Examining associative and non-associative fear memories in the Rat Exposure Test

by

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Abstract

Post-traumatic stress disorder (PTSD) is a debilitating psychological condition which results in a variety of negative symptoms causing significant functional impairment. These symptoms fall into four clusters: avoidance, intrusion, negative cognitions or mood, and changes in arousal. Among those who actively seek treatment for PTSD, only 30% achieve full remission. This disparity represents a need for further research into therapies for PTSD. Predator stress is an animal model of PTSD which exposes a prey animal to a natural predator, creating a situation in which the animal fears imminent injury or death. The rat exposure test (RET) developed by Yang et al. (2004) has been successful in producing defensive behaviors in the mouse during a 5-minute protected exposure to a rat. Long-lasting behavioral changes in the mice, however, have not been assessed in this paradigm. Thus, the goal of these studies was to better assess the RET as a model of PTSD. Experiment 1 examined whether the effects of the RET could be seen up to three weeks post-exposure. The results from this experiment revealed that associative, but not non-associative fear, could be seen up to 21 days post-exposure. Specifically, predator-stressed (PS) animals froze significantly more than control animals upon re-exposure to the fear context. There were no differences observed in non-associative fear memories as measured in the elevated plus maze (EPM), open field (OF), or light/dark (LD) box. After minor changes to the RET protocol were made, the results from Experiment 2 demonstrated that the RET produced associative and non-associative fear memories. Specifically, PS animals froze significantly more than controls during re-exposure to the fear context. In addition, PS animals showed increased anxiety-like behavior in the EPM and LD tests. Finally, Experiment 3 tested the effects of the RET in

female mice, as well as duration of the RET (5 min versus 60 min). The results indicated that there were no differences between PS and control animals regardless of length of exposure. Collectively, these results indicate more refinement needs to be made to the RET to achieve consistent, robust results across experiments.

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Introduction

Post-traumatic stress disorder (PTSD) is a debilitating psychological condition in which a person who endures a traumatic event experiences a multitude of negative psychological, emotional, and physiological symptoms following the event (American Psychiatric Association, 2013). Currently, the prevalence of lifetime PTSD is estimated to be around 9% in Canada (Van Ameringen et al., 2008). This means that a significant proportion of our population will experience PTSD at some point in their life. Symptoms of PTSD often include intrusive memories of the traumatic event, avoidance of activities related to the trauma, persistent generalized hyperarousal, and negative alterations in cognition and mood (American Psychological Association, 2013). These negative psychological symptoms often cause those with PTSD to have problems functioning in society following the traumatic event (Kaniasty & Norris, 2008; Laffaye, Cavella, Drescher, & Rosen, 2008; Solomon & Mikulincer, 2007).

1.1 Post-Traumatic Stress Disorder Symptoms

Historically known as "shell-shock" because of its prevalence in war veterans (Boehnlein & Hinton, 2016), PTSD is the result of experiencing a traumatic event which causes a wide array of negative psychological symptoms. PTSD can occur at any age, and women generally have a higher prevalence rate than men (Breslau et al., 1998; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995; Kilpatrick et al., 2013; Resnick, Kilpatrick, Dansky, Saunders, & Best, 1993). Common events which precipitate PTSD include bodily assault, wartime combat, or sexual assault (Breslau, Davis, Andreski, & Peterson,

1991; Norris, 1992; Perkonigg, Kessler, Storz, & Wittchen, 2000). Events that are common to the human experience, such as death of a loved one or experiencing a job loss, do not constitute a precipitating event to PTSD (American Psychiatric Association, 2013). Exposure to events that would be qualified as extremely traumatic in a community-based setting appears relatively high, with estimates ranging from 39.1% (Breslau et al., 1991) to 81.3% (Stein, Walker, Hazen, & Forde, 1997). From this population, a certain subset of individuals will go on to develop the clinical symptoms of PTSD (Van Ameringen, Mancini, Patterson, & Boyle, 2008).

The diagnosis of PTSD focuses on four behavioural criteria following the experience of a traumatic event: intrusion symptoms, avoidance symptoms, negative alterations in cognition or mood, and alteration of arousal and reactivity. The experience of these symptoms must last longer than one month and cause significant distress or functional impairment, and are not caused by medication, substance use, or illness (American Psychiatric Association, 2013).

The first type of symptom cluster covers a range of symptoms known as intrusion, or involuntary recollection of the traumatic event. This can include recurrent and distressing memories of the event, recurrent and distressing dreams/nightmares of the event, dissociative reactions (flashbacks), intense or prolonged psychological distress with exposure to internal or external cues which represent the traumatic event, and marked physiological reactions to cues which represent the traumatic event (American Psychiatric Association, 2013; Brewin, Dalgleish, & Joseph, 1996; Ehlers, Hackmann, & Michael, 2004; Somer & Ataria, 2015). The emphasis with this symptom cluster is on

spontaneous or triggered memories which can include sensory, physical, or emotional components (American Psychiatric Association, 2013). For example, a person who experiences a motor vehicle accident may have dreams about being in a car crash, or experience involuntary flashbacks to headlights coming towards him/her.

The second type of symptom cluster covers a range of avoidance symptoms, whereby the individual exhibits defensive behaviors meant to mitigate reminders of the traumatic event. These can include refusing to discuss the event, or engaging in avoidance strategies to minimize exposure to physical reminders of the event (American Psychiatric Association, 2013; Békés, Beaulieu-Prévost, Guay, Belleville, & Marchand, 2016). In the example of a person in a motor vehicle accident, he or she may refuse to return to the scene of the crash in an attempt to minimize re-experiencing the trauma.

The third type of symptom cluster references the negative changes in cognition or mood following a traumatic event. These types of symptoms can include: inability to remember an important aspect of the event, exaggerated negative beliefs about oneself or the world, distorted cognitions which cause the person to blame oneself for the event, persistent negative emotional state, diminished interest or participation in significant activities, feelings of detachment, and persistent inability to experience positive emotions (American Psychiatric Association, 2013). These types of symptoms can result in a person developing a sense of depersonalization (Carlson, Dalenberg, & McDadeMontez, 2012), derealization (Armour, Karstoft, & Richardson, 2014), or emotional numbing (Short, Norr, Mathes, Oglesby, & Schmidt, 2016). Exaggerated negative beliefs about

oneself can often be seen in assault or abuse survivors who blame themselves for the attack (Sharma-Patel & Brown, 2016).

The fourth and final type of symptom cluster defines changes in arousal following the traumatic event. These changes can include irritable behavior and violent outbursts, hypervigilance, exaggerated startle response, problems with concentration, and sleep disturbances (e.g., difficulty falling asleep). A person must exhibit two or more of the preceding symptoms in order to receive a diagnosis of PTSD (American Psychiatric Association, 2013)

Collectively, these symptom clusters form the core phenotype seen in PTSD. These symptoms often result in an inability to function in society, leading to feelings of alienation in many sufferers (Kaniasty & Norris, 2008; Laffaye, Cavella, Drescher, & Rosen, 2008; Solomon, Z., & Mikulincer, 2007). Though most individuals who present with the symptoms of PTSD actively seek out some sort of psychotherapy or pharmacotherapy, only about 60 % of patients are responsive to these interventions (Onder, Tural, & Aker, 2006; Zohar et al., 2002), with only 20-30 % of patients achieving full remission (Berger et al., 2009). The disparity in those achieving full remission, coupled with epidemiological studies estimating the lifetime prevalence of PTSD at 9.2 % for the general population in Canada (Van Ameringen et al., 2008) suggests a desperate need for novel, effective treatments of this disorder.

1.2 Rodent Models of PTSD

While there is no animal model that can completely recapitulate the human condition, various animal models have been used to elucidate the molecular mechanisms underlying PTSD (Gan et al., 2016; Kao et al., 2016; Liu et al., 2016; Wolf et al., 2016). One of the benefits of animal models is that researchers can manipulate different substrates within the brain, something that is not feasible with human patients. Unfortunately, there is wide variation among methodologies used to achieve an animal model of PTSD which can lead to varying results across research laboratories. In their review of animal models of PTSD, Yehuda and Antleman (1993) put forth a set of five criteria which would make the "ideal" model of PTSD:

> 1) Even very brief stressors should be capable of inducing biological or behavioural outcomes of PTSD 2) The stressor should be able to produce PTSD in a dose-dependent manner 3) The stressor should be able to produce biological alterations that persist over time or become more pronounced with time 4) The stressor should induce biobehavioural alterations which have the potential for bidirectional expression, and 5) Interindividual variability in response to a stressor should be present as a function of either experience or genetics, or an interaction of the two (p. 336).

Using these criteria to examine various animal models of PTSD, many models fail to reach one or several of these criteria. This marks a clear need for a unified paradigm which is easily accessible and more reliable than current methods.

1.2.1 Fear Conditioning

Despite its limitations, the most common animal model of PTSD used in the literature is fear conditioning (Careaga, Girardi, & Suchecki, 2016; Reznikov, Binko, Nobrega, & Hamani, 2016). Traditional fear conditioning paradigms involve exposing an animal to an aversive stimulus, often paired with a specific context or cue (Mac Callum, Hebert, Adamec, & Blundell, 2014). This type of Pavlovian conditioning can be used to examine the neural basis and substrates of associative memory. Associative fear memories are memories which are inextricably tied to the trauma event (Fifield, Hebert, Angel, Adamec, & Blundell, 2013). In the core symptom clusters of PTSD, associative fear memories are represented by the intrusion and avoidance symptoms mentioned earlier. In rodent models, this can be seen by an increased amount of freezing in response to re-exposure to the trauma context or cue. Freezing is a defensive behavior in rodents and demonstrates the animal perceives a threat to be present (Yang et al., 2004). Thus, measuring freezing behaviors when re-exposing the animal to the trauma context or cue is the basis for determining how fearful a rodent is of a certain environment or cue. If an animal was in a context (or was presented with a cue) in which it previously received a shock (and perceived there was an imminent threat of death or injury), re-exposure of that animal to the same context or cue without the shock should elicit the same defensive response.

Though this paradigm is robust and successful in inducing an associative fear memory, there is little evidence that fear conditioning produces non-associative fear memories (Fogaça, Reis, Paulo, Campos, Guimarães, 2014; Reznikov, Diwan, Nobrega,

& Hamani, 2015). Non-associative fear memories are memories that are not associated specifically with the trauma event which cause psychological distress and negative alterations in cognition and mood (Fifield, Hebert, Angel, Adamec, & Blundell, 2013). Non-associative fear memories are represented by the third and fourth symptom cluster, which often manifest in the form of generalized anxiety and increased hyperarousal. They are not associated with a specific context, and can occur in a situation which is completely separate from the trauma. For example, a war veteran who is no longer in combat can experience increased generalized anxiety when he or she is safely back at home. This represents a major problem with using fear conditioning as a model of PTSD, as non-associative fear memories are a defining characteristic of the disorder. Thus, a more comprehensive rodent model of PTSD is needed which can induce both associative (ex: reexposure stress) as well as non-associative (ex: longer duration in closed arms in the elevated plus maze) fear memories. One such model is predator stress.

1.2.2 Predator Stress

Originally validated by Adamec and Shallow (1993), predator stress is a model of PTSD which produces enduring changes in both associative and non-associative fear memories (Adamec, Fougere, & Risbrough, 2010; Adamec, Toth, Haller, Halasz, & Blundell, 2012; Adamec, Walling, & Burton, 2004; Clay et al., 2011; Fifield et al., 2014). Predator stress involves exposing a prey animal to a natural predator, or predator cue, typically in an inescapable context. This extremely stressful and potentially life-threatening situation causes a predictable fight-or-flight response in the prey animal. This acute, personally threatening and immediately distressing event may be similar to an

event which would cause PTSD in a human. Predator-stress models have been successful in modelling the associative and non-associative symptoms of PTSD in rodents, showing increases in all four types of core symptoms in animals exposed to a predator (Adamec et al., 2010; Adamec et al., 2012; Clay et al., 2011).

While the diversity in predator-stress paradigms represents how effective this paradigm is at modelling PTSD-like symptoms in animals, there is no one agreed upon methodology to achieve the most consistent results. For instance, many researchers have been successful in modelling PTSD in rats by using a predator-scent stress paradigm (Cohen, Kozlovsky, Matar, Zohar, & Kaplan, 2014; Fenchel et al., 2015; Manjoch et al., 2016; Mayer, Matar, Kaplan, Zohar, Cohen, 2014). This paradigm relies on olfactory cues, known as chemosignals, specific to predator urine. Chemosignals are a type of pheromone, which is an invisible scent produced by an animal that transmits information (Wernecke et al., 2015). Many animals rely on their sense of smell to determine what is in their surrounding environment and whether there is any danger present. Specific chemosignals found in cat urine can signal to the rat that there is a threat nearby, allowing it the chance to escape before it is detected (Apfelbach, Blanchard, Blanchard, Hayes, McGregor, 2005). Other "protected" exposures to predator stress include exposure to predator fur (Blanchard, Griebel, & Blanchard, 2003) or placing the prey animal in an activity ball, allowing the predator to jostle it around without threat of harming it (Burgado et al., 2014). The results from these methodologies are not always replicable (Olford, Adamec, & Bundell, 2009). Differences may be due to the type of predator used (e.g., domestic or feral cat), or the state of the predator (well feed or food deprived) (Adamec et al., 2014; Chen, Shen, Liu, & Li, 2014).

We, and others (Lima, Baldo, Canteras, 2016; Rorabaugh et al., 2015; Zoldaz, Park, Fleshner & Diamond, 2015), have used a predator stress model which involves direct, unprotected exposure of a rodent (mouse or rat) to a cat. While Adamec, Kent, Anisman, Shallow, and Merali (1998) and Fifield et al., (2014) have reported robust changes in associative and non-associative fear memories, many other published studies were unable to replicate these effects (Adamec, 2001; Adamec et al., 2001; Adamec et al., 2006; Adamec, Blundell & Burton, 2006; Adamec, Walling & Burton, 2004; Clay et al., 2011; Fifield et al., 2015).

One possible explanation for the discrepancies with predator exposure, specifically when using only a single cat, is a fatigue effect in the predator. The first few exposures to the rat are novel, but each subsequent exposure habituates the cat to the presence of the rat. This can lead to changes in behavior, such as the cat being awake and inquisitive for the first few trials, but soon bored and uninterested in later exposures. Because it is often not feasible with available resources to use multiple different cats for multiple studies to ensure novel reactions to prey, it is our belief that using a more accessible predator, such as a rat, may result in more consistency across prey exposures. A rat/mouse interaction paradigm has the potential to be less expensive, more consistent, and more robust than other types of predator stress paradigms that have been discussed.

1.3 The Rat Exposure Test

The rat-exposure test (RET) was first developed by Yang et al. (2004) in order to evaluate mouse defensive behaviors. Defensive behaviors are innate, unconditioned

responses that are elicited in a perceived threatening situation by the prey animal (Yang et al., 2004). These defensive behaviors are designed to increase the animal's chance of escape and survival when exposed to a predator. Examples of mouse defensive behaviors include freezing, defensive burying, and avoidance (Yang et al, 2004). These defensive behaviors are indicators that the animal perceives a situation as potentially life-threatening, and thus are an indicator of a paradigms' construct validity (ensuring the situation we assume to be stressful for the animal actually is stressful).

The RET as developed by Yang et al. (2004) consists of a two-compartment exposure box, connected by a Plexiglas chamber to the mouse's "home cage". The exposure cage consists of two compartments, divided by a wire mesh screen. This allows the rat and mouse to investigate each other and ensures the transfer of chemosignals across compartments while protecting the mouse from any attack by the rat. The "home cage" of the mouse was a separate box from the exposure cage that contained bedding from the mouse's own cage in the colony room (thus making the new environment similar to the original home cage).

For their initial study, Yang et al. (2004) used amphetamine-injected Long-Evans rats for predators and BALB/C mice as prey. The rats were injected with amphetamine to ensure mobility and inquisitive behavior across trials. On exposure day, a mouse was placed in the exposure cage and either a live rat or a stuffed toy rat control was immediately introduced behind the wire mesh screen. The mouse could investigate the rat in the exposure cage, or return to its home cage through a tunnel connecting the two boxes. Yang et al. (2004) discovered that in response to an introduction of a live rat, mice

in the experimental condition subsequently demonstrated significant amounts of defensive behaviors, including freezing and avoidance. Follow–up studies by different laboratories on this type of predator-stress exposure have also successfully replicated these findings (Amaral, Santos-Gomes, & Nunes-de-Souza, 2010; Blanchard et al., 1998; Campos, Amaral, Rico, Miguel, & Nunes-de-Souza, 2013). Taken together, these results demonstrate that the rat-exposure test is an effective way to induce predator-stress in mice.

1.4 Overview of the Current Research

Building on the current and past literature, our lab sought to examine the use of predator-stress as a model for PTSD. Specifically, we were interested in determining if the RET could be used to model the core symptoms seen in PTSD. Though previous studies have examined mouse behaviors during the exposure, to our knowledge, no study has been conducted which investigates the enduring effect of the RET after the exposure. The RET has many benefits compared to other traditional predator-stress paradigms including a lower cost and lower time investment. Many examples of predator stress involve chronic exposure of a rodent to a predator, using multiple trials a day over the course of a week (Genovese, Johnson, Tobin, & Gauchan, 2014). There are many instances of PTSD, however which derive from a single traumatic event (American Psychiatric Association, 2013). If the RET is successful in producing long-lasting changes in the anxiety phenotype of the mouse, it has the potential to be more time efficient, cost efficient, and consistent than other methods of predator stress currently

used in research. Through a series of three experiments, our research aimed to determine the viability of the RET as a model of PTSD.

A brief pilot experiment gave promising evidence that the RET was successful in producing both associative and non-associative fear, measured by reexposure and anxiety-like behavior (ALB) as well as startle testing. However, there were many problems with the initial experiment protocol (e.g,: recording cameras too far away from subject, too much glare from lighting apparatus, etc.), which needed to be amended in subsequent experiments.

First, we assessed whether the effects of the RET are long-lasting. Because a diagnosis of PTSD can only be made after symptoms have persisted for one month, it is crucial for any animal model of PTSD to have similarly enduring consequences. Accordingly, we were interested in determining if the effects of the RET can be seen up to three weeks post-exposure, as other predator stress methods have done in the past (Adamec et al., 1993; Adamec at al., 2014).

We then made minor adjustments in the protocol for Experiment 2. We implemented calorie restriction and handling in the rats, in an attempt to make them less anxious and more exploratory. We then conducted a standard RET to determine if these changes produced a decrease in the variability across animals.

With the knowledge that females are often overrepresented in cases of psychological illness, we then determined the effects of the RET in female mice for Experiment 3. While most studies are done using male mice, it is important that a model be reflective of

the population with the highest incidence rate in order to develop an effective course of treatment. We also were interested in determining whether increasing the length of exposure from acute (5 minute) to chronic (60 minute) would have any effect on stress behaviors in the mouse.

2.0 Materials and Methods

2.1 Animals

A total of 160 male and female (male N = 96, female N = 64), approximately 7 to 8 week-old C57BL/6 mice (Charles River Laboratories, St Constant, QC, Canada), were used for these experiments. Mice were group housed with 2 to 4 conspecifics per cage depending on the experiment, and had *ad libitum* access to food and water in standard laboratory conditions on a 12 hour light-dark cycle, with lights on at 7:00AM. Prior to and during the course of experiments, all animals were handled daily for identification marking with non-toxic markers and routine husbandry duties during the light-phase of their diurnal cycle. Mice were transported from the animal housing facility to an anteroom, adjacent to the testing room and allowed to habituate to the new location for a minimum of 30 minutes prior to training and testing. All behavioural testing and experimental manipulations to animals were conducted during the light-phase of their diurnal cycle.

A total of 20 male, approximately 200-300 gram Long-Evans rats (Charles River Laboratories, St Constant, QC, Canada) were used as predator stimuli for these experiments. Rats were housed singly with *ad libitum* access to food and water in

standard laboratory conditions on a 12 hour reverse light-dark cycle (lights on at 7:00PM), to ensure exposure to the mice was during the active (dark) phase of their diurnal cycle. In Experiment 1, rats were food deprived 24 hours before exposure. In Experiments 2 and 3, rats were calorie restricted to 85% of their body weight for five days prior to exposure; see section 2.3 "Calorie restriction" below for full details. Additionally, rats were handled for five days prior to exposure. Handling consisted of lifting and petting rats for approximately one minute in the colony room under a red light. Similar to the habituation protocol for the mice, rats were also habituated for 30 minutes to the testing room in Experiments 2 and 3 before any behavioral manipulations and exposures occurred.

All procedures and protocols for experiments and animal housing were followed pursuant to the guidelines of the Canadian Council on Animal Care and Memorial University of Newfoundland's Animal Care Committee.

2.2 Experiments

2.2.1. Experiment 1 – Testing the longevity of the anxiety phenotype produced by predator exposure.

Mice were randomly assigned to one of two conditions, control (N = 16) or predator stress (N = 16). This experiment was conducted to test whether associative and non-associative fear memories were present at three weeks post-exposure. All mice were habituated to the exposure chamber for 5 days. For details on the habituation trials, see section 2.4.1 entitled "Habituation". Rats used in the experiment were food deprived for 24 hours prior to exposure to the RET chamber. On

Day 6, mice in the PS condition underwent a five-minute protected exposure to a rat. Mice in the control condition underwent a five-minute exposure to a control stuffed toy that resembled a rat. For details on the exposure, see section 2.4.2 entitled "Exposure". Beginning on Day 27, all mice were examined on a variety of behavioral tests for associative and non-associative fear memories. On day 27, mice were re-exposed to the exposure chamber. On days 28-30, all mice were tested in the elevated plus maze (EPM), open field (OF), and light/dark box (LD). On Day 31, all mice underwent acoustic startle testing. For details on the behavioral tests, please see section 2.4.3. See Figure 1 below for a timeline of the experiment.

2.2.2. Experiment 2 – Amendments to the original RET paradigm. After reviewing footage from both Experiment 1 as well as the pilot data, it appeared the rats were often too startled themselves to move for the duration of the exposure. We implemented a handling and habituation procedure in order to reduce anxiety in the rats and ensure they would not freeze during the exposure to the mouse. We also started a longer calorie-restriction paradigm in the rats. This was done after observation in the lab that hungry rats are much more active and travel further distances than satiated rats. Details on the calorie restriction (section 2.3 "calorie restriction") and habituation protocol (see section 2.4.1 "habituation") can be found below. Mice were randomly assigned to either control (N = 16) or predator stress (N = 16) groups. On days 1-5, mice were brought up into the testing room and were habituated to the RET box, see section 2.4.1 "habituation". On the 6th day of the experiment, all animals were exposed to either a stuffed rat toy or a live rat, depending on the condition. For a full explanation of exposure protocol see section 2.4.2 "exposure". After a 48-hour delay, animals were then tested on

day 8-12 for associative and non-associative fear memories, see section 2.4.3 “behavioural testing”.

2.2.3. Experiment 3 – The effect of the RET in female mice. Following the success of Experiment 2, Experiment 3 was conducted in order to investigate the effect of the RET in female mice (male mice were used in Experiments 1 and 2). Additionally, we aimed to investigate whether a chronic model (60 minute exposure) would produce more of an anxiety phenotype in the mouse than our usual acute (5 minute) exposure. Thus, 64 female animals were used, divided into four groups: five-minute exposure controls (N = 16), five-minute exposure predator stressed (N = 16), 60-minute exposure controls (N = 16), and 60-minute exposure predator stressed (N = 16). On days 1-5 mice and rats were habituated as described in Experiment 2, see section 2.4.1 “habituation”. Rats were also calorie restricted as they were in during Experiment 2. A full explanation of this protocol can be found in section 2.3 “calorie restriction”. On days 1-5, mice were habituated to the testing room and RET chamber as described in section 2.4.1 “habituation procedure”. On the 6^{th} day, mice were either exposed to a live rat or stuffed toy, depending on the condition (see section 2.4.2 “exposure” for details). After a 48-hour delay in which mice sat in their colony room untouched, testing began on day 8 through day 12, see section 2.4.3 “behavioural testing” for details. A full timeline of the experiment can be found in Figure 3.

2.3 Calorie restriction

Rats were weighed and had food intake monitored for five days prior to exposure to the mice to establish an average baseline for each animal. Once the baseline was recorded, animals were then calorie restricted to 85% of their projected body weight for their age, using guidelines from Charles River Laboratories. Once animals attained 85% of their projected body weight, their weight was maintained at this level for the remaining days prior to exposure.

2.4. Behavior

2.4.1. Habituation procedure. For each experiment, mice were habituated to the testing room and the RET box for 5 days prior to exposure. The RET chamber is a standard 48 cm x 26 cm x 20 cm laboratory sized rat cage made of Plexiglas with a clear divider installed in the middle. The divider had holes which allow for scent transference and investigation of the mouse by the rat during exposure. Mice were brought into the lab anteroom and allowed to sit for 30 minutes prior to habituation. After 30 minutes, mice were removed from their home cage and brought into the testing room via an empty transfer cage and were placed into the RET box for five minutes. After five minutes, mice were returned to their home cages in the lab. The RET box was cleaned with 10% ethanol in between each animal.

Rats underwent the same habituation procedure in Experiments 2 and 3. During the 5 days before exposure, to ensure there was no scent crossover between rats and mice during habituation, all mice were habituated and returned to the colony room before rats

were brought upstairs to the laboratory. In addition, rats were habituated to a separate RET box which is identical in form but used only for rat habituation to ensure there was no confounding scent remaining in the box when the mice were habituated the next day. Rats were brought upstairs to the laboratory and allowed to rest in the testing room for 30 minutes prior to habituation. Rats were then placed into the RET box directly from their home cages and allowed to explore the chamber for five minutes. At the end of the five minutes, rats were removed from the RET box and returned to their home cages.

2.4.2 Exposure. As described previously, mice and rats were habituated to the RET box for five minutes daily for five days prior to exposure. On the 6^{th} day, mice were brought up in separate cohorts, with controls run first. After a period of 30 minutes, control animals were placed into the RET box for five minutes with a stuffed control rat on the opposite side of the plexigas divider. Mice were removed and returned to their home cages after five minutes. After all control mice had been tested, they were returned to the colony room. Rats were then brought up and placed in the testing room. Experimental mice were brought up and allowed to sit for a habituation period of 30 minutes in the anteroom, adjacent to the testing room. Rats were then placed in the RET box on the rat side prior to the introduction of a mouse. Mice were brought into the testing room as in to the habituation procedure, using a transfer cage to transport them from the home cage to the RET box. Mice were then placed on the mouse side of the RET chamber and covered with a Plexiglas top. After five minutes (or 60 minutes for Experiment 3), mice were removed from the box and put back into their home cages. New rats were used every four trials, to ensure the rats remained engaged.

2.4.3 Behavioral Testing. After exposure to the rats, mice were subjected to a battery of behavioral tests to investigate anxiety-like behavior and hyperarousal. Testing included re-exposure to the stressor context (RET box), EPM, OF, L/D box and acoustic startle. Each test was run sequentially on separate days, to ensure there was no fatigue effect on the mice.

2.4.3.1 Re-exposure. Forty-eight hours (or 3 weeks in the case of Experiment 1) after the initial exposure to the rats, mice were re-exposed to the RET box in order to assess associative fear memories. Similar to the habituation protocol, mice were placed via a transfer cage in the RET box for five minutes without a stuffed control or live rat on the rat side. Re-exposures were recorded with a video camera mounted on a tripod facing the RET box. Freezing frequency was recorded. Freezing was operationally defined as total absence of movement, apart from respiration, for >1s.

2.4.3.2 Elevated Plus Maze. The day after re-exposure, mice were tested on the elevated plus maze (EPM), which is a behavioural measure of anxiety in rodents. The EPM consisted of four arms joined together in the centre by a 5-cm platform to form the shape of a plus. Each arm was 5 cm wide, 30 cm long, and was elevated 46 cm above the ground. Two of the arms opposite each other were open (had no sides), while the other two arms were closed (had walls 14 cm high and open at the top). These walls did not extend into the centre of the maze. The maze was painted flat enamel grey. At the beginning of each five-minute trial, mice were placed in the centre of the maze facing the same open arm. All trials were videotaped for later analysis. Open and closed arm time were analyzed with Ethovision video tracking software.

2.4.3.3 Open Field. The OF test consisted of an opened top wooden box (48 cm long x 48 cm wide x 48 high) painted flat enamel grey. The center of the wooden box was marked with red masking tape, with edges 10cm away from the periphery to form a center square. At the beginning of each five-minute trial, mice were placed in the centre of the open field. All trials were videotaped for later analysis. Time spent in the center square was analyzed via Ethovision.

2.4.3.4 Light/dark box. The apparatus consisted of two boxes connected by a chamber painted flat enamel grey. Each box was 19.1 cm long, 19.1 cm wide and 14 cm high. The dark chamber was covered with a solid top. The light chamber was covered with a clear Plexiglas top with ventilation holes. Testing took place in a darkened room with a 100-watt light placed above the light chamber. At the beginning of each 5-minute trial, mice were placed in the light box and allowed to move freely between the two boxes. All trials were videotaped for later analysis. Time and entries into the light side (operationalized as all 4 paws in the light chamber) were scored by experimenters blind to rodent condition.

2.4.3.5 Acoustic startle testing. Startle testing took place in San Diego Instruments standard startle chamber. Within the chamber mice were placed in a cylindrical small animal chamber enclosure measuring 12.7 cm long and 3.7 cm in diameter. The animal enclosure was mounted on top of a piezo electric transducer that produced an electric signal sampled by a computer. This provided a measure of mouse activity. Mice were acclimated to the startle chamber for 5 minutes, during which the startle chamber was dark and emitted a background of 50DB white noise. Immediately

following acclimation, mice were exposed to 30 pulses of 50 ms bursts of white noise of 120db rising out of the 50db background. There was a 30 s inter-trial interval between noise bursts. Analysis included the maximal transducer output (V_{max}) within the 150 ms recording window and the transducer output at the beginning of the noise burst (V_{start}). For each trial, peak startle amplitude was calculated as V_{max}-V_{start} and divided by the mouse's body weight in kg, giving peak startle amplitude in volts/kg.

2.5 Statistics

Independent samples t-tests were used when comparing two groups in Experiment 1 and 2. Multiple two-way analysis of variance (ANOVA) tests were used for Experiment 3 when comparisons across multiple groups were necessary. Follow-up comparisons using t-tests were used. Some analyses did not include all subjects owing to losses due to mechanical or technical errors (e.g,, corrupted video file, etc.).

3.0 Results

3.1 Five-minute protected exposure of a mouse to a rat results in associative but not non-associative fear memories 21 days post-exposure.

To determine if the effects of the RET were long-lasting, mice were either exposed to a live rat or stuffed control on day 6 and tested for associative and non-associative fear at day 27, for a 21-day retention period.

3.1.1 Rat-Mouse Interaction

As with the initial pilot test of the RET, mice that were exposed to a live rat froze more times [Figure 4a; (M = 8.8125, SD = 2.971)] during the exposure than mice exposed to a stuffed control (M = 3.937, SD = 2.594), $t(30) = -4.94$, $p < .05$. This demonstrates that the presentation of a live rat to a mouse is sufficient to produce significant defensive behaviors during the exposure.

3.1.2 Associative fear memory

To determine whether associative fear memories are robust and long-lasting, mice were re-exposed to the fear context 21-days post-exposure. A two-tailed independent samples t-test determined that predator stressed mice still froze significantly more times (M=16.06, SD=0.6) than control mice (M =9.47, SD =0.55) [Figure 4b; $t(30) = 1.70$, $p < .05$], even on day 27. Thus, a five-minute protected exposure to a rat was sufficient to produce long-lasting associative fear memories in the mouse.

3.1.3 Non-associative fear memory

Mice were then tested for non-associative fear memories using the standard battery of anxiety tests (EPM, OF, L/D) and hyperarousal (acoustic startle). There were no differences between PS and control mice in the EPM on open arm duration, (PS M =18.31, SD = 10.41, Control M = 18.71, SD = 13.74, [Figure 4c; $t(27) = 0.32$, $p = 0.75$]; see Table 1 for full details. There were also no observed differences in time spent in center during the OF test (PS M = 56.03, SD = 25.42; Control M =49.19, SD =19.45, [Figure 4d; $t(29) = 0.84$, $p = 0.41$] (Table 2 for full details), nor were there any

differences in number of entries into the light side of the L/D box (PS M =6.94, SD =2.54, Control M =8.06, SD = 1.98, [Figure 4e; t(30) = 1.4, p =0.17] or acoustic startle max startle amplitude (PS M = 31898.38, SD = 14448.59, Control M = 29355.97, SD = 18169.09, [Figure 4f; t(30) = -.433, p = .66]. Taken together, these results indicate that the RET may not be an effective way to produce long-lasting non-associative fear memories in the mouse.

3.2 5-minute protected exposure of a mouse to a rat results in associative and non-associative fear memories.

As mentioned previously, a change in protocol was implemented in order to decrease the variability across rats seen in the pilot experiments and Experiment 1. These changes included calorie restriction to keep the rats active, as well as habituation and handling to decrease the level of anxiety in the rat, thus promoting more free-roaming investigative behaviour. To determine whether the revised RET paradigm would recapitulate symptoms of PTSD seen in humans, mice were exposed to a rat for 5 minutes and their subsequent associative and non-associative fear memories were assessed beginning 48 hours after the initial exposure.

3.2.1 Rat-mouse interaction

A two-tailed independent t-test demonstrated that during initial exposure, mice exposed to a live rat froze significantly more times (M = 7.75, SD = 2.67) than mice that were exposed to a stuffed control (M = 2.3125, SD = 1.25), [Figure 5a; t(30)= 8.272, p <

.05]. Consistent with Experiment 1, this suggests mice in the PS condition did indeed find the stimulus of the live rat to be a stressful condition.

3.2.2. Associative fear memory

Upon re-exposure to the fear context, predator-stressed animals (M = 18.81, SD = 10.87) froze significantly more than control animals, (M = 11.06, SD = 7.52) [Figure 5b; $t(30)= 2.35$, $p < .05$]. This is consistent with Experiment 1 and demonstrates that a five-minute protected exposure of a mouse to a rat is sufficient to produce associative fear memories in the mouse upon reexposure to the fear context.

3.2.3 Non-associative fear memory

To test non-associative fear memories, both groups of animals were run through the EPM, OF, LD and acoustic startle on days 9-12, respectively. The results determined that control mice spent more time in the open arms (duration in seconds) (M = 23.24, SD = 18.27) than PS mice (M = 11.38, SD = 11.42), [Figure 5c; $t(30) = 2.20$, $p < .05$], regardless of total distance travelled measured in cm (M = 1769.12, SD = 157.44; M =1874.27, SD = 141.30, $t(30) = 1.99$, $p < .05$); see Table 3 for full details. An independent t-test also determined PS mice made fewer entries into the light side of the L/D box (measured in seconds) (M=3.69, SD=0.16) than control mice (M=6.31, SD=0.22), [Figure 5d; $t(30) = 38.67$, $p < 0.01$], and PS mice also spent less time overall in the light side (M=31.94, SD =1.43) than control animals (M=55.81, SD=1.53), $t(30) = 45.57$, $p < 0.01$. No differences were observed between groups in the time in the center of the open field (PS M = 76.14, SD=38.05, Control M =75.59, SD=21.67, [Figure 5e; t(30)

= 0.051, p < .05)] (see table 4 for full details), or acoustic startle (PS M = 53.48, SD = 23.98, Control M = 44.93, SD = 14.74, [Figure 5f; t(30) = -1.25, p = > .05]. Overall, these data suggest that a five-minute protected exposure of a mouse to a rat produces both associative and non-associative fear memories in the mouse.

3.3 Female mice exposed to a live rat show no increases in associative or non-associative fear memories, regardless of length of exposure

Following the results from Experiment 1 and 2, we examined whether the RET would induce similar associative and non-associative fear in female mice. We were also interested in comparing our acute 5-minute exposure with a chronic 60-minute exposure. Due to mechanical issues, acoustic startle testing was not completed.

3.3.1 Rat-mouse interaction

The results showed no difference between PS and control mice during initial exposure. A two-way ANOVA found no main effect of group on amount of freezing [F(1, 60) = .641, p > .05] during the initial exposure. The results also showed no main effect of length of exposure on amount of freezing, [F(1,60) = .250, p > .05], or an interaction between condition and length of exposure, [F(1,60) = 1.96, p > .05]. The lack of defensive behaviors by the mouse during the initial exposure suggests that female mice (PS M = 4.59, SD = 2.68, Control M = 4.09, SD = 2.31) respond differently than male mice (PS M = 8.81, SD = 2.97, Control M = 3.93, SD = 2.59) to a rat, with both PS and control groups of female mice looking similar to the control males.

3.3.2 Associative fear memories

Given that PS mice did not show defensive behaviors during the initial rat exposure, it is not surprising that there were no difference between PS and control groups on amount of freezing during re-exposure. Results from a two-way ANOVA revealed no significant main effect of group on amount of freezing during re-exposure [$F(1, 60) = 2.23$, $p = .14$]. There was also no significant main effect of length of exposure on amount of freezing during re-exposure [Figure 6b; $F(1, 60) = .03$, $p = .86$]. Finally, there was no condition by duration effect, [$F(1, 60) = 1.07$, $p = .31$] These data suggest that with the present methodology, female mice exposed to a rat do not produce associative fear memories.

3.3.3 Non-associative fear memories

There was also no main effect found for group on open arm duration in the EPM, [$F(1, 53) = .59$, $p > .05$], nor was there a main effect for length of exposure [$F(1, 53) = .02$, $p > .05$]. Finally, no interaction was seen for condition by length of exposure [$F(1, 53)= 1.83$, $p > .05$] on open arm duration in the EPM. There was no observed main effect of group [Figure 6d; $F(1, 59) = .769$, $p > .05$], nor length of exposure [$F(1, 59) = 2.598$, $p > .05$] on center duration in the OF test. There was also no interaction seen between condition and length of exposure, [$F(1, 59) = .11$, $p > .05$]. Finally, there were no main group effects [Figure 6e; $F(1, 59) = 1.749$, $p > .05$] or length of exposure [$F(1,59) = .070$, $p > .05$] on entries into the light side in the LD box. There was no interaction seen between predator stress by length of exposure [$F(1, 59) = .002$, $p > .05$]. Overall, these data suggest female mice exposed to a rat do not develop non-associative fear memories.

3.3.4 Comparing the effects of Experiment 2 with Experiment 3

Experiment 3 indicated female mice do not respond to the RET stress in a similar manner to male mice. As both experiments used similar procedures, exploratory analysis was conducted to examine any differences between male and female mice on reexposure and ALB testing. Exploratory analysis confirmed female mice responded the same way during exposure and testing phase as male control mice. This demonstrates our exploratory statistics are consistent with the impression given by the data of the two experiments.

4.0 Discussion

Building on data compiled from several studies using the RET (Furuya-da-Cunha, de Souza, & Canto-de-Souza, 2016; Wall, Blanchard, Yang, & Blanchard, 2004; Yang et al., 2004) as well as pilot data from our lab, we sought to determine whether the RET could be used as an animal model of PTSD. Experiment 1 was successful in demonstrating that associative fear memories produced by the RET are enduring for 3-weeks post-exposure. After introducing calorie restriction and handling of the rats, the results from Experiment 2 illustrate a five-minute protected exposure of a mouse to a rat is sufficient to produce associative and non-associative fear memories at 48-hour post-exposure. The results from Experiment 3 demonstrate that female mice do not respond to the RET stress in the same way that male mice respond. Taken together, these results suggest that the RET recapitulates some of the symptoms of PTSD and is a procedure that may produce effects that are dependent on the sex of the stressed animal.

4.1 Examining the long-term behavioural effects of the RET

As PTSD symptoms must be present for at least one month before diagnosis, it is important that a complete model of PTSD represent the chronic nature of the illness. Previous research has demonstrated that the effects of predator stress can be seen up to four months post-exposure (Fifield, Hebert, Angel, Adamec, & Blundell, 2013; Zoldaz, Conrad, Fleshner, & Diamond, 2008; Zoldaz, Park Fleshner, & Diamond, 2015). In light of this, the goal of the first experiment was to determine if the RET produced long-lasting associative and non-associative fear memories. Consistent with the literature, PS mice exhibited associative fear memories, measured as increased freezing when re-exposed to the RET chamber, compared to controls 21 days post-stress. In contrast, PS mice did not show increased non-associative memories as measured using the EPM, OF, or LD compared to controls. Similarly, PS mice did not show hyperarousal compared to controls. This was somewhat surprising as pilot data from our lab has shown associative and non-associative fear memories persist for at least 1 week post-RET. One possibility is that associative fear may be a more robust form of memory than non-associative fear. This is consistent with other research on rodent models of PTSD that have found differences in the development of associative and non-associative fear memories. Indeed, Sauerhöfer et al. (2012) have found evidence that changes in associative and non-associative fear, as well as hyperarousal, can develop independently. Their study using a classic foot-shock paradigm determined that immediate shock presentation induces lower contextual fear and prevents the development of non-associative fear memories. These results may demonstrate that associative and non-associative fear memories depend on different neural substrates that may lead to associative fear memories being more robust

than non-associative fear memories, as our results have shown. Indeed, many treatments for human patients with PTSD rely on extinction therapy, or re-exposure to the fear context (or imagining the fear context) in order to diminish general anxiety and other behavioural outcomes of the traumatic event (Marin et al., 2016; Pradhan, Gray, Parikh, Akkireddi, & Pumariega, 2015). It remains to be seen whether the procedural changes implemented in Experiment 2, that successfully produced non-associative fear memories at 3-5 days post-exposure, would be sufficient to also produce changes in non-associative fear memories that last for 3 weeks.

4.2 Procedural changes to the RET paradigm are sufficient to produce both associative and non-associative fear memories in the mouse

After reviewing footage from both Experiment 1 as well as the pilot data, it appeared the rats were often too startled themselves to move for the duration of the exposure. We implemented a handling and habituation procedure in order to reduce anxiety in the rats and ensure they would not freeze during the exposure to the mouse. We also started a longer calorie-restriction paradigm in the rats. This was done after observation in the lab that hungry rats are much more active and travel further distances than satiated rats. As we were not able to obtain a site licence for the use of amphetamine, it was not possible to inject rats with amphetamine as was done in the initial Yang et al. (2004) study. Thus, calorie restriction offered a novel way we could ensure our rats would be actively investigating the mice upon exposure.

The results from Experiment 2 suggest that the RET is sufficient to produce both associative and non-associative fear memories in the mouse when the interval between exposure and testing is short. This result is in line with a large body of research which suggests predator-stress produces both associative and non-associative fear memories in a prey animal upon exposure to a predator (Adamec, Kent, Anisman, Shallow, & Merali, 1998; Fifield et al., 2014). The results indicated a strong re-exposure effect, indicating the animal remembered the aversive context from two days prior. There was also evidence of non-associative fear, measured by ALB tests. PS mice spent significantly more time in the closed arms of the EPM and had a smaller open arm ratio compared to control mice. Additionally, PS mice also spent significantly less time in the light side of the LD box, and made fewer entries into the light side. These results demonstrate an anxiety phenotype produced in the mice which were exposed to a rat. Importantly, a manipulation check done during data analysis revealed a strong freezing effect of mice in the PS condition during the initial exposure. This defensive behaviour indicates the mice felt threatened during the exposure to the rat, confirming that a rat stimulus is indeed aversive. The results from this study confirm that the RET is successful in producing both associative and non-associative fear memories from an acute five-minute protected exposure lasting at least five days post-stressor.

4.3 Examining the effects of the RET in female mice

The results of Experiment 3 demonstrate that female mice do not show differences in associative or non-associative fear memories in response to the RET. There were no significant differences seen across re-exposure, EPM, OF, or LD testing. Importantly,

during the initial exposure, PS mice did not freeze more than controls. One possible explanation for the lack of fear memories is that the females did not see the rat as a fearful stimulus. To check this, we examined freezing behavior of the mouse during the initial exposure. Consistent with a lack of fear, female mice exposed to the rat did not show increased freezing compared to controls. Furthermore, when compared to PS males, PS females froze less during the initial exposures, as well as during the re-exposure to the RET context. This likely explains why there were no differences in subsequent non-associative fear in female mice (regardless of exposure duration). It should be noted that various studies of sex differences on stress and memory have yielded inconclusive results as well, with some studies finding females are more responsive to stress, yet others finding the opposite effect (Andreano & Cahill, 2009; Bangasser & Shors, 2007; Conrad et al., 2004; Kuhlmann et al., 2005; Maeng et al., 2010; Merz et al., 2010; Park et al., 2008; Schoofs & Wolf, 2009; Shors, 2002; Shors et al., 2007; Waddell et al., 2008; Wolf et al., 2001; Wood & Shors, 1998). Many researchers have proposed that this may be a function of different hormone levels in females versus males (Glover et al., 2012; Merz et al., 2012; Milad et al., 2009a, 2010; Zeidan et al., 2011), or a similar stressor having a significantly different impact across sexes, an effect that can be seen in humans (Zolaz & Diamond, 2013). As our study did not examine the effects of the estrous cycle across weeks of testing, it is difficult to say whether this had an impact on our results.

To our knowledge, there has not yet been a study on the effect that a chronic rat exposure has on the mouse in the RET. Our study determined that lengthening the exposure to 60 minutes did not yield a more significant anxiety effect in female mice.

However, the effect of lengthening the exposure time has not been studied in male mice, which seem to more reliably show stress effects following the RET. It is of interest to note that a longer exposure may have actually had a habituation effect on PS mice (though not statistically significant, the data show this effect is trending towards significance). Rat activity significantly decreases following the initial exposure, and in most trials the rat fell asleep before 60 minutes had elapsed. In response, the mice appeared to acclimate to the situation and engaged in grooming behavior, an indication they did not feel threatened.

4.4 Limitations and Considerations

These results seem to indicate that more work needs to be done to thoroughly map a procedure for running the RET. Experiment 1 produced strong associative fear memories in the PS male mice 21-days post exposure. Similarly, Experiment 2 demonstrated consistent strong associative and non-associative fear memories in the PS mice 48 hours after initial exposure. The lack of differences found between female PS and female control mice indicates appropriate stress parameters from males cannot be applied to females. This is an area which warrants further investigation in the future. In addition, the male data in conjunction with the female data suggest that behavior during the exposure phase is predictive of the long-term effects of the stressor. Males showed freezing during the exposure phase and subsequently showed freezing during re-exposure, while the females did not show freezing in either instance. As many authors have described previously, minor changes in lighting, room temperature, or distance from the testing apparatus to the recording camera can all have effects on an animal's behaviour

(Ding et al, 2015; Thompson, Grabowski-Boase, & Tarantino, 2015). This may give us some idea why the results from these experiments were variable.

Some researchers have argued that many tests used on mice were originally developed for rats, and thus are not reliable in evaluating anxiety in the mouse. One such test is the Open Field assay, developed in 1932 (Hall & Ballechey, 1932). This measure was designed based on the innate behavior of a rodent to gravitate towards enclosed spaces rather than open ones and its instinct to stay close to the edges of a perimeter in order to avoid predation (Barnett, 1963; Grossen and Kelley, 1972; Postet al., 2011). Though this measure has been reliably substantiated in rats for several decades (Bruhwyler, 1990; Gentsch et al., 1987; Nichols and Schreur, 1987), its use for measuring anxiety-like behavior in mice remains unclear. Many studies using typical anxiolytic drugs on mice fail to see any change in the center time in the OF test (Birkett et al., 2011; Crabbe et al., 1998; Crawley, 1981; Fahey et al., 1999; Heredia et al., 2013a; Lalonde and Strazielle, 2010; Lopez et al., 1988; Novas et al., 1988; Seredenin et al., 1990). Indeed, Thompson, Grabowski-Boase, and Tarantino (2015) injected C57BL/6 mice with increasing doses of prototypical anxiolytics (chlordiazepoxide, diazepam, or buspirone) and failed to see any change in center time during the OF test. These data may explain why there was no effect in all three experiments on the OF measure.

There have also been studies which call into question the strain effect of mice used in these studies. Many researchers have reported that C57BL/6 mice have a lower baseline level of anxiety than other strains (Carola et al., 2002; Crawley, 2007; Crawley and Davis, 1982; Griebel et al., 2000; Lepicard et al., 2000; Ohl et al., 2001; Rogers et al.,

1999; Tarantino et al., 2000), which may make it difficult to detect any changes in ALB. If C57BL/6 mice have lower baseline anxiety than other strains, it may be difficult to compare across experiments using different strains of mice. The original RET used BALB-C (high anxiety) mice, while our experiments used male and female C57BL/6 (low anxiety) mice. This strain difference seen in previous studies may explain why the mice in our experiments were less anxious than those in the original study.

Future studies using the RET should also consider the impact of avoidance flight behaviour. If an escape route is available to a mouse upon exposure to a predator, it will typically use the escape route to avoid the interaction altogether (Yang et al., 2004). If flight is not an option (no escape route available), the animal will then freeze in defence (Kavaliers & Choleris, 2001). The reason we may not have seen high levels of freezing in our experiments is the fact that our RET box has ample room for the mouse to escape contact with the rat. While scoring the videos, observers noticed that the mouse would often run to the opposite corner of the box in an attempt to avoid contact with the rat. If the rat approached the corner they had run to, they then fled to the opposite corner. This flight behavior masks any defensive freezing, and may explain why there were low levels of freezing overall across these experiments. Future studies using this particular RET box may do well to score avoidance behaviors such as flight in addition to freezing in order to truly capture the full scope of stress in the mouse.

4.5 General Conclusions

Collectively, the evidence presented here agrees with previous studies done on both the RET as well as other predator-stress paradigms. The results from Experiment 1 agree with previous studies which have seen long-lasting associative fear for up to 3 weeks post-exposure (Adamec at al., 1993). As well, results from Experiment 2 conclusively demonstrate that the RET is capable of producing both associative and non-associative fear memories in an acute manner in male mice. Experiment 3 has shown that female mice do not respond to the RET stress in a similar manner to male mice – which may be useful to other researchers looking to further investigate the use of the RET. To our knowledge, this research is also the first to examine the effect of an acute (five minute) versus a chronic (60 minute) exposure, with results indicating there is no potentiating effect of a longer exposure on associative and non-associative fear memories. However, this manipulation should be studied in male mice in the future. These results may be of use to researchers who are investigating a rodent model of PTSD that is easier to access than other models and has the potential to recapitulate the symptoms seen in PTSD.

References

Adamec, R., Fougere, D., & Risbrough, V. (2010). CRF receptor blockade prevents initiation and consolidation of stress effects on affect in the predator stress model of PTSD. *International Journal of Neuropsychopharmacology, 13*(6), 747-757

Adamec, R., Kent, P., Anisman, H., Shallow, T., Merali, Z. (1998). Neural plasticity, neuropeptides and anxiety in animals--implications for understanding and treating affective disorder following traumatic stress in humans. *Neuroscience and Biobehavioral Reviews, 23*(2), 301-318.

Adamec, R. E., & Shallow, T. (1993). Lasting effects on rodent anxiety of a single exposure to a cat. *Physiology and Behavior, 54*(1), 101-109.

Adamec, R., Toth, M., Haller, J., Halasz, J., Blundell, J. (2012). A comparison of activation patterns of cells in selected prefrontal cortical and amygdala areas of rats which are more or less anxious in response to predator exposure or submersion stress. *Physiology & Behavior, 105*(3), 628-638.

Adamec, R., Walling, S., Burton, P. (2004). Long-lasting, selective, anxiogenic effects of feline predator stress in mice. *Physiology & Behavior, 83*(3), 401-410.

Amaral, V., Santos Gomes, R., Nunes-de-Souza, R. (2010) Increased corticosterone levels in mice subjected to the rat exposure test. *Hormones and Behavior, 57*, 128–133.

Andreano J.M., Cahill L. (2009). Sex influences on the neurobiology of learning and memory. *Learning and Memory, 16,* 248-266.

Apfelbach, R., Blanchard, C., Blanchard, R., Hayes R., McGregor, I. (2005) The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neuroscience & Biobehavioral Reviews, 29*, 1123–1144.

Bangasser D.A., and Shors T.J. (2007). The hippocampus is necessary for enhancements and impairments of learning following stress. *Nature Neuroscience, 10,* 1401-1403.

Blanchard, D. C., Griebel, G., Blanchard, R. J. (2003). Conditioning and residual emotionality effects of predator stimuli: Some reflections on stress and emotion. *Progress in Neuro- Psychopharmacology & Biological Psychiatry, 27*(8), 1177-1185.

Blanchard, R., Hebert, M., Ferrari, P., Palanza, R., Figueira, D., Blanchard, C., Parmigiani, S. (1998). Defensive behaviors in wild and laboratory (Swiss) mice: the mouse defense test battery *Physiolgy & Behavior, 65,* 201–209.

Bulos, E. M., Pobbe, R. H., Zangrossi, H. J. (2015). Behavioral consequences of predator stress in the rat elevated T-maze. *Physiology & Behavior, 146,* 28-35.

Burgado, J., Harrell, C. S., Eacret, D., Reddy, R., Barnum, C. J., Tansey, M. G., Neigh, G. N. (2014). Two weeks of predatory stress induces anxiety-like behavior with

co-morbid depressive-like behavior in adult male mice. *Behavioural Brain Research, 275,* 120-125.

Campos, K., Amaral, V., Rico, J., Miguel, T., Nunes, R., & Nunes-de-Souza, R. (2013). Ethopharmacological evaluation of the rat exposure test: a prey-predator interaction test. *Behavioral Brain Research, 240,* 160–170.

Careaga, M. L., Girardi, C. N., & Suchecki, D. (2016). Understanding posttraumatic stress disorder through fear conditioning, extinction and reconsolidation. Neuroscience and Biobehavioral Reviews, 71, 48-57.

Chen, L., Shen, B., Liu, D., & Li, S. (2014). The effects of early-life predator stress on anxiety- and depression-like behaviors of adult rats. *Neural Plasticity,* 201-204.

Clay, R., Hebert, M., Gill, G., Stapleton, L. A., Pridham, A., Coady, M., & Blundell, J. (2011). Glucocorticoids are required for extinction of predator stress-induced hyperarousal. *Neurobiology of Learning And Memory, 96(2),* 367-377.

Cohen, H., Kozlovsky, N., Matar, M. A., Zohar, J., Kaplan, Z. (2014). Distinctive hippocampal and amygdalar cytoarchitectural changes underlie specific patterns of behavioral disruption following stress exposure in an animal model of PTSD. *European Neuropsychopharmacology, 24*(12), 1925-1944.

Conrad C.D., Jackson J.L., Wieczorek L., Baran S.E., Harman J.S., Wright R.L., & Korol D.L. (2004). Acute stress impairs spatial memory in male but not female

rats: influence of estrous cycle. *Pharmacology, Biochemitry, and Behavior, 78,* 569-579.

Costanzi, M., Cannas, S., Saraulli, D., Rossi-Arnaud, C., Cestari, V. (2011). Extinction after retrieval: Effects on the associative and nonassociative components of remote contextual fear memory. *Learning & Memory, 18*(8), 508-518.

Daeng, B. H., Wardhana, A. W., Widodo, A., Sujuti, H., Mintaroem, K., Widjajanto, E. (2015). Plasma corticotropine releasing hormone (CRH) level difference between Wistarrats exposed to acute stress due to predator and to the psychological stress device. *ASEAN Journal of Psychiatry, 16*(2), 193-202.

Ding, X., Zhao, Y., Liu, S., Zhao, T., Zhu, L., Suo, W. Z., & Fan, M. (2015). An improved elevated platform for simultaneously assessing rodent locomotor activity and anxiety. *CNS Neuroscience & Therapeutics, 21*(6), 536-538.

Fenchel, D., Levkovitz, Y., Vainer, E., Kaplan, Z., Zohar, J., & Cohen, H. (2015). Beyond the HPA-axis: The role of the gonadal steroid hormone receptors in modulating stress-related responses in an animal model of PTSD. *European Neuropsychopharmacology, 25*(6), 944-957.

Fifield, K., Hebert, M., Angel, R., Adamec, R., & Blundell, J. (2013). Inhibition of mTOR kinase via rapamycin blocks persistent predator stress-induced hyperarousal. *Behavioural Brain Research, 256,* 457-463.

Fogaça, M. V., Reis, F. V., Campos, A. C., & Guimarães, F. S. (2014). Effects of intra-prelimbic prefrontal cortex injection of cannabidiol on anxiety-like behavior: Involvement of 5HT1A receptors and previous stressful experience. *European Neuropsychopharmacology, 24*(3), 410-419.

Furuya-da-Cunha, E. M., de Souza, R. R., & Canto-de-Souza, A. (2016). Rat exposure in mice with neuropathic pain induces fear and antinociception that is not reversed by 5-HT2C receptor activation in the dorsal periaqueductal gray. *Behavioural Brain Research, 307,* 250-257.

Gan, P., Ding, Z., Gan, C., Mao, R., Zhou, H., Xu, L., & Zhou, Q. (2016).Corticosterone regulates fear memory via Rac1 activity in the hippocampus. *Psychoneuroendocrinology, 71,* 86-93.

Genovese, R. F., Johnson, C. C., Tobin, C. A., & Gauchan, S. (2014). Multiple presentations reduce the behavioral impact of protected predator exposure in rats. *Behavioural Processes, 108,* 105-109.

Glover E.M., Jovanovic T., Mercer K.B., Kerley K., Bradley B., & Ressler K.J., Norrholm S.D. (2012). Estrogen levels are associated with extinction deficits in women with posttraumatic stress disorder. *Biological Psychiatry, 72,* 19-24.

Hall, C., & Ballechey, E. (1932). A study of the rat's behavior in a field: a contribution to method in comparative psychology. *University of California Publications in Psychology, 6,* 1–12.

Harvey, B. H., Brand, L., Jeeva, Z., & Stein, D. J. (2006). Cortical/hippocampal monoamines, HPA-axis changes and aversive behavior following stress and restress in an animal model of post-traumatic stress disorder. *Physiology & Behavior, 87*(5), 881-890.

Hoffman, A. N., Lorson, N. G., Sanabria, F., Foster Olive, M., & Conrad, C. D. (2014). Chronic stress disrupts fear extinction and enhances amygdala and hippocampal Fos expression in an animal model of post-traumatic stress disorder. *Neurobiology of Learning and Memory, 112,* 139-147.

Kao, C., He, Z., Zannas, A. S., Hahn, O., Kühne, C., Reichel, J. M., Turck, & C. W. (2016). Fluoxetine treatment prevents the inflammatory response in a mouse model of posttraumatic stress disorder. *Journal of Psychiatric Research, 76,* 74-83.

Kavaliers, M., & Choleris, E. (2001). Antipredator responses and defensive behavior: Ecological and ethological approaches for the neurosciences. *Neuroscience and Biobehavioral Reviews, 25*(7-8), 577-586.

Kuhlmann S., Piel M., & Wolf O.T. (2005). Impaired memory retrieval after psychosocial stress in healthy young men. *Journal of Neuroscience, 25,* 2977-2982.

Lima, M. X., Baldo, M. C., & Canteras, N. S. (2016). A role for the anteromedial thalamic nucleus in the acquisition of contextual fear memory to predatory threats. *Brain Structure & Function,* doi:10.1007/s00429-016-1204-2

Liu, M., Xu, F., Tao, T., Song, D., Li, D., Li, Y., & Liu, X. (2016). Molecular mechanisms of stress-induced myocardial injury in a rat model simulating posttraumatic stress disorder. *Psychosomatic Medicine, 78*(8), 888-895.

Mac Callum, P. E., Hebert, M., Adamec, R. E., & Blundell, J. (2014). Systemic inhibition of mTOR kinase via rapamycin disrupts consolidation and reconsolidation of auditory fear memory. *Neurobiology of Learning and Memory, 112*, 176-185.

Maeng L.Y., Waddell J., & Shors T.J. (2010). The prefrontal cortex communicates with the amygdala to impair learning after acute stress in females but not in males. *Journal of Neuroscience, 30*, 16188-16196.

Manion, S. T., Figueiredo, T. H., Aroniadou-Anderjaska, V., Braga, & M. M. (2010). Electroconvulsive shocks exacerbate the heightened acoustic startle response in stressed rats. *Behavioral Neuroscience, 124*(1), 170-174.

Manjoch, H., Vainer, E., Matar, M., Ifergane, G., Zohar, J., Kaplan, Z., & Cohen, H. (2016). Predator-scent stress, ethanol consumption and the opioid system in an animal model of PTSD. *Behavioural Brain Research, 306*, 91-105.

Marin, M., Song, H., VanElzakker, M. B., Staples-Bradley, L. K., Linnman, C., Pace-Schott, E.F., & Milad, M. R. (2016). Association of resting metabolism in the fear neural network with extinction recall activations and clinical measures in trauma -exposed individuals. *The American Journal of Psychiatry, 173*(9), 930-938.

Matar, M. A., Zohar, J., Kaplan, Z., & Cohen, H. (2009). Alprazolam treatment immediately after stress exposure interferes with the normal HPA-stress response and increases vulnerability to subsequent stress in an animal model of PTSD. *European Neuropsychopharmacology, 19*(4), 283-295.

Mayer, T. A., Matar, M. A., Kaplan, Z., Zohar, J., & Cohen, H. (2014). Blunting of the HPA-axis underlies the lack of preventive efficacy of early post-stressor single-dose Delta-9-tetrahydrocannabinol (THC). *Pharmacology, Biochemistry and Behavior, 122,* 307-318.

McGregor, I. S., Schrama, L., Ambermoon, P., & Dielenberg, R. A. (2002). Not all 'predator odours' are equal: Cat odour but not 2,4,5 trimethylthiazoline (TMT; fox odour) elicits specific defensive behaviours in rats. *Behavioural Brain Research, 129*(1-2), 1-16.

Merz C.J., Tabbert K., Schweckendiek J., Klucken T., Vaitl D., Stark R., & Wolf O.T. (2010). Investigating the impact of sex and cortisol on implicit fear conditioning with fMRI. *Psychoneuroendocrinology, 35*, 33-46.

Merz C.J., Tabbert K., Schweckendiek J., Klucken T., Vaitl D., Stark R., & Wolf O.T. (2012). Neuronal correlates of extinction learning are modulated by sex hormones. *Social Cognitive and Affective Neuroscience, 7,* 819-830.

Milad M.R., Igoe S.A., Lebron-Milad K., & Novales J.E. (2009). Estrous cycle phase and gonadal hormones influence conditioned fear extinction. *Neuroscience, 164,* 887-895.

Milad M.R., Zeidan M.A., Contero A., Pitman R.K., Klibanski A., Rauch S.L., & Goldstein J.M. (2010). The influence of gonadal hormones on conditioned fear extinction in healthy humans. *Neuroscience, 168,* 652-658.

Olford, Adamec, & Blundell. (2009). Using Predator Scent Stress to Develop a Preclinical Model of Posttraumatic Stress Disorder (Unpublished Honors Thesis). Memorial University of Newfoundland, Newfoundland and Labrador, Canada.

Olson, V. G., Rockett, H. R., Reh, R. K., Redila, V. A., Tran, P. M., Venkov, H. A., & Raskind, M. A. (2011). The role of norepinephrine in differential response to stress in an animal model of posttraumatic stress disorder. *Biological Psychiatry, 70*(5), 441-448.

Park C.R., Zoladz P.R., Conrad C.D., Fleshner M., & Diamond D.M. (2008). Acute predator stress impairs the consolidation and retrieval of hippocampus-dependent memory in male and female rats. *Learning and Memory, 15,* 271-280.

Pradhan, B., Gray, R., Parikh, T., Akkireddi, P., & Pumariega, A. (2015). Trauma interventions using mindfulness based extinction and reconsolidation (TIMBER©) as monotherapy for chronic PTSD: A pilot study. *Adolescent Psychiatry, 5*(2), 125-131.

Rajbhandari, A. K., Baldo, B. A., & Bakshi, V. P. (2015). Predator stress-induced CRF release causes enduring sensitization of basolateral amygdala norepinephrine systems that promote PTSD-like startle abnormalities. *The Journal of Neuroscience, 35*(42), 14270-14285.

Rasmusson, A. M., & Charney, D. S. (1997). Animal models of relevance to PTSD. In R. Yehuda, A. C. McFarlane, R. Yehuda, A. C. McFarlane (Eds.). Psychobiology of Posttraumatic Stress Sisorder, 332-351.

Reznikov, R., Binko, M., Nobrega, J. N., & Hamani, C. (2016). Deep brain stimulation in animalmodels of fear, anxiety, and posttraumatic stress disorder. *Neuropsychopharmacology, 41*(12), 2810-2817.

Reznikov, R., Diwan, M., Nobrega, J. N., & Hamani, C. (2015). Towards a better preclinical model of PTSD: Characterizing animals with weak extinction, maladaptive stress responses and low plasma corticosterone. *Journal of Psychiatric Research, 611*, 58-165.

Rorabaugh, B. R., Krivenko, A., Eisenmann, E. D., Bui, A. D., Seeley, S., Fry, M. E., & Zoladz, P. R. (2015). Sex-dependent effects of chronic psychosocial stress on myocardial sensitivity to ischemic injury. *Stress: The International Journal on The Biology of Stress, 18*(6), 645-653.

Sauerhöfer, E., Pamplona, F. A., Bedenk, B., Moll, G. H., Dawirs, R. R., von Hörsten, S., & Golub, Y. (2012). Generalization of contextual fear depends on associative rather than non-associative memory components. *Behavioural Brain Research, 233*(2), 483-493.

Schoofs D., & Wolf O.T. (2009). Stress and memory retrieval in women: no strong impairing effect during the luteal phase. *Behavioral Neuroscience, 123*, 547-554.

Shors T.J. (2002). Opposite effects of stressful experience on memory formation in males versus females. *Dialogues of Clinical Neuroscience, 4,* 139-147.

Shors T.J., Mathew J., Sisti H.M., Edgecomb C., Beckoff S., & Dalla C. (2007). Neurogenesis and helplessness are mediated by controllability in males but not in females. *Biological Psychiatry, 62,* 487-495.

Toth, M., Flandreau, E. I., Deslauriers, J., Geyer, M. A., Mansuy, I. M., Pich, E. M., & Risbrough, V. B. (2016). Overexpression of forebrain CRH during early life increases trauma susceptibility in adulthood. *Neuropsychopharmacology*, *41*(6), 1681-1690.

Waddell J., Bangasser D.A., & Shors T.J. (2008). The basolateral nucleus of the amygdala is necessary to induce the opposing effects of stressful experience on learning in males and females. *Journal of Neuroscience, 28,* 5290-5294.

Wall, P. M., Blanchard, R. J., Yang, M., & Blanchard, D. C. (2004). Differential effects of infralimbic vs. ventromedial orbital PFC lidocaine infusions in CD-1 mice on defensive responding in the mouse defense test battery and rat exposure test. *Brain Research*, *1020*(1-2), 73-85.

Wernecke, K. E. A., D. Vincenz, S. Storsberg, W. D'Hanis, J. Goldschmidt, M, & Fent. 2015. Fox urine exposure induces avoidance behavior in rats and activates the amygdalar olfactory cortex. *Behavioural Brain Research 279,* 76-81.

Wolf O.T., Schommer N.C., Hellhammer D.H., McEwen B.S., & Kirschbaum C. (2001). The relationship between stress induced cortisol levels and memory differs between men and women. *Psychoneuroendocrinology, 26,* 11-720.

Wolf, E. J., Sadeh, N., Leritz, E. C., Logue, M. W., Stoop, T. B., McGlinchey, R., & Miller, M. W. (2016). Posttraumatic stress disorder as a catalyst for the association between metabolic syndrome and reduced cortical thickness. *Biological Psychiatry*, *80*(5), 363-371.

Wood G.E., & Shors T.J. (1998). Stress facilitates classical conditioning in males, but impairs classical conditioning in females through activational effects of ovarian hormones. *Proceedings of the National Academy of Sciences, 95*, 4066-4071.

Yehuda, &Antelman. (1993). Criteria for rationally evaluating animal models of posttraumatic stress disorder. *Biological Psychiatry, 33,* 479-486.

Zeidan M.A., Igoe S.A., Linnman C., Vitalo A., Levine J.B., Klibanski A., Goldstein J.M., & Milad M.R. (2011). Estradiol modulates medial prefrontal cortex and amygdala activity during fear extinction in women and female rats. *Biological Psychiatry*, *70*, 920-927.

Zohar, J., Matar, M. A., Ifergane, G., Kaplan, Z., & Cohen, H. (2008). Brief post-stressor treatment with pregabalin in an animal model for PTSD: Short-term anxiolytic effects without long-term anxiogenic effect. European Neuropsychopharmacology, 18(9), 653-666.

Zoladz, P. R., Conrad, C. D., Fleshner, M., & Diamond, D. M. (2008). Acute episodes of predator exposure in conjunction with chronic social instability as an animal model of posttraumatic stress disorder. *Stress, 11*(4), 259-281.

Zoladz, P. R., Park, C. R., Fleshner, M., & Diamond, D. M. (2015). Psychosocial predator-based animal model of PTSD produces physiological and behavioral sequelae and a traumatic memory four months following stress onset. *Physiology & Behavior, 147,* 183-192.

Figure Captions

Fig 1. The timeline for Experiment 1.

Fig 2. The timeline for Experiment 2.

Fig 3. The timeline for Experiment 3.

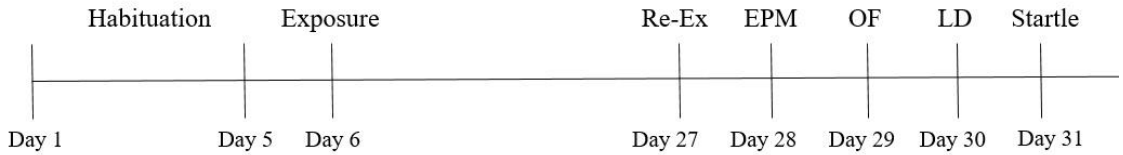
Fig 4a-f. The presentation of a live rat to a mouse is sufficient in producing significant anxiety during the exposure. As with the initial pilot test of the RET, mice that were exposed to a live rat froze significantly more (M = 8.8125, SD = 2.971) during the exposure than mice exposed to a stuffed control (M = 3.937, SD = 2.594), t(30) = -4.94, p < .05. A five-minute protected exposure of a rat to a mouse is sufficient to induce long-lasting associative fear memories within the mouse. A two-tailed independent samples t-test determined that predator stressed mice still froze significantly more (M=16.06, SD=0.6) than control mice (M=9.47, SD =0.55), t(30) = 1.70, p <.05 even on day 27. PS mice were no different than control mice on time spent in the closed arms during the EPM test. An independent samples t-test revealed PS mice were not statistically different in time spent in the closed arms than control mice, (PS M =207.84, SD=24.67, Control M = 205.04, SD =21.58, t(27) = 0.32, p = 0.75. PS and control mice showed no difference in center duration during the OF test. An independent samples t-test demonstrated no observed differences in time spent in center during the open field test (PS M = 56.03, SD = 25.42; Control M =49.19, SD =19.45, t(29) = 0.84, p =0.4. No differences were seen between PS and control mice on number of entries into the light side of the LD box. An independent samples t-test revealed PS mice and control mice did not differ on number of entries made into the light side of the LD box, (PS M =6.94, SD =2.54, Control M =8.06, SD = 1.98, t(30) = 1.4, p =0.17.

Figure 5a-f. A 5-minute protected exposure of a rat to a mouse is sufficient to produce defensive behaviours in the mouse during the exposure. A two-tailed independent t-test demonstrated that during initial exposure, mice exposed to a live rat froze significantly more (M = 7.75, SD = 2.67) than mice that were exposed to a stuffed control (M = 2.3125, SD = 1.25), t(30)= 8.272, p < .05. A 5-minute protected exposure of a rat to a mouse is sufficient in producing associative fear memories in the mouse as measured by freezing at 48 hours post-exposure. Upon reexposure to the fear context, predator-stressed animals froze significantly more (M = 11.06, SD = 7.52) than control animals 18.81, SD = 10.87), t(30)= 2.35, p <.05. Control mice spent more time in the open arms (M = 23.24, SD = 18.27) than PS mice (M = 11.38, SD = 11.42), t(30) = 2.20, p = .036, regardless of total distance travelled. PS mice make fewer entries into the L side of the LD box following the RET. An independent t-test determined PS mice made fewer entries into the light side of the L/D box (M=3.69, SD=0.16) than control mice (M=6.31, SD=0.22), t(30)

= 38.67, $p < 0.0001$. No differences were observed in the OF test following the RET. PS mice and control mice did not significantly differ on time spent in the center of the OF test following exposure to a rat, (PS M = 76.14, SD=38.05, Control M =75.59, SD=21.67, $t(30) = 0.051$, $p = 0.96$).

Figure 6a-e. A five-minute protected exposure of a rat to a female mouse does not produce defensive behaviours during the exposure, regardless of length of exposure. Results demonstrated mice in the PS condition (M = 4.593, SD = 2.696) versus control condition (M = 4.093, SD = 2.305) did not significantly differ on freezing behaviour during the initial exposure, indicating they did not view the rat as a threatening stimulus. The RET does not produce non-associative fear memories in the female mouse, regardless of length of exposure. Results from a one-way ANOVA revealed no significant main effect of PS on amount of freezing during reexposure [$F(3, 60) = 2.120$, $p = .151$]. There was also no significant main effect or interaction effect of length of exposure on amount of freezing during reexposure [$F(3, 60) = .012$, $p = .914$]. No differences were observed in EPM open arm duration between PS and control animals [$F(3, 53) = .311$, $p = .579$]. There was no observed main effect or interaction for either predator stress [$F(3, 59) = .769$, $p = .384$] nor length of exposure [$F(3, 59) = 2.598$, $p = .112$] on center duration in the OF test. No main effect was observed for predator stress [$F(3, 59) = 1.749$, $p = .191$] or length of exposure [$F(3,59) = .070$, $p = .792$] on entries into the light side in the LD box.

Figure 1:



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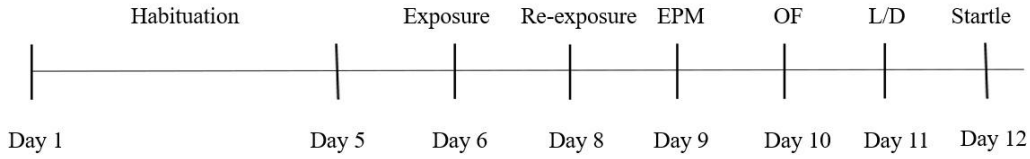

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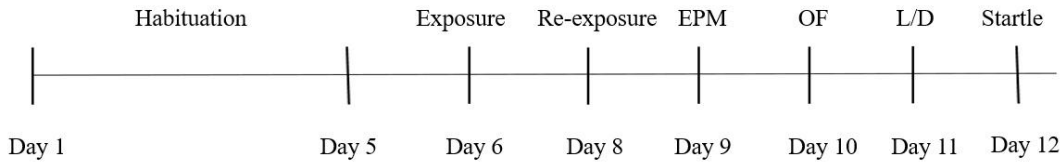

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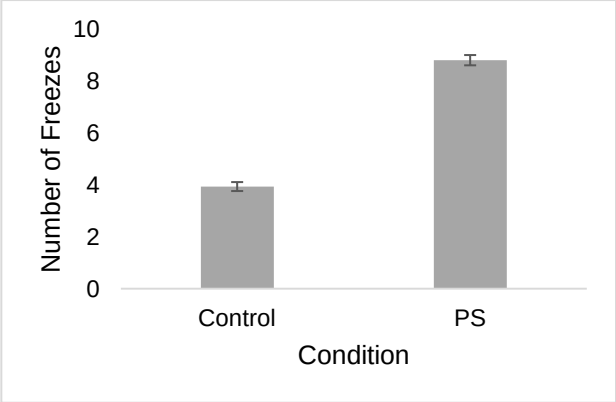

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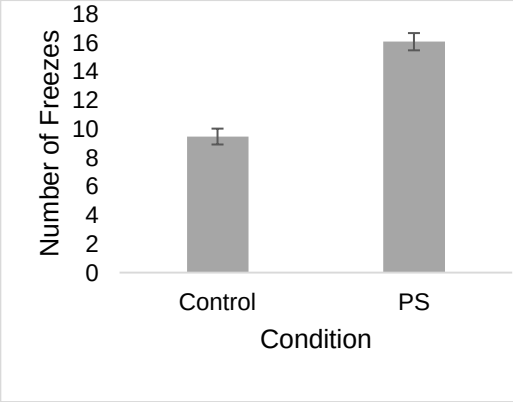

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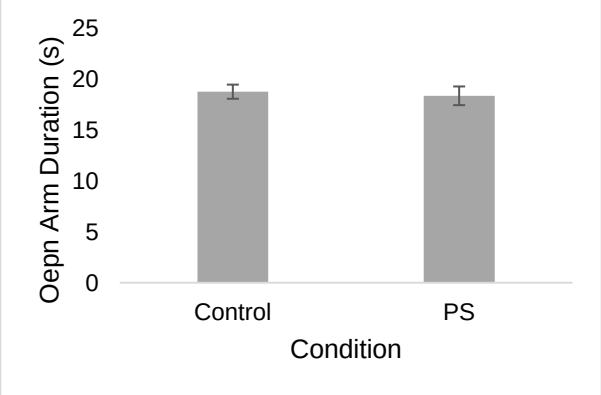

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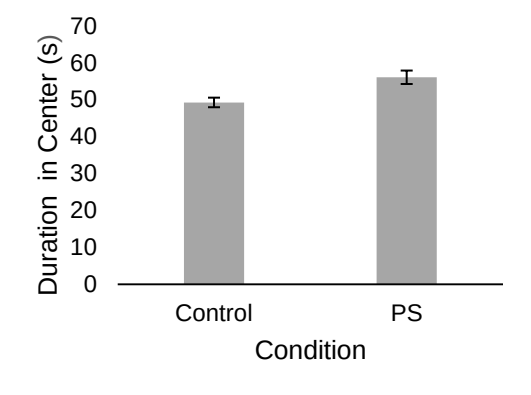

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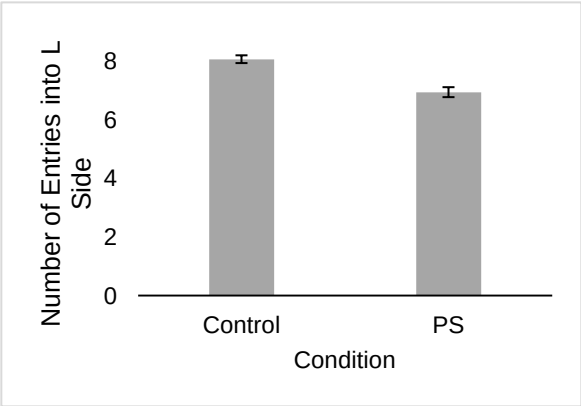

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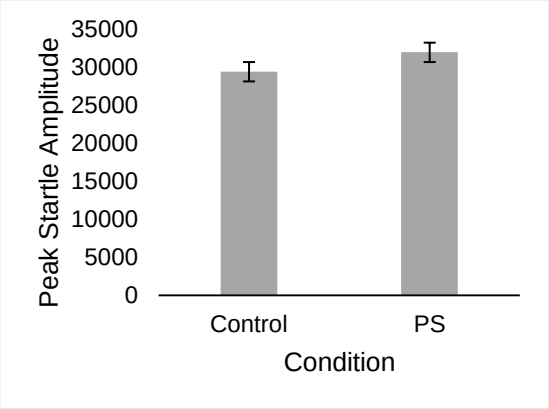

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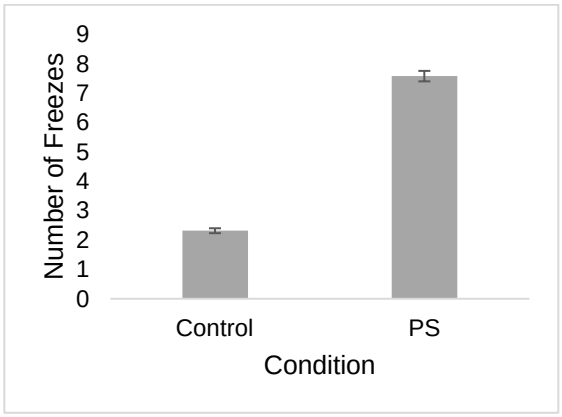

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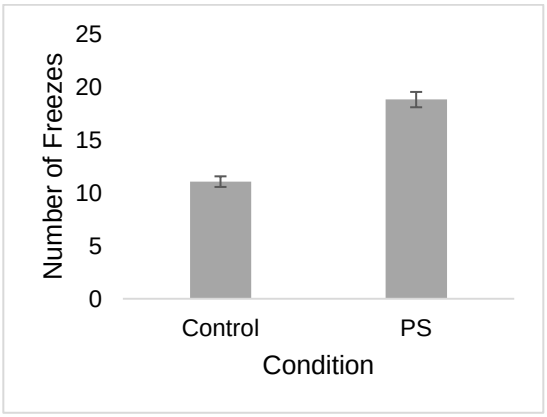

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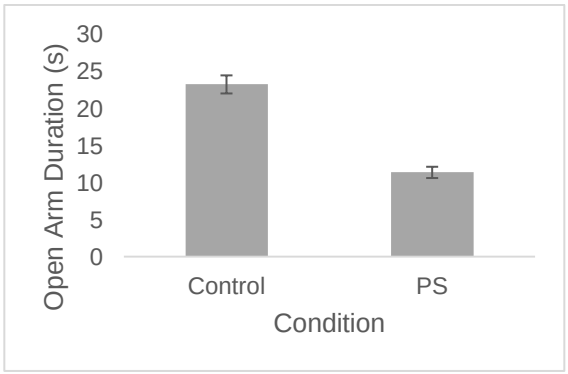

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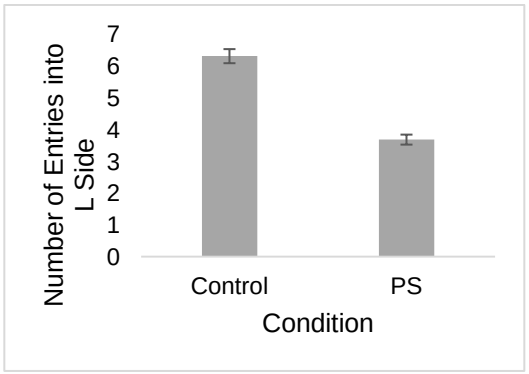

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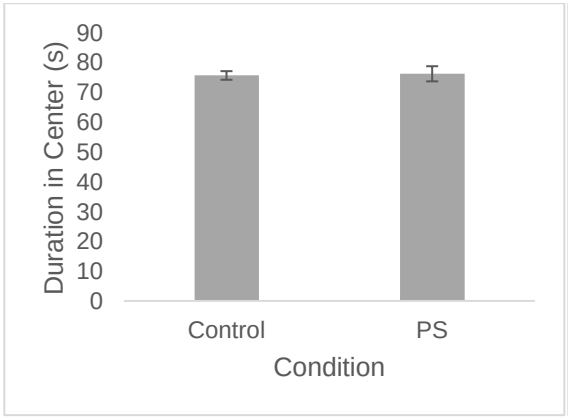

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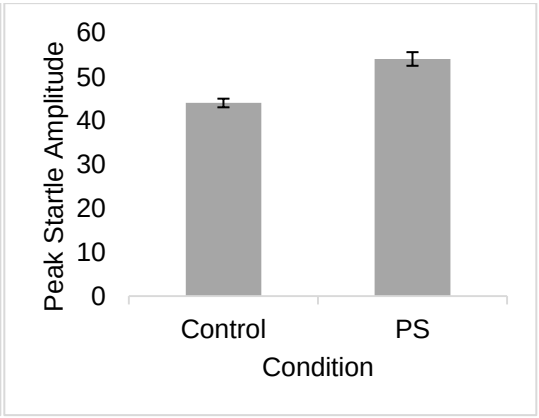

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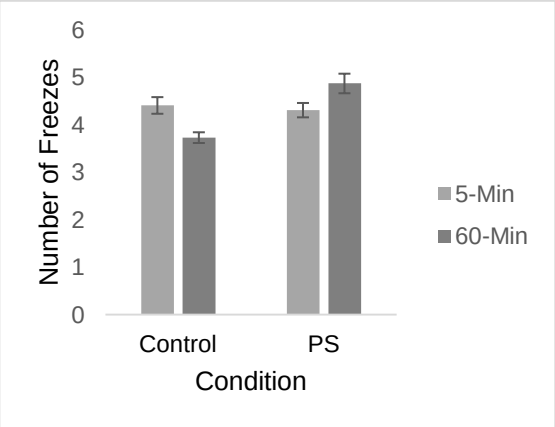

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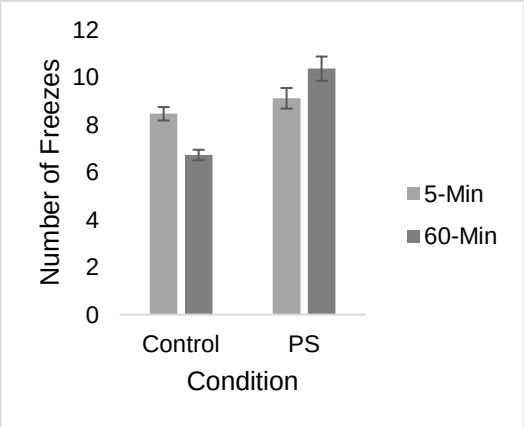

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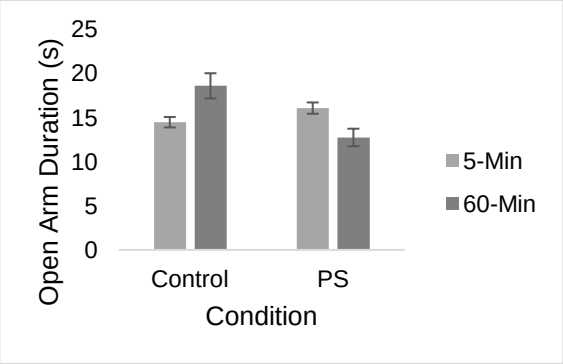

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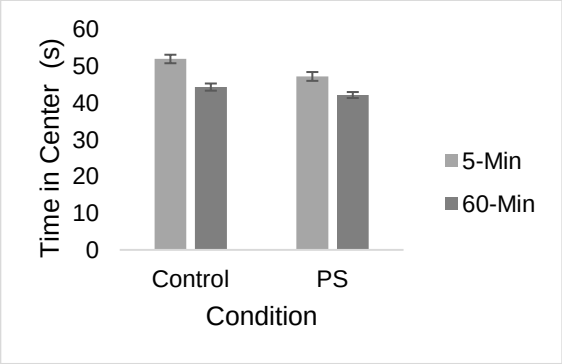

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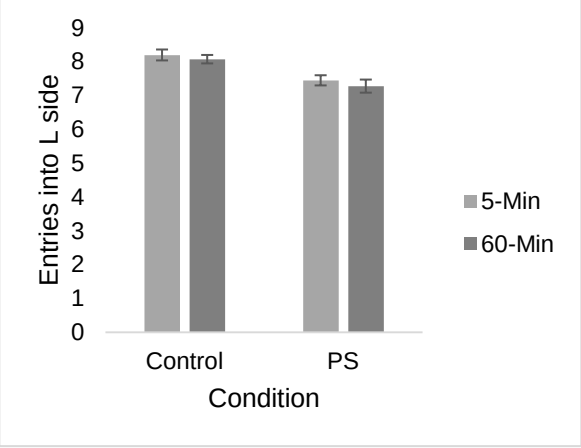

Table 1

Results from the EPM – Experiment 1

Condition	Mean total distance (cm)	Mean closed duration (s)	Mean open duration (s)	Mean open arm ratio
Control	2628.92	205.04	18.71	.08
PS	2640.17	207.83	18.31	.08

Table 2

Results from the OF – Experiment 1

Condition	Mean total distance (cm)	Mean center duration (s)	Proportion in center
Control	2936.39	49.18	0.16
PS	2918.39	56.00	0.19

Table 3

Results from the EPM – Experiment 2

Condition	Mean total distance (cm)	Mean closed duration (s)	Mean open duration (s)	Mean open arm ratio
Control	1786.176	211.31	23.24	0.09
PS	1714.71	223.53	11.38	0.04

Table 4

Results from the OF – Experiment 2

Condition	Mean total distance (cm)	Mean center duration (s)	Proportion in center
Control	2516.52	75.58	0.36
PS	2383.18	76.14	0.37