ANXIETY AND FEAR AS A FUNCTION OF THREAT CERTAINTY AND SEX DIFFERENCES

by
Viktoria Taskov

Submitted in partial fulfillment of the
requirements for Departmental Honors in
the Department of Neuroscience
Texas Christian University
Fort Worth, Texas

May 7th, 2018

ANXIETY AND FEAR AS A FUNCTION OF

THREAT CERTAINTY AND SEX

DIFFERENCES

Project Approved

Supervising Professor: Brenton Cooper, Ph.D.

Department of Psychology

Anna Petursdottir, Ph.D.

Department of Psychology

Sarah Hill, Ph.D.

Department of Psychology

TABLE OF CONTENTS

ABSTRACT

Anxiety is an increasingly widespread mental health issue affecting a significant portion of the United States population. Further research in the field of mental health is beneficial to understanding the mechanisms that drive anxiety, and to discovering novel, therapeutic interventions. Using a rodent model to conduct this research is practical due to the similarities between the rat brain and human brain. We examined anxiety- and fear-related responses in both male and female subjects that are subjected to either unpredictable or predictable threat; unpredictable threats generate a state of anxiety, and predictable threats produce fear. Threat predictability was manipulated by administration of temporally inconsistent, or temporally consistent foot shocks in an operant chamber. Equal numbers of males and females were included within each group and the rats were randomly assigned to either the temporally consistent or temporally inconsistent footshock condition. Animals were tested over the course of three days; Day 1 is the contextual conditioning test day wherein the animal is exposed to the novel environment and the initial presentation of the foot shocks. Day 2 is the memory test day, where animals are returned to the test chamber, but no shock is given; Day 3. Day 3 is the reinstatement test where the animal is placed back into the chamber and one footshock is administered. Anxiety and fear were assessed by measuring rearing (anxiety-related) and freezing (fear-related) behaviors in the test chamber on each test day. Treatment and sex differences in anxiety and fear were generated by varying threat predictability. These results will provide insights into the role of potential sex differences in anxiety and fear-related behaviors.

INTRODUCTION

Mental health illnesses are an issue of increasing prevalence in today's society. There are numerous estimates which have been found to be clearly indicative of this. One study found that approximately 50% of the adult population in the United States meets the requirements and criteria to be diagnosed with a mental health disorder at some point over the duration of their life. (Kessler, Chiu, Demler, & Walters, 2005) Many times, these disorders have no known cure, and the treatments necessary to maintain an average lifestyle and minimize the symptoms can be expensive. The total costs incurred for one year of mental health services was estimated to be $57.5 billion which is equivalent to approximately $1,931 per patient. (National Institute of Mental Health, 2006) The cost of treatment may be one of the major deterrents preventing people from seeking treatment.

Another major deterrent could be the stigma that is associated with mental illness. One study estimates that approximately forty-five percent of people surveyed believe that mental illness is physical, and only about seven percent believe that it can be overcome (Seeman et. al., 2016). The implications of such statistics can be devastating to a patient suffering from these illnesses and can negatively impact many aspects of their life. Much of this bias is due to the fact that there is so little known about such ailments. It is of utmost importance for there to be further research in these fields in order to be able to better understand how to treat these disorders in novel ways to alleviate the suffering of this large population of people. Better understanding of such diseases could also aid in removing the stigma that is currently often associated with the affected portion of the population.

One of the most effective and ethical ways to further the research in these fields is by the use of animal models for human disorders. Human subjects are not an ethical option when it comes to studying mental health. The nature of this research can be quite invasive and could potentially harbor negative effects for the subjects involved. However, if a study is able to reveal results which aid in the construction of a parallel animal model to a human disorder, this opens up the door of being able to discover novel interventions and better understanding of the neural mechanisms which underlie the illness. In using animal models, however, we can encounter several problems. One of the major roadblocks is that many of the diseases we are attempting to study do not have clear biomarkers used for diagnosis. One of the criteria that an animal model must meet in order to qualify as appropriate for conducting research upon, the animals must present with similar symptoms to the human disorder. The subjects should experience side effects to the administered model which mimic the symptoms traditionally be seen in humans affected by this illness.

As one could imagine, creating a model which mimics symptoms could be especially difficult when studying ailments which alter emotional state. Emotional state can be difficult to identify in animals as they cannot verbally declare their current affective state. This poses a problem for many researches in this line of work.). A variety of behavioral paradigms have been developed to quantify an animals emotional state (on a wide spectrum from arousal to distress) based on behavior. This information has been enriched by research exploring vocalization patterns of animals (Brudzinsky, 2010); the specific types and acoustic features of the animal's call provide further information on which to base the assessment of the animal's emotional state.

Differentiating Anxiety and Fear:

In examining fear and anxiety, both are negative emotional states which are reflected in the behavior of the animal. These behaviors lie upon a continuum and are widely accepted to be distinguished based on to imminence and certainty of the perceived threat. Anxiety and fear are very different in that they lie on opposite ends of this spectrum which we are observing. Evidence from a variety of studies shows that there are different, but highly-related, neural systems which mediate anxiety and fear. (Blanchard, 1993) In order to understand the nature of this research, we must first examine the differences between these two emotional states.

Fear is elicited when the threat is certain and the timing of the threat is predictable and well known. The affective state that fear promotes is marked by the actions flight, fight, or freezing. The responses to such a threat differ as a function of environment in which the threat is presented, for example, if there is a possibility of escape. The animal partakes in these behaviors based on a cost/benefit analysis in which it will attempt to maximize its chances of survival. Fight, flight, and freezing are indicators that the rat is partaking in fear-driven behavior. (Blanchard, 1993)

Anxiety, on the other end of the spectrum, is driven by a threat that has an unknown or unpredictable timing, or even a perceived threat that may never manifest. This affective state is often marked by heightened vigilance and assessment of surroundings, and suppression of non-defensive behaviors, in attempts to determine the likelihood of danger. (Davis, 2006) Unlike the fear related behaviors which are clear and obvious, anxiety-related behaviors are covert and much more difficult to distinguish. This is an affective state in which the animal is experiencing conflict: to either avoid the stressor, or approach and explore the source of the threat. (McNaughton & Corr, 2004) Diffuse cues, or situations, indicating an uncertain threat will elicit a state of anxiety.

Sex-Related Differences in Affective States:

Sex differences in the prevalence of affective disorders in humans are well documented. Women have higher incidence of posttraumatic stress disorder (PTSD), major depression, and anxiety disorders (Kessler et al., 2005), whereas men have a higher incidence of autism, attention deficit hyperactivity disorder, schizophrenia, and Parkinson's. It is estimated that women comprise approximately 60% of individuals with generalized anxiety disorder specifically. Most rodent studies have been conducted using male subjects, and those conducted in humans disregard the effects of sex differences (Cover et al., 2014). As this research pertains to anxiety, female subjects are critical to include as they are rarely studied in this paradigm and yet are susceptible to these disorders.

One of the reasons to which these differences can be attributed is hormonal differences. Males and females have different sex hormones which play a role in and have influence on the brain and the periphery. Circulating sex hormones over the course of the life, prompts morphological and physiological changes (Merz & Wolf, 2016). These differences stem from the hypothalamic-pituitary-adrenal axis (HPA axis) which is the major source of the sex hormones which drive differences in male and female reproductive cycles.

I sought to determine whether there are sex differences in response to threat that is either certain or uncertain. I measured rearing and freezing behavior of the subjects as indicators of anxiety and fear, respectively before and after the presentation of temporally consistent or inconsistent footshocks in a fear conditioning chamber. The following day fear memory was measured by exposing the animals to the conditioning chamber. The last day animals were re-exposed to the context and a single reinstatement shock was delivered to determine whether there were sex or threat certainty differences in the reinstatement of the fear memory.

METHODS

Subjects:

The experimental subjects consisted in total of forty-eight adult Long Evans rats (Harlan Laboratories, IN). All the animals were housed in standard polycarbonate cages. The room in which they were kept was temperature and humidity controlled and was on a 12:12 hour light/dark schedule with lights on beginning at 0800 each day. Food and water were available to the animals at all times. The rats within this study were assigned to one of two groups: the random footshock condition or the consistent footshock condition. There were twenty-four subject in each condition, both divided evenly between males and females. Thus we had twelve male and twelve female subjects in each of the footshock conditions. Subjects were pair--housed until one week prior to testing when they were moved to individual housing. While the rats were individually housed PVC pipe and scratch pads were placed inside each of the animals' cage in order to provide environmental enrichment.

Apparatus:

The conditioning sessions were carried out in an operant test chamber (Med Associates, St. Albans, VT) housed in a sound attenuating cubicle. The construction of the conditioning chamber is as follows: the rear and front wall (which additionally consists of the chamber door), as well as the ceiling, were composed of Plexiglas. The other walls were constructed from stainless steel plates. Above the floor, there were a series of stainless steel rods, 0.5 cm in diameter with 1.5 cm between rods. Beneath the stainless steel rods, a stainless steel pan filled with bedding was placed for each session. The computer recording the sound and video data was connected to a standalone aversive scrambler (Med Associates, St. Albans, VT) which was located outside of the sound attenuating chamber.

In order to capture the video recordings for analysis of the subject behavior- freezing and rearing- a webcam (Logitech, Fremont, CA) was placed at the top of the operant chamber. It was fixed into place just above the ceiling of the chamber, but still inside of the sound attenuating cubicle.

Random Footshock Condition:

The experiments were carried out on three consecutive days. The conditions were kept as constant as possible in terms of experimenters present and time of day that the sessions were held. On the first test day, the subject was placed into the chamber and allowed an exploratory period of two mins before the first mild footshock (0.5 mA, 0.5s). From that point forward, pseudo-randomized subsequent footshocks were separated by a variable 30-100 second inter-shock interval. Each of the subjects was delivered six shocks before the trial ended after 9 min. After testing, each subject was promptly returned to their cage and ultimately to the animal colony room. On the second day of testing, the Contextual Memory Test, the rats were placed into the chamber and allowed to explore for the full duration of the nine minute session with no shock delivery. The same actions were carried out between animals as day one. On the third and final day of the testing, Reinstatement Day, the subjects were placed into the chamber and allowed an initial exploratory period of two minutes before a single footshock (0.5 mA, 0.5 s) The rats remained in the chamber for the remainder of the session (7 min) and were administered no further shocks. Following each session, the animals were returned to their cages once again and the chamber was cleaned with 70% ethanol, dried, and the bedding in the pan below the shock grid was replaced.

Consistent Footshock Condition:

The experiments were carried out on three consecutive days. The conditions were kept as constant as possible in terms of experimenters present and time of day that the sessions were held. On the first test day, the subjects was placed into the chamber and allowed an exploratory period of two mins before the first footshock (0.5 mA, 0.5 s). From that point forward, the subsequent footshocks were separated by a fixed 60 second inter-shock interval. After testing, each of the subjects experienced six footshocks before the trial ended. The rat was exposed to the shock environment for a total of nine minutes. Each subject was afterward promptly returned to their cage and ultimately to the animal colony room. On the second day of testing, the Contextual Memory Test, the rats were placed into the chamber and allowed to explore for the full duration of the nine minute session with no shock delivery. The same actions were carried out between animals as day one. On the third and final of the testing, Reinstatement Day, the subjects were placed into the chamber and allowed an initial exploratory period of two minutes before a single footshock (0.5 mA, 0.5 s). The rats remained in the chamber for the remainder of the session (7 min) and were administered no further shocks. Following each session, the animals were returned to their cages once again and the chamber was cleaned with 70% ethanol, dried, and the bedding in the pan below the shock grid was replaced.

Behavioral and Statistical Analysis:

Analysis of subject behavior was based on the occurrence of two specific behaviors: rearing and freezing. Rearing behavior was used to quantify anxiety-related behaviors- an increase in rearing behavior indicates an increasingly anxious state. As with freezing, rearing was scored for all three days. Rearing was defined as an exploratory behavior in which rats lifted both front limbs off the chamber floor. Rearing was scored in 20 s bins, and the number of rearing occurrences within each bin was counted. The data were independently scored by two observers. A minimum of 90% inter-observer agreement was required and data were rescored if the inter-rater agreement did not reach the threshold. Rearing was then converted to percent rearing for the initial baseline exploratory period and additionally following the initial onset of shock. A 2x(2, 3, or 4) repeated measures ANOVA was used to compare time spent rearing. The data were analyzed by dimensions of sex (male vs. female), shock condition between subjects (random vs. consistent), and baseline rearing vs. rearing after the first footshock within subjects. Rearing usually terminates after the second shock, therefore additional time points were not analyzed for this behavior.

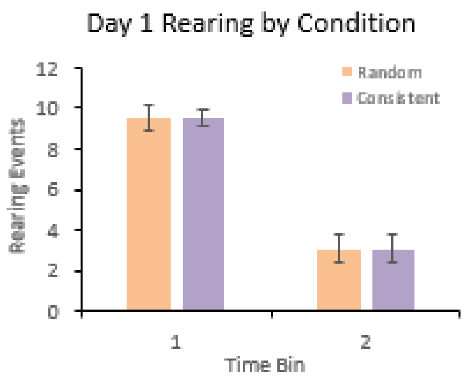
Freezing behavior was used to quantify fear-related behaviors. For all three test days, freezing was scored. Freezing was defined as a complete cessation of all movement outside of respiration. The freezing was assessed in eight second intervals, and cessation of movement for any 4 continuous s during the 8 s bin was classified as the occurrence of freezing during the time bin. The data were coded by two independent observers, and the percent agreement was calculated with a minimum of 90% agreement required. In cases where 90% was not reached, the data were recoded. The levels of freezing were then converted to percent time spent freezing for both the initial two minute baseline exploratory period and then additionally for each intertrial interval after the administration of each shock. A 2x(2, 3, or 4) repeated measures ANOVA was used to compare time spent freezing. The data were analyzed by dimensions of sex (male vs. female), shock condition between subjects (random vs. consistent), and as a function of time (6 intershock intervals).

RESULTS

In all three test days, a three way ANOVA did not reveal significant interaction effects, therefore the data for condition and sex were analyzed separately. During the fear conditioning training, there were no statistically significant differences between the groups over time, and thus the results were analyzed within groups over time. A subset of the data were analyzed by a second blind observer, as mentioned earlier, and the overall percent agreement between the sets of data was 91.67%. A repeated measures ANOVA with parameters 2x(2,3, or 4) was used to analyze the data. As these graphs are examined, one must keep in mind what time frame of the experiment each time bin is representative of. For this purpose, additional graphics further demonstrating this have been included as well.

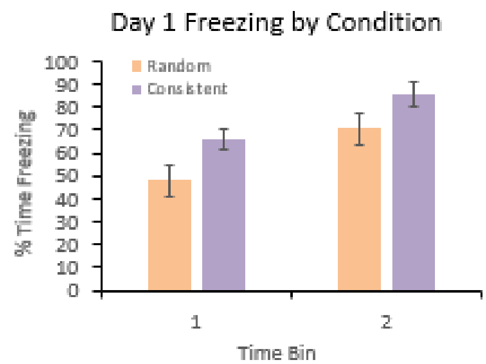
Day 1:

On Day 1, mild footshocks are delivered beginning at 2 min in either a consistent of pseudo-randomized pattern. As we look at the data within condition, Figure 1 depicts the instances of rearing on Day 1 of the experiments, and Figure 2 demonstrates the percent time spent freezing on Day 1. Figure 3 demonstrates which sections of time are represented by each time bin for the rearing and figure 4 for the freezing. Freezing is not coded for the first 2 minutes of Day 1 because the subjects will not engage in fear-related behaviors until they are presented an aversive stimulus in the environment. 2x2 ANOVA's were used for both rearing and freezing in this case. The results here are not statistically significant between the two conditions, but there is a difference over time. After the administration of the shock, the animals decrease in their instances of rearing. Additionally, after the presentation of the shock at 2 min, the animals begin to engage in freezing behavior. This demonstrates that the subjects are shifting from an anxious disposition to a fearful one. This demonstrates that the mild footshock stimulus is eliciting the proper response.



Time- $F(1,43) = 101.49, p < .001$
Condition- $F(1,43) = 0$, n.s.

Figure 1. Bar graph showing rearing events during Day 1 Unsignaled Footshock for both the random and consistent footshock conditions. The 2 time bins are before and after the footshock administered at 2 mins. There is no statistically significant difference between the rearing between the conditions over time.



Time - $F(1, 45) = 31.55, p < .001$
Condition - $F(1,45) = 4.79, p < .05$

Figure 2. Bar graph shows percent time spent freezing during Day 1 Unsignaled Footshock for both the random and consistent footshock conditions. The 2 time binds are before and after the footshock administered at 2 mins.

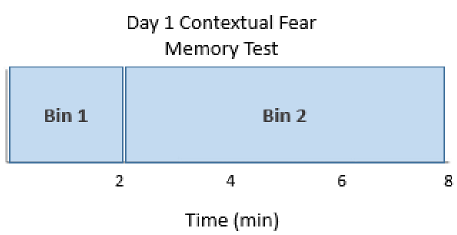


Figure 3. Chart shows which time frames of the duration of the experiment each time bin is representative of in the rearing analysis. Time bin 1 is before shock administration and time bin 2 is after.

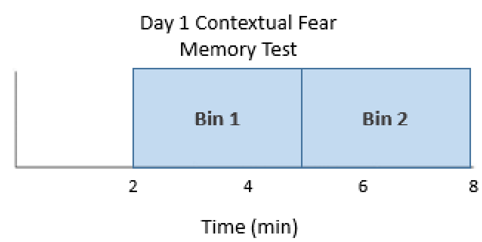
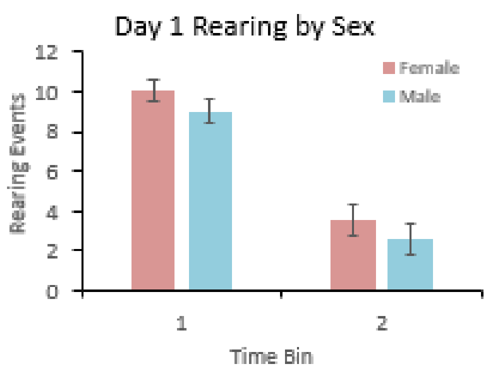


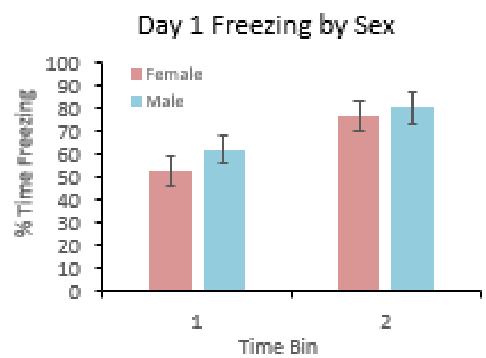
Figure 4. Chart shows which time frames of the duration of the experiment each time bin is representative of in the freezing analysis. Time bin 1 is the 3 min following the shock administration and time bin 2 is the 3 min following.

As we look at the data within sex, Figure 5 shows the instances of rearing on Day 1 of the experiments, and Figure 6 illustrates the percent time spent freezing on Day 1. Figure 5 illustrates which sections of time are represented by each time bin for the rearing and Figure 6 for the freezing. Freezing is not coded for the first 2 minutes of Day 1 because the subjects will not engage in fear-related behaviors until they are presented an aversive stimulus in the environment. 2x2 ANOVA’s were used for both rearing and freezing in this case. The results are nearly identical to those for condition. The results here are not statistically significant between the males and females, but there is a difference over time. After the administration of the shock, the animals decrease in their instances of rearing. Additionally, after the presentation of the shock at 2 min, the animals begin to engage in freezing behavior. This demonstrates that the subjects are shifting from an anxious disposition to a fearful one. This demonstrates that the mild footshock stimulus is eliciting the proper response.



Time- $F(1,43) = .003$, n.s.
Sex - $F(1,43) = 3.41$, $p < .071$

Figure 5. Bar graph showing rearing events during Day 1 Unsignaled Footshock for both male and female rats in the study. The 2 time bins are before and after the footshock administered at 2 mins. There is no statistically significant difference between the rearing in either time bin between the sexes.

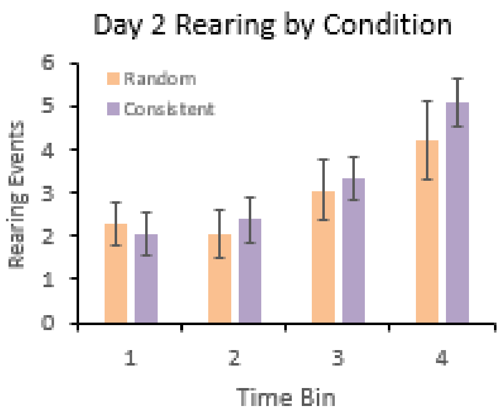


Time- $F(1, 45) = 31.55$, $p < .001$
Sex - $F(1,43) = 0.62$, n.s.

Figure 6. Bar graph shows the percent time spent freezing during Day 1 Unsignaled Footshock for both male and female rats in the study. The 2 time binds are before and after the footshock administered at 2 mins. There is no statistically significant difference between the freezing in either time bin between the sexes.

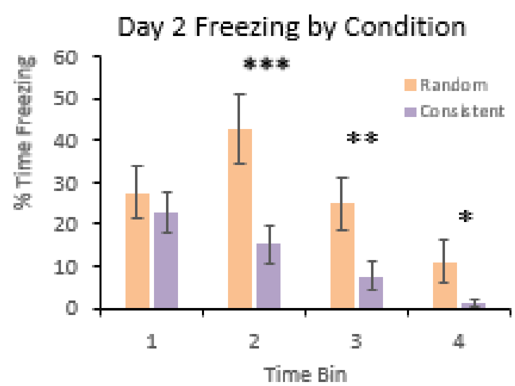
Day 2:

On Day 2, there are no footshocks administered. Looking first within condition, Figure 7 shows the instances of rearing on Day 2 of the experiments, and Figure 8 displays the percent time spent freezing on Day 2. Figure 9 illustrates which sections of time are represented by each time bin for the rearing and figure 10 for the freezing. 2x4 ANOVA's were used for both rearing and freezing in this case. Over the course of the experiment, there is no statistically significant differences in rearing between the conditions, but over time there is a trend towards animals beginning to engage in rearing behavior again. In analyzing the freezing, however, there are statistically significant differences in three of the four time bins. The subjects in the random footshock condition were freezing significantly more than those in the consistent footshock condition. Overall, the results from Day 2 demonstrates that extinction of learning is properly occurring as expected when the threatening stimulus is no longer being presented in this environment.



Time- $F(3,132) = 13.0$, $p < .001$
Condition- $F(3,132) = 0.47$, n.s.
Time x Condition- $F(3,132) = 0.47$, n.s.

Figure 7. Bar graph showing rearing events during Day 2 Context Test for both the random and consistent footshock conditions. The time bins are separated into 2 mins per bin in order to analyze trends in rearing over time. There is no statistically significant difference in the rearing between the two conditions over time.



Time - $F(2.4, 110.31) = 14.64$, $p < .001$
Condition $F(1,46) = 6.04$, $p < .02$
Condition x Time $F(2.4, 110.31 = 3.42$, $p < .029$

Figure 8. Bar graph showing percent time spent freezing during Day 2 Context Test for both the random and consistent footshock conditions. The time bins are separated into 2 mins per time bin to analyze trends in freezing over time. The subjects in the random condition persisted longer in freezing.

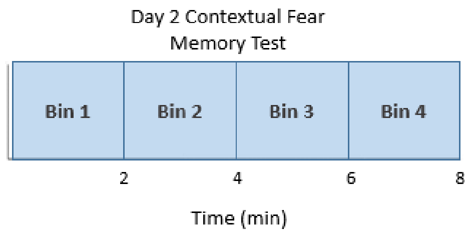


Figure 9. Chart shows which time frames of the duration of the experiment each time bin is representative of in the rearing analysis. Each time bin represents consecutive 2 min intervals.

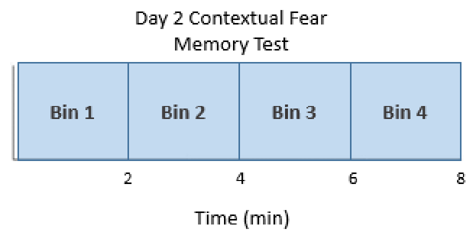
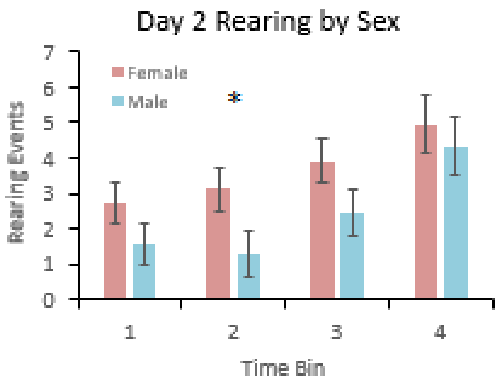


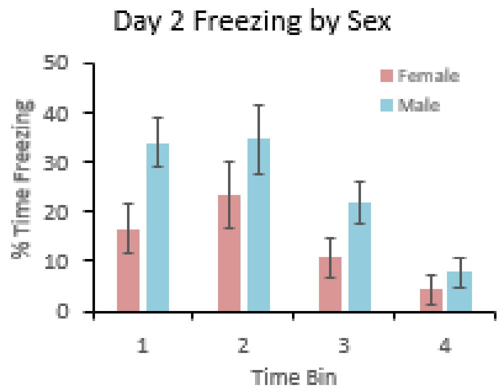
Figure 10. Chart shows which time frames of the duration of the experiment each time bin is representative of in the rearing analysis. Each time bin represents consecutive 2 min intervals.

Looking now within sex, Figure 11 demonstrates the instances of rearing on Day 2 of the experiments, and figure 12 demonstrates the percent time spent freezing on Day 2. Figure 9 demonstrates which sections of time are represented by each time bin for the rearing and figure 10 for the freezing. 2x4 ANOVA's were used for both rearing and freezing in this case. In one of the four time bins, the females were rearing significantly more than the males. The other time bins are trending towards the same. There are no statistically significant differences in freezing between the sexes, but there are trends present. The data is trending towards males freezing more than females. This is a trend that could not be significant unless the sample sizes of the experiment were much larger than the ones used here. Over time, the subjects increase in instances of rearing and decrease in time spent freezing. Again, this demonstrates that extinction of learning is properly occurring as expected when the threatening stimulus is no longer being presented in this environment.



Time- $F(3,132) = 13.0$, $p < .001$
Sex- $F(1,44) = 4.27$, $p < .05$
Time x Sex- $F(3,132) = 0.60$, n.s.

Figure 11. Bar graph showing rearing events during Day 2 Context Test for both male and female subjects in the study. The time bins are separated into 2 mins per bin in order to analyze trends in rearing over time. Females exhibited more rearing behavior than males.

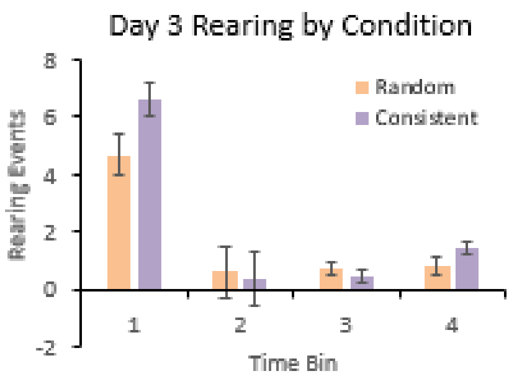


Time - $F(2.4, 107.08) = 13.96$, $p < .001$
Sex $F(1,46) = 2.97$, $p < .09$
Sex x Time $F(2.32, 107.08) = 1.08$, n.s

Figure 12. Bar graph showing percent time spent freezing during Day 2 Context Test for both male and female subjects in the study. The time bins are separated into 2 mins per bin in order to analyze trends in rearing over time. Males trended towards exhibiting more freezing behavior and persisting longer in freezing over time.

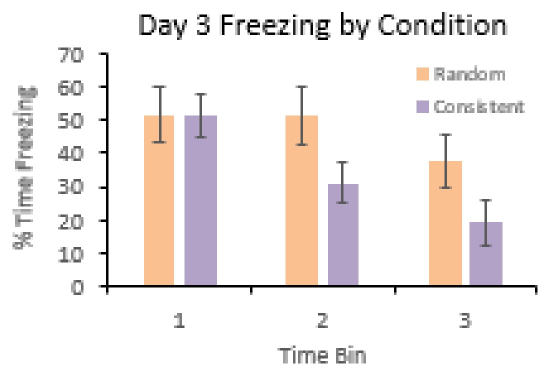
Day 3:

On Day 3, a single, mild footshock is delivered at 2 min following exposure to the testing chamber. As we look at the data within condition, Figure 13 illustrates the instances of rearing on Day 3 of the experiments, and Figure 14 shows the percent time spent freezing on Day 3. Figure 15 demonstrates which sections of time are represented by each time bin for the rearing and figure 16 for the freezing. A 2x4 ANOVA was used for rearing data analysis, and a 2x3 ANOVA was used for freezing data analysis in this case. With the rearing, it is obvious that after the reinstatement of the footshock, the rearing decreases significantly, thus the animals are accurately remembering the fearful events that occurred on Day 1. Freezing is not displayed for the first 2 minutes of Day 3 because the subjects did not engage in freezing behaviors demonstrating that the extinction learning has occurred. After presentation of the shock, the animals once again begin to engage in freezing behavior. There is a trend towards the random animals freezing more and persisting longer in the freezing behavior. This difference might be significant with larger sample sizes. There were no statistically significant differences in the data.



Time- $F(3,135) = 48.09$, $p < .001$
Time x Condition- $F(3,135) = 2.77$, $p < .05$

Figure 13. Bar graph showing rearing events during Day 3 Reinstatement Test for both the random and consistent footshock conditions. The time is split into 5 interval time bins in order to analyze trends in rearing over time. There is no statistically significant difference in the rearing between the two conditions over time.



Time - $F(1.74, 78.342) = 22.82$, $p < .001$
Condition $F(1,45) = 1.75$, n.s.
Condition x Time $F(1.74, 78.342) = 5.42$, $p < .01$

Figure 14. Bar graph showing percent time spend freezing during Day 3 Reinstatement Test for both the random and consistent footshock conditions. The time bins are separated into 3 mins per bin in order to analyze trends in freezing over time. Subjects in the random condition trended towards exhibiting more freezing behavior and persisting longer in freezing behavior over time.

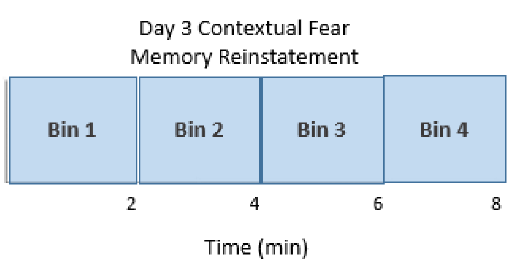


Figure 15. Chart shows which time frames of the duration of the experiment each time bin is representative of in the rearing analysis. Each time bin represents consecutive 2 min intervals.

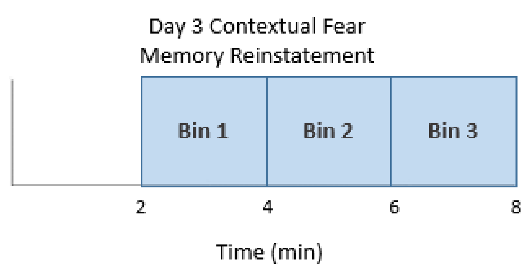
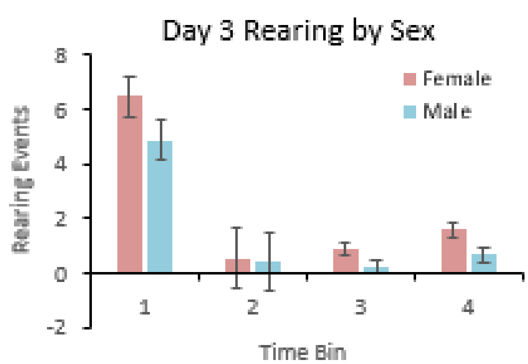


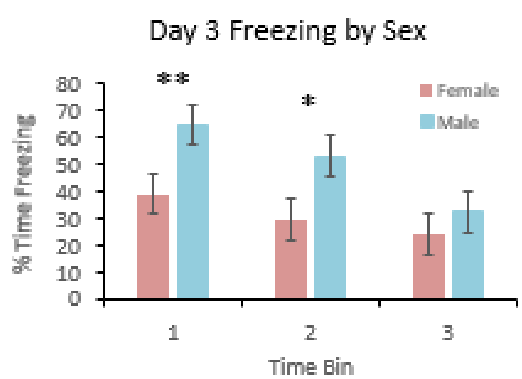
Figure 16. Chart shows which time frames of the duration of the experiment each time bin is representative of in the rearing analysis. The first 2 min are not included in the data, but each time bin represents consecutive 2 min intervals after the footshock presented at 2 min.

Looking now within sex, Figure 17 shows the instances of rearing on Day 3 of the experiments, and Figure 18 displays the percent time spent freezing on Day 3. Figure 15 illustrates which sections of time are represented by each time bin for the rearing and figure 16 for the freezing. A 2x4 ANOVA was used for rearing data analysis, and a 2x3 ANOVA was used for freezing data analysis in this case. The results for rearing by sex, look very similar to those by condition. There were no statistically significant differences, but after the presentation of the shock, the subjects greatly decrease in instances of rearing. Once again, this is indicative that they are properly remembering the fearful events of Day 1. Freezing is not displayed for the first 2 minutes of Day 3 because the subjects did not engage in fear-related behaviors if extinction learning has properly occurred on Day 2. With freezing, there were statistically significant differences in two of the three time bins. In both the 2 mins immediately following presentation of the reinstated footshock, and the two min immediately after, males were freezing significantly more than females. The third time bin is also trending towards the same pattern.



Condition- $F(1,45) = 1.98$, n.s.
Time x Sex- $\mathbf{F}(3,135) = 3.37$, $p < .02$

Figure 17. Bar graph showing rearing events during Day 3 Reinstatement Test for both the male and female subjects in the study. The time is split into 5 interval time bins in order to analyze trends in rearing over time. There is no statistically significant difference in the rearing between the sexes over time.



Time - $F(1.74, 78.342) = 22.82$, $p < .001$
Sex $F(1,45)$ 4.05, $p < .05$
Sex x Time $F(1.74, 78.342) = 3.85$, $p < .05$

Figure 18. Bar graph showing percent time spend freezing during Day 3 Reinstatement Test for both the male and female subjects in the study. The time bins are separated into 3 mins per bin in order to analyze trends in freezing over time. Males exhibited significantly more freezing behavior and persisted far longer in freezing over time.

DISCUSSION

First we analyze the findings from Day 1, Unsignaled Footshock, by condition. With the incidences of rearing, there were no statistically significant differences between the two conditions, but there was a difference over time. After the first presentation of the mild footshock, the subjects have a major decrease in rearing activity. This indicates a change in the animals' disposition. After the presentation of the shock they are transitioning from engaging in anxiety-related behavior to fear-related behavior. With the percent time spent freezing, again, there are no statistically significant differences between the two groups, but there is a change over time. After the presentation of the shock, and over the course of the experiment, the subjects increase in freezing. This is, once again, representative of the temporal transition towards fear-dependent response to the mildly aversive stimuli.

As we move on to the findings from Day 1, Unsignaled Footshock, by sex we see a very similar pattern. There were no statistically significant differences between the males and females in the experiment, and we observe the same changes over time. The subjects are rearing less, after the onset of the threatening stimuli, and freezing more. This also supports the same idea that the subjects are learning about the context and about the stimuli that are being presented to them. This acquisition and learning of the context and stimuli are critical to the remainder of the experiment as the subjects' behavior for the other two days of the experiment is based on the learning and acquisition that occurs on Day 1.

During the contextual memory test, there are some group differences. On Day 2, Context Test, the subjects in the Consistent Footshock group reacts differently upon re-exposure to the threatening context than the subjects in the Pseudo-Randomized Footshock group. Specifically with the incidences of rearing, there are no statistically significant differences between the two conditions. Over the course of the experiment, the subjects in both conditions trend towards an increase in rearing. This demonstrates a shift away from fear-dependent behaviors. However, the freezing between the two conditions on Day 2 shows significant differences. Between minutes 3 and 8 of the experiment, animals in the Pseudo-Randomized Footshock condition spend significantly more time freezing than those in the Consistent Footshock condition. This means that they engaged longer, and persisted longer, in fear-related behaviors suggesting that they have a stronger fear memory trace than the animals in the Consistent Footshock condition.

The findings of Day 2, as far as freezing between the conditions goes, are not what we had anticipated. We anticipated that the random footshock exposure would elicit greater anxiety responses, rather than enhanced fear-related memory formation. While the patterns observed in this experiment are not consistent with the original research hypothesis, they are consistent with findings regarding the Rescorla-Wagner learning theory. This theory states that unpredictable stimuli are more readily acquired into fear memory due to the "element of surprise". Unfortunately, even a mild footshock, regardless of the method and frequency of delivery, if far too threatening to elicit anything other than a fear-dependent response. This is something that we had not anticipated being a problem, but is also a point of reference for future directions for this project.

There were also differences on Day 2, Context Test, between sex. Between minutes 2 and 4 of the experiment the female subjects had higher incidences of rearing then the male subjects. There were not statistically significant differences in the other time bins for Day 2, but there is a trend towards all of the time bins reflecting the same pattern. These differences would become more obvious if the experiment included larger sample sizes. If there were more animals per condition and thus more data, it would help accentuate these gaps.

The overall trends of Day 2 over time demonstrate that Day 2 has properly extinguished the learning that occurred on Day 1 of the experiment. As the time of Day 2 continues, there is an increase in rearing behavior once more, and a decrease in freezing behavior overall. This pattern is seen in subjects in both conditions and of either sex. This is critical to the success of the experiment because we are able to properly demonstrate extinction learning by the end of Day 2. When the animals are no longer being presented the threatening stimuli in the environment, they begin to dissociate the two.

On Day 3, Reinstatement Test, by condition we are able to see that there are no statistically significant differences between the two conditions, but after the presentation of the single shock at 2 min the subjects have a rapid decline in instances of rearing. This demonstrates accurate retrieval of the events on Day 1. The freezing on Day 3 between conditions also does not show any statistically significant differences, but it does trend towards the same findings from Day 2. There is a trend towards the subjects in the Pseudo-Randomized Footshock condition freezing more than those in the Consistent Footshock condition.

The final set of data, Day 3, Reinstatement Test, by sex reinforces the sex differences seen on Day 2. With rearing activity, there are no statistically significant differences between the males and females, but the data is trending toward the results seen on Day 2 with the females rearing more than the males. Again, to tease out these differences larger sample sizes would be necessary. Overall, after the reinstatement of the footshock, the animals decrease in rearing activity. With freezing, between minutes 2 and 6 of the experiment, the male subjects were freezing significantly more than their female counterparts. The data were once again trending towards this same pattern in the other time bins as well.

The overall trends seen in Day 3, Reinstatement Test, demonstrate that the subjects are recalling the events of Day 1, and responding appropriately. The temporal trends of Day 2 indicate that the days prior have elicited a proper response.

To recap, some of the major findings of this study include those pertaining to the sex differences. In the data, there are several time points over the course of the experiment where there are significant differences in the behaviors of the male and female rats. These instances are indicative of a stronger fear-memory trace in male rats. This leads us to the conclusion that there are sex differences in reinstatement of fear memory, and these differences are likely related to amygdala-dependent fear memory and aggressive behavior in males.

If the project were to be repeated or replicated, a shift of the paradigm for inducing anxiety would be something to consider. One alternate set-up would be to train the rats that are assigned to the "anxiety condition" to two distinct environments: one negative (i.e. a single mild footshock), and one positive (i.e. given food). The subjects could then be placed into a third, but distinctly hybrid environment (hybrid of the positive and negative environments). This would induce anxiety as the animals are unsure if they are currently in the positive environment or the negative.

Future Directions:

In addition, if the experiment were to be replicated, larger sample sizes could also be used in order to help emphasize and make statistically significant some of the trends that we see in the graphs currently. With larger sample sizes, this would hopefully bring down the standard deviation and better help illustrate more differences between the sexes and conditions. If more animals were used, there might even be a 3-way interaction between sex, certainty of threat, and behavioral responses across time.

REFERENCES

Kessler, R. C., Chiu, W. T., Demler, O., & Walters, E. E. (2005). Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the national comorbidity survey replication. *Archives of General Psychiatry, 62*(6), 617-627.

U.S. Department of Health and Human Services, National Institutes of Health, National Institute of Mental Health. (2006). *Mental Healthcare Cost Data for All Americans* (2006). Retrieved from http://www.nimh.nih.gov/statistics/4COST_AM2006.shtml

Seeman, N., Tang, S., Brown, A.D., Ing, A. (2016). World survey of mental illness stigma. *Journal of Affective Disorders*, 190, 115-121.

Brudzynski, S.M. (2010). Vocalizations as an ethotransmitter: introduction to the handbook of mammalian vocalizations. In Brudzynski, S.M. *Handbook of mammalian vocalizations* (477-486). London, UK: Elsevier

Merz, C. J., Wolf, O. T. (2017). Sex differences in stress effects on emotional learning. *Journal of Neuroscience Research,* 95, 93-105.

Cover, K.K., Maeng, L.Y., Lebron-Milad, K., Milad, M.R. (2014). Mechanisms of estradiol in fear circuitry: implications for sex differences in psychopathology. *Translational Psychiatry*, 4,e422.

Davis, M. (2006). Neural systems involved in fear and anxiety measured with fear-potentiated startle. *American Pschologist*, 61(8), 741-756.

McNaughton, M., & Corr, P. J. (2004). A two-dimensional neuropsychology of defense: fear/anxiety and defensive distance. *Neuroscience and Biobehavioral Reviews, 28*, 285–305

Blanchard, R. J., Yudko, E. B., Rodgers, R. J., & Blanchard, D. C. (1993). Defense system psychopharmacology: an ethological approach to the pharmacology of fear and anxiety. *Behavioural Brain Research*, 58, 155–165.