ovariectomized females primed to be immediately responsive to males during cohabitation, and the other intact females that could then go on to become pregnant. In the future, given that experiment 5 looked at partner-directed USVs, we intend to now perform a stranger-directed USV comparison. In this case, the USVs of the partner vocalizing to a stranger will be played back, and set against the USVs of a stranger vocalizing to another stranger who is not the subject female. In this case, the subject female may not show any systematic differences either which will suggest that stranger-directed USVs are not systematically different from each other for different subjects. Then finally, we intend to play back a stranger vocalizing to its partner and compare to a stranger vocalizing to a stranger that is not the subject. If the female subject still prefers the stranger-directed sounds, then it is likely that stranger-directed sounds contain some acoustic cues that are distinct and systematically arousing for voles. If on average the same preference is not seen across all the subjects, then that stands in contrast to the finding of a preference for stranger vocalizing to subject's odor (compared to partner vocalizing to subject's odor). The latter result may be due to individual recognition of the partner leading to a preference to pay attention to the stranger.

In summary, our studies show that olfactory cues contain information specific to the individual that is used by males to identify their female mates. Meanwhile, our results hint at the

possibility that call production by males in response to these odors is context-based, which is then received by females who can act accordingly and use the information to recognize the situation they are in to locate their mates. (Sangiamo et al., 2020).

While we found significant results in each of our experiments to reach these preliminary conclusions, several limitations should be noted. One limitation is that the number of animals in each experiment was low. By having more repeats i.e. using more male subjects our data will be more statistically better powered.

Another limitation was the difficulty of effectively attenuating extraneous environmental sounds, since they were not performed in an anechoic chamber. As a result, the audio recordings of the USVs exhibited a low signal to noise ratio, and a lot of the noise was not able to be completely removed during the data analysis. Consequently, certain parameters had to be included into the analysis, such as rejecting any whistle whose durations were less than 3 ms, that is the default used for cleaner recordings (Liu et al., 2003). Furthermore, the calls had to lie between 10 kHz to 140 kHz, and anything recorded outside this range was also labelled as noise and excluded. On introduction of these parameters, the spectrograms of any “calls” that were subsequently excluded were visualized to ensure that no real USV had been removed. While there was a lot of background noise, the biggest source of noise was animal movement within the chamber and an echoing of sound within the room, and the sound from the animal playing

with the petri dish. While paper bedding was specifically used in favor of Bed-o-cobbs Laboratory Animal Bedding with the hopes of reducing sound due to animal movement, this was still not enough. Currently, we are using an alternative way of analyzing vocalizations via DeepSqueak, as highlighted in the methods section. We were unable to complete our analysis of the USV data by the time of this thesis submission due to the pandemic. However, this direction looks promising in attenuating noise computationally rather than experimentally, which was a difficult task. Ongoing work is now looking at the acoustic features of male USVs, which could lead to further insights about the communicative salience of USVs. We plan to explore the calls spectrotemporal properties to further understand the different syllable types employed by voles in their auditory communication, and how they may vary. This will be first done by deriving other acoustic features, such as frequency trajectories (Chong et al., 2019). This would also include studying the median frequency distribution in each vocalization condition, and looking at the inter-call period and call-bandwidth statistics. The inter-call period is the time between two consecutive calls and is useful since whistles are usually seen in bouts. The call bandwidth is the range of frequencies that make up the whistle. Furthermore, we will look at the amount of jump calls, which are characterized by sudden frequency changes in the call. These measures will further provide insight into the type and composition of different syllables that make up vole USVs, as well as the context in which they are produced. These investigations could help us in even understanding whether the USVs produced in response to the different contexts might convey a negative or positive affective state (Sangiamo et al., 2020).

In conclusion, we hope that the current dissection of the communicative salience of each sensory modality will lead to improved ethological models when using prairie voles to investigate other neuroscientific questions. We hope that further investigations into vocalizations will spark an understanding of the type of information conveyed by these cues and how they are perceived and used in mate recognition.

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