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Operant Discrimination Of Emotional Calls In Rats: Are There Biological Constraints?

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Abstract – Rats emit 50 kHz ultrasonic vocalizations (USVs) in positive contexts, while they produce 22 kHz USVs in negative contexts. 50 kHz USVs are possibly associated with positive affective states, while 22 kHz USVs with those of negative, and such associations may influence the feasibility of discrimination learning using USVs. Here, we investigated whether operant conditioning which utilizes USV stimuli is affected by presumed innate connections between 50 kHz USVs and positive, and 22 kHz USVs with negative affective states. We trained rats to discriminate 50 kHz and 22 kHz USVs in two conditions. In the congruent condition, 50 kHz USVs were paired with a positive event, while 22 kHz USVs with a negative one. In the incongruent condition, 22 kHz USVs were paired with a positive and 50 kHz USVs with a negative outcome. Rats were trained to serially reverse the learning conditions four times in total. Results show that the number of lever presses to reach the learning criteria did not significantly differ between the congruent and the incongruent conditions, both in the discrimination and reversal learning phases. These results suggest that rats did not learn the congruent condition faster than the incongruent condition, nor did the congruent condition more strongly hamper the acquisition of reversal learning than the incongruent condition. Namely, rats did not respond differently to the congruent and the incongruent conditions. The presumed innate connections (50 kHz USVs with positive, 22 kHz USVs with negative affective states) do not appear to substantially influence the feasibility of discrimination and serial reversal learning which utilizes USV stimuli.

Keywords – Rats, Ultrasonic vocalization (USV), Operant conditioning, Biological predisposition

Adult rats emit two types of ultrasonic vocalizations (USVs): 50 kHz USVs and 22 kHz USVs (Sales, 1972). 50 kHz USVs and 22 kHz USVs are considered to have contrasting emotional valences based on the contexts in which these USVs are emitted. Rats emit 50 kHz USVs in positive and appetitive contexts including juvenile rough-and-tumble play (Knutson et al., 1998), mating (McIntosh & Barfield, 1980; Thomas & Barfield, 1985), and in anticipation of various rewards (e.g., food and electrical stimulation of the brain; Burgdorf et al., 2000). Contrarily, 22 kHz USVs occur in negative and aversive contexts, such as social defeat (Corrigan & Flannelly, 1979; Thomas et al., 1983), before and after electric shock (Cuomo et al., 1988; Tonoue et al., 1986), exposure to predators (Blanchard et al., 1991), and in response to cues associated with aversive events (e.g., drugs with aversive effects; Burgdorf et al., 2001). 50 kHz and 22 kHz USVs differ, not only in contexts they are produced, but also in behaviors they induce. Playback of 50

kHz USVs elicit social approach behavior (Willadsen et al., 2014; Wöhr & Schwarting, 2007), while 22 kHz USVs induce freezing and avoidance behavior in recipients (Brudzynski & Chiu, 1995; Wöhr & Schwarting, 2007). Also, 50 kHz and 22 kHz USVs can transmit the affective state of the sender to the receiver (Brudzynski, 2013), causing emotional contagion in the recipient (Saito et al., 2016). Taken together, the evidence suggests that both 50 kHz and 22 kHz USVs express affective states of vocalizers and may even predict reward or punishment for receivers.

As described above, it has been considered that 50 kHz USVs are associated with positive affective states and appetitive events, while 22 kHz USVs with negative affective states and aversive events (Brudzynski, 2013; Knutson et al., 2002). Such internal connections may influence the feasibility of learning when USV stimuli are to be associated with rewarding or aversive events. This assumption is related to biological preparedness which proposes that organisms are prepared to associate certain events over others (Seligman, 1970). Although the associative learning theory assumed that the laws of learning were universal and independent of specific stimuli, responses, and reinforcers, studies revealed that organisms could be prepared for or constrained from forming specific associations (Burgos, 2015; Mazur, 2017; Sosa, 2024). For example, Garcia & Koelling (1966) demonstrated that gustatory stimuli were more readily associated with sickness, while audiovisual stimuli were more easily associated with peripheral pain, compared to the reversed associations (i.e., gustatory-pain and audiovisual-sickness associations). Such evidence suggests that in some learning situations, innate predispositions can play a significant role to bias learning. Although cautious interpretations are required, studying such learning bias would contribute to deeper understandings of how animals' behaviors are controlled by competing influences of ontogeny (learning) and phylogeny (heredity) (Mazur, 2017).

Rat USVs can be a good study system for biological predispositions on learning, considering presumed innate connections between USVs and rewarding/aversive events. In fact, previous studies suggest the existence of biological predisposition which facilitates the association of aversive events (e.g., foot-shock) with 22 kHz USVs (Bang et al., 2008; Endres et al., 2007; Kim et al., 2010; Parsana et al., 2012). For example, Endres et al. (2007) conducted a classical fear conditioning experiment in which conditioned stimuli (i.e., playback of 22 kHz USVs, 50 kHz USVs, modified USVs, 22 kHz tones, and white noise) were paired with electric foot shock. Results show that rats quickly acquired conditioned freezing to natural 22 kHz USV stimuli, but this response was not specific to 22 kHz USVs. During the extinction phase, however, there was a marked difference between 22 kHz USVs and the other stimuli. When rats were trained with 22 kHz USV stimuli, they exhibited better retention, delayed extinction, and better long-term memory of conditioned freezing response than in case of other stimuli.

Although there is growing understanding about the predisposition to associate 22 kHz USVs and aversive events, to our knowledge, few studies have been conducted to examine whether 50 kHz USVs are more readily associated with rewarding events than 22 kHz USVs, not vice versa, due to the lack of effective experimental paradigms like fear conditioning. Moreover, there is a limitation in previous classical conditioning studies regarding the use of natural behavior as a response. Since 50 kHz and 22 kHz USVs evoke different behavioral responses in freely behaving animals, it would be difficult to directly compare the properties of these USVs by measuring natural responses. Thus, it is necessary to utilize learned behavior which can be reinforced by both positive and negative reinforcers, to assess whether 50 kHz USVs are more likely to be associated with a positive event, 22 kHz USVs with that of negative, than the reversed associations. If the successive behaviors after the presentation of 50 kHz and 22 kHz USVs are the same kind of learned behavior (e.g., lever pressing), we can directly examine the effect of USV presentation by comparing the number of that behavior between the two USV conditions. In line with this, Saito et al. (2016) conducted a cognitive bias task. They trained rats to respond differently to two distinct auditory stimuli, each of which signaled either a positive or a negative outcome. Once trained, they tested rats with an ambiguous sound cue to examine whether rats interpret the cue as positive or negative by calculating bias in the response rate between the two levers. Results showed that rats responded to ambiguous cues as positive after hearing 50 kHz USVs and as negative after hearing 22 kHz USVs. This operant conditioning study successfully compared 50 kHz and 22 kHz USVs simultaneously and showed that USVs evoke emotional contagion which influences learned behavior. Therefore, we applied the operant conditioning

paradigm to assess if connections between 50 kHz USVs and positive affective states, 22 kHz USV and those of negative influence the feasibility of discrimination learning using USVs.

In this study, we investigated whether operant conditioning which utilizes USV stimuli is affected by presumed innate connections (50 kHz USVs with positive, 22 kHz USVs with negative affective states). We tested the hypothesis using the paradigm of a two-choice operant task with two discriminative stimuli and two response levers. There were two trial types: reward and avoidance trials. In reward trials, reward stimuli were presented, and animals were conditioned to press the reward lever to obtain the reward. Contrarily, in avoidance trials, rats had to press the avoidance lever to avoid punishment and skip to the next trail in response to avoidance stimuli. Pressing the incorrect lever was punished by the presentation of aversive noise in both reward and avoidance trials. The task consisted of two conditions: the congruent and the incongruent condition. In the congruent condition, the emotional valence of USV and the reinforcer were congruent, in which 50 kHz USVs (positive) were assigned to the reward stimuli (positive) and 22 kHz USVs (negative), the avoidance stimuli (negative). In contrast, the emotional valence of USV and the reinforcer were inverted in the incongruent condition, namely, 22 kHz USVs served as reward stimuli and 50 kHz USVs, as avoidance stimuli.

In Experiment 1, we examined whether learning is facilitated when learning content is consistent with the presumed innate connections. We predicted that rats would learn in the congruent condition faster than the incongruent condition. In Experiment 2, we further exploratorily tested if the previous experience of learning using USVs (the congruent or incongruent condition in the discrimination learning phase in Experiment 1) would affect the following learning process, using the serial reversal learning paradigm. For Experiment 2, we hypothesized that rats would retain the memory of the congruent condition better than the incongruent condition, and that this would hinder the acquisition of reversal learning process. Together, we investigated whether operant conditioning which utilizes USV stimuli is biased by presumed innate connections between USVs and positive/negative affective states.

General Method

Ethics Statement

All procedures including the housing were conducted under the experimental implementation regulations of the University of Tokyo and were approved by the animal experimental committee at the University of Tokyo, Graduate School of Arts and Sciences (Permission Number: 27-8).

Subjects and Housing

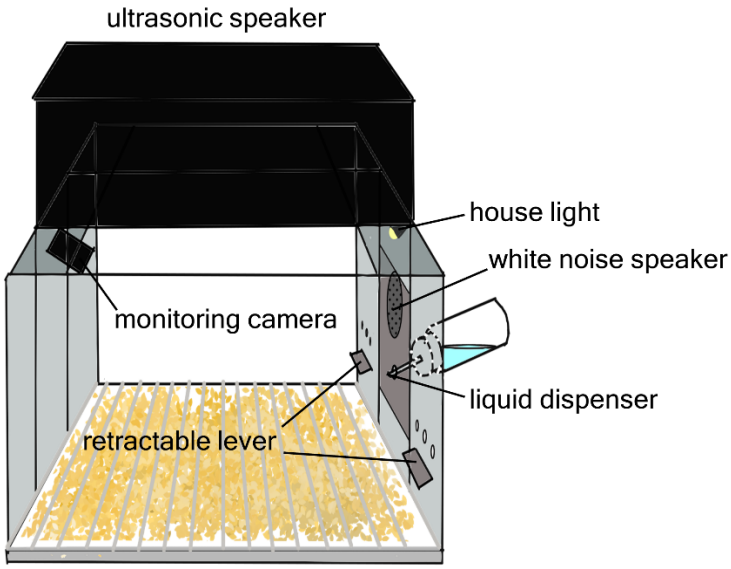
Subjects were nine male Sprague-Dawley (SD) rats (*Rattus norvegicus*) (SLC Japan Inc., Hamamatsu, Japan). Rats were seven to nine months of age at the start of the experiments and were used for the training. They were experimentally naïve and had no prior experience of similar experimental settings or procedures. All subjects were housed in individual plastic cages (Interior: 37.5 cm L × 23.5 cm W × 20 cm H) with wood chip bedding under a 12:12 h light/dark cycle (lights on at 8:00 am) in a temperature- and humidity-controlled room. Rats were individually housed because they were under food restrictions and accurate measurements of their food intake were required during the learning tasks. The individual cages were arranged in a rack, allowing visual, auditory, and olfactory contact with conspecifics to minimize stress. Before the experiments, rats were handled for approximately five minutes and fed *ad libitum* (Lab Diet, PMI Nutrition International, St. Louis, USA) for seven days to obtain their free-feeding weights (603.3 ± 46.0 g (*M* ± *SD*)). During the experiment, each rat's body weight was kept at approximately 85% of the free-feeding weight, following at least one week of gradual food restriction. Tap water was freely available except during training sessions. All experiments took place during the light phase of the cycle.

Apparatus

Experiments took place in an operant chamber (Interior: 30.5 cm L × 24.1 cm W × 29.2 cm H, ENV-007-CT, Med Associates, St. Albans, USA), enclosed within a sound-attenuating box. The chamber was equipped with an ultrasonic speaker (Center Speaker NS-C210, Yamaha, Hamamatsu, Japan), a monitoring camera (Debut Video Capture Software, NCH Software, Canberra, Australia), two retractable levers, a liquid dispenser, a white noise speaker and a house light positioned above the dispenser and a grid floor (Figure 1). The frequency range of the NS-C210 speaker was 65 Hz to 45 kHz at the -10 dB point and extended up to 100 kHz at the -30 dB point, relative to the peak frequency response. The speaker reasonably covers the frequency range of USVs, with an acceptably flat response in the relevant bands. The ultrasonic speaker and the monitoring camera were located on the top of the chamber, while the liquid dispenser and the white noise speaker were set between the two levers.

Figure 1

The Operant Chamber Used in this Study



Note. The operant chamber was equipped with an ultrasonic speaker, a monitoring camera, two retractable levers, a liquid dispenser, a white noise speaker, and a house light.

Recording and Editing of Ultrasonic Vocalization Stimuli

Before the behavioral experiment, we recorded 50 kHz and 22 kHz USVs from four rats, which were not used for the training. These stimulus rats from whom USVs were recorded were unfamiliar with the subject rats used for the training. Two rats were introduced in a sound-attenuated box equipped with a recording system (UltraSoundGate 116Hb with CM16/CMPA and Avisoft-RECORDER USGH, Avisoft Bioacoustics, Glienicke, Germany) and a monitoring camera (Debut Video Capture Software). The frequency range of the USG amplifier was 20 Hz to 140 kHz, defined at the -3 dB point relative to the peak frequency response. The CM16 microphone had a frequency range of 10 kHz to 140 kHz at the -3 dB point relative to its best frequency. Its frequency response was within ±3 dB around 22 kHz (±1/3 octave) and within ±2 dB around 50 kHz (±1/3 octave), indicating satisfactory performance for recording USVs. Rats were naïve to each other and they started to emit USVs soon after the first encounter in the box.

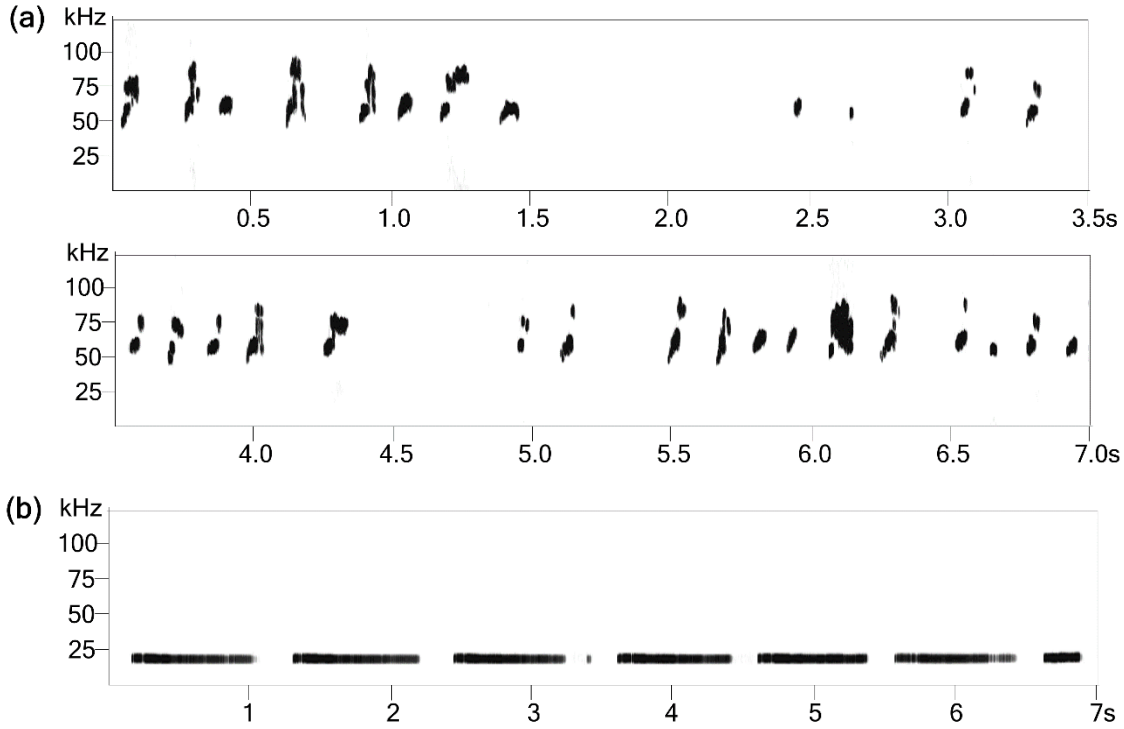
After recording, we edited the recordings and made USV stimuli for the training. Sound analysis software (Avisoft-SASLab Pro, Avisoft Bioacoustics; GoldWave, GoldWave Inc.) were used to make USV stimuli. First, USVs in the recordings were classified as either 50 kHz or 22 kHz by visual inspection of the

sound spectrogram (FFT length: 512 points; Resolution: 488 Hz). Second, some parts of the recording were selected to make USV stimuli that have a duration of 7.0 seconds and contain multiple syllables (50 kHz USV: 28.5 ± 2.4 syllables (Figure 2a); 22 kHz USV: 7.5 ± 0.8 syllables (Figure 2b) (*M* ± *SD*)). Total duration of USV syllables per stimulus file were 4.8 ± 0.6 s for 22 kHz USV stimuli and 1.4 ± 0.3 s for 50 kHz USV stimuli (*M* ± *SD*)). We made three files from each recording of one pair of rats, and thus obtained six files for both types of USV playback stimuli, respectively. Each file contained USV syllables which were emitted from only one pair of rats and the order of emitted syllables was not changed from the original recordings. Third, background noise was reduced to as low as possible using functions of software. Consistent noise was removed by "Noise Reduction" function in GoldWave. Sporadic and brief noise was manually reduced using the standard eraser cursor on the spectrogram and "Remove erased spectrogram sections from waveform" function in Avisoft-SASLab Pro. Finally, the sound pressure level of USV stimuli was adjusted so that each stimulus would be played at about 66 dB sound pressure level (SPL) on average (50 kHz USV stimuli: 66.0 ± 1.5 dB; 22 kHz USV stimuli: 66.4 ± 1.4 dB (*M* ± *SD*)).

USV stimuli were converted from digital to analog signals (CED MICRO3 1401, A-M Systems, Sequim, USA), amplified (Integrated Amplifier A-10, Pioneer, Tokyo, Japan), and then played through a speaker which was placed on the top of the operant chamber. Before starting each session, we checked the output of USV stimuli using a bat detector (Ultra Sonic Detector Ver 6, YS Design Studio, Tokyo, Japan).

Figure 2

Examples of USV Stimuli



Note. Examples of USV stimuli used in the behavioral experiment. For each spectrogram, the x-axis represents time (s), and the y-axis frequency (kHz). (a) a sample of 50 kHz USV stimuli which contains 30 syllables of short duration. (b) a sample of 22 kHz USV stimuli composed of seven syllables of long duration. Both (a) and (b) had a duration of 7.0 s.

Experiment 1

In Experiment 1, we tested if connections between 50 kHz USVs and positive, 22 kHz USVs and negative affective states influence the feasibility of discrimination learning using USVs. Discrimination

learning consisted of two conditions: congruent and incongruent. In the congruent condition, 50 kHz USVs were paired with a positive outcome, while 22 kHz USVs with a relatively negative, which was consistent with the presumed innate connections. In the incongruent condition, the relationship between USV type and valence of outcome was inverted from the original one (i.e., 22 kHz USVs paired with positive outcome, while 50 kHz USVs with negative one). We predicted that the congruent condition would be learned faster than the incongruent condition.

Method

Discrimination Learning Training

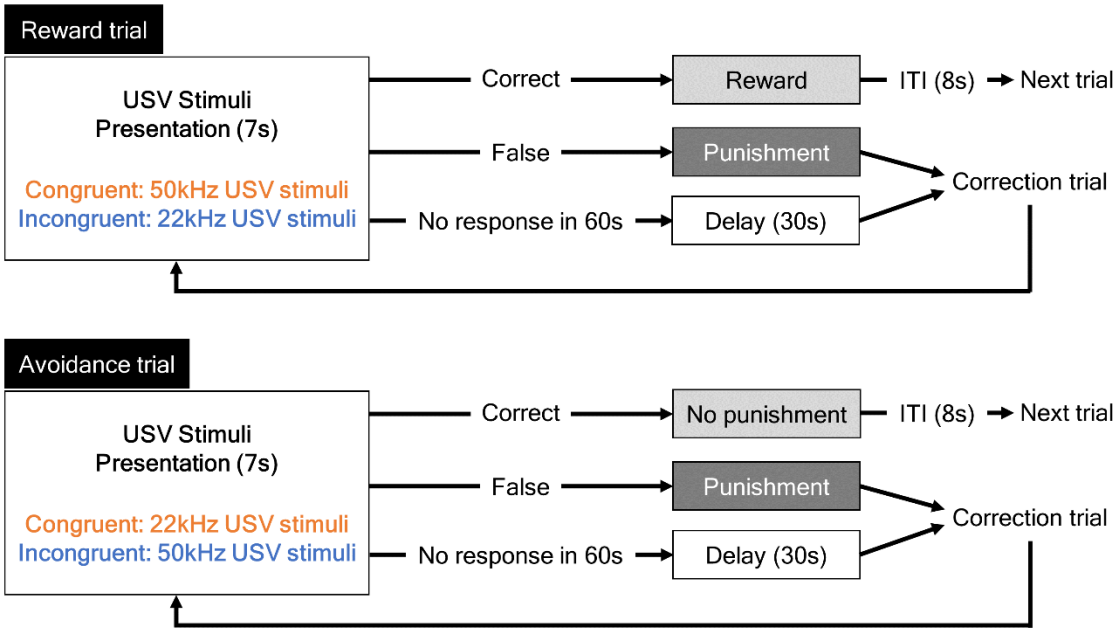
Prior to the training, rats were placed inside the operant chamber for 60 min per day to habituate to the testing environment. During this time, they were also trained to press the left and right levers to gain access to reward (20% sucrose solution dispensed for 3.0 seconds) until their responses were within three seconds.

During the discrimination learning training phase, rats learned discriminative responses to 50 kHz and 22 kHz USVs by pressing the left and right levers (Figure 3). Rats were trained in either of the two conditions: the congruent condition or the incongruent condition. In the congruent condition, 50 kHz USVs were paired with reward and 22 kHz USVs with punishment avoidance. In contrast, in the incongruent condition, 22 kHz USVs were paired with reward and 50 kHz USVs with punishment avoidance. Five rats were trained in the congruent condition and four rats were trained in the incongruent condition, respectively. Trials were designed according to Saito et al. (2016). There were two types of trials in each session: reward trials and avoidance trials (Figure 3). For example, in the congruent condition, rats were trained to press one lever to obtain a reward when 50 kHz USV stimuli (7.0 s, stimulus file counterbalanced) were presented. This trial was referred to as a reward trial and the lever, the reward lever. In the same session, rats were also trained to press the other lever when 22 kHz USV stimuli (7.0 s, stimulus file counterbalanced) were presented. This trial was referred to as an avoidance trial and the lever, the avoidance lever. No reward was presented in avoidance trials, while rats had to press the avoidance lever to move on to the next trial in which they might be able to get a reward. Which of the left and right levers was assigned the reward or avoidance lever was counterbalanced between subjects. In both reward and avoidance trials, pressing the incorrect lever led to the punishment (blackout and 95 dB SPL white noise presented for 30 s) and the same trial was repeated to reform the incorrect response (correction trial). Correction trials continued until rats pressed the correct lever. If rats did not press either of the levers in 60 s, the levers would be retracted, and the response for the trial was counted as an omission. Correction trials were also inserted after omissions.

Each session lasted for 60 min and each rat had one training session per day. A session consisted of a maximum of 200 trials. Reward and avoidance trials were presented in a pseudo-random order that the same trial type did not repeat more than three trials. Trials were separated by an inter-trial interval (ITI) of 8.0 s. If the rat was able to finish 200 trials before the 60 min limit, the session ended there.

Figure 3

Design of the Trials



Note. In the congruent condition, the discriminative stimuli for the reward trial were 50 kHz USVs, and stimuli for the avoidance trial were 22 kHz USVs, and vice versa in the incongruent condition. In the reward trial, the correct response was to press the reward lever. In the avoidance trial, the correct response was to press the avoidance lever.

Analysis and Statistics

As for the measure of learning acquisition, the number of lever presses to achieve the learning criteria was used. The learning criterion was defined as correct response rates exceeding 85% for both reward and avoidance trials for two consecutive sessions. Although the number of sessions to the criteria is commonly used to compare learning performance (e.g., Cook et al., 2004; Daniel & Schluessel, 2020), it was difficult to use this measure in this study, because the number of trials completed per session was not consistent across sessions. Typically, rats did not finish 200 trials within the 60-min limit during earlier sessions, while they managed to complete 200 trials before the limit in later sessions. Another commonly used measure, the number of trials to the criteria (e.g., Eichenbaum et al., 1986; Schrijver et al., 2004) was also difficult to use in this study, because the task had omissions. Including these omissions in measures of learning speed would be inappropriate, as rats did not experience the association between response and outcome during these trials. Thus, we decided to use the number of lever presses to the criteria (i.e., correct plus incorrect lever presses) as the most appropriate measure of learning speed (c.f., Tait & Brown, 2007). We compared the average number of lever presses needed to achieve the learning criteria between the congruent and the incongruent conditions by Wilcoxon rank sum test. In this analysis, all the lever presses to the learning criteria were included. We also compared the average number of reward lever presses to the learning criteria (correct response rates for reward trials exceeded 85% for two consecutive sessions) between the two conditions. Omissions were excluded from the analysis. Data were analyzed using R (3.3.2) (R Core Team, 2016). Detailed results of the learning task and R code for the analysis are available as online supplementary material.

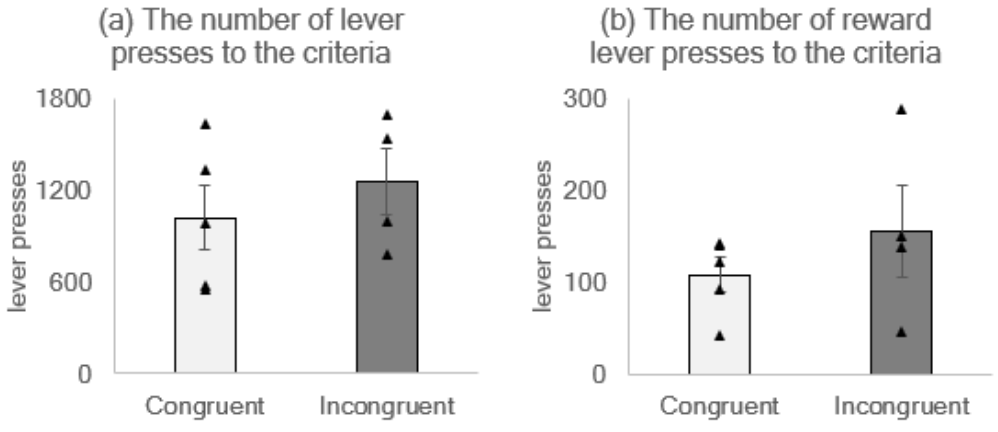
Results and Discussion

All nine rats achieved the learning criteria. The average number of lever presses (including both the reward and avoidance lever presses) to the learning criteria did not significantly differ between the congruent and the incongruent conditions (congruent condition (n = 5): 1016.2 ± 210.2, incongruent condition (n = 4): 1254.3 ± 216.7 ($M \pm SE$); Wilcoxon rank sum test: W = 6, p = .41; Figure 4a).
In both the congruent and the incongruent condition, rats quickly learned the reward lever pressing, while they acquired the avoidance lever pressing much more slowly. The number of avoidance lever presses to the learning criteria (correct response rates for avoidance trials exceeded 85% for two consecutive sessions) was 500.0 ± 106.7 for the congruent condition (n = 5), 624.3 ± 107.5 for the incongruent condition (n = 4) ($M \pm SE$). These numbers are almost equivalent to half of the average number of lever presses to the learning criteria (including both the reward and avoidance lever presses) and showed a similar trend. Therefore, we did not perform separate analysis on avoidance lever presses. On the other hand, the reward lever presses were acquired early in the learning process. Thus, we compared the number of reward lever presses to the learning criteria (correct response rates for reward trials exceeded 85% for two consecutive sessions) between the two conditions. There was no significant difference in the average number of reward lever presses to the criteria between the congruent and the incongruent condition (congruent condition (n = 5): 108.8 ± 18.7, incongruent condition (n = 4): 156.5 ± 49.5 ($M \pm SE$); Wilcoxon rank sum test: W = 6, p = .41; Figure 4b).

These results are inconsistent with the hypothesis that rats learn the congruent condition faster than the incongruent condition. This might suggest that the presumed innate connections (50 kHz USVs with positive, 22 kHz USVs with negative affective states) did not influence the feasibility of discrimination learning which utilizes USV stimuli. However, USVs may influence the learning process in a more elusive way. For example, rats quickly acquired conditioned freezing to both 50 kHz and 22 kHz USVs (Bang et al., 2008; Endres et al., 2007), but some differences were found in more detailed inspection. Endres et al. (2007) report that the association between 22 kHz USVs and aversive stimuli was more resistant to extinction than the other stimuli including 50 kHz USVs. Bang et al. (2008) found asymmetrical stimulus generalization in favor of biological predisposition to associate 22 kHz USVs and aversive events; there was less generalization of fear to the CS− (cues unpaired with foot shock) when 22 kHz USVs served as the CS+ (cues co-terminated with foot shock) than when 22 kHz USVs served as the CS－.

Figure 4

Results of the Discrimination Learning Training



Note. (a) represents the average number of lever presses to the learning criteria in the congruent and the incongruent conditions. (b) shows the average number of reward lever presses to the learning criteria in the congruent and the incongruent conditions. Shaded bars indicate the congruent condition while filled bars indicate the incongruent condition. Error bars represent *SE*. Each triangle indicates an individual subject.

Experiment 2

In Experiment 1, we found that rats did not learn the congruent and the incongruent condition at a different learning rate. However, rats may retain the learned content better when it is consistent with the presumed innate connections (50 kHz USVs with positive, 22 kHz USVs with negative affective states). This hypothesis is related to the study by Endres et al. (2007), which revealed that the association between 22 kHz USVs and aversive events was retained better and more resistant to extinction. They indicate that rats have a preparedness to acquire 22 kHz USVs as danger-predicting cues, which makes it difficult to extinguish associations between 22 kHz USVs and aversive events.

In Experiment 2, we exploratorily tested if the previous experience of learning using USVs (the congruent or the incongruent condition in the discrimination learning phase in Experiment 1) would affect the following learning process using the serial reversal learning paradigm. In a serial reversal learning paradigm, animals may progressively improve their performance across reversals in a learning-to-learn manner, or they may take longer across reversals due to proactive interference. (Shettleworth, 2010). Under the influence of proactive interference, previously learned associations inhibit the acquisition of a new association (Shettleworth, 2010).

We hypothesized that rats would retain the memory of the congruent condition better than the incongruent condition, and that this would hamper the acquisition of reversal learning process. Thus, we predicted that reversal from the congruent to the incongruent condition is more difficult compared to reversal from the incongruent to the congruent condition.

Methods

Serial Reversal Learning Training

The serial reversal learning phase started immediately after rats achieved the learning criteria in the discrimination learning training in Experiment 1. Rats were trained to serially reverse the discrimination until the end of the fourth reversal learning phase (Table 1). If the learning criteria were not achieved in 45 sessions, training was terminated there. For rats that experienced the congruent condition in the discrimination learning phase (Congruent start: CS), the first reversal was the incongruent condition, the second the congruent, the third the incongruent, the fourth the congruent, and vice versa for those that learned the incongruent condition in the discrimination learning phase (Incongruent start: IS). This means that, in each reversal, eliciting USV stimuli in the reward and avoidance trials were reversed, but the reward and avoidance levers were fixed throughout the whole process of learning (Figure 3). The subjects, the apparatus, the task design and the learning criteria were the same as in Experiment 1.

Table 1

Design of the Reversals

	Congruent Start (CS)	Incongruent Start (IS)
Discrimination (Experiment 1)	Congruent	Incongruent
1st reversal	Incongruent	Congruent
2nd reversal	Congruent	Incongruent
3rd reversal	Incongruent	Congruent
4th reversal	Congruent	Incongruent

Note. Rats were trained to serially reverse the congruent and the incongruent condition until the end of the fourth reversal learning phase. For rats that experienced the congruent condition in Experiment 1 (Congruent start: CS), the first reversal in Experiment 2 was the incongruent condition, the second the congruent, the third the incongruent, and the fourth the congruent. For those that learned the incongruent condition first (Incongruent start: IS), the first reversal was the congruent condition, the second the incongruent, the third the congruent, and the fourth the incongruent.

Analysis and Statistics

Firstly, we tested the same hypothesis as in Experiment 1 which predicted that the congruent condition would be learned faster than the incongruent condition. Due to the reasons stated in Experiment 1, the number of lever presses was used as a measure of learning acquisition. The average number of lever presses to the learning criteria and the average number of the reward lever presses to the criteria were compared between the congruent and the incongruent condition using Wilcoxon signed-rank test. For these analyses, the average number of lever presses in the first and the third reversal phase was compared with the average number of lever presses in the second and the fourth reversal phase within each subject. These analyses also examined whether rats would retain the memory of the congruent condition better than the incongruent condition, because in the serial reversal learning phase, learning the congruent condition is the same as reversing from the incongruent condition to the congruent condition, and vice versa.

We also compared the average correct reaction time for both reward and avoidance trials between the congruent and the incongruent condition by Wilcoxon signed-rank test. For these analyses, the average latency to both the correct reward lever pressing and the correct avoidance lever pressing were compared between the congruent and the incongruent conditions within each subject. The correct lever pressings in the last two sessions in each reversal phase (two sessions that accomplished the learning criteria) were used to calculate the average correct reaction time.

Additionally, to examine the formation of learning set, we analyzed the whole learning process, from the discrimination learning phase in Experiment 1 to the fourth reversal learning phase. We examined whether the number of lever presses to the criteria differed between the learning phases using Friedman's test. Wilcoxon signed-rank test with Holm's correction was used to assess differences between each learning phase. Data were analyzed using R and MATLAB R2019a (MathWorks, Natick, U.S.A.). Detailed results of the learning task and R code for the analysis are available as online supplementary material.

Results and Discussion

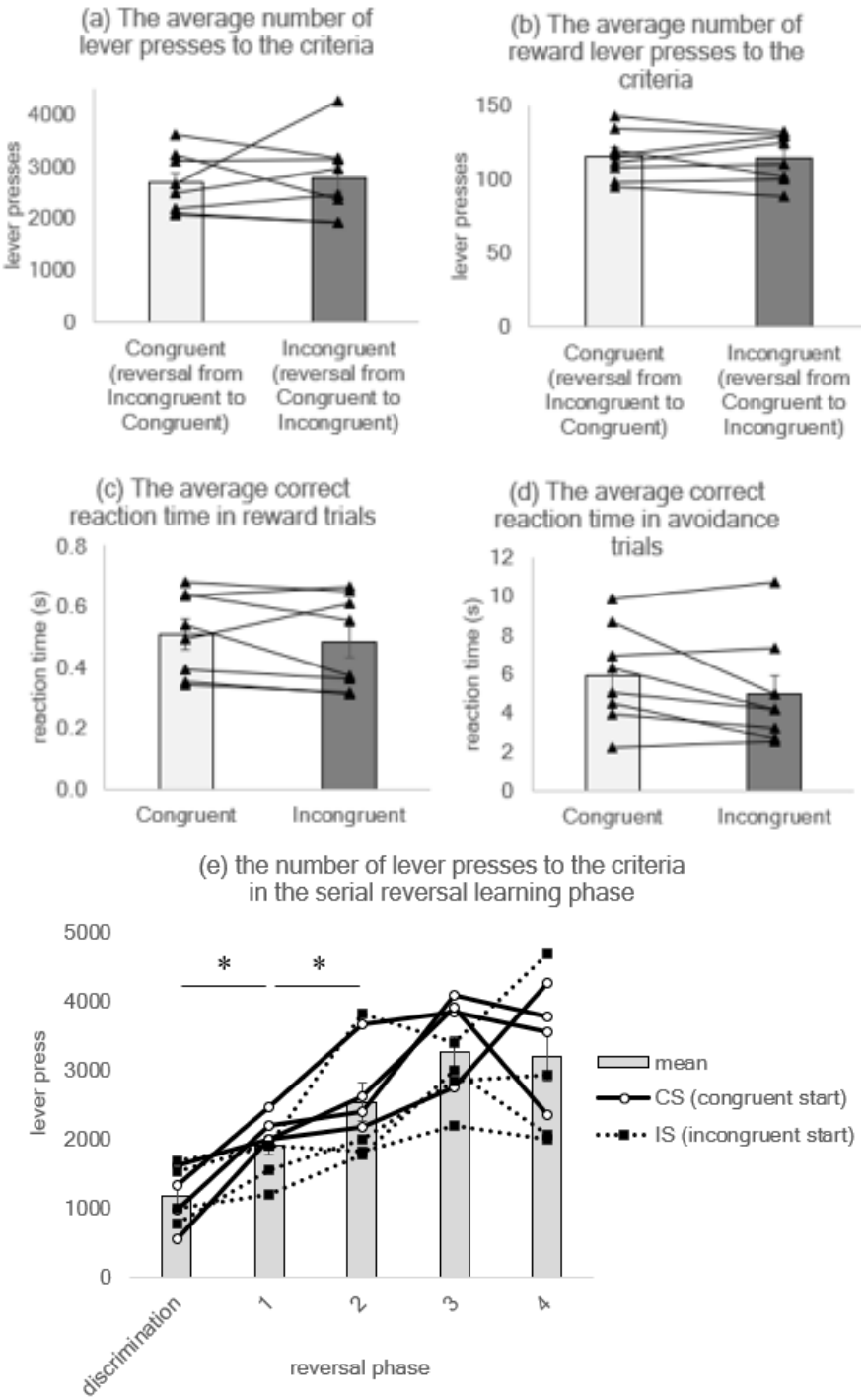
Eight out of nine subjects completed the serial reversal learning training. One rat could not achieve the learning criteria in the second reversal phase in 45 sessions. Training ended there for that subject and the subject was excluded from further analysis. In three sessions (two sessions in the second reversal learning phase of one subject and one session in the third reversal learning phase of another subject), the liquid dispenser was not able to deliver rewards from the middle of the session due to mechanical troubles. Lever presses in these sessions are included in the analyses below.

The average number of lever presses to the learning criteria in the serial reversal learning phases did not significantly differ between the congruent and incongruent conditions (congruent condition (n = 8): 2674.5 ± 203.7, incongruent condition (n = 8): 2772.0 ± 273.2 (M ± SE); Wilcoxon signed-rank test: V = 17, p = .95; Figure 5a). Also, there was no significant difference in the average number of reward lever presses to the criteria between the congruent and the incongruent condition (congruent condition (n = 8): 115.9 ± 5.8, incongruent condition (n = 8): 114.9 ± 5.9 (M ± SE); Wilcoxon signed-rank test: V = 20, p = .84; Figure 5b). Consistent with Experiment 1, animals did not learn the congruent condition significantly faster than the incongruent condition. These results also indicate that the number of lever presses to the learning criteria for reversing from the congruent to the incongruent condition did not differ from those for reversing from the incongruent to the congruent condition. This implies that the congruent condition is no more resistant to extinction than the incongruent condition.

Also, we analyzed the average correct reaction time of the two conditions because rats may have responded faster in the congruent condition than in the incongruent condition. There was no significant difference between the congruent and the incongruent condition in the average correct reaction time in reward trials (congruent condition (n = 8): 0.51 ± 0.05 (s), incongruent condition (n = 8): 0.48 ± 0.05 (s) (M ± SE); Wilcoxon signed-rank test: V = 26, p = .30; Figure 5c), nor in avoidance trials (congruent condition (n = 8): 5.93 ± 0.89, incongruent condition (n = 8): 4.98 ± 0.98 (M ± SE); Wilcoxon signed-rank test: V = 29, p = .15; Figure 5d).

Figure 5

Results of the Serial Reversal Learning Training



Note. (a) represents the average number of lever presses to the learning criteria. (b) shows the average number of reward lever presses to the learning criteria. (c) represents the average correct reaction time in reward trials, while (d) shows the average correct reaction time in avoidance trials. In (a) to (d), each pair of triangles connected by a line represent each individual. (e) shows the number of lever presses to the learning criteria in the serial reversal learning phase. Solid lines indicate Congruent Start (CS) animals. Broken lines indicate Incongruent Start (IS) individuals. Error bars represent *SE*. * indicates $p < .05$.

The number of lever presses needed to achieve the learning criteria was significantly different between the learning phases (Friedman's test: $X^2 = 26.7$, $p < .001$; Figure 5e). Individual comparisons using Wilcoxon signed-rank test with Holm's correction revealed significant differences between the discrimination learning and the first reversal learning phase (discrimination learning phase ($n = 8$): 1189.9 ± 148.9, first reversal learning phase ($n = 8$): 1905.4 ± 136.0 ($M \pm SE$); $V = 0$, $p = .031$; Figure 5e), between the first and the second reversal learning phase (second reversal learning phase ($n = 8$): 2534.4 ± 279.6 ($M \pm SE$); $V = 1$, $p = .047$; Figure 5e) and a marginal difference between the second and the third reversal learning phase (third reversal learning phase ($n = 8$): 3250.1 ± 235.0 ($M \pm SE$); $V = 3$, $p = .078$), although no significant difference was observed between the third and the fourth reversal learning phase (fourth reversal learning phase ($n = 8$): 3203.1 ± 360.8 ($M \pm SE$); $V = 22$, $p = .64$).

Consistent with Experiment 1, these results indicate that rats did not learn the congruent condition faster than the incongruent condition, nor did the congruent condition more strongly hinder the acquisition of reversal learning than the incongruent condition. In addition, animals did not press the correct lever faster in the congruent condition than in the incongruent condition. Namely, rats did not respond differently to the congruent and the incongruent conditions in all aspects. Converging the evidence, it seems likely that the presumed innate connections (50 kHz USVs with positive, 22 kHz USVs with negative affective states) did not influence the feasibility of discrimination and serial reversal learning which utilizes USV stimuli.

General Discussion

Contrary to our predictions, rats did not learn the congruent condition faster than the incongruent condition (Experiment 1). Also, the congruent condition did not hinder subsequent learning more strongly than the incongruent condition. Animals did not press the correct lever faster in the congruent condition than in the incongruent condition (Experiment 2). These results are inconsistent with studies which suggest that the association between 22 kHz USVs and aversive events may be facilitated/enhanced by biological predispositions (innate connections between 22 kHz USVs and negative affective states) (Bang et al., 2008; Endres et al., 2007; Kim et al., 2010; Parsana et al., 2012).

These results may together indicate that the presumed innate connections (50 kHz USVs with positive, 22 kHz USVs with negative affective states) did not influence the feasibility of discrimination and serial reversal learning which utilizes USV stimuli. This may be because it is difficult to detect the impact of presumed innate connections (50 kHz USVs with positive, 22 kHz USVs with negative affective states) on learning. Several classical conditioning studies suggest that USVs influence the learning process in an elusive way. For example, rats quickly acquired conditioned freezing to both 50 kHz and 22 kHz USVs (Bang et al., 2008; Endres et al., 2007), but the impact of biological constraints were only observed in further inspections (i.e., extinction, asymmetrical stimulus generalization). Biological constraints may not be strong enough to influence the number of lever presses to the learning criteria or correct lever pressing latency in operant conditioning. This indicates that rats were able to perform flexible lever-pressing responses toward USVs which were repeatedly presented in an operant chamber. In accordance with this, Saito et al. (2019) has shown that rats relatively quickly (i.e., 16.5 ± 3.9 days) learned to discriminate between 50 kHz USVs and 22 kHz USVs by pressing corresponding levers. USVs presented as discriminative stimuli might become rather neutral signals through extensive training. In future investigations, it would be helpful to examine whether USVs generally lose original valences for such experienced animals or flexible responses to USVs are only limited to operant chamber situations and they would recover typical responses in a more naturalistic playback conditions such as a radial maze.

The characteristics of the operant conditioning task employed in our study may have made it difficult to detect the influence of presumed innate connections on learning. First, USV stimuli were short and USV types were frequently switched between trials in our study. This may have weakened the impact of the USVs on emotion and behavior. Typical USV playback method is to present a few minutes of stimuli containing dozens of USV syllables, with an interval of a few minutes (Brudzynski & Chiu, 1995; Wöhr & Schwarting, 2007). In Saito et al. (2016) which has shown that USV playback modulates learned behavior (lever pressing), the same type of USV stimuli is also presented for extended periods of time. Thus, USVs

may need to be presented for a relatively long time to exert affective or behavioral influence. Moreover, short USV stimuli were presented hundreds to thousands of times before achieving the learning criteria in our study. Considering it has been demonstrated that USV stimuli are susceptible to habituation (Schönfeld et al., 2020; Wöhr & Schwarting, 2012), such repeated stimulus presentation may have suppressed natural emotional responses to USVs. In line with this, 50 kHz USV playback experiments which measured approach behavior to the sound source in a radial maze consistently report that responses were most evident during the initial presentation and attenuated afterwards (Schönfeld et al., 2020; Wöhr & Schwarting, 2012). Such habituation has also been observed in playback experiments in an operant chamber (Willuhn et al., 2014), suggesting that the habituation phenomena is independent of types of experimental apparatus used. Moreover, Berz et al. (2021) suggested that habituation depends on the subject's internal state and match of internal states between the first and second USV exposures would be essential for habituation to occur. Because an operant chamber as used in our study creates a highly controlled environment with little interference from external stimuli, animals' internal states during operant tasks are likely to match across sessions. The combination of repeated USV playback exposure and a highly controlled operant chamber environment, likely stabilizing animals' internal states, may have enhanced habituation to USVs. However, it is of note that compared to Wistar rats which are typically used for USV playback studies, SD rats appear to be more resistant to the habituation phenomena (Berz et al., 2021). Thus, future experiments which expose rats to a thousand USV playbacks and observe whether any emotional or behavioral responses remain would be highly beneficial.

Moreover, too large a difference between the values of pressing the two types of levers may have greatly affected learning and masked the impact of the USVs on behavior. There were two types of levers in our experimental paradigm: reward and avoidance lever. While pressing the reward lever leads to immediate reward, pressing the avoidance lever leads only to avoidance of punishment. Regardless of which USV stimulus was presented, rats consistently pressed the reward lever with much shorter latency (about one-tenth) than they pressed the avoidance lever, with no significant difference between the congruent and incongruent condition. These results indicate that rats were highly motivated to press the reward lever, on the other hand, they pressed the avoidance lever very reluctantly. Also, rats learned to press the reward lever much more rapidly than to press the avoidance lever. Rats quickly achieved the learning criteria for reward trials, but they needed several times as many trials to achieve the learning criteria for avoidance trials. It is likely that what was actually learned was to restrain from pressing the reward lever in avoidance trials. This may have distorted the design of the learning task in a different direction than it was supposed to, making it unable to compare the positive and negative properties of USVs. The usage of sucrose rewards might also have enlarged the discrepancy between the values of pressing the two types of levers. In line with this, Montanari et al. (2020) has shown that USV playbacks affected cocaine intake of rats but not sucrose intake, suggesting that rewarding property of sucrose is so strong that it could overcome the affective properties of USVs.

Regardless of the direction of reversal, learning tended to slow down during the serial reversal learning phases (i.e., subjects needed more trials as reversals progressed). This may also be related to a large difference in the reinforcing values of the reward and avoidance levers. In serial reversal learning experiments which involves repeated reversals of the reward contingency, rats can improve their performance over successive reversals by reducing errors in a learning-to-learn manner (Castañé et al., 2010; Pubols Jr., 1957). However, depending on the task and learning phases (especially in earlier reversals), rats may slow down learning (Gatling, 1952; Pubols, Jr., 1957). The difficulty of reversal learning task is that animals need to inhibit previously rewarded responses and activate previously unrewarded responses. Such proactive interference from previously learned associations inhibits animals from learning reversals (Shettleworth, 2010), resulting in extended learning. In serial reversal learning phases in our study, the reward and avoidance levers were fixed, while eliciting USV stimuli in the reward and avoidance trials were reversed. Thus, it is very likely that rats were reinforced to always press the reward lever, when they did not acquire correct responses yet, immediately after the reversal. This perseverance to press the reward lever may have suppressed the acquisition of reversals. Also, reversals of the reward contingency of USV stimuli may have inhibited reversal learning as well. Rats needed to avoid

pressing the reward lever when they heard USV stimuli that previously led to a reward. Such experiences with USV stimuli may have proactively interfered with reversals. Although learning clearly slowed down until the second reversal, from the third reversal onward, individual differences became greater, with some individuals learning faster. These fast-learning subjects might have started learning how to reverse the discrimination. Similarly, Gatling (1952) suggested that although there was a large interference that slowed down learning in earlier reversals, learning increasingly speeded up in later reversals.

Future challenges include improving the paradigm and increasing the generalizability of the findings. First, difference between the values of pressing the two types of levers should be as small as possible. To this aim, reinforcing values of avoiding punishment need to be large enough without inhibiting learning. A possible task is to deliver punishments such as foot shocks in avoidance trials, when some time has elapsed without lever pressing. This would also reduce the number of trials to the criteria, mitigating habituation to USV stimuli playback. Second, it might be effective to increase the time between USV stimulus presentation and lever presentation to lengthen the time subjects hear USV stimuli. Third, it might be necessary to include conditions where neutral sounds are paired with positive/negative events and compare them to the conditions which include USVs. If rats acquire conditions with USV stimuli at a different learning rate than they learn conditions with neutral stimuli, we can assume that predispositions to associate USVs with positive/negative events bias learning. Fourth, it would be necessary to fix the number of trials per session to more accurately compare learning rates across conditions. This would allow comparison of the number of sessions instead of lever presses to the criteria. It would then be possible to look at the learning curve to inspect the acquisition process in more detail. Also, reducing the number of trials per session and the maximum duration of a training session would help keep rats' engagement to the learning task. The fact that one subject was excluded from the training due to delayed acquisition in Experiment 2 may indicate individual differences in motivation that could have affected the learning outcome. Together with the modifications mentioned above to make the task easier, alleviating the burden of training would be helpful. Last, the sample size of our study was relatively small, and this may have underpowered the statistical robustness of the results by increasing the risk of a Type II error. Future studies with a larger sample size and an improved experimental paradigm as above would contribute to the better understanding of whether the presumed innate connections between USVs and positive/negative affective states affect learning using USVs.

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Data Availability: The data supporting the findings of this study are available as online supplementary material at the OSF project (https://osf.io/2v5d6/overview?view_only=f6f4a2d9ece74de9bb48f11b30c565d7). Additional data are available from the corresponding author upon request

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