Shocking News: A Rat's Tail of Uncertainty

An Evaluation of Anxiety Responses Using a Pseudo-Randomized Unsignaled Footshock Paradigm

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INTRODUCTION

Anxiety disorders are a widespread and serious health concern currently affecting approximately 18% of the adult population per year (Kessler, et al., 2005). Thus, there is a strong need to develop and improve therapeutic treatments for anxiety. This pilot study focuses on the refinement of behavioral measures of anxiety in an animal model by assessing freezing, rearing, and ultrasonic vocalizations rats in an elevated state of anxiety. Moreover, because sex differences in the prevalence of affective disorders in humans are well documented, we included both male and female rats to investigate sex-related differences in behavior within this paradigm.

The ultrasonic vocalizations (USVs) of rats are produced at frequencies above the level of human hearing. USVs are often used as a tool to assess the emotional state of rats. Previous research has identified two main call types for rats: 22 kHz (related to strongly negative emotion) and 50 kHz (Brudzynski & Panksepp, 2008). 50 kHz calls can then be further broken down into constant frequency (CF) and frequency modulated (FM) subtypes. FM calls are produced with a bandwidth greater than 15 kHz; these calls are related to positive emotional states. Whereas, CF calls are produced with a constant frequency and a bandwidth less than 10 kHz. Our lab hypothesizes that CF 50 kHz calls are expressions of anxiety in rat (Taylor, Urbano, & Cooper, 2017)

Our lab has previously explored the vocalizations of rats across a continuum of negative affective state (i.e., from anxiety to fear) within a single testing session using a sequence of temporally consistent mild footshocks. The current pilot experiment explores USV production in male and female rats when the temporal predictability was reduced by randomizing the time between footshocks. We utilized an unpredictable footshock paradigm with the goal of increasing or prolonging a state of anxiety as compared to our previous procedure. In this paradigm, shocks were administered across three successive days: on Day 1, mild footshocks were administered in a pseudo-randomized pattern, on Day 2, subjects were returned to the same context but did not receive footshocks, and on Day 3, a single reinstatement shock was administered. In addition to USVs, rearing and freezing behavior were also recorded and used to assess anxiety and fear. To explore sex differences, both male and female rats were tested in this paradigm. These results could aid in the construction of a more efficient animal model to use in research for the study of anxiety disorders and potential therapeutic interventions.

COMPARATIVE RESULTS
Predictable Footshock Interval

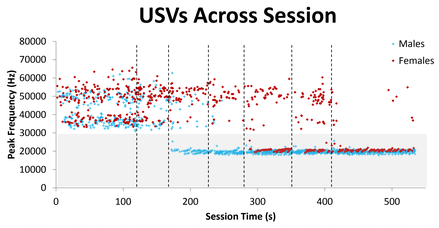

Figure 1. Scatterplot showing all CF 50 and 22 kHz USVs produced during *predictable* footshock session. Each dot represents one call produced during the session. CF 50 kHz call production decreases as 22 kHz call production increases. Dotted vertical lines indicate the times of footshock administration. The grey box designates calls in the 22 kHz category.

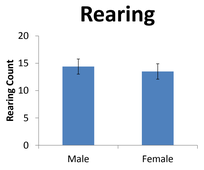

Figure 2. No sex-related differences were observed for rearing during *predictable* footshock, $t(12) = 0.456$, $p = .653$. Bars show total mean and SE.

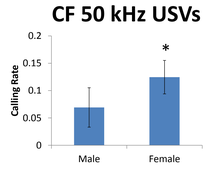

Figure 3. Female rats produce a higher rate of CF 50 kHz than male rats during predictable footshock, $F(1, 18) = 4.643$, $p = .045$. Analysis conducted with repeated measures ANOVA within session between sex.

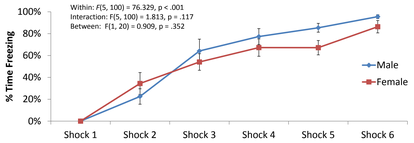

Figure 4. No sex-related differences were observed in freezing during *predictable* footshock. A repeated measures ANOVA indicated no difference in freezing behavior between groups across the inter-shock interval.

Data from Taylor, et al., *in prep*

METHODS

Subjects

Adult Long Evans rats (male, N=6; female, N=6)

Experimental Design

I. Pseudo-randomized footshock
- Mild footshock (0.5 mA, 0.5 s)
- 2 min pre- and post-shock
- 30 - 100 s inter-shock interval

II. Context Test
- 9 min exposure to footshock environment
- No shock administration

III. Single Footshock
- 2 min pre-shock

Figure 5. Experimental design schematic.

USV Recording Procedure

- Sessions were continuously recorded with an UltrasoundGate Condenser Microphone (CM 16; Avisoft Bioacoustics)
- Digitized and saved for later call classification (250 kHz sample rate, 16 bit, Avisoft Recorder)
- Spectrograms for each session were calculated with SASLab Pro (Avisoft Bioacoustics) using 1024 FFT length, 100% frame size, FlatTop window, and 96.87% overlap

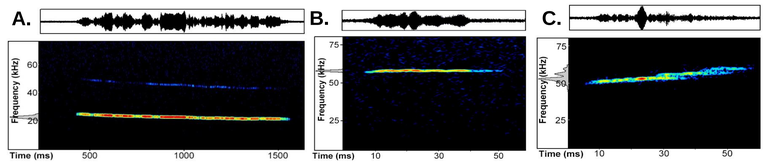

Figure 6. Representative spectrograms showing examples of USVs. Displayed are (A) 22 kHz, (B) CF 50 kHz (< 5 kHz bandwidth, no FM), and (C) FM Step 50 kHz (<15 kHz bandwidth, step-wise FM) USVs. Call categorization was performed by experienced coder based on template and described criteria.

Rearing and Freezing

I. Rearing
- Floor contact with hind limbs only.
- Counted by blind observer per 20 sec
- Used as index of risk assessment when threat is uncertain

II. Freezing
- Lack of all but respiratory movement.
- Coded by blind observer per 8 sec interval.
- Used as an index of fear when threat is certain or imminent.

RESULTS

DAY 1

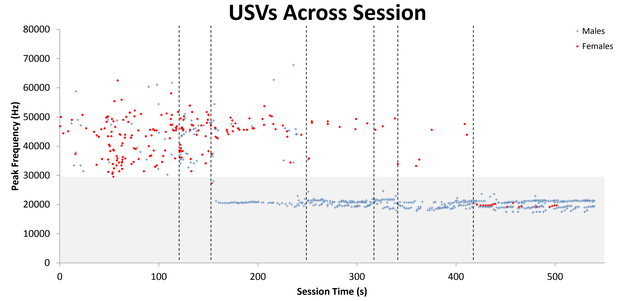

Figure 7. Scatterplot showing all CF 50 and 22 kHz USVs produced during Day 1. Each dot represents one call produced during the session. CF 50 kHz call production decreases as 22 kHz call production increases. Dotted vertical lines indicate the times of footshock administration. The grey box designates calls in the 22 kHz category.

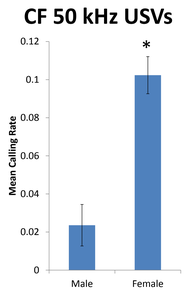

Figure 8. Female rats produced a higher rate of CF 50 kHz than male rats during Day 1, $t(8) = 4.531$, $p = .002$. Bars show mean and SE.

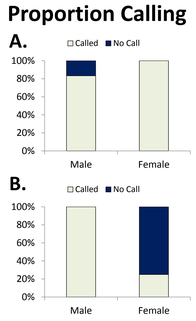

Figure 9. The proportion of subjects in each group that vocalized was compared using Fisher's Exact test. Similar calling behavior was observed for (A) CF 50 kHz USVs, $p = 1.000$, but a higher proportion of males produced 22 kHz USVs, $p = .033$ (B).

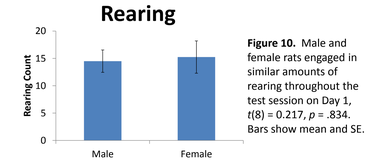

Figure 10. Male and female rats engaged in similar amounts of rearing throughout the test session on Day 1, $t(8) = 0.217$, $p = .834$. Bars show mean and SE.

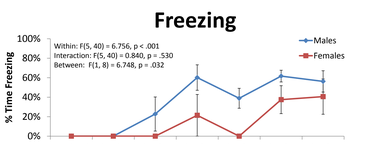

Figure 11. Males spent more time freezing than female rats throughout the session. A repeated measures ANOVA indicated a significant difference in the freezing behavior between groups across the inter-shock intervals.

DAY 2

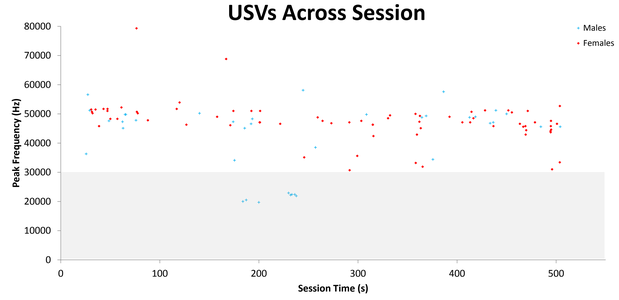

Figure 12. Scatterplot showing all CF 50 and 22 kHz USVs produced during Day 2. Each dot represents one call produced during the session. Both male and female rats produced CF 50 kHz USVs throughout the session, but few 22 kHz USVs were observed. The grey box designates calls in the 22 kHz category.

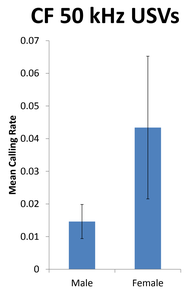

Figure 13. Male and female rats produced CF 50 kHz USVs at a similar rate, $t(5) = 1.225$, $p = .275$. Bars show mean and SE.

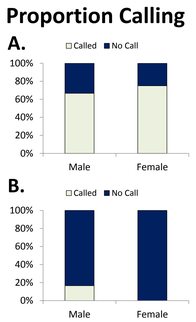

Figure 14. The proportion of subjects in each group that vocalized was compared using Fisher's Exact test. Similar calling behavior was observed for (A) CF 50 kHz USVs, $p = 1.000$, and (B) 22 kHz USVs, $p = 1.000$.

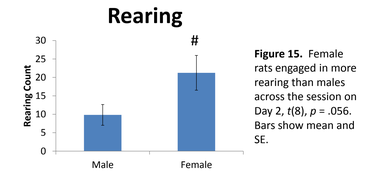

Figure 15. Female rats engaged in more rearing than males across the session on Day 2, $t(8)$, $p = .056$. Bars show mean and SE.

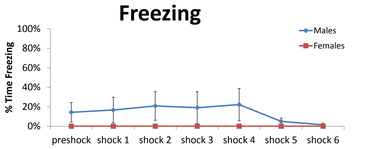

Figure 16. Male, but not female rats exhibited freezing behavior during the context test on Day 2. Because freezing was only observed in male rats, no further analysis was conducted.

DAY 3

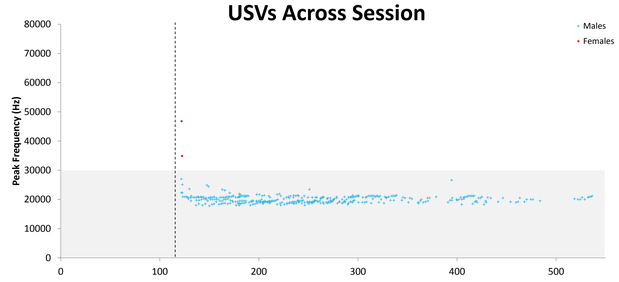

Figure 17. Scatterplot showing all CF 50 and 22 kHz USVs produced during Day 3. Each dot represents one call produced during the session. No USVs were produced prior to footshock administration. Few CF 50 kHz USVs were produced during the session. Dotted vertical line indicates time of footshock administration. The grey box designates calls in the 22 kHz category.

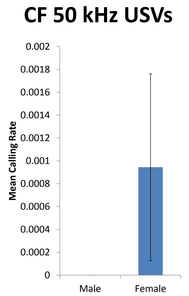

Figure 18. Only one rat (female) produced CF 50 kHz USVs during the session. No further analysis was conducted. Bar shows mean and SE.

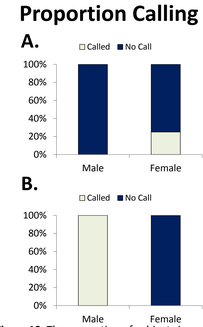

Figure 19. The proportion of subjects in each group that vocalized was compared using Fisher's Exact test. Similar calling behavior was observed for (A) CF 50 kHz USVs, $p = 1.000$, but a higher proportion of males produced 22 kHz USVs, $p = .005$ (B).

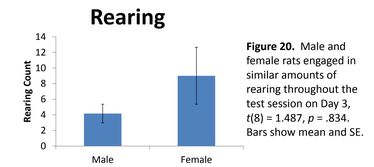

Figure 20. Male and female rats engaged in similar amounts of rearing throughout the test session on Day 3, $t(8) = 1.487$, $p = .834$. Bars show mean and SE.

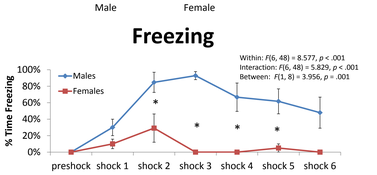

Figure 21. Male rats spent significantly more time freezing than female rats throughout the session as indicated by repeated measures ANOVA. Notably, male rats exhibited enhanced freezing behavior as compared to Day 1. Only 2:4 female rats engaged in any freezing behavior.

SUMMARY AND DISCUSSION

- Male rats transitioned more quickly toward fear-related behavior, and female rats may have engaged in prolonged anxiety-related behavior:
  - **Day 1**
    - Male rats exhibited extended freezing and a greater likelihood to produce 22 kHz USVs, whereas female rats exhibited a higher rate of CF 50 kHz USVs and persisted longer in calling.
    - However, rearing behavior was similar between male and female rats.
  - **Day 2**
    - Male, but not female rats engaged in freezing and produced 22 kHz USVs, whereas female rats engaged in greater risk-assessment behavior (rearing).
    - However, the rate and proportion of rats producing CF 50 kHz USVs did not differ between male and female rats.
  - **Day 3**
    - Male rats exhibited extended freezing, and only male rats produced 22 kHz USVs.
    - It is difficult to draw any conclusions regarding the response of females during the single footshock session due to minimal levels of USV production or freezing.
- Compared to consistent intervals, unpredictable footshock intervals:
  - Elicits less fear as expressed by time freezing and the production of 22 kHz USVs.
  - Elicits exaggerated sex-related differences in the patterns of anxiety and fear.
  - However, an accurate comparison cannot be made without concurrent testing and a larger sample size.
- This pilot experiment highlights the importance of utilizing both male and female subjects within animal models of emotion because sex-related differences exist in emotional responses in both human and non-human animals alike. Furthermore, it suggests that predictability is an important modulator of defensive behavior along a continuum including anxiety and fear, and additional research with this paradigm may enrich our understanding of emotionally-motivated vocalizations in rats.

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