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Ultrasonic vocalizations near 30 kHz may indicate excitement rather than distress in female Wistar rats

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ABSTRACT

Rats emit ultrasonic vocalizations (USVs), sometimes referred to as 50-kHz vocalizations, during activities such as play and lower-frequency USVs, sometimes referred to as 22-kHz vocalizations, when experiencing distress. Definitions of 22-kHz vocalizations vary in terms of which frequencies should be included, with differing upper limits (e.g., 25 kHz, 30 kHz, or 33 kHz). In the current experiments, we examined the extent to which USVs near the upper frequency limit may represent milder anxiety than lower-frequency vocalizations. In Experiment 1, rats received both gentle tickling and simulated rough-and-tumble play with a human hand, while low-frequency (i.e.,18–33 kHz) and high-frequency (i.e., 34–90 kHz) USVs were recorded; rough-and-tumble play elicited significantly more low-frequency USVs (median 29.5 kHz), but also significantly more high-frequency USVs (median 50.7 kHz). In Experiment 2, rats were presented with 23-kHz, 32-kHz, and 52-kHz USVs while foraging in a T-maze. The 23-kHz USV elicited the most freezing, while the 32-kHz USV elicited significantly less freezing than either the 23- or the 52-kHz USV. Taken together, these results suggest that within the range of frequencies referred to as 22-kHz vocalizations, lower-frequency USVs may indicate a more negative affective state than higher-frequency ones. As USV production can serve as an indicator of rat welfare, an understanding of what frequencies represent positive versus negative affective states is crucial.

1. Introduction

Rats emit two classes of ultrasonic vocalizations (USVs) which appear indicative of their affective states: higher frequency ones sometimes termed 50-kHz vocalizations (Panksepp and Burgdorf, 2003) and lower frequency (but still ultrasonic) ones sometimes referred to as distress calls or 22-kHz calls (Blanchard et al., 1991). High-frequency USVs are elicited by play, either with a conspecific or with a human hand (e.g., Panksepp and Burgdorf, 2000; Schwarting et al., 2007; Cloutier et al., 2013) or appetitive stimuli such as food (Opiol et al., 2015), amphetamine (Wright et al., 2010), or cocaine (Browning et al., 2011). Anticipation of appetitive social stimuli may also elicit 50-kHz vocalizations (Simola and Granon, 2019); a predictive stimulus (e.g., a human hand) or context may serve as a Pavlovian conditioned stimulus eliciting 50-kHz vocalizations as a conditioned response (Panksepp and Burgdorf, 2000).

22-kHz USVs, conversely, are elicited by stressors such as electric shock (de Vry et al., 1993), loud noise, social defeat, or the presence or scent of a predator (Brudzynski, 2019) as well as anticipation of aversive stimulation (Simola and Granon, 2019). These vocalizations have been compared to, and share numerous properties with, human crying (e.g., expression of anxiety which can be decreased with anxiolytic drugs; a sex difference in which more crying/22-kHz vocalizations are emitted by females; Brudzynski, 2019).

In addition to being elicited by different stimuli, the two classes of USVs communicate different affective states to conspecifics (Simola and Granon, 2019), are especially likely to occur in social contexts (Brudzynski, 2019), and have distinct effects on the behavior of rats that hear them. For example, Wohr and Schwarting (2007) found that rats approached 50-kHz but not 22-kHz USVs in a radial arm maze, and that the 22-kHz USVs reduced locomotor activity. Similarly, Inagaki and Ushida (2021) reported that 50-kHz USVs induced exploration while 22-kHz USVs induced anxiety-related behaviors, and Burman et al. (2007) reported that 22-kHz but not 50-kHz USVs inhibited emergence from a wooden box.

A commonly measured behavioral response to 22-kHz USVs (as well as other aversive stimuli) is immobility or freezing. For example, Wohr and Schwarting (2007, 2008) reported increased immobility or

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decreased locomotion in rats hearing 22-kHz USVs. Other research suggests this immobility may be a learned response rather than an innate one. Some social learning (Kim et al., 2010) or associative learning between a rat's own 22-kHz calls and a fearful affective state (Parsana et al., 2012) may be required in order for immobility to occur reliably in response to distress USVs.

Higher-frequency USVs, while sometimes termed 50-kHz USVs (e.g., Panksepp and Burgdorf, 2000), include USVs with peak frequencies from 30 kHz up to 90 kHz (Wöhr, 2018; Opiol et al., 2015). These USVs may take a wide variety of spectrographic forms including trills (i.e., rapid frequency oscillations), step-up and step-down calls (i.e., USVs that instantly jump to a higher or lower frequency), split calls (i.e., USVs with a lower-frequency middle component that includes a harmonic), and flat calls of relatively unchanging frequency (Wright et al., 2010.). While the significance of every type of USV is not known, evidence suggests that different forms of high-frequency calls may be elicited by different social contexts or stimuli. For example, Wright et al. (2010) reported more flat calls from rats recorded alone and more trill calls from pair-tested subjects, and Opiol et al. (2015) reported more frequency-modulated USVs in rats anticipating food but more flat calls after the food had been delivered. Additionally, some types of USVs may occur during specific types of locomotion. For example, split calls were common when rats jumped, while trill USVs were common during walking (Burke et al., 2017).

Lower-frequency USVs, sometimes called 20-kHz (Panksepp and Burgdorf, 2003) or 22-kHz calls (Wöhr and Schwarting, 2007), which tend to be flat in shape and of relatively long duration (Blanchard et al., 1991), also include a range of frequencies. However, the defined range of frequencies that constitute distress calls varies. For example, distress calls have been defined as vocalizations between 20 and 25 kHz (Wright et al., 2010), 20 and 30 kHz (Inagaki and Ushida, 2021), 22 and 28 kHz (Mittal et al., 2020), or all USVs up to 33 kHz (Schwarting et al., 2007). The somewhat varied definitions of both high- and low-frequency USVs mean that USVs in the 30-kHz range are ambiguous in terms of indicating a positive or negative affective state. In our laboratory, such USVs were regularly recorded during heterospecific play with a human hand; however, no other behavioral indicators of distress occurred during these sessions, and rats approached, crawled over, and chased the hand rather than retreating from it.

The current experiments explored whether USVs near 30 kHz might represent an affective state intermediate between enjoyment and distress. In Experiment 1, rats were subjected to either gentle tickling or rough-and-tumble heterospecific play while low- (i.e.,18–33 kHz) and high-frequency (i.e., 34–90 kHz) USVs were recorded. Based on behavioral observations made during previous lab experiments, we hypothesized that rough-and-tumble play with a human hand would elicit more lower-frequency USVs, but similar higher-frequency USVs as gentler heterospecific play. In Experiment 2, we played 23-, 32-, and 52-kHz USVs as rats engaged in a foraging task, with the hypothesis that the 23-kHz, but not the 32 kHz USV, would elicit increased latency to eat and elicit more freezing behavior than the 52 kHz USV.

2. Experiment 1

2.1. Materials and methods

All procedures in both experiments were approved by the University of Alaska Anchorage Institutional Animal Care and Use Committee (IACUC; IRBNet Protocol # 1683228).

2.2. Animals and husbandry

Eight female 5-month-old Wistar rats (Charles River, Wilmington, MA) served as subjects. Rats were housed in groups of four in pairs of plastic 41 cm × 21 cm x 20 cm cages (Allentown Caging, Allentown, NJ) connected by 7.75 cm diameter, 35 cm long sections of acrylonitrile butadiene styrene (ABS) pipe. Pine shavings (GEM Shavings, LLC, Auburn, WA) and shredded paper were used as bedding, and subjects were exposed to a 12 hr light/dark cycle (lights on from 19:00–07:00) in a climate-controlled (i.e., approximately 22 ℃, 20–23 % humidity) room. Subjects received Mazuri Rodent Pellets (PMI Nutrition International, LLC, Brentwood, MO) and tap water ad libitum as well as feeding enrichment consisting of uncooked whole grain pasta, dog food/treats, or fresh vegetables approximately 5 times per week.

Subjects had previously served in another experiment in which USVs were elicited by conditioned (i.e., the presentation of a stationary human hand) and unconditioned (i.e., simulated rough-and-tumble play with a hand) stimuli. In order to maximize vocal responsiveness to heterospecific play, subjects were first socialized as described by Panksepp and Burgdorf (2000), and, at approximately four weeks of age, subjects were kept in isolation for two days, during which time they experienced play with a human hand. This method results in lasting acceptance of heterospecific play.

2.3. Experimental treatment

Heterospecific play took place in a 50 × 40 × 21 cm plastic tub (Maryland Plastics, Federalsburg, MD). USVs were recorded with a Wildlife Acoustics (Wildlife Acoustics, Inc Maynard, MA) Song Meter SM3 Bioacoustics Recorder equipped with a Wildlife Acoustics SMM-U2 Ultrasonic Microphone (attached to a corner of the tub with tape), and analyzed with Kaleidoscope Pro Version 5.3.9 (Wildlife Acoustics, Inc Maynard, MA) software.

In counterbalanced order, on a single day, rats were presented with two sessions of heterospecific play, each consisting of three manual stimulations of the same body areas: neck, back, and belly, with body areas within each session presented in a single order for all subjects. During the Gentle session, rats were tickled by an experimenter's hand, but their movements were not constrained (i.e., the hand followed them around the enclosure). During the Rough-and-Tumble session, the rats were gently immobilized. For back stimulation, researchers pressed down between the rat's shoulder blades firmly enough to prevent the rat from moving away while tickling, similar to the "push and drill" technique described by Schwarting et al. (2007). For neck stimulation, rats were restrained by having their heads gently grasped from the front with a thumb and index finger while the remaining fingers tickled the neck area. For belly stimulation, rats were pinned on their backs during tickling, as would occur during play with a conspecific (e.g., Cloutier et al., 2013). Each tickling technique lasted 15 s, followed by a 15-second period of no stimulation before the next tickling technique began.

2.4. Results and discussion

Fig. 1 presents the raw average numbers of low (18–33 kHz) and high (34–90 kHz) USVs recorded during rough and gentle play sessions. For analysis, data were subjected to a square root transformation to correct for positive skew. Low- and high-frequency USVs were each analyzed with a separate repeated-measures 2 (Roughness: Rough or Gentle) x 3 (Body Area: Back, Neck, or Belly) ANOVA. When examining low-frequency USVs, the main effect of roughness was significant, F (1, 7) = 6.538, p = .038; rougher tickling elicited more low-frequency USVs. The effect of body area and interaction between roughness and body area were not statistically significant. For high-frequency USVs, the main effect of roughness was significant, F (1, 7) = 44.753, p < .001; rougher tickling elicited more high-frequency USVs. The effect of body area was not statistically significant. However, the interaction between roughness and body area was statistically significant, F (2, 14) = 9.409, p = .003; the rough session was especially effective at eliciting high-frequency USVs during belly stimulation (i.e., when the rat was pinned on its back). Additionally, low and high USVs were positively correlated during rough sessions for back (r = .92, p = .001) and neck (r = .95, p = .003) stimulation and during gentle tickling sessions for back

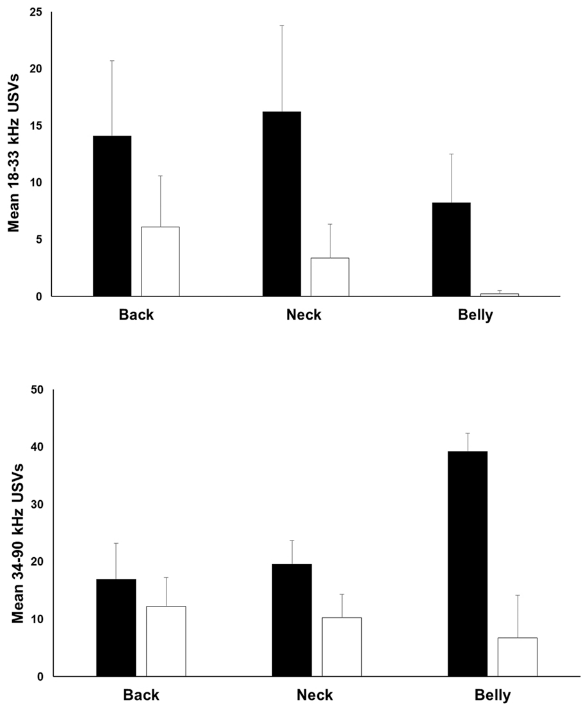

Fig. 1. Effect of simulated rough-and-tumble play (black bars) versus gentle tickling (white bars) on average numbers of 18–33 kHz (top panel) and 34–90 kHz (bottom panel) USVs produced during 15 s periods of stimulation. Error bars indicate *SEM*.

($r = .79$, $p = .02$) stimulation.

These results show that USVs which have been defined as indicative of distress are readily elicited by play. However, we observed no other behavioral evidence of distress, in either the gentle or the rough session. Rats approached and chased the hand in between 15-s tickling techniques, and during the 15 s of immobilization in the rough session, struggling was minor or absent completely, and subjects never bit or emitted any audible vocalizations which indicate aggression (Brudzynski, 2019). In fact, subjects remained docile and playful while engaged with the researchers' hand and were easily restrained. The apparent lack of fear in the rougher session resembles results from Cloutier et al. (2012), who reported that repeatedly restraining rats on their backs resulted in a similar preference for a human hand as gentler tickling and did not appear to be aversive. In the present study, the greater number of high-frequency USVs recorded during rougher sessions provide additional evidence that playful restraint was not necessarily aversive to the subjects.

Additionally, the fact that the number of low-frequency USVs were positively correlated with the number of high-frequency USVs during two of the three stimulation techniques in the rough session seems inconsistent with the idea that the low-frequency USVs indicated distress. Stressors such as bright light and predator odor suppress high-frequency USVs in rats (Panksepp and Burgdorf, 2010), and previous research has argued that a negative correlation found between 50-kHz and 22-kHz vocalizations emitted during playful handling provided evidence that these two types of calls represent opposite and incompatible emotional states (Burgdorf et al., 2005). Another experiment reported no correlation between 50-kHz and 22-kHz vocalizations during heterospecific play (Mällo et al., 2007). While the playful handling was very similar across these studies and the current one, possible explanations for the varying correlations include the types of rats (i.e., outbred in the current study but selectively bred for 50-kHz calls in Burgdorf et al.) or the range of frequencies included in the measurement of 22-kHz vocalizations (i.e., 18–33 kHz in the current study versus 20–25 kHz in Mällo et al.).

3. Experiment 2

3.1. Materials and methods

3.1.1. Animals and husbandry

Subjects in Experiment 2 included the 8 subjects from Experiment 1 plus 4 additional females from the same cohort. The 4 new subjects were excluded from the first experiment because they had previously been found to emit relatively few USVs, but they were socialized in the same manner as the other 8 subjects. Subjects were housed and fed as in Experiment 1 (i.e., ad libitum access to both food and water).

3.2. Apparatus

USV stimuli were selected from recordings obtained during initial socialization of the subjects in the current experiments (i.e., when subjects were singly housed at approximately four weeks of age). The same Wildlife Acoustics Song Meter SM3 Bioacoustics Recorder used in Experiment 1 was used in recording these, and Kaleidoscope Pro Version 5.3.9 software was used to examine and select the USVs. A Binary Acoustic Technologies (BAT) AT100 Ultrasonic Transmitter was used to present stimulus USVs to subjects.

Three USVs similar to each other in shape (i.e., relatively flat) but differing in average frequency were chosen. The first USV was the lowest USV found and was approximately 23 kHz, which would be classified as a distress call by all definitions we reviewed (e.g., Wright et al., 2010; Inagaki and Ushida, 2021; Mittal et al., 2020; Schwarting et al., 2007). It should be noted, however, that no other behavioral indicators of distress were observed from the subject when these calls were recorded. Additionally, previous research has indicated that low-frequency USVs may be more common early in socialization with a human hand (Cloutier et al., 2013). The second USV was an approximately 32-kHz USV, which lies within some but not all distress call operational definitions. The third USV was approximately 52 kHz, clearly higher frequency than any

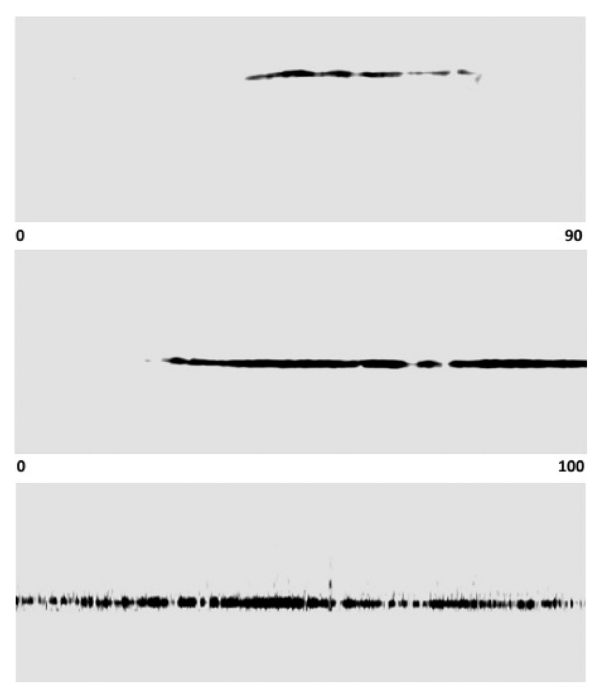

Fig. 2. Spectrographic forms of the three rat ultrasonic vocalizations (USVs) obtained during heterospecific play with 4-week-old female Wistar rats and played continuously on a loop for subjects during Experiment 2. Top, middle, and bottom USVs were 52 kHz, 32 kHz, and 23 kHz, respectively. X-axis represents time in milliseconds.

distress call definition. These USVs are displayed in Fig. 2 and were presented continuously, on a loop, for the duration of each subject's testing.

A T-Maze (start area 50 cm × 16 cm; each arm 109 cm × 9.5 cm) made from black polyvinyl chloride (PVC) was used for the foraging task, and sessions were recorded with a video camera (Sony Handycam©, HDR-PJ10) mounted on a tripod above the T-Maze. The BAT AT100 Ultrasonic Transmitter was placed at the far end of one randomly selected arm of the maze, and a RadioShack Sound Level Meter was placed at the far end of the opposite arm. The decibel meter was selected as a control stimulus due to being similar to the transmitter in size, shape, and color. Golden Puffs Malt-O-Meal Cereal (Post Consumer Brands, LLC; Lakeville, MN) pieces placed in small plastic condiment containers served as reinforcers for entering either arm of the maze. The free software Solomon Coder (Version: beta 19.08.02; Peter, 2019; https://solomon.andraspeter.com/) was used to facilitate scoring of video footage.

3.3. Procedure

Subjects were habituated to eating in the T-maze apparatus for three days prior to presentation of the USVs. Day one of habituation consisted of allowing each subject to explore the maze undisturbed. Subjects were placed in the starting area, a guillotine door was raised after 10 s, and the rats were left to explore for five minutes. The ultrasonic transmitter and decibel meter were present but turned off. On the second and third day, food was added by baiting each arm of the maze with one piece of cereal inside of a condiment container immediately in front of the decibel meter and transmitter. Subjects were exposed to five trials per day in which they were again released into the start area, held there by a closed guillotine door for 10 s, and then allowed to explore until both pieces of cereal were consumed. All subjects consumed both pieces in less than 2 min on every trial of day three.

During testing, each subject experienced three trials with both arms baited, with a different frequency USV presented at 65 dB from one arm of the maze throughout the duration of each trial. The order of USV presentation was counterbalanced, and two subjects experienced each of the six possible orders. Placement of the transmitter and decibel meter were randomized for each subject on each trial. As in habituation, each trial began with the rat being held for 10 s inside the start area of the maze, with the transmitter presenting the USV stimulus continuously on a loop from the time the rat was placed in the maze until the end of each trial. Trials ended when the subject consumed both pieces of cereal, or when 120 s had elapsed, whichever came first. The maze was wiped with damp and then dry paper towels between subjects. Subjects were returned to the colony room after each trial, and testing was conducted so that all 12 rats completed one trial before any experienced the next; in this manner, approximately 45 min elapsed between each subject's trials. All 36 trials were conducted in a single afternoon.

The dependent variable in the current experiment was freezing, defined as standing motionless (on either two or four feet) except for movements required for respiration. Scoring began once the guillotine door was lifted. Freezing was scored continuously using 1-s intervals and the free behavioral scoring software, Solomon Coder. Observers scored freezing as occurring during an interval if the rat met the definition at any point during the interval, otherwise a nonoccurrence was scored. The number of occurrence intervals were then divided by the total number of intervals for the trial to give an approximation of the percentage time spent freezing. The subjects were expected to show more freezing with 23-kHz USV presentation, and less with 32- and 52-kHz USVs.

3.4. Data analysis and interobserver agreement

Interobserver agreement (IOA) was calculated for 100 % of sessions by having two observers simultaneously but independently collect data on occurrences and nonoccurrences of freezing. For each subject and USV level, IOA was calculated three ways to determine reliability across response opportunities and second-by-second scoring. First, total-count IOA was calculated for occurrences of freezing. For each session, the lower number of occurrences recorded was divided by the higher number of occurrences recorded. Second, total-count IOA was calculated for nonoccurrences of freezing. For each session, the lower number of nonoccurrences recorded was divided by the higher number of nonoccurrences recorded. Third, interval-by-interval IOA was calculated for both occurrences and nonoccurrences of freezing. Each session was divided into 1-s intervals. For each interval, an agreement was scored if both observers recorded that freezing occurred (i.e., occurrence) or if both observers recorded that freezing did not occur (i.e., nonoccurrence). If one observer recorded the occurrence of freezing and the other observer recorded a nonoccurrence of freezing, a disagreement was scored for that interval. The total number of intervals scored as agreements were then divided by the total number of intervals scored as agreements or disagreements. All quotients were multiplied by 100 to yield percentages.

3.5. Results and discussion

Fig. 3 represents the overall (average) percentage and range of 1-second intervals with freezing during low (23-kHz), medium (32-kHz), and high (52-kHz) USV frequencies. Table 1 represents interobserver agreement (IOA) scores within and across all subjects and USV levels for freezing data. With respect to second-by-second scoring, overall IOA scores were at or above 80 % for 10 of 12 subjects, which is typically considered acceptable for human observers (Cooper et al., 2020). Across USV levels, mean IOA was approximately 87 % (range, 76.8–99.7 %), and, across subjects, mean IOA was approximately 87 % for low-USV sessions (range, 72.9–99.1 %), 87 % for medium-USV sessions (range, 76.6–100 %), and 87 % for high-USV sessions (range, 70.1–100 %). With respect to independent response opportunities, overall IOA scores for occurrences and nonoccurrences were at or above 80 % for 7 of 12 subjects and 10 of 12 subjects, respectively. Across USV levels, mean total-count IOA was approximately 75 % for occurrences (range, 16.7–96.1 %) and 86 % for nonoccurrences (range, 57.3–99.7 %). Across subjects, mean total-count IOA for occurrences was approximately 75 % for low-USV sessions (range, 0.0–100 %), 69 % for medium-USV sessions (range, 0.0–100 %), and 84 % for high-USV sessions (range, 0.0–100 %). For nonoccurrences, mean total-count IOA was approximately 83 % for low-USV sessions (range, 50–99.1 %), 90 % for medium-USV sessions (range, 79.7–100 %), and 91 % for high-USV sessions (range, 79.1–100 %).

Despite acceptable average IOA, scores for occurrences were very

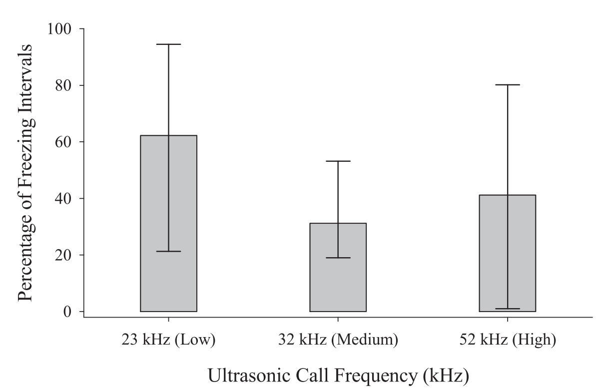

Fig. 3. Overall (average) percentage of 1-second intervals with freezing across 23 kHz (low), 32 kHz (medium), and 52 kHz (high) USV levels. The minimum number of intervals with freezing, maximum number of intervals with freezing, and range of intervals with freezing are represented for each USV level by the error bars.

Table 1
Percentage of interobserver agreement within and across subjects and conditions.

Subject	Conditions			Overall Average
	Low	Medium	High	
Adore				
Occurrences	73.33	80.00	87.50	80.28
Non-occurrences	76.47	94.29	91.55	87.44
Both	78.57	86.05	89.38	84.67
Alaska				
Occurrences	50.00	0.00	0.00	16.67
Non-occurrences	96.77	89.66	88.89	91.77
Both	90.63	89.66	88.89	89.73
Aquaria				
Occurrences	100.00	86.96	86.67	91.21
Non-occurrences	100.00	96.25	97.01	97.75
Both	94.55	97.00	85.00	92.18
Jinkx				
Occurrences	0.00	100.00	100.00	66.67
Non-occurrences	99.08	100.00	100.00	99.69
Both	99.08	100.00	100.00	99.69
Jujubee				
Occurrences	92.23	79.66	95.70	89.20
Non-occurrences	50.00	81.25	81.82	71.02
Both	89.19	76.58	87.39	84.39
Katya				
Occurrences	88.24	100.00	100.00	96.08
Non-occurrences	93.10	100.00	100.00	97.70
Both	90.91	100.00	100.00	96.97
Kim Chi				
Occurrences	84.44	74.55	95.52	84.84
Non-occurrences	58.82	79.71	93.62	77.38
Both	80.00	80.00	70.27	76.76
Latrice				
Occurrences	97.56	76.79	86.30	86.88
Non-occurrences	93.55	80.60	79.59	84.58
Both	90.99	79.09	83.93	84.67
Shangela				
Occurrences	98.06	95.24	88.46	93.92
Non-occurrences	75.00	94.34	79.07	82.80
Both	94.50	83.19	83.04	86.91
Trixie				
Occurrences	80.00	66.67	90.63	79.10
Non-occurrences	76.36	88.24	96.43	87.01
Both	72.90	80.95	84.96	79.60
Valentina				
Occurrences	81.25	66.67	80.00	75.97
Non-occurrences	93.75	87.50	95.35	92.20
Both	78.69	84.55	86.27	83.17
Yvie				
Occurrences	25.00	0.00	93.33	39.44
Non-occurrences	84.62	87.27	0.00	57.30
Both	80.49	87.27	93.33	87.03

low in at least one USV condition for the three subjects that exhibited the lowest amounts of freezing (i.e., Alaska, Jinkx, and Yvie). For that reason, these three rats were removed from the statistical analyses below; however, removing their data did not change the pattern of significant differences (data available from first author).

Percentages of intervals in which freezing was observed (first author's scores) were analyzed using a 3 (Frequency: Low, Medium, or High) × 6 (Order of USV presentation).

repeated measures ANOVA with frequency as a within-subjects variable and order as a between-subjects variable. Neither the effect of order, $F(5, 3) = 0.97$, $p = .55$, nor the order by frequency interaction $F(10, 6) = 1.14$, $p =$[TS 0.46ere statistically significant. The effect of frequency, however, was significant, $F(2, 6) = 24.65$, $p = .001$, $\eta p^2 = .89$. To determine where significant differences between the USV frequencies were, three paired sample *t* tests were conducted. When comparing percentages of intervals with freezing during presentation of the low ($M = 62.22$ %, $SEM = 8.54$) versus the medium frequencies ($M = 28.44$ %, $SEM = 5.11$), a significant difference was observed $t(8) = 7.11$, $p = .0001$. A significant difference was also found when comparing the low ($M = 62.22$ %) versus high ($M = 40.11$ %, $SEM = 8.94$) conditions $t(8) = 5.56$, $p = .0005$. A significant difference was also found between the medium and high conditions $t(8) = 2.48$, $p = .038$.

As predicted, subjects exhibited the highest levels of freezing during presentation of the lowest frequency. The medium, 32-kHz frequency, on the other hand, elicited the lowest amounts of freezing on average despite falling within a range frequently considered indicative of distress. The effect of the medium versus high condition was also significant; more freezing occurred during the 52-kHz presentation than during the 32-kHz presentation. A possible explanation for this could be that while generally associated with positive affect, 50-kHz vocalizations may also indicate aggression (Panksepp and Burgdorf, 2000). Taken together, the current results do not support the idea that 32-kHz USVs communicate distress the way that 23-kHz ones do.

4. General discussion

In the current research, we examined rat production of and reaction to relatively low USVs that are sometimes defined as distress calls. In Experiment 1, rats produced more relatively low USVs (median frequency 29.5 kHz) but also more high (median frequency 55.7 kHz) USVs in response to simulated rough and tumble play than to gentle tickling. In Experiment 2, presenting 23-kHz but not 32-kHz USVs elicited increased immobility compared to a 52-kHz condition; the 32-kHz USV elicited the lowest amount of freezing. Taken together, these results suggest that moderately low USVs near 30 kHz may not represent the same negative affective state as lower-frequency USVs, although they are often included in definitions of what constitutes a distress call.

One limitation of the current study is that only female rats were used; female rats emit slightly higher-frequency USVs than male rats (Blanchard et al., 1992). Therefore, the USVs emitted during Experiment 1 may differ from what would have occurred with male subjects. Another limitation concerning Experiment 1 is that some vocalizations were counted as both low- and high-frequency. This is difficult to avoid, as many USVs contain a wide range of frequencies (Wright et al., 2010). For example, a "split" vocalization containing a middle component of 30 kHz and end components of 50 kHz would have been counted in both high and low frequency categories in Experiment 1. Therefore, while Fig. 1 accurately depicts how often low and high frequency USVs were detected, the total numbers of USVs produced appear higher than they were. While this may be considered to be a limitation of the current data collection methodology, the fact that relatively low USVs were produced within a few milliseconds of high-frequency ones may also provide further evidence that low-frequency USVs are not always indicative of distress.

Rat USVs can be useful indicators of welfare or stress. For example, Cloutier et al. (2013) suggested that tickling or heterospecific play with humans could be beneficial, particularly for laboratory rats housed alone, based in part on the USVs emitted by subjects anticipating human handling. Similarly, LaFollette et al. (2018) recommended tickling as a way to improve interactions between pet store rats and employees. In this research, production rate of 50-kHz USVs appeared related to temperament in that rats emitting low amounts of 50-kHz USVs behaved more fearfully during testing. The current research suggests that production of USVs near 30 kHz during heterospecific play may not be cause for concern or indicative of true distress as would be the case for USVs closer to 22 kHz.

Author note

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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