# COMPARISON OF MALE AND FEMALE AVOIDANCE, DARTING, AND FREEZING BEHAVIOR WITHIN THE SPS MODEL

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the Degree in Master of Science in Neuroscience

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# ABSTRACT

Post-traumatic stress disorder (PTSD) is more likely to develop in women compared to men after trauma exposure, however, PTSD symptoms manifest differently in males and females, with females showing stronger avoidance symptoms. A core symptom of PTSD is re-experiencing the traumatic event in the form of persistent fear memories, also known as extinction retention deficits. Rat models are a common way of exploring extinction retention deficits, however, most of these behavioral paradigms are not effective in female rats. Previous studies have found that female rats prefer to try to escape an aversive stimulus by darting, while male rats prefer to freeze in place. However, the relationship between avoidance and fear memory within PTSD is still something that is not well understood. To address this issue, I ran a series of experiments to determine the effects of SPS on fear memory and avoidance behaviors in male and female rats. The findings indicate that SPS females engaged in multiple defensive behaviors in response to threat, while SPS male rats exhibited freezing behavior in response to a feared stimulus. Overall, these findings raise the possibility that female rats exhibit multiple behavior strategies when presented with threat in comparison to males. These findings further implicate the importance of exploring sex differences in stress effects on specific behaviors to better treat both men and women.

# Chapter 1

# INTRODUCTION

Post-traumatic stress disorder (PTSD) is a debilitating disorder that has a prevalence rate of 7.8% within the US population (Kessler, Sonnega et al.1995) and is associated with extensive socioeconomic costs (Kessler 2000, Dams, Rimane et al. 2020). Women are two to three times more likely than men to develop PTSD after a traumatic experience (Olff 2017; Pooley, Benjamin et al. 2018). Symptoms manifest differently between men and women. Previous studies have found that women tend to show more avoidance behavior (Turgeon, Marchand et al. 1998; Cameron and Hill 1989; Hourani et al. 2015). This is described as trying to limit contact with situations that trigger anxiety, fear, memories, or thoughts associated with the traumatic event (Tull, Hahn et al. 2011). A core symptom of PTSD is the persistent recall of traumatic memories (i.e. persistent fear memory or extinction retention deficits) (5th ed.; DSM–5; American Psychiatric Association, 2013). Extinction retention deficit refers to memories that are resistant to inhibition, updating, or extinction (Giustino and Maren, 2015; Bergstrom, 2016; Careaga et al., 2016). However, the interplay of avoidance and fear memory within PTSD is still something that is not well understood.

Rat models are a useful way of understanding the underlying neurobiological mechanisms of PTSD and can employ methods that cannot be used in humans. Unfortunately, most rat models of traumatic stress were developed in male rats (Armario, Escorihuela et al. 2008; Yamamoto, Morinobu et al. 2009; Bowers and Ressler 2015; Deslauriers, Toth et al. 2018) and do not work in female rat model systems. To understand the mechanisms via which sex differences to traumatic stress manifest, we need to identify how animal models of traumatic stress can be utilized to work in female rat model systems or develop new traumatic stress models. It is important to have an rat model where the effects of traumatic stress on defense behaviors can be consistently observed in female rats for validity and for results to be replicated in future studies. It is also important to understand potential sex differences in behaviors to develop diagnostic and therapeutic techniques that effectively treat both sexes (Pooley, Benjamin et al. 2018).

To address this discrepancy, I ran a series of experiments using rat models to identify a behavioral paradigm where the effects of traumatic stress on extinction retention deficits (or persistent fear memory) in female rats can be observed. To accomplish this goal, I analyzed the effects of single prolonged stress (SPS) in different behavioral paradigms that measure avoidance. SPS is a valid model of traumatic stress and mimics many neurochemical and neurobiological symptoms observed in PTSD patients (Liberzon et al. 1997; Yamamoto et al. 2008; Pooley, Benjamin et al. 2018). These effects include enhanced negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis, arousal, and glucocorticoid (GR) receptor expression. SPS can be combined with fear conditioning to examine the effects of traumatic stress on emotional memory. This is done by presenting an unconditioned stimulus (US) (foot shock) and pairing it with a conditioned fear stimulus (CS) (tone) in SPS-exposed and control rats (Knox, Nault et al. 2012; Yamamoto, Morinobu et al. 2007). The amount of time a rat freezes in response to a CS is used as a measure of fear memory (Blanchard, Flannelly et al. 1986; Frendt and Fanselow 1999; Maren 2011; Lang and Davis 2006; Mendes-Gomes, Motta et al. 2020). Extinction training and testing is used to examine extinction retention deficits (VanElzakker, Dahlgren et al. 2014). During extinction training and testing a series of the same CS is presented in the absence of the US. The purpose of extinction training is for the rat to learn that the CS will not be followed by the US. The purpose of extinction testing is to determine if the rat remembers extinction or fear. SPS male rats consistently freeze more during an extinction test (i.e. persistent fear memory or extinction retention deficits) (Liberzon, Krstov et al. 1997; George, Rodriguez-Santiago et al. 2015; Ferland-Beckham et al. 2021). While SPS consistently induces extinction retention deficits in male rats (Ferland-Beckham et al. 2021), there are inconsistent results in female rats (Biddle and Knox 2021; Keller, Schreiber et al. 2015;). This inconsistency could be due to limitations within the SPS model and/or could mean the behavioral paradigms used to measure fear response are not appropriate for females.

Darting may be a female-specific defense behavior and refers to rapid movement in response to an aversive stimulus (Gruene et al. 2015; Shansky 2018); this behavior may be indicative of the rat's attempt to avoid or escape the aversive stimulus. A study by Gruene et al. found that female rats displayed significantly more darting behavior compared to males during fear conditioning and extinction sessions (Gruene et al. 2015). Gruene et al. 2015 also found that females generally exhibit lower freezing behavior, which could mean that females prefer to actively avoid aversive stimuli rather than freeze (Gruene et al. 2015). The findings of Gruene et al., have highlighted the importance of examining traumatic stress effects in behavioral paradigms that involve female specific behaviors (e.g. darting, avoidance) so as to potentially capture traumatic stress effects on emotional memory in female rats.

The experiments I conducted for my thesis were designed to investigate active avoidance and passive/inhibitory avoidance in SPS and control rats. We first examined the effect of SPS on avoidance induced by a tone (Experiment 1, cued avoidance (CA)) and then inhibitory avoidance (IA) (Experiment 2). While previous experiments have examined cued/inhibitory avoidance in males (Fadok et al. 2017; Lee and Noh, 2016), few studies have been conducted on females (Shansky 2015; Gruene et al. 2015). For CA and IA experiments we also measured freezing and darting. All experiments were done in females and then replicated in males if there was an observed effect in female rats. If SPS increases avoidance behavior in female rats and not male rats, then in both the CA and IA I expected to observe increased avoidance behavior in female rats in these paradigms.

# Chapter 2

# METHODS AND MATERIALS

## 2.1 Animals

40 female and 16 male Sprague-Dawley rats were used for this study. The number of rats used per independent group was based on similar previous studies (Knox, Nault et al. 2012; Roldan et al. 200; Sabban et al. 2018). Rats were obtained from Charles River Inc. Upon arrival, adult female, and male rats (PD 53-56, approximately 150-250g) were housed in same-sex pairs. Rats were given ad libitum access to food and water and kept on a 12-hour light/dark cycle before and during behavioral assessment testing. Rats were allowed an acclimatization period of at least seven days prior to any experimental protocols. Following SPS, rats were housed individually. SPS was run during the inactive cycle (between the hours of 7 AM - 7 PM) and behavioral paradigms were run during the active cycle (between the hours of 12:00 AM - 12:00 PM). SPS was conducted during the inactive cycle to maximize the stressful nature of the protocol. Behavior was conducted during the dark cycle because rats are naturally active (Yang, Weber et al. 2008; Heller, Millar et al. 2000); which allows us to obtain the most accurate behavioral data in response to SPS treatment.

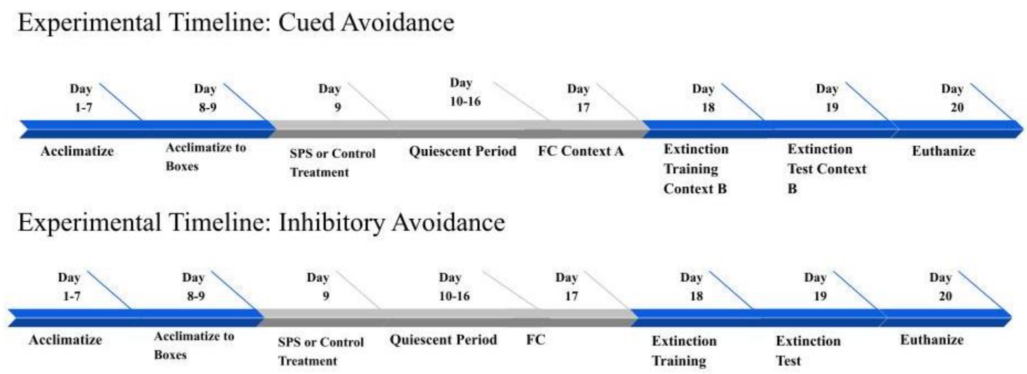


Figure 1. Experimental Timelines: Individual behavior timelines for cued avoidance (CA) and inhibitory avoidance (IA).

**2.2 SPS and control treatments**

Rats were subjected to SPS, which is defined as a single exposure of restraint, forced swim, and ether (Liberzon, Krstov et al. 1997; Liberzon, Abelson et al 1999). After the initial stress, rats are then subjected to a post-stress incubation period, after which they exhibit multiple effects that model PTSD (Yamamoto, Morinobu et al. 2008; Bowers and Ressler 2015; Deslauriers, Toth et al. 2018). Rats were transported to a novel room and restrained for 120 minutes, subjected to forced swim for 20 minutes in 24 °C water in groups of 3-6 Rats. Rats were then recuperated for 15 minutes and then exposed to ether until general anesthesia was induced in groups of 3-6 rats. For the control stress procedure, rats were transported to a novel room for the duration of SPS. Both SPS and control rats were then subjected to a 7-day post-stress incubation period where they were housed individually with ad libitum access to food and water.

### 2.3 Behavioral Procedures

For CA, fear conditioning was conducted in a distinct context (i.e. Context A) and consisted of five CS-unconditioned stimuli (US) pairings. The CS was a tone (2.9kHz, 10s, 81dB) that co-terminated with a US foot shock (1mA, 1s). Extinction training (30 CS-only presentations) was conducted 24h later in a novel context (Context B). Extinction testing (10 CS-only presentations) was conducted 24h after extinction training in Context B. Both extinction training and testing were conducted in passive avoidance boxes where rats try to avoid the chamber where the CS is presented. The passive avoidance box is divided into a light/threat side (left) and a dark/non-threat side (right) with a sliding door in the middle. Rats initially started off on the dark/non-threat chamber of the box with the door closed until the CS was presented in the dark/non-threat chamber. After this the door was opened, which allowed rats to transition between the chamber in which the CS was presented and the chamber where the CS was not presented.

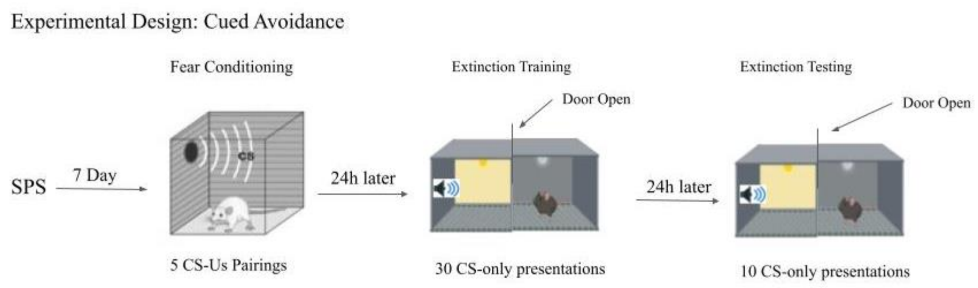


Figure 2. Experimental Design CA: Seven days after SPS treatment rats were put in context A and exposed to fear conditioning where they receive 5 CS-US pairings. 24h later they are put into context B for extinction training and exposed to 30 CS-only presentations. 24h later for extinction testing rats were put in context B and exposed to 10 CS-only presentations

For IA, during fear conditioning rats were placed in the light/non-threat side of a passive avoidance chamber that was connected to a dark chamber via a doorway. Upon entering the chamber, the doorway closed, and rats were administered 1 US foot shocks (0.7 mA, 1s). The initial shock occurred 30s after the rat has entered the chamber. Extinction training was conducted 24h later by placing the rat in the light/non-threat chamber. The door opens at the beginning of the recording allowing the rat to move freely. The same procedure was employed for extinction testing which was conducted 24h after extinction training

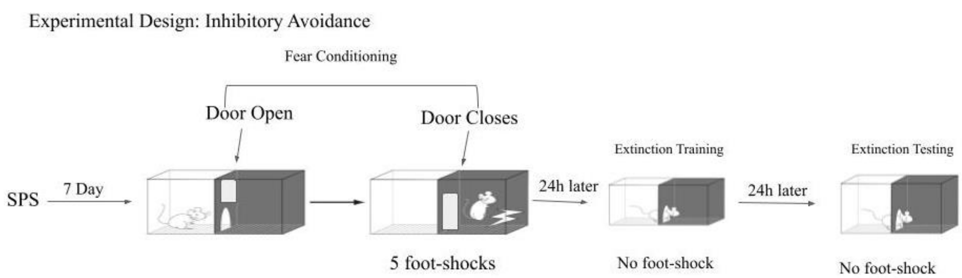


Figure 3. Experimental Design IA: 7 days after SPS treatment rats are exposed to fear conditioning where they received 5 foot-shocks. 24h later is extinction training where there is no foot-shock. 24h later for extinction testing there is no foot-shock.

## 2.4 Data and Statistical Analysis

### 2.4.1 Behavioral Analysis

Cameras were mounted to the ceiling of Context A and both chambers of Context B. They were used to record all behavior that was scored with Any-Maze software (Stoelting Inc. Kiel, WI, USA). Any-Maze was used to score behavior indicative of trying to predict and escape the aversive stimulus (avoidance), levels of fear (freezing), and escape tendency (darting). These multiple behaviors were used to measure how SPS affects different aspects of defense behavior induced by presentation of cued and contextual threats. Freezing has been used to measure fear and extinction memory (Blanchard, Flannelly et al. 1986, Frendt and Fanselow 1999, Maren 2011, Lang and Davis 2006, Mendes-Gomes, Motta et al. 2020). Darting is a defense behavior that may be displayed more in female rats (Gruene, Flick et al. 2015; Shansky 2018; Colom-Lapetina,  et al. 2019; Greiner, Muller et al. 2019). Time spent and entries made into different sides of a passive avoidance box were used to measure avoidance behavior (Ganon-Elazar and Akirav 2012; Le Dorze, Borreca et al. 2020).

Freezing was defined as a complete lack of movement, except for breathing, for greater than two seconds and was calculated as a percentage of the total time recorded (Knox, Nault, et al. 2012) and scored in 30s bins. This was to examine the acquisition of fear memory during fear conditioning, acquisition of extinction during extinction training, and level of fear vs. extinction memory retrieval during extinction testing.

Darting during fear conditioning was defined as the sudden movement of an rat in response to a threat. Darting was scored as the maximum speed of the rat measured in 5-second bins.

For CA, avoidance was measured as the time the rat spent outside of the threat chamber and represents the rats' attempt to actively avoid the chamber in which the auditory fear-CS is presented. For IA, avoidance was measured as the time it takes for the rat to make its first entry from the light/non-threat chamber into the dark/threat chamber. Typically, nocturnal animals, like a rat, avoid well-lit areas, especially during their active cycle to avoid predators (Stephan 1983). Rats will quickly enter a dark arena unless the dark arena is paired with an aversive event (e.g. foot shock).

The time it takes to make the first entry and the time spent outside the threat chamber is a form of passive/inhibitory avoidance, because rats can avoid the dark/threat arena by not entering the arena. Time spent on the threat and non-threat side of the chamber, and number of entries and exits were recorded. Duration within the threat and non-threat chamber was averaged into a single variable for all sessions. The number of entries into the threat and non-threat chamber was calculated as the total number of entries and exits for the entire test session.

**2.4.2 CA**

Freezing during fear conditioning was analyzed using a stress (SPS vs. Control) x FC trial (baseline, trial 1-5) factor design with the repeated measure being FC trial. For darting during fear conditioning three pre-CS bins, two bins for the 10s CS presentation, and three bins post-CS presentations were averaged across all CS-US pairings. For this measure we used a stress (SPS Vs. Control) x bin (pre-CS, during CS, post-CS) factor design.

For extinction training freezing was analyzed using a stress (SPS vs. Control) x EXT trial (baseline, trial 1-30) factor design. For extinction testing freezing was measured using a stress (SPS vs. Control) x EXT trial (baseline, trial 1-10) factor design. Our lab has shown that consistent changes in darting behavior are observed when a rat cannot escape from an aversive context (unpublished data). Because of this observation, we decided not to analyze darting behavior during extinction training and testing because rats have another means of escaping the threat context/chamber.

During extinction training and testing rats could avoid the context in which the CS was presented (threat chamber) and we used this as a measure of avoidance. This could not be done during fear conditioning because rats could not avoid the context in which fear conditioning was occurring. For extinction training we used a stress (SPS vs. Control) x trial (baseline, 1-30) factor design to analyze time spent in the non-threat/light chamber (our measure of avoidance). For extinction testing we used a stress (SPS vs. Control) x trial (baseline 1-10) factor design to analyze time spent in the non-threat/light chamber. Total avoidance during extinction training and testing were analyzed using an independent t-test (SPS vs. Control) with Bonferroni corrections applied where necessary.

**2.4.3 IA behavior**

IA refers to the time a rat takes to enter a chamber that has been paired with a foot-shock. The rats first dark/threat entry during fear conditioning, extinction training, and extinction testing was analyzed using a stress (SPS vs. Control) x session (FC, Extinction Training, Extinction testing) factor design. In addition to avoidance, we also measured freezing during fear conditioning, extinction training, and extinction testing. Freezing was averaged into 30s bins and analyzed separately using a stress x bin factor design for fear conditioning, extinction training, and extinction testing. Darting behavior was also recorded, but only during fear conditioning, because our lab has shown that consistent changes in darting behavior are observed when a rat cannot escape from an aversive context (laboratory observation). During fear conditioning, maximum velocity in 5s bins was converted to z-scores. The frequency of z-scores that were 1 or greater was calculated and subjected to t-test (SPS vs. control).

For all factor designs, main and interactions were analyzed using analysis of variance, and main and simple comparisons were analyzed using t-tests with

Bonferroni corrections applied where needed. A p-value less than 0.05 was used to determine statistical significance.

Chapter 3

RESULTS

### 3.1 Experiment 1: Cued Avoidance

#### 3.1.1 Freezing

During fear conditioning, a mixed-measure ANOVA found a significant main effect of trial [$F_{(5,30)}$=25.038, p<.001] which suggest that both SPS (n = 4) and control (n = 4) female rats acquired conditioning freezing. There were no significant effects of stress (main or interaction) for freezing during fear conditioning (ps > .05) (Figure 4), which suggests SPS and control rats acquired conditioned freezing in an equivalent manner. For darting during fear conditioning, we found a main effect of stress ($F_{(7,42)}$ =2.350, p=.040) with SPS rats showing more darting behavior towards the end of the CS tone and just after the CS tone. (Figure 5).

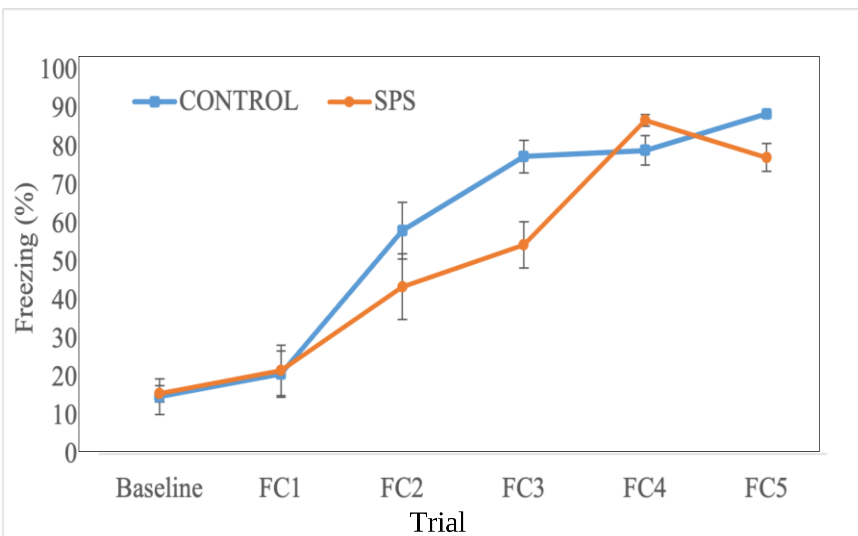

Figure 4. Effect of SPS on Freezing CA During Fear Conditioning: During fear conditioning rats were placed in Context A. When the test starts a tone was play that co-terminates with a foot shock. 5 CS-US pairings were presented, and duration of freezing was measured. A mixed-measure ANOVA revealed that SPS and controls acquired fear condition at the same rate [$F_{(5,30)}$=25.038, p<.001] but there was no effect of stress (ps>.05) .

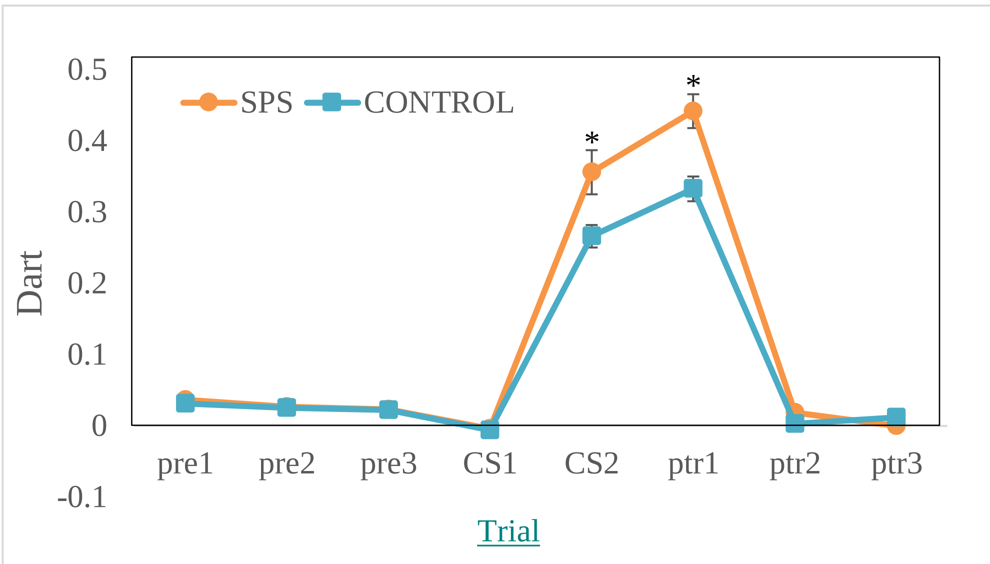


Figure 5. Effect of SPS on Darting CA Fear Conditioning: During fear conditioning rats were placed in Context A. When the test starts a tone was played that co-terminates with a foot shock. 5 CS-US pairings were presented, and the maximum speed of the rat within a 5s bin was recorded. A mixed-measure ANOVA revealed there was a main effect of stress during bins ($F_{(7,42)}$ =2.350, p=.040) with SPS rats showing more darting.

During extinction training, a mixed-measure ANOVA found that there was a significant main effect of trial for the cubic trend component for freezing [$F_{(1,6)}$=9.205, p=.023]. This effect reflected that all rats showed fear memory retrieval. (i.e. enhanced freezing), but decreased freezing across trials (i.e. acquisition of extinction). There was no main or interaction effects of stress (ps>.05) (Figure 6).

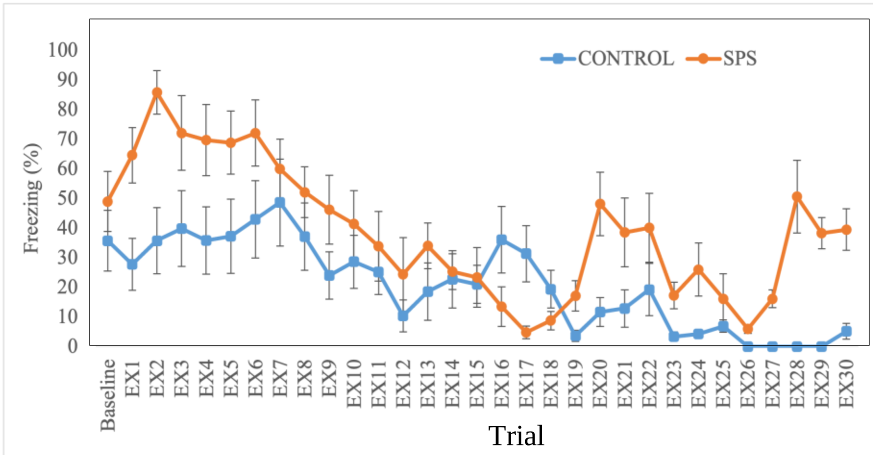


Figure 6. Effect of SPS on Freezing During Extinction Training CA: During extinction training rats were placed on the non-threat side of the chamber. The door opens and a series of 30-CS only presentations were played. The duration of freezing was recorded. A mixed-measure ANOVA revealed that SPS and controls showed fear memory retrieval as shown by main effect of trial on the cubic trend component [$F_{(1,6)}$=9.205, p=.023] but no effect on stress.

During extinction testing, a mixed-measure ANOVA found that there was significant main effect of trial for the quadratic trend component [$F_{(1,6)}$=8.403, p=.027]. This effect reflected that all rats showed fear memory retrieval. (i.e. enhanced freezing), but decreased freezing across trials. There was no stress effect (ps>.05) (Figure 7).

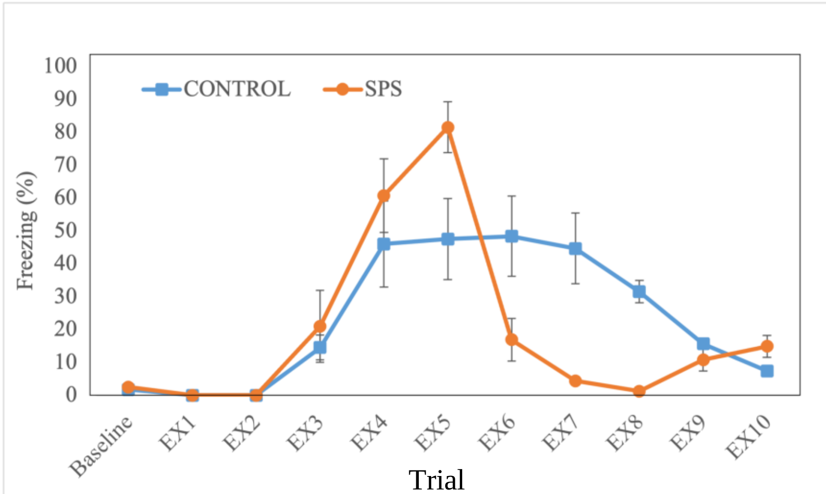


Figure 7. Effect of SPS on Freezing During Extinction Testing CA: During extinction testing rats were placed on the non-threat side of the chamber. The door opens and a series of 10-CS only presentations were played. The duration of freezing was recorded. A mixed-measure ANOVA found that there was significant main effect of trial for the quadratic trend component [$F_{(1,6)}$=8.403, p=.027]. This effect reflected that all rats showed fear memory retrieval but decreased freezing across trials. There was no effect on stress (ps>.05).

### 3.1.2 Avoidance

We only examined avoidance during extinction training and testing because during fear conditioning the rat cannot avoid the context in which the tone is presented. During extinction training a mixed-measure ANOVA found no significant difference between SPS and controls for total entries made to either side (ps>.05) (Figure 8). A mixed-measure ANOVA suggests a significant period x stress x side interaction [$F_{(30,360)}$ =1.499 , p=0.048] with SPS rats spending more time in the non-threat chamber, however, a post-hoc test found no significant difference between SPS and control rats (ps>.05) (Figure 9). During extinction testing, a mixed-measure ANOVA found there was a significant interaction between period x stress x side

[$F_{(1,12)}$=6.060, p=.030] with control rats spending more time on the threat side and SPS rats preferring to stay on the non-threat side [$T_{(86)}$=2.719, p=.008] (Figure 10).

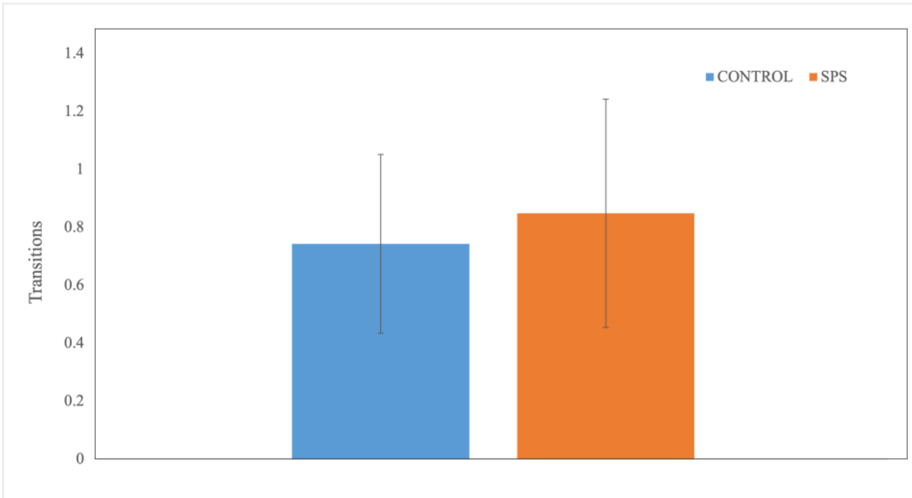


Figure 8. Effect of SPS on Non-Threat Side Entries During Extinction Testing CA: During extinction training rats were placed on the non-threat side of the chamber. The door opens and a series of 30-CS only presentations were played. The number of entries to the non-threat chamber for control and SPS rats is shown. A mixed-measure ANOVA revealed no significant difference between SPS or control for threat entries (ps>.05).

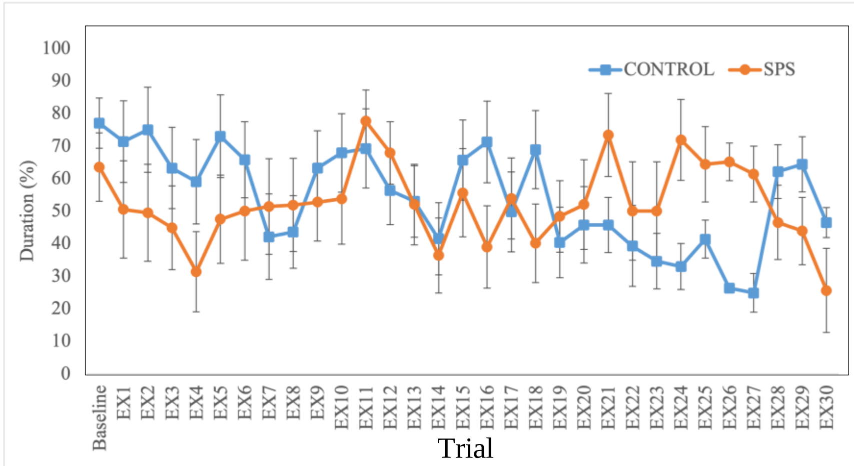


Figure 9. Effect of SPS on Non-Threat Side Duration During Extinction Training CA: During extinction training rats were placed on the non-threat side of the chamber. The door opens and a series of 30-CS only presentations were played. The duration on the non-threat side for control and SPS rats is shown here. A mixed-measure ANOVA revealed an interaction between time period, stress, and side [$F_{(30,360)}$ =1.499 , p=0.048] but a post-hoc test found no difference between SPS and control rats.

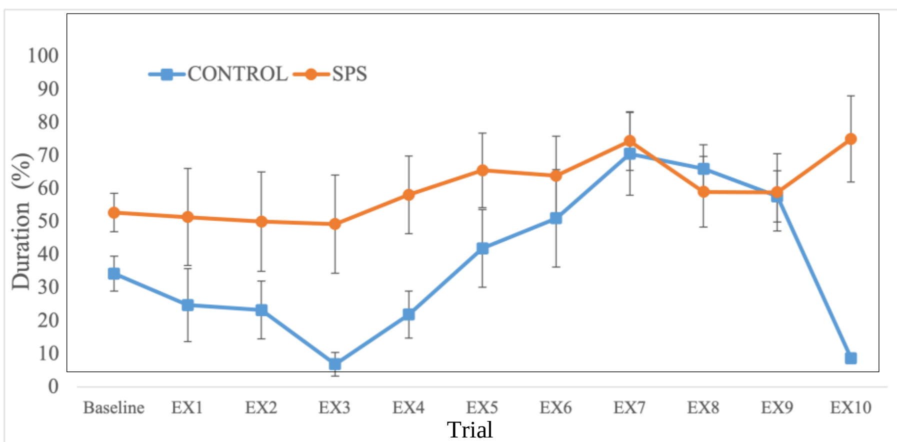


Figure 10. Effect of SPS on Non-Threat Side Duration During Extinction Testing CA: During extinction testing rats were placed on the non-threat side of the chamber. The door opens and a series of 10-CS only presentations were played. The duration of control and SPS rats on the threat side is shown here. A mixed-measure ANOVA revealed an interaction between time period, stress, and duration of time spent on either side

[$F_{(1,12)}$=6.060, p=.030] with SPS rats spending more time on the non-threat side.

SPS had effects on avoidance during extinction testing, but results were difficult to interpret for extinction testing. There were interesting results for darting behavior in the CA experiment, however, SPS did not affect the retention of extinction when freezing was used as the dependent variable; a finding that replicated previous results from the lab (Keller et al. 2015). Because these results were difficult to interpret, we did not pursue these experiments any further and did not attempt to perform these experiments in male rats.

**3.2 Experiment 2: IA**

Initially, one-shock fear conditioning for IA did not result in significant avoidance behavior in female rats. This was confirmed using a mixed-measure ANOVA (ps>.05) (Figure 11). We decided to switch the design for IA to include five 1mA, 1s shocks for fear conditioning. This did result in avoidance behavior in females (SPS = 8, Control = 8). We then used this design in males (SPS = 8, Control = 8) and analyzed the data of males and females together by including a sex factor in all factor designs.

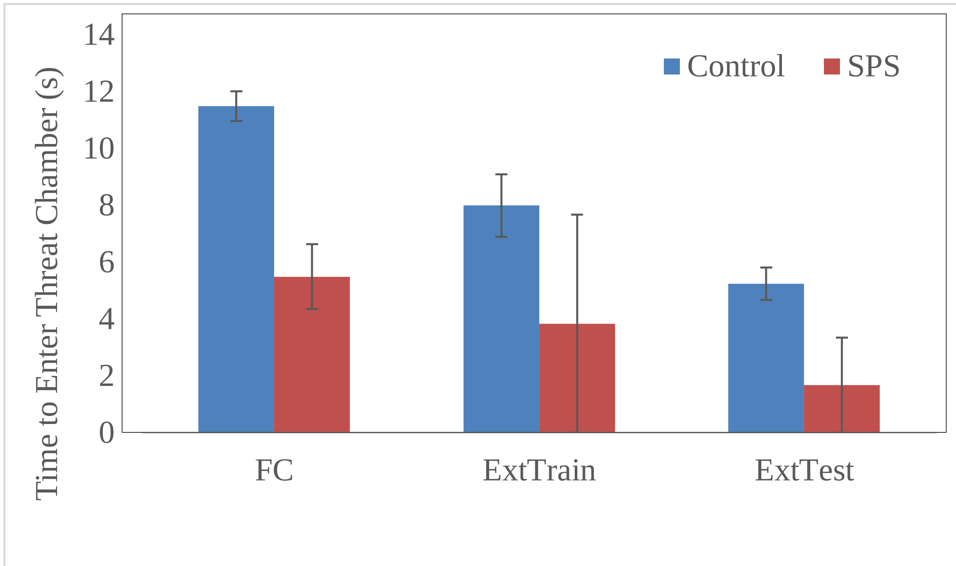


Figure 11. Effect of SPS on Avoidance During All Behavior Sessions IA: The rats first threat entry during fear conditioning, extinction training, and extinction testing were recorded. Here, we are comparing SPS vs. Controls first threat entry (i.e. IA). There was no effect on stress (ps>.05).

**3.2.1 Avoidance**

A mixed-measure ANOVA found that there was an overall significant main effect of sex [$F_{(1,44)}$=14.025, p=<.001] with male rats showing more IA than female rats across all behavioral sessions, though this was more pronounced for extinction training and testing (Figure 12). A post-hoc test confirmed that male rats showed significantly more IA than females during extinction training and testing (extinction training: male vs. female $T_{(16)}$=2.879, p=.011 extinction testing: $T_{(17)}$=2.011, p=.030). A post-hoc test confirmed that there was no significant difference between SPS and control females and no difference between SPS and control males for IA during extinction training and testing (ps>.05). Using a stress x bin x sex factor design to analyze avoidance throughout the entire IA sessions (as opposed to time the animal makes the first entry into the threat chamber), we found a significant stress x sex interaction on time spent in the threat chamber during extinction training [$F_{(1,44)}$=6.693, p=.013] with control males spending the shortest time in the threat chamber when compared to SPS males and control females [$T_{(452)}$=8.341, p<.001] (Figure 13). There was a significant interaction between sex x stress on transitions between the two chambers [$F_{(1,44)}$=6.764, p=.013] with control females transitioning more than control males [$T_{(718)}$= 9.611, p<.001] (Figure 14). During extinction testing, there was a significant interaction between stress x sex on percent time in the threat chamber [$F_{(1,44)}$=6.576, p=.014] with control males spending more time in the threat chamber than SPS males [$T_{(467)}$=8.724, p<.001] (Figure 15). A post-hoc analysis revealed no significant difference between SPS and control females (ps>.05).

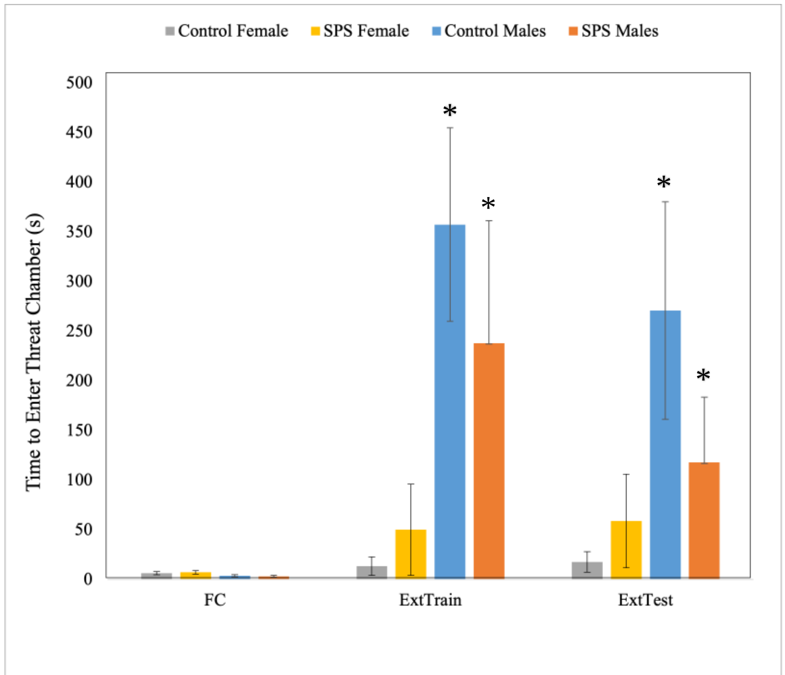


Figure 12. Effect of SPS on Avoidance in Males and Females During All Behavior Sessions IA: The rats first threat entry during fear conditioning, extinction training, and extinction testing were recorded. Here, we are comparing SPS vs. Controls as well as Male vs. Female first threat entry (i.e. IA). A mixed-measure ANOVA concluded that there was an overall significant difference between males and females for their first dark entry [$F_{(1,44)}$=14.025, p=<.001] with both SPS and control males taking longer to enter the threat zone during fear conditioning, extinction training, and extinction testing. Asterisk denotes main effect of sex.

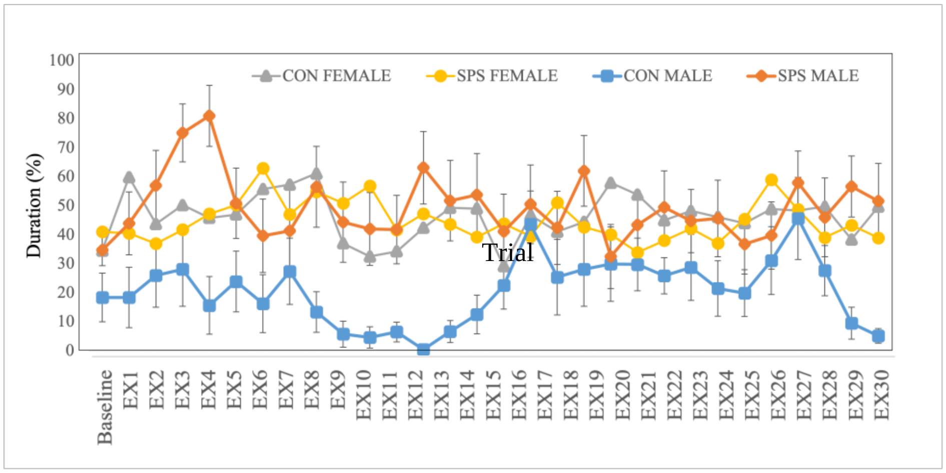


Figure 13. Effect of SPS on Threat Side Duration in Males and Females During Extinction Training IA: During extinction training rats were placed in the non-threat chamber. The door opens and the rat is allowed to freely move between the two chambers. Duration on the threat chamber is measured here. A mixed-measure ANOVA showed a significant effect between stress and sex on time spent in the threat chamber [$F_{(1,44)}$=6.693, p=.013] with SPS males avoiding the threat chamber.

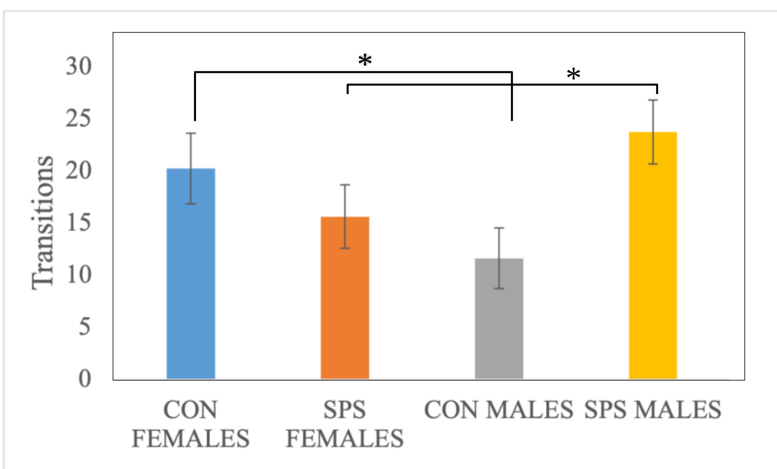

Figure 14. Effect of SPS on Threat Side Entries Males and Females During Extinction Testing IA: During extinction testing rats were placed on the non-threat side. The door opens and the rat is allowed to freely move between the two chambers. Number of entries to the threat chamber were recorded here. A mixed-measure ANOVA revealed an interaction between sex and transiting between the two chambers with control females transitioning more than control males and SPS males transitioning more than SPS females [$F_{(1,44)}$=6.764, p=.013]. Asterisk denotes main effect of sex.

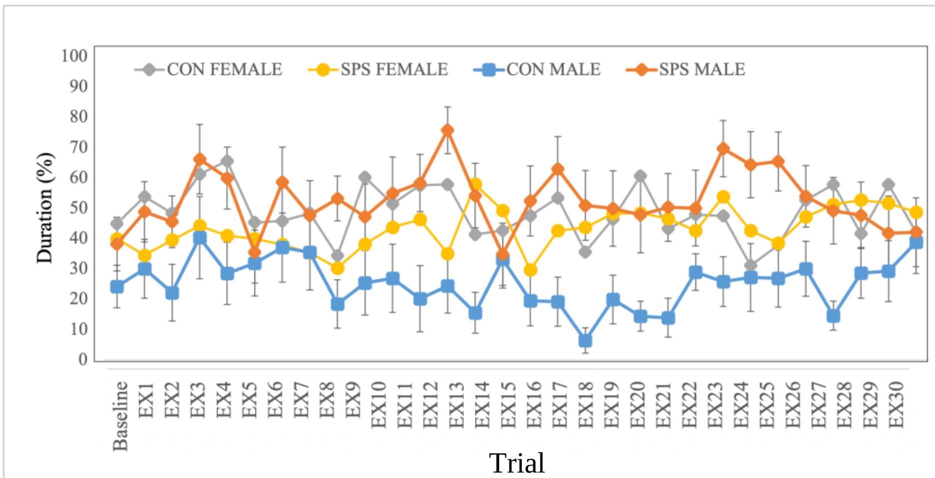


Figure 15. Effect of SPS on Threat Side Duration Males and Females During Extinction Testing IA: During extinction testing rats were placed on the non-threat side. The door opens and the rat is allowed to freely move between the two chambers. Duration on the threat chamber was recorded here. A mixed-measure ANOVA showed that there was a significant effect between stress and sex on duration in the threat chamber [$F_{(1,44)}$=6.576, p=.014] with control males spending the most amount of time in the threat chamber.

### 3.2.2 Freezing:

For fear conditioning, using a mixed-measure ANOVA there was a significant main effect for the linear trend component [$F_{(1,44)}$=163.885, p<.001], but not stress or sex effects (ps > .05) suggesting that male and females acquired contextual freezing at an equivalent rate (Figure 16). During extinction training there was a significant main effect for the linear trend component [$F_{(1,44)}$=29.835, p<.001]. This effect reflected that all rats showed fear memory retrieval. (i.e. enhanced freezing), but decreased freezing across trials (i.e. acquisition of extinction). There was no significant difference between male and females and no effect of stress on behavior (ps>.05) (Figure 17). During extinction testing, there was a significant main effect for the linear trend component [$F_{(1,44)}$=5.266, p=.027] reflecting that all rats showed fear memory retrieval. (i.e. enhanced freezing), but decreased freezing across trials (i.e. acquisition of extinction). There was no significant difference between males and females and no effect of stress on behavior (ps>.05). (Figure 18).

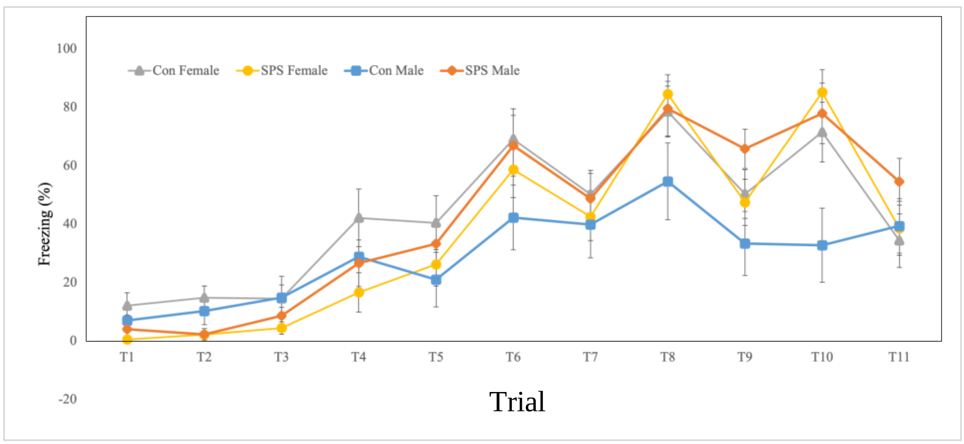

Figure 16. Effect of SPS on Freezing in Males and Females During Fear Conditioning IA: During fear conditioning the door opened and the rat can move to the threat chamber. Once entering the threat chamber, the door closes, and the rat is shocked 5 times. Freezing duration was measured here. A mixed-measure ANOVA found no significant difference between males and females in terms of freezing (p=.509). The linear trend component [$F_{(1,44)}$=163.885, p<.001] suggests that male and females acquired contextual freezing at an equivalent rate].

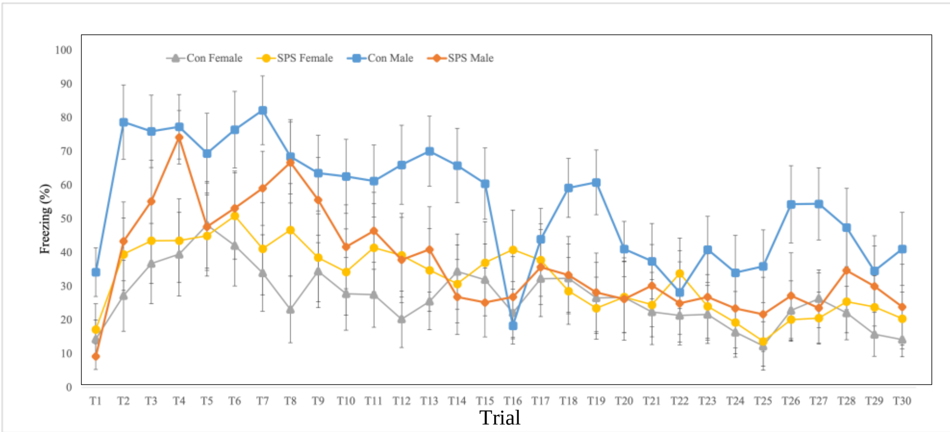


Figure 17. Effect of SPS on Freezing in Males and Females During Extinction Training IA: During extinction training rats were placed on the non-threat side. The door opens and the rat is allowed to freely move between the two chambers. Freezing duration was measured here. A mixed-measure ANOVA analysis found no difference between males and females (ps>.05). No effect of stress on freezing (ps>.05)

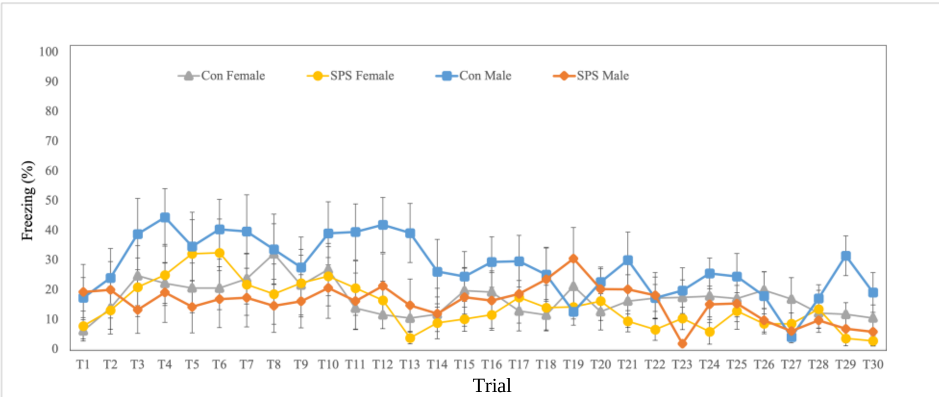

Figure 18. Effect of SPS on Freezing Males and Females During Extinction Testing IA: During extinction testing rats were placed on the non-threat side. The door opens and the rat is allowed to freely move between the two chambers. A mixed-measure ANOVA analysis found no significant difference between males and females (ps>.05). No effect of stress on freezing (ps>.05).

### 3.2.3 Darting

Darting was only measured during fear conditioning because it was not possible to distinguish between darting and avoidance during extinction training and testing when the rat has another side to escape to. Using a mixed-measures ANOVA we found no significant sex or stress interaction (ps>.05) (Figure 19).

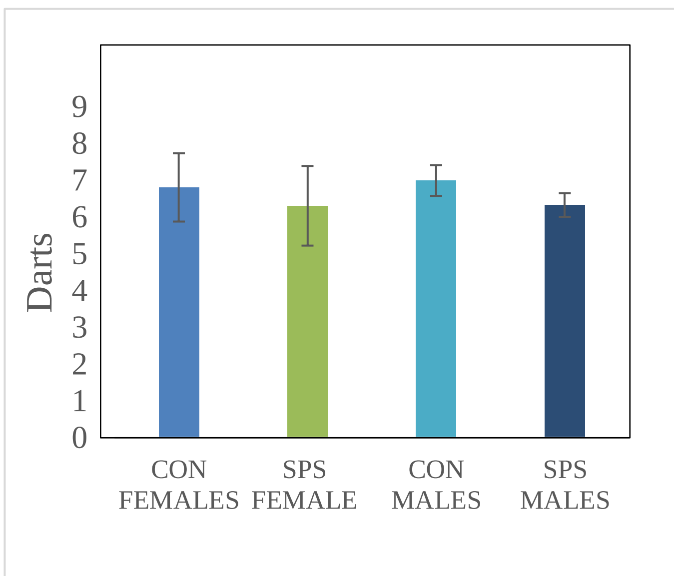

Figure 19. Effect of SPS on Darting Males and Females During Fear Conditioning IA: During fear conditioning. the door opened and the rat can move to the threat chamber. Once entering the threat chamber, the door closes, and the rat is shocked 5 times. A within-subjects ANOVA analysis found no significant stress or sex interactions ($p>.05$).

# Chapter 4

## DISCUSSION

Overall, this study demonstrates that SPS can be demonstrated to induce persistent fear memory in female rats, but only in terms of avoidance behavior. We did not observe extinction retention deficits for conditioned freezing. In Experiment 1 there was no significant difference between SPS and control groups during fear conditioning but a main effect of during bins with SPS rats darting more. This finding suggests that female rats may be switching from freezing to darting behavior. There was no difference between control and SPS rats for freezing during extinction training and extinction testing. This finding suggests that conditioned freezing may not be affected by SPS in female rats. This observation: this is consistent with previous studies that found SPS did not induce extinction retention deficits in cued avoidance females (Keller et al. 2017). These findings also indicate that females may be resilient to the effects of SPS (Keller et al. 2017). In contrast to this, we found an interesting difference for avoidance behavior. During extinction training results were inconclusive on whether there was a significant difference between SPS and control rats. During extinction testing control rats spent most of their time on the threat side. This finding suggests that SPS rats stayed on the non-threat side to avoid the potential aversive stimulus during extinction testing.

For Experiment 2, we observed SPS effects in both sexes. During fear conditioning, SPS females/males froze more compared to control females/males. This finding suggests that SPS influences freezing behavior. For darting, there was no significant difference between males and females during fear conditioning. This suggests that darting is not specifically a female defense response. For avoidance, we observed a significant difference when male and female rats made their first dark entry into the threat chamber. Males showed more IA than females by taking longer to enter the threat chamber. Males also made fewer transitions and spent more time in the threat chamber during extinction training than control females. This observation raises the possibility that females were trying to avoid the aversive stimulus by actively avoiding it by moving.

The results of these experiments reveal that females switch behaviors more than males do according to their environment. When put into a context with no way to escape, we observed that both male and female rats will switch between darting and freezing behavior. However, when put into a context where there is a means of escaping to another side, female rats prefer to travel between compartments or stay in the non-threat chamber, whereas males prefer to freeze. These sex differences in behavior may be due to females being able to learn and retain associations better than males (Dalla and Shors 2009). Previous research has suggested that the high estrogen levels in females promote acquisition, while high testosterone disrupts learning and has a major role in the development of sex differences in numerous learning paradigms (Leuner et al. 2004; Dalla and Shors, 2009; Gupta et al. 2001). Another explanation could be that females are more active than males because they must be more exploratory to search for food needed to sustain themselves and their offspring (Rosenfeld, 2017).

Limitations to this study include that for all experiments, vaginal swabs were not used because they may introduce a confound that interacts with SPS (Nahvi et al. 2021). This is supported by research showing that SPS effects are not observed in male rats that were exposed to anogenital swabbing (Keller, Screiber et al. 2015). Future experiments examining the effects of SPS in female rodents may want to use a different method of measuring estrogen levels such as measuring estrogen levels in the blood or using fewer vaginal swabs to estimate stages of estrous (Nahvi et al. 2021). Another limitation is that we were not able to compare males and females during CA or analyze darting during extinction training and testing for CA and IA. Future studies should include this to obtain more complete data.

## Chapter 5

## CONCLUSION

The finding that females prefer to actively avoid aversive contexts further highlights that males and female respond differently to the stress. This presents implications for future studies using both female and male rats, specifically, that freezing or darting alone is not a complete measure of learned fear in females. Females also displayed more switching between defense behaviors compared to males, indicating that they are learning and adapting to their environment. These sex differences in learning how to predict and avoid the aversive stimulus is influenced by a combination of hormones, genes, and the environment (Dalla and Shors 2009; Beiko et al. 2004). The active avoidance displayed in SPS/female rats in response to a cued CS demonstrates their ability to learn/avoid the CS-US association quicker than males. This result is replicated when exposed to context alone and further demonstrates that females can learn and adapt to their environment quicker than males. The results of this research emphasize the need for identifying the neurobiological cause of these sex differences in behavior as well as adjusting diagnostic and treatment protocols to serve men and women with PTSD.

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## Appendix A

## IACUC APPROVAL

**University of Delaware**
**Institutional Animal Care and Use Committee**
**Request to Amend an Animal Use Protocol**

RECEIVED
AUG 08 2019
IACUC (JH)

| | |
|---|---|
| **Title of Protocol:** Paradoxical effects of estrogens in fear memory | |
| **AUP Number: 1349-2019-A** | **← (4 digits only)** |
| **Principal Investigator**: Dayan Knox | |

**Requested Changes**

**I am requesting a change to:** ***(Check <u>all</u> that apply)***

- ☐ Animal Species *(Complete Section 1)*
- ☐ Animal Numbers *(Complete Section 2)*
- ☐ Animal Procedures *(Complete Section 3)*
- ☒ Therapeutic or Experimental Agents *(Complete Section 4)*
- ☐ Pain Category *(Complete Section 5)*
- ☐ Use of Biological Material, Hazardous Agents or Radiation *(Complete Sections 4 & 6)*
- ☐ Other *(Specify)* Click here to enter text.

*(Complete Section 7)*

**Changes MUST NOT be initiated until IACUC approval is granted**

**Official Use Only**

IACUC Approval Signature: [illegible], DVM

Date of Approval: 9/18/19