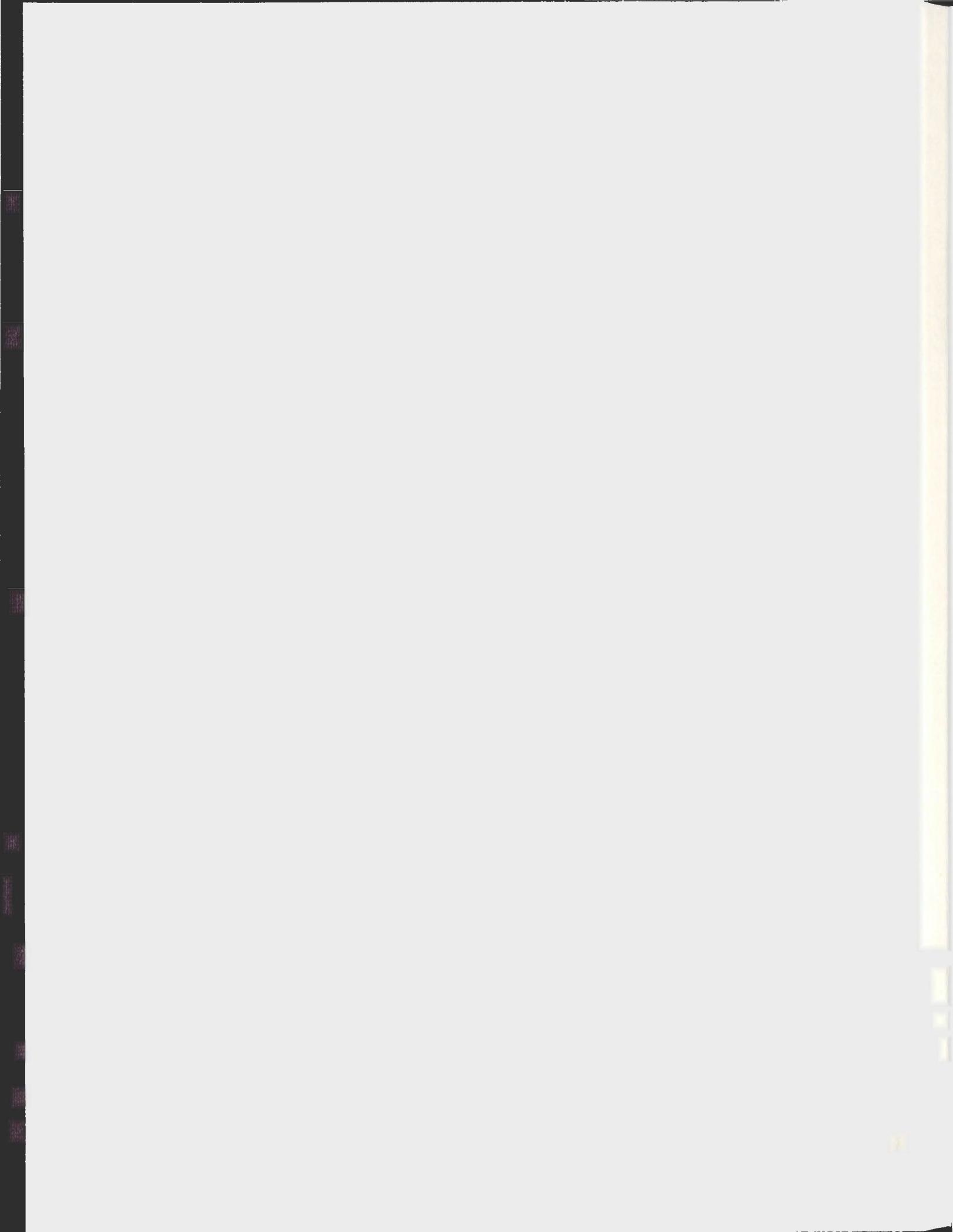


GLUCOCORTICOIDS ARE REQUIRED FOR EXTINCTION  
OF PREDATOR STRESS-INDUCED HYPERAROUSAL

RACHEL LAURA MURIEL CLAY





**Glucocorticoids are required for extinction of predator stress-induced  
hyperarousal.**

By

©Rachel Laura Muriel Clay

A thesis submitted to the

School of Graduate Studies

in partial fulfillment for the degree of

Master's of Science

Psychology

Experimental Psychology

Behavioural Neuroscience

Memorial University of Newfoundland

June 28, 2010

St. John's

Newfoundland

## Abstract

Extinction of fearful behavior induced by severe stress was studied using predator stress. Predator stress involves a ten minute unprotected exposure of a rodent to a cat which induces long-lasting changes in anxiety-like behaviours and hyperarousal (increased acoustic startle response) (Adamec & Shallow, 1993; Adamec et al., 2001; Cohen et al., 2004). In the present set of experiments, three questions were addressed using predator stressed mice. First, can predator stress-induced fear memories be extinguished? Second, is the extinction of predator stress-induced fear memories glucocorticoid-dependent? Finally, is re-exposure to the predator stress context necessary to see glucocorticoid's effects on predator stress-induced fear memories?

Extinction was induced by re-exposing mice to the predator stress room in the absence of the cat. This repeated re-exposure to predator stress context increased activity in the predator stress context implying extinction of predator stress-induced immobility, a context-dependent fear memory. Repeated re-exposures to the predator stress context also decreased subsequent hyperarousal (acoustic startle response) and generalized anxiety-like behaviour. These fearful behaviors were predator stress context independent, having been tested in environments different from the cat exposure room. Furthermore, blocking glucocorticoid synthesis with metyrapone during repeated exposures to the predator stress context had no effect on activity. Therefore, reducing corticosterone levels did not affect extinction of the predator stress-induced, context-dependent fear memory. However, metyrapone given during repeated exposures to the predator stress context prevented extinction of predator stress-induced hyperarousal. These results suggest that extinction of predator stress-induced, context-independent fear memory is dependent on

the presence of endogenous corticosterone during the extinction trials. Finally, re-exposure to the predator stress context was found to be necessary to see glucocorticoid's effects on predator stress-induced fear memories. This was determined by repeated injection of metyrapone over days without re-exposure to the predator stress context.

## Acknowledgements

I dedicate this work to my family, friends and pets; for their constant love and support throughout my time working on my thesis and beforehand. Without you guys I wouldn't have made it this far!

I would like to thank my supervisors, Dr. Robert Adamec and Dr. Jacqueline Blundell for their seemingly endless patience, and support in all things. I would also like to thank Dr. Vincent LoLordo for introducing me to the amazing world of academic research and for giving me the direction and confidence to go as far as I already have, and hopefully further.

A special thank-you goes to Dr. Charles Malsbury for acting as a member of my supervisory committee and being a steadfast well of knowledge of which I could draw from. A Big thank-you to Dr. Mark Hebert, for being a sounding board for ideas and always bringing humor into the lab.

My sincere gratitude goes out to the School of Graduate Studies for financial support by means of a Graduate Fellowship and also my supervisors (again) for pitching in that extra bit which permitted me to complete my thesis without excessive debt.

I also owe a huge amount to Dr. Mark Hebert and Greg Gill who both made data collection and analysis less painful, and much more amusing. I would like to thank the many undergraduate students who I have had the opportunity to work with for all their important contributions.

Last, but not least, I would like to thank the many staff at all the local coffee shops, for always providing me with the necessary caffeine to stay awake and focused on working, regardless the time of day.

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## List of Abbreviations

5-HT	5-Hydroxytryptamine (Serotonin)
ACTH	Adrenocorticotrophic hormone
ALB	Anxiety-like behaviour
ANOVA	Analysis of variance
BLA	Basolateral Nucleus of the Amygdala
CeA	Central Nucleus of the Amygdala
CRH	Corticotrophin Releasing Hormone
EPM	Elevated Plus Maze
GR	Glucocorticoid Receptor
HB	Hole Board
HC	Handled Control
HPA	Hypothalamic-Pituitary-Adrenal
L/D	Light/Dark Box
mPFC	Medial Prefrontal Cortex
MR	Mineralcorticoid Receptor
PS	Predator Stress
PFC	Prefrontal Cortex
PSE	Predator Stress Extinction
PSME	Predator Stress Metyrapone Extinction
PSMn	Predator Stress Metyrapone No-Extinction
PSVE	Predator Stress Vehicle Extinction
PSVn	Predator Stress Vehicle No-Extinction
PTSD	Posttraumatic stress disorder

## 1.0 Introduction

### 1.1 Posttraumatic Stress Disorder (PTSD)

Exposure to severe stressors such as traumatic physical or psychological experience may result in the development of affective disorders. One such disorder, Posttraumatic Stress Disorder (PTSD), is characterized by persistent re-experiencing of the trauma, avoidance of trauma-associated stimuli, a general numbing, and hyperarousal (DSM-IV-TR, 309.81). Hyperarousal, as measured by the acoustic startle response, is elevated in PTSD patients (Garrick et al., 2001; Ladwig et al., 2002). Between 6.8 – 15% of North Americans develop PTSD following a traumatic event (Kessler et al., 2005). The importance of studying PTSD has increased with the recent terrorist attacks such as September 11, 2001 in New York, which increased its prevalence (Galea et al., 2002; Kessler & Wang., 2008).

The inability to extinguish intense fear memories is an important clinical problem in psychiatric disorders such as PTSD (Morgan et al., 1995; Fyer, 1998; Yang, Chao, & Lu, 2006). Treatments for these types of disorders often rely on progressive extinction of these fear memories (Bentz et al., 2010). While treatments are beneficial for some PTSD patients, they are not effective in all cases. Thus, the goal of this set of experiments is to use an animal model of PTSD to identify factors that modulate progressive extinction of fear memories produced by severe stress. Knowledge of such factors may help design more effective extinction treatments for PTSD.

## **1.2 Hypothalamic-pituitary-adrenal Axis**

Given that anxiety disorders, and PTSD in particular, have a large stress component, it is not surprising that dysfunction of the stress system is associated with these disorders. Selye (1956) was the first to demonstrate a common pathway of physiological activity in response to stress. This pathway was later dubbed the hypothalamic-pituitary-adrenal (HPA) axis. During a stressful event, cells of the paraventricular nucleus of the hypothalamus respond by secreting corticotrophin releasing hormone (CRH) into capillaries in the median eminence of the hypothalamus. CRH released into this portal capillary system stimulates neurosecretory cells in the anterior pituitary which in turn release adrenocorticotropin hormone (ACTH). From there, ACTH travels through the blood stream and acts on the cortex of the adrenal gland where it stimulates secretory cells to release glucocorticoids (i.e. corticosterone in animals, cortisol in humans) into the general circulatory system (Carroll et al., 1976). ACTH and glucocorticoids subsequently act to decrease activity of paraventricular neurons, negatively influencing their own release. This is often referred to as the negative feedback loop (Sapolsky et al., 1985).

### ***1.2.1 PTSD and HPA axis***

Alterations in the HPA axis have been identified in stress-related disorders such as PTSD. Despite some inconsistencies in the literature, multiple studies in several laboratories have shown that individuals with PTSD have reduced circulating levels of cortisol compared to healthy controls (Mason et al., 1986; Yehuda et al., 1990; Kellner et al., 1997; Heim et al., 1998 Kellner et al., 2000; Yehuda, 2002;

Yehuda 2009). In particular, patients with PTSD show an enhancement of the negative feedback effect of cortisol on the pituitary and a general increase in reactivity of other tissues such as the hypothalamus and adrenal gland. These findings imply that although cortisol surges are possible in these individuals, they will be quickly depressed back to baseline (Yehuda, 2002). These results have been exhibited in both urinary (Mason et al., 1986; Yehuda et al., 1990) and salivary samples (Kellner et al., 1997). Furthermore, individuals who go on to develop PTSD have been found to have lower levels of cortisol immediately following a trauma (Yehuda, Shalev & McFarlane, 1998; Delahanty, Raimonde & Spoonster, 2000; Resnick et al., 1995), implying that cortisol levels may have been lower prior to traumatic exposure and hence represent a pre-existing risk factor for PTSD development (Yehuda, 2002).

Treatment with cortisol in humans has ameliorated some symptoms in disorders involving emotional memories such as PTSD and phobias (Aerni et al., 2004; Soravia et al., 2006). Specifically, oral administration of a low dose of cortisol in PTSD patients decreases the intensity of re-experiencing the traumatic event and reduces the incidence of nightmares (Aerni, et al., 2004; Soravia et al., 2006). Later it was shown that low doses of cortisol decreased symptoms in chronic PTSD patients (Schelling et al., 2006). It is unclear from these studies, however, if cortisol was acting by inhibiting retrieval, facilitating extinction or blocking reconsolidation. Thus, clarifying the role of glucocorticoids in psychiatric disorders such as PTSD may be useful in development of new treatments that are more efficacious.

### **1.3 Animal Models of PTSD**

Animal models are useful because they permit: 1) exposure to a severe stressor in a controlled fashion; 2) study of the effect of stress on affect as it develops; and 3) study of pharmacological and other treatments which may be difficult to test in humans, but can be easily evaluated in animals. Although it is not possible to model all aspects of PTSD in animals, several experimental paradigms have been developed which demonstrate PTSD-like symptoms. The two discussed here are the fear conditioning and predator stress paradigms.

#### ***1.3.1 Fear Conditioning***

The fear conditioning paradigm is most commonly used to model the intrusive fear memories associated with PTSD. Fear conditioning occurs when a neutral stimulus (i.e., tone or context) elicits defensive behaviours (i.e., freezing,) if the neutral stimulus was previously paired with an aversive stimulus (i.e., shock; Dexter & Merrill, 1969). This is an appropriate model of PTSD because not only does it demonstrate a learned fear association as seen in PTSD patients, but also demonstrates a long lasting persistence of these fear memories (Rothbaum & Davis, 2003; Orr et al., 1993; 2000).

##### ***1.3.1.1 Fear Conditioning, Consolidation, and Glucocorticoids***

Consolidation of a memory is the process by which a labile short-term memory trace is transferred into a fixed long-term memory (de Quervain et al., 2009). For consolidation to occur, de-novo protein synthesis and long-term changes in

synaptic plasticity are required (Kandel, 2001). Glucocorticoids are involved in the consolidation of memories of emotionally arousing events (McGaugh, & Roozendaal, 2002; de Quervain et al., 2009). Administration of corticosterone to rodents (the rodent equivalent of cortisol) enhances fear memory consolidation (Sandi, & Rose, 1994; Roozendaal, 2002; McGaugh, & Roozendaal, 2002; Abercrombie et al., 2003; Okuda, Roozendaal, & McGaugh, 2004), while blockade of corticosterone (or cortisol) synthesis with metyrapone impairs memory consolidation in both animals and humans (Maheu, Joober, & Lupien 2004). Furthermore, Cordero and Sandi, (1998) demonstrated that rats which were fear conditioned with a low intensity shock and then injected with corticosterone immediately afterwards, showed more freezing than controls 24 h and 7 days following conditioning. These glucocorticoid effects tend to be biphasic whereby low to moderate doses enhance, while high doses inhibit consolidation of fear memory (Pugh et al., 1997; Abrari et al., 2009). Overall, these data suggest that glucocorticoids play an important role in the consolidation of a shock-induced fear memory.

#### *1.3.1.2 Fear Conditioning, Extinction and Glucocorticoids*

Glucocorticoids also play a role in the consolidation of extinction memory. Extinction is defined as a reduction in conditioned fear response(s) when the conditioned stimulus is repeatedly presented in the absence of the unconditioned stimulus (Quirk & Mueller, 2008). Following fear conditioning training, animals returned to the training context without shock exhibit increased freezing when compared to non-shocked controls, indicating fear memory. However, if these fear

conditioned animals are repeatedly re-exposed to the context without shock, freezing to the context decreases, suggesting a decrease in fear of the context (Rescorla, 1996; Milad et al., 2009). Many studies demonstrate that extinction is not only the result of forgetting or of memory erasure, but also involves the formation of new associations that compete with prior fear-conditioned associations, hence new extinction memories (Rescorla, 1996).

As in consolidation of fear memories, glucocorticoids modulate consolidation of extinction memory (Myers & Davis, 2002). Consistent with the human data described above (Aerni et al., 2004; Soravia et al., 2006), recent rodent studies have shown that administration of corticosterone during reactivation of a shock-induced conditioned fear memory (re-exposure to the training context without shock) facilitates extinction of the fear memory (Cai et al., 2006; Yang et al., 2006; Abrari et al., 2008). Moreover, block of corticosterone synthesis with metyrapone prevents extinction (Barrett & Gonzalez-Lima 2004; Yang et al., 2006; Blundell et al., in preparation). It is important to note that reactivation of the fear memory must be paired with corticosterone or metyrapone to have any effect on fear memory extinction (Cai et al., 2006; Blundell et al., in preparation). Repeated injection of corticosterone or metyrapone alone after fear conditioning is without effect on subsequently reactivated fear response. Furthermore, the block of extinction in metyrapone-treated animals can be rescued with an exogenous injection of corticosterone (Blundell et al., in preparation). In light of these data, Cai et al., (2006) and others (Yang et al., 2006; Abrari et al., 2008; Blundell et al., in preparation) propose that the glucocorticoid surge during reactivation of a fear

memory is necessary for the extinction of the fear memory. In the absence of the glucocorticoid surge, fear memories persist which may ultimately lead to at least one of the core symptoms of PTSD, the persistent fear memories. This is consistent with the human data showing decreased cortisol levels in patients with PTSD (Mason et al., 1986; Yehuda et al., 1990; Kellner et al., 1997; Heim et al., 1998 Kellner et al., 2000; Yehuda, 2002; Yehuda 2009). While the fear conditioning data highlight the importance of glucocorticoids in context-specific fear memories, they do not address another core symptom of PTSD, hyperarousal, nor do they address the associated symptom of generalized anxiety.

#### *1.3.1.3 Limitations of Fear Conditioning as a Model of PTSD*

To date, preclinical models of PTSD have focused on fear conditioning due to its methodological simplicity and demonstration of fear memories which is one PTSD-like symptom. The disadvantages of fear conditioning is that it does not involve exposure to a truly life-threatening event nor mimic other PTSD symptoms such as persistent generalized hyperarousal (Pitman, 1997), or increased anxiety-like behaviour (Pitman, Orr & Shalev, 1993).

#### *1.3.2 Predator Stress*

Predator stress is an ecologically relevant model of PTSD that models effects of a life threatening event on PTSD relevant symptoms of hyperarousal (enhanced acoustic startle response) and anxiety-like behaviour. The predator stress paradigm allows us to determine if pharmacologically targeting extinction not only effects

subsequent context-specific symptoms, but also more generalized cue-independent symptoms of hyperarousal and anxiety.

Predator stress is both fear provoking and stressful (Adamec et al., 1998; Blanchar, et al., 1998; Dielenberg, Carrive, & McGregor, 2001a; McGregor et al., 2002). Predator stress (PS) typically involves a short (5-10 min) unprotected exposure of a rodent to a predator (i.e. cat) or predator odor (Adamec & Shallow, 1993; Cohen & Zohar, 2004; Adamec, Walling & Burton 2004; Muñoz-Abellán et al., 2008; Muñoz-Abellán, Armaraio & Nadal, 2009 ). This “traumatic” event is more ecologically valid than fear conditioning as it presents the animal with an event (exposure to a predator or predator cues) that they could possibly encounter in nature (Adamec & Shallow 1993; Cohen & Zohar, 2004; Muñoz-Abellán et al., 2008). Also, predator stress paradigms reliably induce hyperarousal (enhanced acoustic startle response) which closely parallels symptoms seen in human patients with PTSD (Adamec, Blundell & Burton, 2003; Adamec et al., 2006a; Adamec, Head, Soreq & Blundell, 2008; Cohen & Zohar, 2004). Furthermore, predator stress causes a long-lasting increase in anxiety-like behaviour as measured in the elevated plus maze, light/dark box, and hole board (Adamec & Shallow, 1993; Adamec, Walling & Burton, 2004; Cohen & Zohar, 2005; Adamec, Head, Soreq & Blundell, 2008). Increased generalized anxiety is co-morbid with PTSD (Pitman, Orr & Shalev, 1993). Importantly, common pharmacological treatments for PTSD (e.g. sertraline) are efficacious in reducing anxiety-like behaviours and hyperarousal following predator stress (Matar et al., 2006; Zohar et al., 2008; Adamec et al., 2004; Adamec et al., 2007).

### *1.3.2.1 Predator stress, Consolidation, and Glucocorticoids*

Like fear conditioning, consolidation of predator stress-induced fear memories also involves glucocorticoids. In particular, glucocorticoids participate in consolidation of predator stress-induced hyperarousal and generalized anxiety. Of importance, predator stress increases release of stress hormones. Muñoz-Abellán et al., (2008) found that both predator urine and fur odor exposure increased plasma levels of corticosterone and ACTH in rats, which remained elevated above controls 120 min after exposure. Similarly, cat exposure elevated plasma corticosterone peaking at 30 min post exposure and persisting to 180 min after cat exposure in rats (Adamec et al., 2006a). Moreover, blocking both glucocorticoid and mineralcorticoid receptors immediately after cat exposure interfere with consolidation of hyperarousal and anxiety (Adamec et al. 2007). Overall, these data suggest that glucocorticoids play a key role in the consolidation of predator stress fear memories.

### *1.3.2.2 Predator stress, Extinction, and Glucocorticoids*

Despite the merits of predator stress as a model of hyperarousal and anxiety aspects of PTSD, extinction of a predator stress-induced fear memory has not been assessed. Thus, the first goal of these experiments was to determine if the memory of the predator stress encounter (predator stress-induced contextual fear memory) can be extinguished. In study 1, predator stressed (cat exposed) mice were repeatedly re-exposed (once a day for 5 days) to the predator stress context (in the absence of the cat) and hyperarousal and anxiety-like behaviour were assessed one week later. Consistent with the fear conditioning data, predator stressed mice repeatedly re-

exposed to the predator stress context showed increased activity in the context across days, suggesting extinction of the predator stress-induced contextual fear memory. Even more interesting is the fact that repeated re-exposure to the predator stress context extinguished predator stress-induced hyperarousal (reduced startle to control levels), a context-independent fear memory. Hyperarousal is context-independent because it is measured in an environment very different from the cat exposure room (stress context).

Given that extinction of a contextual shock-induced fear memory is glucocorticoid-dependent (Cai et al., 2006; Yang et al., 2006; Abrari et al., 2008), the second goal of these experiments was to determine if extinction of predator stress-induced fear memories is also glucocorticoid dependent. In study 2, predator stressed mice were re-exposed (once a day for 5 days) to the predator stress context (without the cat present) after administration of metyrapone, a glucocorticoid synthesis blocker. Metyrapone was given during the first 4 days of re-exposures only. Hyperarousal and anxiety-like behaviour were assessed one week later. Metyrapone had no effect on activity during the repeated exposures to the context, suggesting that glucocorticoid blockade does not affect extinction of the predator stress-induced contextual fear memory. However, metyrapone prevented extinction of predator stress-induced hyperarousal (startle), a context independent fear memory. Study 3 examined whether re-exposure to the predator stress context was necessary to see metyrapone's effects on hyperarousal. After predator stress, metyrapone was injected over 4 days without re-exposure to the predator stress context, and hyperarousal and anxiety-like behaviour were measured one week later. Metyrapone depressed the

startle response, an effect opposite to that seen when given during re-exposure to the predator stress context.

Overall, we show that repeated re-exposure to the predator stress context without the cat present leads to both extinction of a context-dependent, predator stress-induced, fear memory, and also leads to a reduction in hyperarousal, a generalized, chronic, PTSD-like feature. Furthermore, extinction of context-independent predator stress-induced hyperarousal is dependent on endogenous corticosterone during the extinction trials.

## **2.0 Methods**

### **2.1 Study 1 - Extinction Study**

#### **2.1.1 Subjects**

A total of 45 male C57BL/J6 (C57) (Charles River, Canada) mice were used in Study 1. Mice arrived at 7 weeks of age and were housed individually in clear plastic cages with wire covers (42 cm x 25 cm x 20 cm) and provided food and water *ad libitum*. Mice adapted to a 12 hour light/dark cycle (lights off at 7 AM) for one week. The colony rooms for the mice were at the point farthest possible from the room where the cats were housed to ensure isolation from olfactory cues. Prior to treatment, mice were handled daily for 5 days. Handling consisted of the mouse being picked up by the tail and placed on the back of the hand for approximately 30s before being returned to its home cage. Following treatment, predator stressed mice were housed in a separate colony room from mice which were not predator stressed. This

was done to reduce the chance of residual olfactory cues remaining on the mice from the exposure context from stressing the unstressed controls. All procedures involving animals in this study adhered to the guidelines of the Canadian Council on Animal care, and were approved by the Institutional Animal Care committee of Memorial University.

### ***2.1.2 Groups***

There were three groups (n=15): handled control (HC), predator stressed (PS), and predator stressed extinction (PSE). Mice in the handled control (HC) group were not exposed to a cat. Instead they were only handled on predator exposure day, and then remained undisturbed in their home cage until behavioral testing. Predator stressed (PS) mice underwent a 10 min unprotected exposure to a cat, and were then returned to their home cage where they were left undisturbed until behavioral testing. A detailed description of the predator stress exposure can be found in the section entitled "Testing". Predator stressed extinction (PSE) mice were exposed to the cat in the same manner as were the PS mice. Over the 5 days after cat exposure, PSE mice were re-exposed to the exposure context for 10 minutes without the cat present.

Seven days after the final re-exposure (a total of 12 days after predator exposure), all mice underwent several tests of anxiety and hyperarousal including elevated plus maze, hole board test, light/dark box, and response to acoustic startle. Behavioural tests were run over 3 days with hole board and elevated plus maze (EPM) on the first testing day, light/dark box on the second day and acoustic startle

response on the third. A detailed description of the behavioral tests can be found below in the section 2.4.

## **2.2 Study 2 - Metyrapone Study**

### **2.2.1 Subjects**

A total of 60 male C57BL/J6 (C57) (Charles River, Canada) mice were used in Study 2. Mice arrived at 7 weeks of age and were housed individually in clear plastic cages with wire covers (42 cm x 25 cm x 20 cm) and provided food and water *ad libitum*. Mice adapted to a 12 hour light/dark cycle (lights off at 7 AM) for one week and were handled for five days prior to experiment commencement as described in the previous study. The colony rooms for the mice were at the point farthest possible from the room where the cats were housed to ensure isolation from olfactory cues.

### **2.2.2 Groups**

There were three groups (n=20) in this study: predator stress (PS), predator stress extinction plus metyrapone (PSME), and predator stress extinction plus vehicle (PSVE). Mice in the PS group were treated the same as those described in the Extinction study. Briefly, mice in the PS, PSME, and PSVE groups were exposed to a cat for 10 minutes. Following cat exposure, mice in the PS group were returned to their home cages and left undisturbed until behavioral testing. Twenty-four hours after cat exposure, mice in the PSVE and PSME groups were placed back in the predator stress room without the cat. Ninety minutes prior to re-exposure to the cat

room, mice in the PSME and PSVE groups received subcutaneous injections of metyrapone or vehicle, respectively. This procedure was repeated for five days with one exception; PSME and PSVE groups did not receive injections on the fifth re-exposure day.

Seven days after the final re-exposure (or a total of 12 days after predator stress), all mice underwent several tests of anxiety and hyperarousal including elevated plus maze, hole board, dark/light box, and response to acoustic startle. Behavioural tests were run across 3 days with hole board and elevated plus maze (EPM) on the first testing day, light/dark box on the second day and acoustic startle response on the third. A detailed description of the behavioral tests can be found below in the section 2.4.

### ***2.2.3 Drug Administration***

On re-exposure days (1-4), PSME and PSVE mice were injected subcutaneously with either a 50mg/kg dose of metyrapone (Tocris Bioscience; concentration of 5mg/ml dissolved in 5% ethanol in saline, volume dependent on mouse weight, but did not exceed 0.3 ml) or vehicle (5% ethanol in saline) 90 minutes prior to exposure. Amount of vehicle and metyrapone depended on mouse weight and was calculated as if an injection of metyrapone were given, and did not exceed 0.3 ml. The drug solutions were prepared daily (between 8 am and 12 pm) and the metyrapone and vehicle solutions were kept away from light and chilled.

## 2.3 Study 3 - Metyrapone Control Study

### 2.3.1 Subjects

A total of 45 male C57BL/J6 (C57) (Charles River, Canada) mice were used in this study. Mice arrived at 7 weeks of age and were housed individually in clear plastic cages with wire covers (42 cm x 25 cm x 20 cm) and provided food and water *ad libitum*. Mice adapted to a 12 hour light/dark cycle (lights off at 7 AM) for one week. The colony rooms for the mice were at the point farthest possible from the room where the cats were housed to ensure isolation from olfactory cues. Prior to treatment, mice were handled daily for 5 days as described in the Extinction Study.

### 2.3.2 Groups

There were three groups (n=15) in this study: predator stress (PS), predator stress no-extinction metyrapone (PSMn), and predator stress no-extinction vehicle (PSVn). Animals in the PS, PSMn, and PSVn groups underwent an unprotected predator exposure as described in the section 2.4. PS mice were then returned to their home cages until behavioral testing. Mice in the PSMn and PSVn groups received a subcutaneous injection of either metyrapone or vehicle once a day for the following four days predator stress. Except for the daily injections, PSVn and PSMn mice were left undisturbed in their home cages until behavioral testing.

Seven days after the final injection (a total of 12 days after predator exposure), all mice underwent several tests of anxiety and hyperarousal including elevated plus maze, hole board test, light/dark box, and response to acoustic startle. Behavioural tests were run over 3 days with hole board and elevated plus maze

(EPM) on the first testing day, light/dark box on the second day and acoustic startle response on the third. A detailed description of the behavioral tests can be found below in the section 2.4.

### ***2.3.3 Drug Administration***

On injection days, PSMn, and PSVn mice were injected subcutaneously with either a 50mg/kg dose of metyrapone or vehicle (as described in 2.2.3 Drug Administration).

## **2.4 Testing**

Groups were counterbalanced for time of day tested and time of day exposed to a predator. This was done to control for possible variability due to circadian rhythms. Cat exposures were completed between 8 am and 2 pm daily and testing of anxiety-like behaviors were completed between 10 am and 3 pm. Response to acoustic startle response was measured between 9 am and 4 pm.

### ***2.4.1 Exposure Context and Cat and Mouse Behaviors Measured During Cat Exposure***

The exposure room was approximately 2 meters by 1.3 meters and 3.5 meters in height with no windows. The cat was transported to the exposure room at least 30 minutes prior to testing. Between tests, a litter box for the cat was introduced so the cat did not soil the room. The mice were introduced singly into the exposure room via a small grey plastic container 18.5 cm high, 19 cm long and 14.5 cm wide. This

container had a moving panel that forced the mouse forward into the exposure room once the sliding door of the container was drawn aside. The mouse-cat interaction was videotaped for the 10 minute exposure by a camera mounted on a wall of the exposure room. The cat used was an adult male cat and all mice were exposed to the same cat.

Mouse behaviors measured were number of approaches to the cat, and flights from the cat. Cat behaviors measured were number of approaches to the mouse, number of times the cat sniffed the mouse, number of times the cat bit the mouse, number of times the cat physically contacted the mouse with its paw and number of vocalizations. Also, amount of time spent in close proximity to each other and number of times they entered within one square foot of each other were measured. Close proximity was defined as cat and mouse being within one foot of each other. To aide this measurement, the floor of the exposure room was divided into one foot squares with masking tape. Locomotor activity was assessed by counting the number of taped lines the mouse crossed during the 10 min cat exposure.

#### ***2.4.2 Mouse Behaviors During Exposure to the Context without a Cat***

Mouse behavior was videotaped for the 10 minute exposure by a camera mounted on a wall of the exposure room. Locomotor activity was assessed by counting the number of lines the mouse crossed in 10 minutes.

#### ***2.4.3 Modified Hole-board***

The hole board was performed as described previously (Adamec, Walling &

Burton 2004; Adamec et al., 2006b; Adamec et al., 2008; Adamec et al., 2009). The hole board and elevated plus maze were illuminated with red overhead lights to permit videotaping. Illumination levels of red light were: 44 foot candles at the floor of the testing apparatus.

The hole-board test was performed in a 36 cm square, open-topped box, with walls rising 20 cm above the floor of the box. The floor of the box was elevated 6 cm above the ground and was painted with grey enamel, and the walls were painted dark grey. Four holes of 1 cm diameter were located on the floor of the box, each in a separate corner, 9 cm from the wall. White masking tape was used to outline the center of the box, forming a square 4 cm from the walls of the box. Mice were placed in the center of the floor at the beginning of each trial and were then videotaped for the 5 minute trial.

Behaviors of the mice measured included frequency of head dips into the holes, frequency of rears, and amount of time spent in the center of the box as well as in the periphery. Head dips were defined as the mouse sticking its head into one of the four holes. Rears were defined as any instance where the mouse raised itself up on its hind legs, with its forepaws leaving the ground (with the exception of grooming behaviors). Head dips and rears in the hole-board were taken as measures of rodent exploration and activity, respectively (File & Wardill, 1975a; 1975b). Mice were considered in the center when all four paws were within the center area defined by white masking tape, and near the wall when all four feet were within the 4 cm area between the masking tape and the wall.

#### ***2.4.4 Elevated Plus Maze***

The elevated plus maze was performed as described previously (Adamec, Walling & Burton 2004; Adamec et al., 2006b; Adamec et al., 2008; Adamec et al., 2009). Immediately after the hole-board test, mice were transferred by the tail to their home cage and then into the elevated plus maze. The elevated plus maze consisted of four arms arranged in the shape of a plus sign, with two opposite arms “open” and the other two arms “closed”. All arms were 5 cm wide and 30 cm in length from the center, which was 5 cm square. The floor of the maze was wood painted with a grey enamel and was located 46 cm above the ground. The ‘closed’ arms had 14 cm transparent plastic walls surrounding their perimeters, while the ‘open’ arms had a 0.2 cm high lip surrounding their perimeters. Mice were placed in the center facing the same open arm at the start of each trial, which lasted 5 min.

Behaviors quantified included entries and time spent in open and closed arms. Mice were considered to have entered an arm if all four legs were in the arm. Ratio time and ratio entry into open arms are standard measures of rodent anxiety which control for overall activity levels. Ratios are calculated as total time in the open arms divided by the total time in any arm for ratio time, and number of entries into the open arms divided by number of entries into the any arm for ratio entry. Lower ratios indicate higher anxiety. Risk assessment was defined as having at least two hind legs in a closed arm with the nose pointed toward an open arm.

#### ***2.4.5 Light-Dark box***

The light/dark box was performed as described elsewhere (Adamec, Walling

& Burton 2004; Adamec et al., 2006b; Adamec et al., 2008; Adamec et al., 2009).

The light/dark box consisted of two chambers with each chamber measuring 19.1 cm on all sides, with walls 14 cm high. There was a small rectangular tunnel 6.4 cm high by 7.5 cm wide connecting the two chambers. The entire apparatus was made of dark grey plastic. The dark chamber was entirely enclosed with a solid black plastic top.

The light chamber had a plastic transparent cover with ventilation holes. Testing took place in a darkened room with a 100 watt light bulb placed 56 cm above the floor of the light chamber which provided illumination at the intensity of 70 foot candles at the floor of the chamber. Mice were placed in the light chamber facing away from the dark chamber at the start of the test and their activity was videotaped for 5 minutes. Following this the mice were returned to their home cages.

Behavioral measures taken included time spent in each chamber, and number of entries into each chamber (defined as having all four paws in the chamber).

#### ***2.4.6 Startle Testing***

Response to acoustic startle was performed as previously described (Adamec, Walling & Burton 2004; Adamec et al., 2006b; Adamec et al., 2008; Adamec et al., 2009). Startle testing took place in a San Diego Instruments standard startle chamber. During testing, mice were placed into a cylindrical small animal enclosure (measuring 12.7 cm long and 3.7 cm in diameter) within the chamber. The animal enclosure sat atop a piezo electric transducer that produced an electrical signal sampled by a computer, providing a measure of mouse movement. Startle testing was completed in the dark and involved acclimation of the mice to the startle apparatus

with a background of 50 db white noise for 5 minutes. Following acclimation, mice were exposed to 30 pulses of 50 msec bursts of white noise of 105 db rising out of the background. There was a 30 second inter-trial interval. Startle response was measured by computer, as the maximal output of the transducer ( $V_{max}$ ) within a 150 msec recording window. A second value,  $V_{start}$ , was measured just before the pulse. Peak startle amplitude was calculated as  $V_{max} - V_{start}$  for each trial.

### 3.0 Results

#### 3.1 Study 1 - Extinction Study

##### 3.1.1 Cat-Mouse Interaction

There were no differences in behaviour of the cat or mouse across groups on any measure [One -way ANOVAs, all  $F(1,28)$  where all  $p > 0.4$ ]. Therefore, differences in behaviour between groups can be attributed to treatment effects and not to variations across treatment in cat or mouse reaction to each other. See Table 1 for full statistical analysis.

##### 3.1.2 Extinction Trials

Repeated re-exposure to the predator stress room (without the cat present) increased mouse activity in the room across days [repeated measures ANOVA, Day Effect,  $F(4,56) = 4.94$ ,  $p = 0.002$ ]. Taped lines crossed on extinction day 1 were lower than on extinction days 3, 4 and 5. Extinction day 2 lines crossed were lower than those crossed on day 5 (Fisher's LSD,  $p < .05$ , Figure 1). These data suggest that

repeated re-exposure to the predator stress room extinguished a predator stress-induced contextual fear memory expressed as reduced activity. See Table 1 for full statistical analysis.

### ***3.1.3 Startle Response***

Startle response was assessed in HC, PS and PSE groups. The non-normality of the data (Omnibus test = 260.99,  $p < 0.001$ ) required the use of the Kruskal-Wallis non parametric chi square test of median differences across groups. Thus, median peak startle amplitude across 30 trials was compared across groups and an effect of group was found [ $\chi(2)^2 = 15.83$ ,  $p = 0.000$ ; Figure 2]. Consistent with previous studies (Adamec et al., 2008; Adamec, Fougere, & Risbrough, 2009), predator stressed mice (PS) showed enhanced peak startle amplitudes compared to handled controls (HC) (Kruskal-Wallis Multiple-Comparison Z-test,  $z = 3.59$ ,  $p < .001$ ). In addition the startle amplitudes of the mice repeatedly re-exposed to the context (PSE) was not significantly different from handled control levels (Kruskal-Wallis Multiple-Comparison Z-test,  $z = 0.38$ ,  $p > .34$ ) and significantly lower than PS mice (Kruskal-Wallis Multiple-Comparison Z-test,  $z = 3.20$ ,  $p < .001$ , Figure 2). This finding shows that repeated exposure to the predator stress context, reduced predator stress-induced hyperarousal.

Examination of the mean peak startle amplitude for all three groups revealed a decline in startle response (habituation) over trials. Slowed rate of habituation of the startle response occurs in predator-stressed mice also showing enhanced startle

amplitudes (Adamec et al., 2008; Adamec, Fougere, & Risbrough, 2009). Rate of habituation was measured by the trial constant (Tau) estimated from fits of the exponential decay function

$$Y = Y_0 e^{-t/\text{Tau}}$$

to mean peak startle amplitude over trials for each of the three groups [all df adjusted  $r^2 > .82$ , all exponential fits  $F(2, 27) > 75.2$ ,  $p < 0.001$ , all Tau  $> 0$ , t tests  $p < .01$ ]. Y and  $Y_0$  in the function are mean peak startle amplitude, t is startle trial and the parameter Tau is the number of startle trials required for startle amplitude to decline to 37 % of maximum. The program fitting the functions (Jandel Table Curve V4) also estimates standard error (SE) of each Tau value and these SE were used to calculate t tests of Tau differences between groups. Handled control (HC) mice and predator stressed mice repeatedly exposed to the predator stress context (PSE mice) habituated more quickly (smaller Tau values) than predator stressed only (PS) mice (Tau contrasts Planned t tests  $p < 0.03$ ; Figure 3). Therefore, repeated exposure to the predator stress context rescued both the predator stress-induced peak startle response and the delay of startle habituation. See Table 1 for full statistical analysis. (Note in this and subsequent Tau analyses, startle amplitude means over trials were smoothed 20% with a FFT smoothing function provided by the program to improve fit. This smoothing did not distort the data.)

### ***3.1.4 Elevated Plus Maze, Hole Board, and Light/Dark Box***

Anxiety-like behaviour and activity were assessed in the elevated plus maze (EPM), hole board (HB), and light/dark box (L/D). In the EPM, repeated exposure to

the predator stress context (in the absence of the predator) was anxiolytic in PSE mice. There was a main effect of group for ratio time [ $F(2, 42) = 5.73, p = 0.01$ , Figure 4] and ratio entry [ $F(2, 42) = 3.34, p = 0.04$ , Figure 4]. PSE mice spent a higher proportion of time in the open arms of the maze more than PS and HC groups, and entered the open arms more than HC (mean contrasts, Fisher's LSD,  $p < .05$ , Figure 4). Surprisingly, ratio time and ratio entry of PS and HC groups did not differ in the EPM [ $F(2, 42)$  where all  $p > 0.05$ ]. There were no other group differences in behaviour in the EPM [Table 1]. Thus, exposure to a cat did not increase anxiety-like behaviour in the EPM. Furthermore, measures of general activity and anxiety-like behaviour in the hole board (HB) and light-dark box (L/D) did not differ across groups. See Table 1 for full statistical analysis.

## **3.2 Study 2 - Metyrapone Extinction Study**

### **3.2.1 Cat Mouse Interaction**

Similar to Study 1, in the cat-mouse interaction, there were no differences in behaviour of the cat or mouse across groups [Table 1]. Therefore, differences in behaviour between groups are likely not due to variations across treatment in cat or mouse reaction to each other.

### **3.2.2 Extinction Trials**

Consistent with Study 1, repeated exposure to the predator stress context without the predator present increased activity in the context across days [repeated measures ANOVA, main effect of Day  $F(4, 267) = 10.76, p = 0.000$ ]. Lines crossed

on extinction day 1 were lower than all other extinction days and extinction day 2 lines crossed were lower than those crossed on day 5 (Fisher's LSD,  $p < .05$ , Figure 5). However, there was no group effect or interaction with extinction day. Therefore, metyrapone had no effect on locomotor activity during re-exposures to the predator stress context. These data suggest that extinction of a predator stress-induced contextual fear memory may not be corticosterone-dependent. See Table 1 for full statistical analysis.

### ***3.2.3 Startle Response***

Similar to Study 1, the non-normality of the data (Omnibus test 492.90,  $p < 0.001$ ) required the use of the Kruskal-Wallis non parametric chi square test of median differences across groups. Thus, median peak startle amplitude across 30 trials was compared across groups and an overall effect of group was found [ $\chi(2)^2 = 31.93$ ,  $p < 0.001$ ; Figure 6). Kruskal-Wallis Multiple-Comparison Z-tests (all  $z > 2.60$ , all  $p < 0.01$ ) showed that all groups differed from each other. Vehicle-injected predator stress context re-exposed mice (PSVE) showed a depression of startle amplitude below that of PS mice, and metyrapone-injected predator stress context re-exposed mice (PSME) showed an even greater startle response than PS animals. Therefore, repeated exposure to the predator stress context (in the absence of the predator) reduced peak startle amplitude. However, blocking corticosterone with metyrapone during repeated exposure to the predator stress context prevented this reduction in peak startle.

Rate of habituation of peak startle amplitude for all three groups (PS, PSME and PSVE) was assessed by estimating Tau as described above. Rate of habituation was measured by the trial constant (Tau) estimated from fits of the exponential decay function

$$Y = Y_0 e^{-t/\text{Tau}}$$

to mean peak startle amplitude over trials for each of the three groups [all df adjusted  $r^2 > 0.50$  but  $< 0.81$ , all exponential fits  $F(2, 27) > 15.02$ ,  $p < 0.001$ , all Tau  $> 0$ , t tests  $p < .02$ ]. Y, and  $Y_0$  in the function are mean peak startle amplitude, t is startle trial and the parameter Tau is the number of startle trials required for startle amplitude to decline to 37 % of maximum. The program fitting the functions (Jandel Table Curve V4) also estimates SE of each Tau value and these SE were used to calculate t tests of Tau differences between groups. PSVE mice habituated more quickly (smaller Tau value) than PS and PSME mice, which did not differ (Tau contrasts Planned t tests  $p < 0.02$ , Figure 7). Therefore, repeated exposure to the predator-stress context (in the absence of the predator) facilitated habituation of peak startle amplitude. However, blocking corticosterone with metyrapone during repeated exposure to the predator stress context prevented this facilitation. Together, these data suggest that corticosterone participates in the effects of predator stress context re-exposures on both startle amplitude and its habituation. See Table 1 for full statistical analysis.

### *3.2.4 Elevated Plus Maze, Hole board, and Light/Dark Box*

Anxiety-like behaviour and activity were assessed in the elevated plus maze (EPM), hole board (HB), and light-dark box (L/D). Overall, there were no group differences in anxiety-like behaviour across all tests [Table 1]. Therefore re-exposure to the predator stress context with or without metyrapone (PSME, PSVE) was without effect on anxiety-like behaviour in the EPM, HB or L/D tests relative to PS mice.

## ***3.3 Study 3 - Metyrapone Control Study***

### *3.3.1 Cat Mouse Interaction*

Similar to Study 1 and 2, in the cat-mouse interaction, there were no differences in behaviour of the cat or mouse across groups [Table 1]. Therefore, differences in behaviour between groups are likely not due to variations across treatment in cat or mouse reaction to each other.

### *3.3.2 Startle Response*

Similar to Study 1 and 2, the non-normality of the data (Omnibus test = 460.99,  $p < 0.001$ ) required the use of the Kruskal-Wallis non parametric chi square test of median differences across groups. Thus, median peak startle amplitude across 30 trials was compared across groups and an overall effect of group was found [ $\chi(2)^2 = 58.88, p < 0.001$ ]. Predator stressed mice repeatedly injected with metyrapone (4 injections over 4 days) without re-exposure to the context (PSMn) showed a

decreased startle response compared to predator stressed mice (PS) and predator stress mice given repeated injections of vehicle (PSVn). Peak startle amplitude of PSVn mice was also reduced to a level between PS and PSMn mice. (Kruskal-Wallis Multiple-Comparison Z-value test, all  $z > 3.01$ , all  $p < 0.01$ ; Figure 9). These data indicate that reducing corticosterone for four days following exposure to a cat can significantly dampen peak startle amplitude measured one week later.

Rate of habituation of peak startle amplitude for all three groups (PS, PSMn and PSVn) was assessed by estimating Tau as described above. Rate of habituation was measured by the trial constant (Tau) estimated from fits of the exponential decay function

$$Y = Y_0 e^{-t/\text{Tau}}$$

to mean peak startle amplitude over trials for each of the three groups [all df adjusted  $r^2 > 0.77$  but  $< 0.95$ , all exponential fits  $F(2, 27) > 42.08$ ,  $p < 0.001$ , all Tau  $> 0$ , t tests  $p < 0.038$ ]. Y, and  $Y_0$  in the function are mean peak startle amplitude, t is startle trial and the parameter Tau is the number of startle trials required for startle amplitude to decline to 37 % of maximum. The program fitting the functions (Jandel Table Curve V4) also estimates SE of each Tau value and these SE were used to calculate t tests of Tau differences between groups. Unexpectedly, PS mice habituated more quickly (smaller Tau value) than both PSMn and PSVn mice which did not differ (Tau contrasts Planned t tests  $p < 0.038$ , Figure 10). Therefore, repeated vehicle injections (PSVn) and metyrapone injections (PSMn) without room exposure decreased the peak startle amplitude, and delayed habituation beyond that of PS. See Table 1 for full statistical analysis.

### 3.3.3 Elevated Plus Maze, Hole Board and Light-Dark Box

Anxiety-like behavior and activity were assessed in the elevated plus maze (EPM), hole board (HB), and light/dark box (L/D). Overall, there were no group differences on any measure in all three tests [Table 1]. Therefore, repeated injection of vehicle (PSVn) or metyrapone (PSMn) was without effect on anxiety-like behaviour and activity in the EPM, HB or L/D tests relative to PS mice.

## 4.0 Discussion

While there have been studies of extinction of fear learning and its underlying mechanisms (e.g. Cai et al., 2006; Yang et al., 2006; Abrari et al., 2008), extinction of predator stress-induced fear memories has not been examined until the present study. Like fear conditioning, predator stress produces associative, context-dependent fear memories. Unlike fear conditioning, predator stress also produces non-associative fear memories (context-independent fear memories such as hyperarousal and anxiety-like behaviors). These memories are context-independent because tests measuring hyperarousal and anxiety-like behavior take place in environments very different from the predator stress context. The present set of experiments sought to answer three questions regarding extinction of predator stress-induced fear memories of both types. First, can predator stress-induced fear memories be extinguished by re-exposure to the stress context without the cat present? Second, is the extinction of predator stress-induced fear memories glucocorticoid dependent? Finally, is re-

exposure to the predator stress context necessary to see glucocorticoid effects on predator stress-induced fear memories?

#### **4.1 Context-Dependent Fear Memory**

Study 1 showed that re-exposures to the predator stress context without the cat present extinguished a predator stress-induced contextual fear memory. Specifically, repeated re-exposure of predator stressed mice to the predator stress context increased their activity in the context over days (Figure 1). This finding is consistent with shock-induced fear memory extinction, where repeated re-exposure to the shock context (without the shock) leads to decreased freezing behavior across days (Quirk & Mueller, 2008). In contrast to shock-induced fear memories (Cai et al., 2006; Yang et al., 2006; Abrari et al., 2008), extinction of a predator-stress induced contextual fear memory is not glucocorticoid-dependent (Study 2). Predator stressed mice given either metyrapone or vehicle 90 min prior to re-exposure to the predator stress context for four days showed the same increase in activity in the context over days (Figure 5). Thus, reducing corticosterone levels with metyrapone did not alter the extinction of reduced activity.

While it is not surprising that the mechanisms underlying extinction of a predator stress-induced fear memory and shock-induced fear memory are different, other possibilities may explain this discrepancy. For example, extinction of a shock-induced fear memory is often measured as a decrease in freezing to the context over re-exposures (Rescorla, 1996; Cai et al., 2006; Milad et al., 2009). Due to limitations of the set-up of the predator stress room, it was not possible to reliably measure

mouse freezing. Thus, the measure of mobility used in the present studies was the number of lines that the animal crossed during the re-exposure trials. It was assumed that this measure of mobility should increase as freezing to the context decreased. However, this is an indirect measure of freezing. Thus, although unlikely, metyrapone may have affected freezing behaviour during re-exposures to the predator stress context, yet had no effect on number of lines crossed. In future studies, a modification to the predator stress set-up which would allow for better resolution of mouse behaviour (freezing) could address this issue.

#### **4.2 Context-Independent Fear Memory (hyperarousal measured as acoustic startle response)**

Consistent with previous studies using rats (Adamec, Blundell & Burton, 2003; Adamec et al. 2006a) and mice (Adamec, Head, Soreq & Blundell, 2008; Cohen & Zohar, 2004), predator stress lastingly increased hyperarousal, measured as increased startle response to an acoustic stimulus. Increased startle response appeared as increased peak startle amplitude and decreased rate of habituation of peak startle amplitude (delayed habituation) following exposure to a cat. Repeated re-exposure of stressed mice to the predator stress context decreased startle amplitude and increased rate of startle habituation to levels of handled controls (Figures 2 and 3). Therefore, repeated re-exposure to the predator stress context not only extinguished a context-dependent fear memory, but also extinguished predator stress-induced hyperarousal (a context-independent fear memory; Study 1). Overall, these novel findings suggest

that extinction of a context-dependent, predator stress-induced fear memory may also reduce the generalized, persistent, PTSD-like symptom of hyperarousal.

Extinction of context-independent hyperarousal depends on the presence of corticosterone during re-exposure to the predator stress context (Figures 6 & 7, Study 2), unlike extinction of context-dependent fear memory (Figure 5, Study 2). Consistent with Study 1, predator stressed mice re-exposed to the context (and given vehicle) showed extinction of hyperarousal manifested as a decreased peak startle amplitude and faster habituation of startle amplitude relative to predator stressed only mice (Figure 6, 7). Administration of metyrapone to predator stressed mice prior to re-exposure to the stress context blocked this extinction. These novel findings suggest that corticosterone during re-exposure to the predator stress context is critical to extinction of both peak startle amplitude and its habituation.

The effects of metyrapone on hyperarousal are not simply a non-specific lasting drug effect since metyrapone given with or without stress context re-exposure had opposite effects on startle amplitude. In Study 3, predator stressed mice were given four injections of metyrapone or vehicle (once a day for four days) without re-exposure to the predator stress context. Hyperarousal was measured nine days later. Metyrapone depressed peak startle amplitude, an effect opposite to that observed when given during re-exposure to the predator stress context (Figure 8). Thus, reducing corticosterone for four days following exposure to a cat dampened peak startle amplitude measured nine days later. It may be that metyrapone blocked consolidation of predator stress-induced hyperarousal. In rats, consolidation of

predator stress-induced hyperarousal is prevented by blocking glucocorticoid and mineralcorticoid receptors (Adamec et al., 2007).

Overall, these data suggest that glucocorticoids are necessary during predator stress context re-exposures to dampen (extinguish) hyperarousal. In contrast, glucocorticoids play the opposite role, following predator stress without reactivation. It is likely that glucocorticoid interactions with neurochemical and neuroanatomical contexts unique to the re-exposure context or its absence contribute to the different modes of action.

Interestingly, predator stressed mice given repeated injections of vehicle without stress context re-exposure also showed slightly decreased peak startle amplitude compared to predator stressed only mice. However, this decrease was not as large as that seen in mice given metyrapone (Figure 8). Since predator stressed only mice were not handled prior to startle testing, these data suggest that injection or handling in vehicle-treated mice reduced peak startle amplitude. Furthermore, this effect was potentiated by metyrapone. Perhaps the smaller reduction in peak startle amplitude in vehicle-treated mice reflects some countering facilitation by corticosterone. Finally, predator stressed only mice habituated more quickly than both predator stressed mice given metyrapone and predator stressed mice given vehicle. These data suggest that experience with injection (vehicle or metyrapone) or handling increased the trials to habituate in predator stressed animals. It is unclear why repeated handling and injection decreased peak startle amplitude, yet delayed habituation. One might expect that a decrease in startle amplitude would be associated with faster habituation. However, Adamec and colleagues have suggested

that independent substrates are responsible for changes in startle amplitude and startle habituation (Adamec, Blundell & Burton, 2005; Adamec et al., 2007).

#### **4.3 Context-Independent Fear memory (anxiety-like behavior)**

Predator stressed mice repeatedly re-exposed to the predator stress context exhibited decreased anxiety-like behavior in the EPM in comparison to both the predator stressed only and handled control groups (Figure 4). These data suggest that extinction of a predator stress-induced, context-dependent fear memory can decrease subsequent context-independent anxiety-like behavior. However, this effect is more like a general anxiolytic effect than a reduction of predator stress-induced anxiety, because exposure to a cat did not increase all anxiety-like behaviors (Study 1). Surprisingly, handled control mice showed anxiety-like behavior in the EPM resembling that of predator stressed mice. In fact, predator stress did not increase anxiety-like behavior over handled controls in two additional tests of anxiety, HB and L/D box. These data are inconsistent with previous studies which have found increased anxiety-like behavior in these tests following predator stress (Adamec, Walling & Burton 2004; Adamec et al., 2006b; Adamec et al., 2008; Adamec et al., 2009). When present data were compared to these previous studies, it appeared that our handled control mice spent much less time in the light and much more time in the dark of the light/dark box than handled control animals in previous studies. Similarly, our handled control mice much more frequently entered the closed arms of the EPM than in previous studies (Adamec, Walling & Burton 2004; Adamec et al., 2006b;

Adamec et al., 2008). In general, it appears that our handled control mice behaved in these tests as if they had been exposed to a predator.

Several possibilities may explain the discrepancy between the present study and previous ones. First, many studies that show increased anxiety-like behavior following predator stress have used rats instead of mice (Adamec, Bartoszyk, & Burton, 2004; Adamec, Blundell, & Burton, 2006; Cohen et al., 2006). Second, previous studies in mice (Adamec, Walling & Burton 2004; Adamec et al., 2006b; Adamec et al., 2008; Adamec et al., 2009), used larger numbers of mice per group. For example, Adamec et al., (2008) used C57BL/J6 mice in groups of 25 (unlike the 15-20 mice used in the current studies), and found increased anxiety-like behavior in the HB and the EPM. Finally, not all studies using mice have reported changes in all measures of anxiety-like behavior following predator stress (Adamec, Walling & Burton 2004; Adamec et al., 2006b; Adamec et al., 2008; Adamec et al., 2009). At least two studies in mice report a lack of changes in ratio time and ratio frequency in the EPM following predator stress (Adamec, Walling & Burton 2004; Adamec et al., 2009). Moreover, Adamec et al., (2008) found that predator stress depressed ratio entry (frequency) in mice. Also, some studies found changes in anxiety-like behaviour in the HB (Adamec et al., 2008; Adamec et al., 2009) while others have not (Adamec, Walling & Burton 2004). Overall, these data indicate that predator stress-induced changes in anxiety-like behaviour as measured in the EPM, HB and L/D box in mice are inconsistent across studies. Unlike anxiety-like behaviour, hyperarousal in mice is consistently shown following exposure to a predator (or predator odours) (Adamec, Walling & Burton 2004; Adamec et al., 2006b; Adamec et

al., 2008; Adamec et al., 2009; Cohen et al., 2008). Given that hyperarousal, and not anxiety-like behaviour, is a core symptom of PTSD, future studies in mice will focus exclusively on the long-lasting changes in hyperarousal following cat exposure.

Repeated injections of metyrapone following predator stress had no effect on anxiety-like behavior (Study 2 and 3). To our knowledge, this is the first study to examine the effects of multiple injections of metyrapone on anxiety-like behavior in stressed mice. However, our data are consistent with Blundell et al. (in preparation) which showed that a single injection of metyrapone 90 min prior to anxiety testing did not alter anxiety-like behavior. These data highlight the specific role of glucocorticoids in long-lasting changes in hyperarousal in predator stressed mice.

#### **4.4 Metyrapone**

Studies of the role of glucocorticoids in extinction of shock-induced contextual fear memories have employed metyrapone (Yang et al., 2006; 2007; Barrett & Gonzalez-Lima, 2004; Blundell et al., in preparation). Therefore, metyrapone was used in the current study to block corticosterone synthesis during extinction of predator stress-induced fear memories. While the primary action of metyrapone is to decrease glucocorticoid synthesis by inhibiting 11- $\beta$  steroid hydroxylation (Jenkins et al., 1958; Rotllant, & Amario, 2005), metyrapone has additional effects. For example, metyrapone dose-dependently increases circulating ACTH levels (Rotllant et al., 2002), due to reduced negative feedback regulation. Metyrapone also stimulates the systemic release of deoxycorticosterone (a precursor to corticosterone), which can be converted to other neurosteroids (i.e.

tetrahydroxydeoxycorticosterone; Strashmirov & Bohus, 1966). Furthermore, metyrapone blocks synthesis of serotonin in the rat hippocampus (Korte-Bouws et al., 1996). However, this effect was after a 150 mg/kg dose iv and may not be relevant to the present studies which used 50 mg/kg ip. Nevertheless, it is possible that changes in predator stress-induced hyperarousal following metyrapone injections in the current studies may be due to one, or a combination, of these effects.

In future studies, to ensure that the metyrapone-induced changes in hyperarousal are due to block of corticosterone synthesis, we will administer corticosterone following extinction trials in mice previously given metyrapone. Given that exogenous corticosterone rescues metyrapone's block of extinction of a shock-induced fear memory (Yang et al., 2006; Blundell et al., in preparation), we expect that corticosterone will reverse metyrapone's effects on hyperarousal.

Glucocorticoids bind to two different intracellular receptors, low affinity glucocorticoid receptors (GRs) and high affinity mineralcorticoid receptors (MRs). There is evidence that metyrapone may affect extinction of a fear memory by reducing the binding of glucocorticoids to low affinity GRs (McEwen et al., 1986). In light of these data, future experiments will examine the effects of GR or MR block on extinction of a predator stress-induced fear memory. We expect that blocking GRs during reactivation of the predator stress memory will prevent extinction of predator stress-induced hyperarousal.

#### 4.5 Extinction and Functional Neuroanatomy

The functional neuroanatomy of extinction of predator stress-induced fear memories is unknown. However, it has been shown that the right amygdala and ventral hippocampus are involved in behavioural changes produced by predator stress. Lasting potentiation of both ventral hippocampal inputs to the basolateral amygdala and central amygdala outputs to the periaqueductal gray follow consolidation of predator stress-induced fear memories. Moreover, degree of potentiation in both pathways is highly positively predictive of severity of negative affective changes (Adamec, Blundell & Burton, 2005).

These areas (and others) are likely to be involved in extinction of predator stress-induced fear memories, as other data implicate this circuitry in extinction of shock-induced fear memories (for reviews see: Myers & Davis, 2007; Quirk & Mueller 2008). For instance, the amygdala is involved in establishing fear memories and extinction of fear memories (Pare et al., 2004; Davis, 2006). The central nucleus of the amygdala (CeA) is important for emotional output and receives input from a large variety of cortical sources as well as other amygdalar nuclei (Pare & Smith, 1998). The basolateral amygdala (BLA) is important for the consolidation of extinction learning and has been shown to undergo synaptic restructuring following extinction (Chatwal et al., 2005; Markram et al., 2007). Between the BLA and CeA are intercalated cells which provide inhibitory influence from the BLA to the CeA (Pare & Smith, 1993; 1998; McDonald et al, 1996). Also, these cells receive information from the medial prefrontal cortex (mPFC; the infralimbic subregion), the entorhinal cortex, the hippocampus and the subiculum (Canteras & Swanson, 1992;

McDonald & Mascagni, 1997; Likhtik et al, 2005). The mPFC is activated during extinction of a fear memory (Barrett et al., 2003; Phelps et al., 2004; Santini et al., 2004). Moreover, smaller mPFCs are associated with poorer outcomes of extinction of a fear memory (Milad, et al., 2005). Furthermore, lesion studies have shown that the mPFC is necessary for extinction learning and recall of a previously learned extinction memory (Morgan et al., 1993; Quirk et al., 2000, 2003; Milad & Quirk, 2002). In addition to the amygdala and mPFC, the hippocampus is especially important for extinction of contextual fear learning (Duvcarci & Pare, 2007). Pharmacological inactivation of the hippocampus prior to extinction training blocks subsequent extinction learning, demonstrating that hippocampal activity is important for extinction learning (Corcoran et al., 2005). All of these areas work together to reduce output from the CeA, and hence change emotional behaviors following extinction of a shock-induced fear memory (Quirk & Mueller, 2008; Carrive, Lee & Su 2000). Similarly, reduced output of CeA in predator stressed rodents likely reduces effects of stress on affect. Thus, extinction of predator stress-induced fear memories likely involves suppression of CeA output. Therefore, brain areas critical to extinction of a shock-induced fear memory are candidates for neural substrates of extinction of predator stress-induced fear memories. Given the overlap of hippocampal and amygdala circuits in extinction of fear learning and predator stress effects on affect, future studies targeting these areas during extinction of predator stress-induced fear memories are warranted.

#### 4.6 Effects of Glucocorticoids on Extinction Neural Circuitry

Both glucocorticoid receptors (GRs) and mineralcorticoid receptors (MRs) are present in brain areas thought to be involved in shock-induced fear memory extinction. These areas include the amygdala (de Kloet, Joels, & Holsboer, 2005; McEwen, Weiss & Schwartz, 1968), hippocampus (Andreasen & Lambert, 1991), mPFC (Quirk et al., 2006) and other regions (Rodrigues, LeDoux & Sapolsky, 2009). The best support for glucocorticoid involvement in extinction is in the amygdala. Post-training injection of a GR antagonist into the BLA impairs fear memory consolidation and extinction (Roosendaal & McGaugh, 1997; Chatwal et al., 2005; Markrann et al., 2007). Furthermore, intra-amygdalar infusion of RU28362 (GR agonist) facilitates extinction learning, whereas infusion of mifepristone (GR antagonist) into the amygdala blocks extinction learning (Yang et al., 2006). Glucocorticoid administration in the BLA decreases the effects of inhibitory input to BLA neurons (Duvarci & Pare, 2007). This increases the excitability of these BLA neurons, increasing inhibitory input to the CeA and hence changing behavioural output following glucocorticoid administration.

In addition to the amygdala, the effects of glucocorticoids on the hippocampus have been studied extensively. Low levels of glucocorticoid primarily activate MR and increase hippocampal activity (de Kloet et al., 1999), whereas high glucocorticoid levels, which activate both MRs and GRs, inhibit hippocampal activity (Joels & de Kloet, 1992). This suggests that there is an optimal level of glucocorticoid activation in the hippocampus, and that by overshooting this level, consolidation of a contextual fear memory can be inhibited. In addition,

corticosterone may act within the hippocampus to facilitate extinction learning. Administration of glucocorticoids into the hippocampus facilitates extinction learning (Micheau et al., 1982).

Chronic stress or glucocorticoid treatment induces neuronal atrophy and dendritic spine loss in the PFC (Brown, et al., 2005; Cook & Wellman, 2004). Within the infralimbic subregion of the mPFC, dendritic retraction of pyramidal cells is produced by a brief swim stress in mice and is accompanied by resistance to extinction of fear learning (Izquierdo, et al., 2006). This implicates the infralimbic subregion of the mPFC in extinction of fear memories. Stress effects may depend on glucocorticoids since this region is sensitive to GR agonists (Roozendaal, McReynolds & McGaugh, 2004).

To our knowledge, the central action of corticosterone in extinction of a predator stress memory has not been studied. However, areas thought to be involved in extinction of shock-induced fear memory are likely candidates for predator stress extinction. Therefore, future studies targeting GR and MR in amygdala, as well as other brain regions (those described above) during extinction of a predator stress-induced fear memory are warranted. Given that metyrapone blocked extinction of hyperarousal (a context-independent fear memory) and not activity during re-exposures to the predator stress context (a context-dependent memory), we expect that blockade of GR or MR in amygdala (and other regions important in extinction), will only affect extinction of hyperarousal.

#### 4.7 Implications for PTSD

We show that extinction of a context-dependent fear memory can decrease a context-independent fear memory (hyperarousal; Figure 2). This preclinical finding has clinical parallels. In particular, exposure therapies which require progressive extinction of traumatic fear memories decrease symptoms of PTSD including hyperarousal in some affected individuals (Morgan et al., 1995; Bentz et al., 2010). Our data are also consistent with a role for glucocorticoids in susceptibility to, and maintenance of, acquired anxiety disorders such as PTSD. Patients with PTSD have lower circulating levels of cortisol and altered HPA axis activity (Yehuda, 2002), suggesting that corticosteroids may have a protective effect. Blunted cortisol responses following reactivation of the traumatic memories might enhance persistent hyperarousal in affected individuals. Repeated reactivation of fear memories in unaffected individuals, followed by normal cortisol surges, may serve to dampen hyperarousal. If this is true, then our data (see Figure 6) suggest that PTSD patients who exhibit blunted cortisol may benefit from supplemental cortisol treatment during reactivation of their traumatic event(s). Indeed, cortisol administration before exposure therapy in humans is helpful in the treatment of established disorders of emotional memories such as PTSD and phobias (Aerni et al., 2004; Soravia et al., 2006). Alternatively, our data suggest that simply blocking cortisol following exposure to a traumatic event may dampen hyperarousal.

#### **4.8 General Conclusions**

Re-exposure to the predator stress context extinguished a predator stress-induced, context-dependent fear memory, and decreased subsequent hyperarousal (context-independent fear memory). Glucocorticoid synthesis blockade did not affect extinction of a predator stress-induced context-dependent fear memory. However, it did prevent extinction of predator stress-induced hyperarousal. These results suggest that extinction of predator stress-induced, context-independent fear memory is dependent on the presence of corticosterone during the extinction trials.

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**Table 1: Summary of Statistical Tests**

**Extinction Study**

Test Variant	Parameter	Comparison	Mean and Standard Deviation	n of each group	Results
Predator Exposure	Frequency of mouse approaches to the cat	PS vs. PSE	PS: Mean=2.87 SD=1.46 PSE: Mean=3.20 SD=2.45	15	1-way ANOVA: group F(1, 28)=0.20, p=0.65
	Frequency of mouse flights from the cat	PS vs. PSE	PS: Mean=9.73 SD=8.91 PSE: Mean=7.20 SD=7.28	15	1-way ANOVA: group F(1, 28)=0.73, p=0.40
	Frequency of cat approaches to mouse	PS vs. PSE	PS: Mean= 1.47 SD= 1.68 PSE: Mean=1.80 SD=2.08	15	1-way ANOVA: group F(1, 28)=0.23, p=0.63
	Frequency of cat physically contacting the mouse with its paw	PS vs. PSE	PS: Mean= 27.87 SD=35.92 PSE: Mean=28.80 SD=47.19	15	1-way ANOVA: group F(1, 28)=0.04, p=0.95
	Total time cat and mouse were within one square of each other	PS vs. PSE	PS: Mean= 72.14 SD= 84.52 PSE: Mean=81.75 SD= 75.27	15	1-way ANOVA: group F(1, 28)=0.11, p=0.74
	Lines crossed	PS vs. PSE	PS: Mean=107.27 SD=20.91	15	1-way ANOVA: group F(1, 28)=0.62, p=0.44

			PSE: Mean=97.07 SD=45.22		
Re-exposures	Lines crossed	PSE across extinction days	Day 1: Mean= SD= Day 2: Mean= SD= Day 3: Mean= SD= Day 4: Mean= SD= Day 5: Mean= SD=	15	Repeated measures ANOVA: extinction day ( <b>F(4,56) = 4.94, p = 0.002.</b> )
Acoustic Startle Response	Median peak startle amplitude	HC vs. PS vs. PSE	HC: Median= 366 SD= 46.48 PS: Median= 437 SD= 71.65 PSE: Median= 332 SD= 94.89	15	Kruskal-Wallis: <b>H(2)=15.83, p&lt;0.001.</b> Median amplitude contrasts with the Kruskal-Wallis multiple z-test revealed that PS was different from both HC and PSE which did not differ from each other (p<0.01).
	Habituation	HC vs. PS vs. PSE	HC: Tau=11.69 SD= 14.79 PS: Tau=30 SD=26.88 PSE: Tau=10.49 SD=8.87	15	Fit of exponential decay: All exponential fits <b>F(2, 27)&gt;75.2, p&lt;0.001.</b> All Tau >0, p<0.01. Tau planned contrasts t-tests where PS had a greater Tau than HC and PSE which did not differ (p<0.03).
Hole Board	Frequency of head dips	HC vs. PS vs. PSE	HC: Mean=2.80 SD=1.70 PS: Mean=2.73 SD=2.22 PSE: Mean=2.55 SD=1.46	15	1-way ANOVA: group F(2, 42)=0.61, p=0.55
	Frequency of rears	HC vs. PS vs. PSE	HC: Mean=34.07 SD=10.44 PS: Mean=35.13 SD=9.72 PSE: Mean=42.27 SD=10.18	15	1-way ANOVA: group F(2, 42)=2.91, p=0.06
	Total time in center	HC vs. PS vs. PSE	HC: Mean=54.75 SD=14.65 PS: Mean=58.98 SD=23.57 PSE: Mean=48.37 SD=14.40	15	1-way ANOVA: group F(2, 42)=1.31, p=0.28

	Total time in periphery	HC vs. PS vs. PSE	HC: Mean=152.57 SD=33.58 PS: Mean=141.91 SD=36.61 PSE: Mean=165.33 SD=33.84	15	1-way ANOVA: group $F(2, 42)=1.71, p=0.19$
Elevated Plus Maze	Frequency of risk assessment	HC vs. PS vs. PSE	HC: Mean=10.07 SD=2.74 PS: Mean=10.20 SD=4.46 PSE: Mean=9.53 SD=2.69	15	1-way ANOVA: group $F(2, 42)=0.16, p=0.85$
	Total time risk assessment	HC vs. PS vs. PSE	HC: Mean=17.26 SD=7.75 PS: Mean=15.17 SD=8.78 PSE: Mean=16.44 SD=6.67	15	1-way ANOVA: group $F(2, 42)=0.27, p=0.76$
	Ratio time	HC vs. PS vs. PSE	HC: Mean=0.14 SD=0.09 PS: Mean=0.18 SD=0.08 PSE: Mean=0.23 SD=0.06	15	1-way ANOVA: group <b><math>F(2, 42)=5.73, p=0.01</math></b>
	Ratio frequency	HC vs. PS vs. PSE	HC: Mean=0.19 SD=0.12 PS: Mean=0.24 SD=0.08 PSE: Mean=0.29 SD=0.10	15	1-way ANOVA: group <b><math>F(2, 42)=3.34, p=0.04</math></b>
Light/Dark Box	Latency to enter the light	HC vs. PS vs. PSE	HC: Mean=130.40 SD=137.38 PS: Mean=91.33 SD=102.40 PSE: Mean=98.13 SD=110.03	15	1-way ANOVA: group $F(2, 42)=0.47, p=0.63$

Frequency to enter dark	HC vs. PS vs. PSE	HC: Mean=6.80 SD=2.34 PS: Mean=7.33 SD=3.39 PSE: Mean=7.67 SD=3.66	15	1-way ANOVA: group F(2, 42)=0.28, p=0.75
Total time in dark	HC vs. PS vs. PSE	HC: Mean=264.27 SD=26.66 PS: Mean=253.97 SD=29.45 PSE: Mean=347.60 SD=30.14	15	1-way ANOVA: group F(2, 42)=1.28, p=0.29
Frequency to enter light	HC vs. PS vs. PSE	HC: Mean=3.67 SD=3.68 PS: Mean=5.40 SD=3.79 PSE: Mean=6.20 SD=4.68	15	1-way ANOVA: group F(2, 42)=1.51, p=0.23
Total time in light	HC vs. PS vs. PSE	HC: Mean=23.12 SD=26.47 PS: Mean=34.21 SD=25.79 PSE: Mean=40.48 SD=26.26	15	1-way ANOVA: group F(2, 42)=1.69, p=0.20

### Metyrapone Extinction Study

Predator Exposure	Frequency of mouse approaches to the cat	PS vs. PSVE, vs. PSME	PS: Mean=2.50 SD=1.64 PSME: Mean=2.50 SD=1.54 PSVE: Mean=1.65 SD=1.87	20	1-way ANOVA: group F(2, 57)=1.69, p=0.19
	Frequency of mouse flights from the cat	PS vs. PSVE, vs. PSME	PS: Mean=2.00 SD=2.43 PSME: Mean=1.90 SD=1.83 PSVE: Mean=2.00 SD=2.61	20	1-way ANOVA: group F(2, 57)=0.01, p=0.99

Frequency of cat approaches to mouse	PS vs. PSVE, vs. PSME	PS: Mean=1.05 SD=2.33 PSME: Mean=1.20 SD=1.58 PSVE: Mean=1.55 SD=2.50	20	1-way ANOVA: group F(2, 57)=0.28, p=0.76
Frequency of cat sniffing the mouse	PS vs. PSVE, vs. PSME	PS: Mean=0.15 SD=0.49 PSME: Mean=0.74 SD=1.37 PSVE: Mean=0.60 SD=1.14	20	1-way ANOVA: group F(2, 57)=1.65, p=0.20
Frequency of cat biting the mouse	PS vs. PSVE, vs. PSME	PS: Mean=0 SD=0 PSME: Mean= 0.05 SD=0.22 PSVE: Mean=0.05 SD=0.22	20	1-way ANOVA: group F(2, 57)=0.50, p=0.61
Frequency of cat physically contacting the mouse with its paw	PS vs. PSVE, vs. PSME	PS: Mean=2.00 SD=4.51 PSME: Mean=2.65 SD=5.43 PSVE: Mean=5.75 SD=10.11	20	1-way ANOVA: group F(2, 57)=1.58, p=0.21
Frequency of cat vocalizations	PS vs. PSVE, vs. PSME	PS: Mean=10.53 SD=13.80 PSME: Mean=12.70 SD=13.80 PSVE: Mean=6.60 SD=9.07	20	1-way ANOVA: group F(2, 57)=1.24, p=0.30
Total time cat and mouse were within one square of each other	PS vs. PSVE, vs. PSME	PS: Mean=21.99 SD=14.42 PSME: Mean=55.40 SD=121.10 PSVE: Mean=27.70 SD=35.27	20	1-way ANOVA: group F(2, 57)=1.18, p=0.31
Lines crossed	PS vs. PSVE, vs. PSME	PS: Mean=12.12 SD=3.59	20	1-way ANOVA: group F(2,57)=0.13, p=0.88

		PSME	PSME: Mean=11.68 SD=5.81 PSVE: Mean=11.36 SD=4.45		
Re-exposures	Lines crossed	PSVE vs. PSME across extinction days	Day 1: Mean=14.32 SE=0.730 Day 2: Mean=16.77 SE=0.93 Day 3: Mean=17.74 SE=0.76 Day 4: Mean=17.31 SE=0.68 Day 5: Mean=19.20 SE=0.90	20 (PSVE = 19)	Mixed ANOVA: extinction day <b>F(4,56) = 10.76, p = 0.000.</b>
Acoustic Startle Response	Median peak startle amplitude	PS vs. PSVE, vs. PSME	PS: Median= 515 SD= 77.37 PSVE: Median= 444 SD= 111.80 PSME: Median= 557 SD= 111.80	20	Kruskal-Wallis: <b>H(2)=31.93, p&lt;0.001.</b> Median amplitude contrasts with the Kruskal-Wallis multiple z-test revealed that all group were different (p<0.01).
	Habituation	PS vs. PSVE, vs. PSME	PS: Tau= 5.35 SD= 1.62 PSVE: Tau= 3.21 SD= 1.02 PSME: Tau= 5.67 SD= 1.06	20	Fit of exponential decay: All exponential fits <b>F(2, 27)&gt;15.02, p&lt;0.001.</b> All Tau >0, p<0.02. Tau planned contrasts t-tests where PSVE had a smaller Tau than PS and PSME (p<0.02) which did not differ.
Hole Board	Frequency of head dips	PS vs. PSVE, vs. PSME	PS: Mean=3.55 SD=2.19 PSME: Mean=3.35 SD=1.98 PSVE: Mean=4.40 SD=2.35	20	1-way ANOVA: group F(2,57)=1.31, p=0.28
	Frequency of rears	PS vs. PSVE, vs. PSME	PS: Mean=35.10 SD=10.61 PSME: Mean=42.65 SD=12.52 PSVE: Mean=43.40	20	1-way ANOVA: group F(2,57)=2.98, p=0.06.

SD=12.44

	Total time in center	PS vs. PSVE, vs. PSME	PS: Mean=41.36 SD=22.60 PSME: Mean=36.25 SD=17.09 PSVE: Mean=34.89 SD=9.54	20	1-way ANOVA: group F(2,57)=0.78, p=0.46
	Total time in periphery	PS vs. PSVE, vs. PSME	PS: Mean=258.64 SD=22.60 PSME: Mean=263.75 SD=17.09 PSVE: Mean=265.11 SD=9.54	20	1-way ANOVA: group F(2,57)=0.78, p=0.46
Elevated Plus Maze	Frequency of risk assessment	PS vs. PSVE, vs. PSME	PS: Mean=13.73 SD=5.32 PSME: Mean=14.07 SD=4.46 PSVE: Mean=15.40 SD=3.56	15	1-way ANOVA: group (2,42)=0.57, p=0.57
	Total time risk assessment	PS vs. PSVE, vs. PSME	PS: Mean=46.49 SD=19.89 PSME: Mean=49.45 SD=20.73 PSVE: Mean=45.04 SD=9.02	15	1-way ANOVA: group (2,42)=0.25, p=0.78
	Ratio time	PS vs. PSVE, vs. PSME	PS: Mean=0.25 SD=0.18 PSME: Mean=0.25 SD=0.14 PSVE: Mean=0.26 SD=0.13	20 (PS = 18)	1-way ANOVA: group F(2,55)=0.05, p=0.95
	Ratio frequency	PS vs. PSVE, vs. PSME	PS: Mean=0.34 SD=0.17 PSME: Mean=0.35 SD=0.13 PSVE: Mean=0.36	20 (PS = 18)	1-way ANOVA: group F(2,55)=0.14, p=0.87

SD=0.12

Light/Dark Box	Latency to enter the dark	PS vs. PSVE, vs. PSME	PS: Mean=33.98 SD=33.39 PSME: Mean=28.45 SD=25.80 PSVE: Mean=22.54 SD=15.15	20 (PS = 19)	1-way ANOVA: group F(2,56)=0.96, p=0.39
	Frequency to enter dark	PS vs. PSVE, vs. PSME	PS: Mean=7.58 SD=2.34 PSME: Mean=7.10 SD=2.75 PSVE: Mean=7.50 SD=2.11	20 (PS = 19)	1-way ANOVA: group F(2,56)=0.22, p=0.80
	Total time in dark	PS vs. PSVE, vs. PSME	PS: Mean=219.83 SD=37.70 PSME: Mean=222.90 SD=52.09 PSVE: Mean=220.16 SD=37.36	20 (PS = 19)	1-way ANOVA: group F(2,56)=0.03, p=0.97
	Frequency to enter light	PS vs. PSVE, vs. PSME	PS: Mean=5.63 SD=2.91 PSME: Mean=4.35 SD=2.50 PSVE: Mean=5.60 SD=3.35	20 (PS = 19)	1-way ANOVA: group F(2,56)=1.23, p=0.30
	Total time in light	PS vs. PSVE, vs. PSME	PS: Mean=53.57 SD=34.65 PSME: Mean=37.50 SD=23.61 PSVE: Mean=40.76 SD=18.17	20 (PS = 19)	1-way ANOVA: group F(2,56)=2.03, p=0.14

#### Metyrapone Control Study

Predator Exposure	Frequency of mouse approaches to	PS vs. PSVn, vs. PSMn	PS: Mean=1.20 SD=0.68 PSMn: Mean=0.87 SD=0.91	15	1-way ANOVA: group F(2, 41)=0.50, p=0.61
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the cat		PSVn: Mean=1.27 SD=1.10		
Frequency of mouse flights from the cat	PS vs. PSVn, vs. PSMn	PS: Mean=0.33 SD=0.49 PSMn: Mean=0.13 SD=0.35 PSVn: Mean=0.07 SD=0.26	15	1-way ANOVA: group F(2, 41)=1.93, p=0.16
Frequency of cat approaches to mouse	PS vs. PSVn, vs. PSMn	PS: Mean=0.47 SD=1.06 PSMn: Mean=0.07 SD=0.26 PSVn: Mean=0.67 SD=1.29	15	1-way ANOVA: group F(2, 41)=0.89, p=0.42
Frequency of cat sniffing the mouse	PS vs. PSVn, vs. PSMn	PS: Mean=0.53 SD=1.12 PSMn: Mean=0.20 SD=0.41 PSVn: Mean=0.07 SD=0.26	15	1-way ANOVA: group F(2, 41)=1.77, p=0.18
Frequency of cat physically contacting the mouse with its paw	PS vs. PSVn, vs. PSMn	PS: Mean=4.60 SD=7.84 PSMn: Mean=0.87 SD=2.59 PSVn: Mean=4.20 SD=11.23	15	1-way ANOVA: group F(2, 41)=60, p=0.55
Frequency of cat vocalizations	PS vs. PSVn, vs. PSMn	PS: Mean=5.07 SD=6.82 PSMn: Mean=2.87 SD=5.37 PSVn: Mean=3.00 SD=4.54	15	1-way ANOVA: group F(2, 41)=0.63, p=0.54
Frequency of cat and mouse being within one square of each other	PS vs. PSVn, vs. PSMn	PS: Mean=2.87 SD=2.39 PSMn: Mean=2.13 SD=1.30 PSVn: Mean=3.46 SD=1.85	15	1-way ANOVA: group F(2, 41)=1.31, p=0.28

	Total time cat and mouse were within one square of each other	PS vs. PSVn, vs. PSMn	PS: Mean=31.22 SD=28.89 PSMn: Mean=20.58 SD=16.94 PSVn: Mean=29.82 SD=24.55	15	1-way ANOVA: group F(2, 41)=0.84, p=0.44
	Lines crossed	PS vs. PSVn, vs. PSMn	PS: Mean=13.87 SD=4.39 PSMn: Mean=13.33 SD=4.28 PSVn: Mean=14.91 SD=5.86	15	1-way ANOVA: group F(2, 42)=1.15, p=0.33
Acoustic Startle Response	Median peak startle response	PS vs. PSVn, vs. PSMn	PS: Median= 520 SD= 84.52 PSVn: Median= 464 SD= 134.16 PSMn: Median= 376 SD= 88.99	15	Kruskal-Wallis: <b>H(2)=58.88, p&lt;0.001</b> . Median amplitude contrasts with the Kruskal-Wallis multiple z-test revealed that all group were different from each other (p<0.01).
	Habituation	PS vs. PSVn, vs. PSMn	PS: Tau= 6.11 SD= 0.53 PSVn: Tau= 10.91 SD= 2.40 PSMn: Tau= 11.58 SD= 4.26	15	Fit of exponential decay: All exponential fits <b>F(2, 27)&gt;42.08, p&lt;0.001</b> . All Tau >0, p<0.038. Tau planned contrasts t-tests where PS had a smaller Tau than PSVn and PSMn (p<0.038).
Hole Board	Frequency of head dips	PS vs. PSVn, vs. PSMn	PS: Mean=7.60 SD=3.36 PSMn: Mean=10.13 SD=3.04 PSVn: Mean=8.13 SD=4.19	15	1-way ANOVA: group F(2,41)=2.20, p=0.12
	Frequency of rears	PS vs. PSVn, vs. PSMn	PS: Mean=39.93 SD=13.87 PSMn: Mean=42.47 SD=11.74 PSVn: Mean=36.60 SD=10.92	15	1-way ANOVA: group F(2,41)=1.01, p=0.35
	Total time in	PS vs.	PS: Mean=67.86	15	1-way ANOVA: group F(2,41)=0.37, p=0.70

	center	PSVn, vs. PSMn	SD=17.94 PSMn: Mean=67.94 SD=19.50 PSVn: Mean=62.96 SD=17.27		
	Total time in periphery	PS vs. PSVn, vs. PSMn	PS: Mean=131.05 SD=17.56 PSMn: Mean=132.37 SD=24.59 PSVn: Mean=139.64 SD=19.67	15	1-way ANOVA: group F(2,41)=0.36, p=0.70
Elevated Plus Maze	Frequency of risk assessment	PS vs. PSVn, vs. PSMn	PS: Mean=12.13 SD=3.02 PSMn: Mean=13.60 SD=2.35 PSVn: Mean=10.73 SD=2.71	15	1-way ANOVA: group F(2,41)=2.83, p=0.07
	Total time risk assessment	PS vs. PSVn, vs. PSMn	PS: Mean=34.37 SD=19.19 PSMn: Mean=33.99 SD=11.86 PSVn: Mean=27.81 SD=8.13	15	1-way ANOVA: group F(2,41)=0.75, p=0.48
	Ratio time	PS vs. PSVn, vs. PSMn	PS: Mean=0.21 SD=0.14 PSMn: Mean=0.21 SD=0.09 PSVn: Mean=0.14 SD=0.08	15	1-way ANOVA: group F(2,41)=1.95, p=0.16
	Ratio frequency	PS vs. PSVn, vs. PSMn	PS: Mean=0.27 SD=0.13 PSMn: Mean=0.28 SD=0.08 PSVn: Mean=0.23 SD=0.10	15	1-way ANOVA: group F(2,41)=0.90, p=0.42
Light/Dark Box	Latency to enter the dark	PS vs. PSVn, vs.	PS: Mean=16.45 SD=11.40	15	1-way ANOVA: group F(2,41)=0.12, p=0.87

	PSMn	PSMn: Mean=12.23 SD=9.51 PSVn: Mean=11.71 SD=11.44		
Frequency to enter light	PS vs. PSVn, vs. PSMn	PS: Mean=8.07 SD=2.79 PSMn: Mean=8.87 SD=3.42 PSVn: Mean=6.27 SD=3.19	15	1-way ANOVA: group F(2,41)=1.93, p=0.16
Total time in light	PS vs. PSVn, vs. PSMn	PS: Mean=65.35 SD=36.63 PSMn: Mean=71.20 SD=26.63 PSVn: Mean=49.14 SD=29.18	15	1-way ANOVA: group F(2,41)=2.35, p=0.11

## Figure Captions

- Figure 1: Extinction Study: Mean + SEM of lines crossed across all 5 extinction days. An “a” indicates a significant difference from Extinction Day 1 ( $p < 0.05$ ). A “b” indicates a significant difference from Extinction Day 2 ( $p < 0.05$ ). As can be seen, animals showed a significant increase in lines crossed over extinction days.
- Figure 2: Extinction Study: Median peak startle amplitudes + SEM in arbitrary units are plotted over three groups: handled controls (HC), predator stressed (PS) and predator stressed + extinction (PSE). Medians marked with the same letter do not differ, medians marked with different letters differ ( $p < 0.05$ ). HC and PSE did not differ, whereas PS mice showed an elevated startle amplitude ( $p < 0.05$ ). As can be seen, extinction training (PSE) returned startle response to the level of controls (HC).
- Figure 3: Extinction Study: Trial constants ( $Tau$ ) + SE are plotted over three groups: handled controls (HC), predator stressed (PS) and predator stressed + extinction (PSE).  $Tau$  values marked with the same letter do not differ,  $Tau$ 's marked differently differ. HC and PSE did not differ, whereas PS mice showed elevated trials to habituate ( $p < 0.05$ ). As can be seen, extinction training (PSE) returned trials to habituate ( $Tau$ ) to the level of controls (HC).
- Figure 4: Extinction Study: Mean + SEM of elevated plus maze behaviours are plotted over three groups: handled controls (HC), predator stressed (PS) and predator stressed + extinction (PSE). The upper panel shows ratio time in the open arms data and the lower panel shows ratio frequency to enter the open arms data. For a given plot, means marked with the same letter do not differ, means marked with different letters differ ( $p < 0.05$ ). HC and PS did not differ, whereas PSE mice showed an elevated ratio time and ratio frequency ( $p < 0.05$ ). Therefore, extinction training increased ratio time and ratio frequency to enter the open arms relative to PS controls, an anxiolytic effect.
- Figure 5: Metyrapone Extinction Study: Mean + SEM of lines crossed on each of 5 extinction days are plotted. An “a” indicates a significant difference from Extinction Day 1 ( $p < 0.05$ ). A “b” indicates a significant difference from Extinction Day 2 ( $p < 0.05$ ). Plotted separately are two extinction groups: predator stressed + vehicle + extinction training (PSVE) and predator stressed + metyrapone + extinction (PSME). As can be seen, all animals showed a significant

increase in lines crossed over extinction days and groups did not differ.

- Figure 6: Metyrapone Extinction Study: Median peak startle amplitudes + SEM in arbitrary units are plotted over three groups: predator stressed (PS), predator stressed + vehicle + extinction training (PSVE) and predator stressed + metyrapone + extinction (PSME). Medians marked with a different letter differ from each other ( $p < 0.05$ ). As can be seen, vehicle + extinction training (PSVE) decreased peak startle amplitude, whereas metyrapone + extinction (PSME) elevated peak startle amplitude relative to PS controls. Therefore, metyrapone blocked the effect of extinction on peak startle amplitude.
- Figure 7: Metyrapone Extinction Study: Trial constants ( $Tau$ ) + SE are plotted over three groups: predator stressed (PS), predator stressed + vehicle + extinction training (PSVE) and predator stressed + metyrapone + extinction (PSME). Tau values marked with a different letter differ from each other ( $p < 0.05$ ), tau values marked with a similar letter do not differ. Vehicle + extinction (PSVE) decreased trials to habituate, whereas metyrapone + extinction (PSME) and PS controls did not differ. Therefore, metyrapone blocked the effect of extinction on startle habituation.
- Figure 8: Metyrapone Control Study: Median peak startle amplitudes + SEM in arbitrary units are plotted over three groups: predator stressed (PS), predator stressed + vehicle + no extinction (PSVn) and predator stressed + metyrapone + no extinction (PSMn). Medians marked with a different letter differ from each other ( $p < 0.05$ ). As can be seen, vehicle + no extinction training (PSVE) decreased peak startle amplitude and metyrapone + no extinction (PSME) decreased it even further.
- Figure 9: Metyrapone Control Study: Trial constants ( $Tau$ ) + SE are plotted over three groups: predator stressed (PS), predator stressed + vehicle + no extinction (PSVn), and predator stressed + metyrapone + no extinction (PSMn). Tau values marked with a similar letter do not differ, Tau values marked with a different letter differ ( $p < 0.05$ ). Both PSVn and PSMn showed equally increased trials to habituate ( $p < 0.05$ ) relative to PS controls.

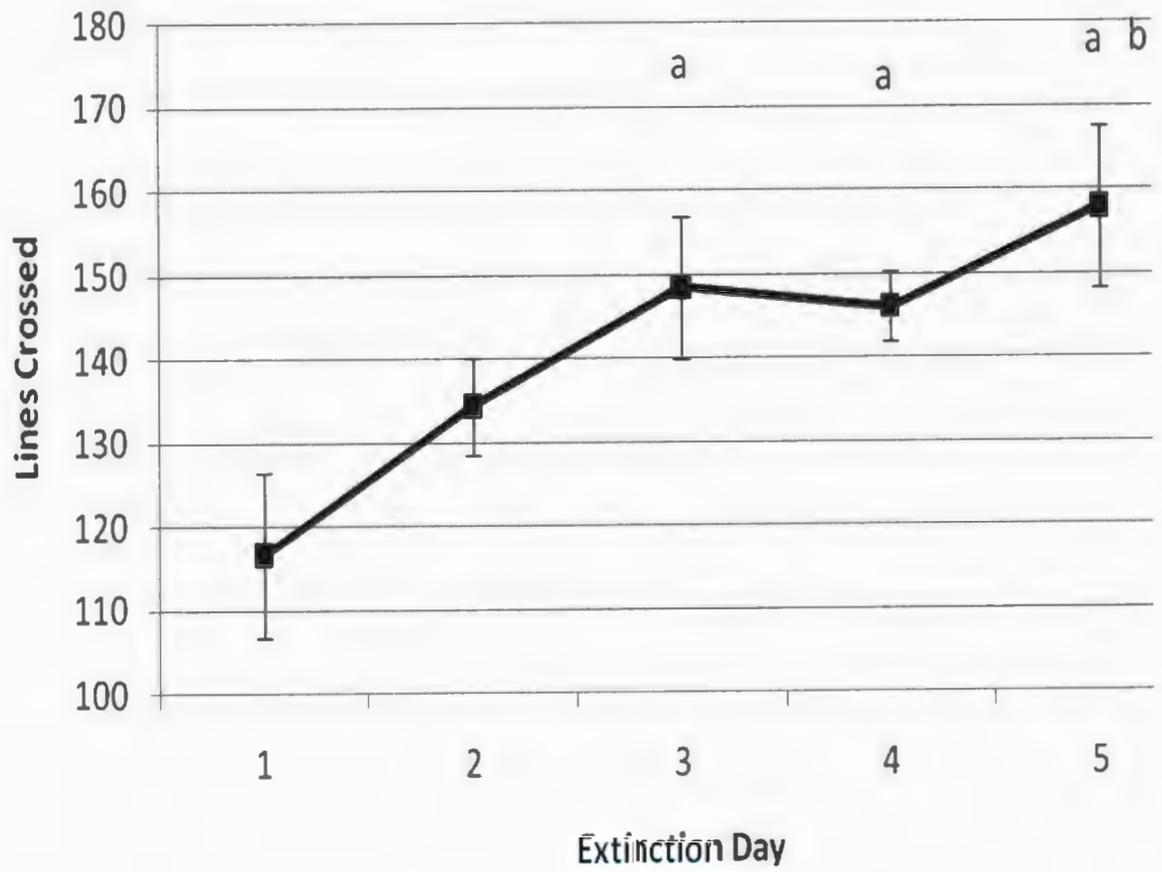


Figure 1

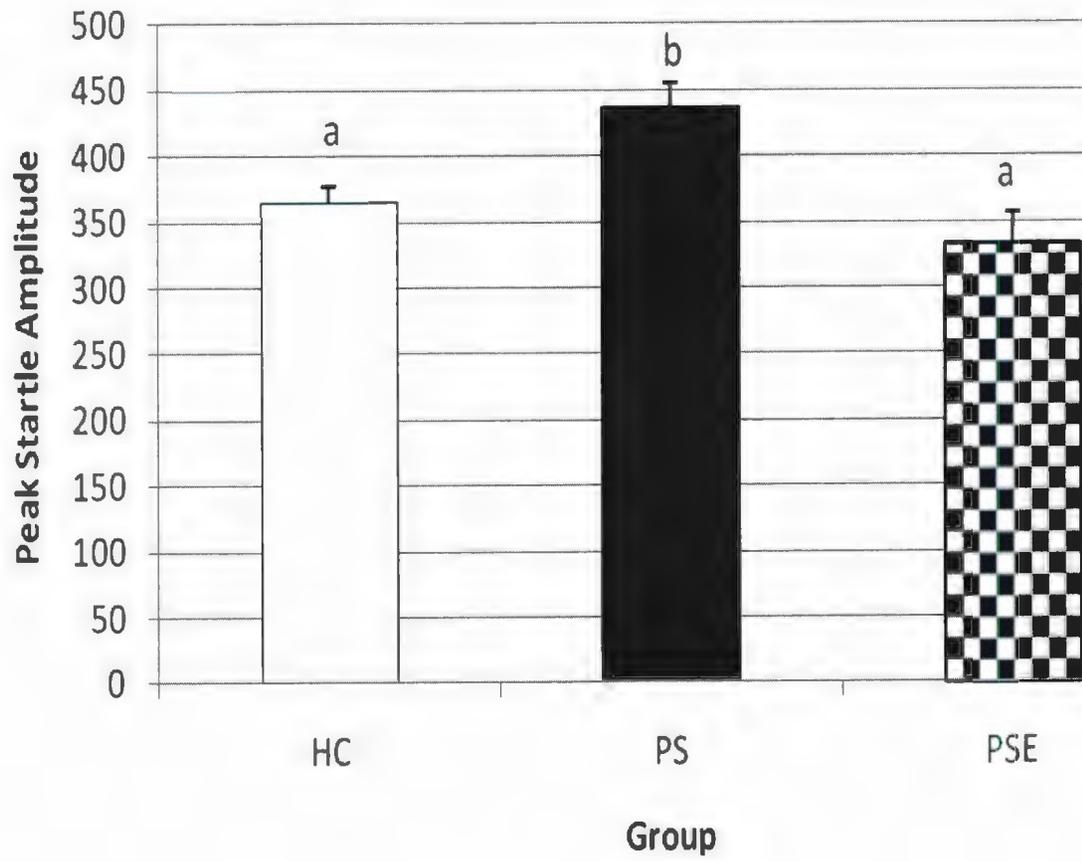


Figure 2

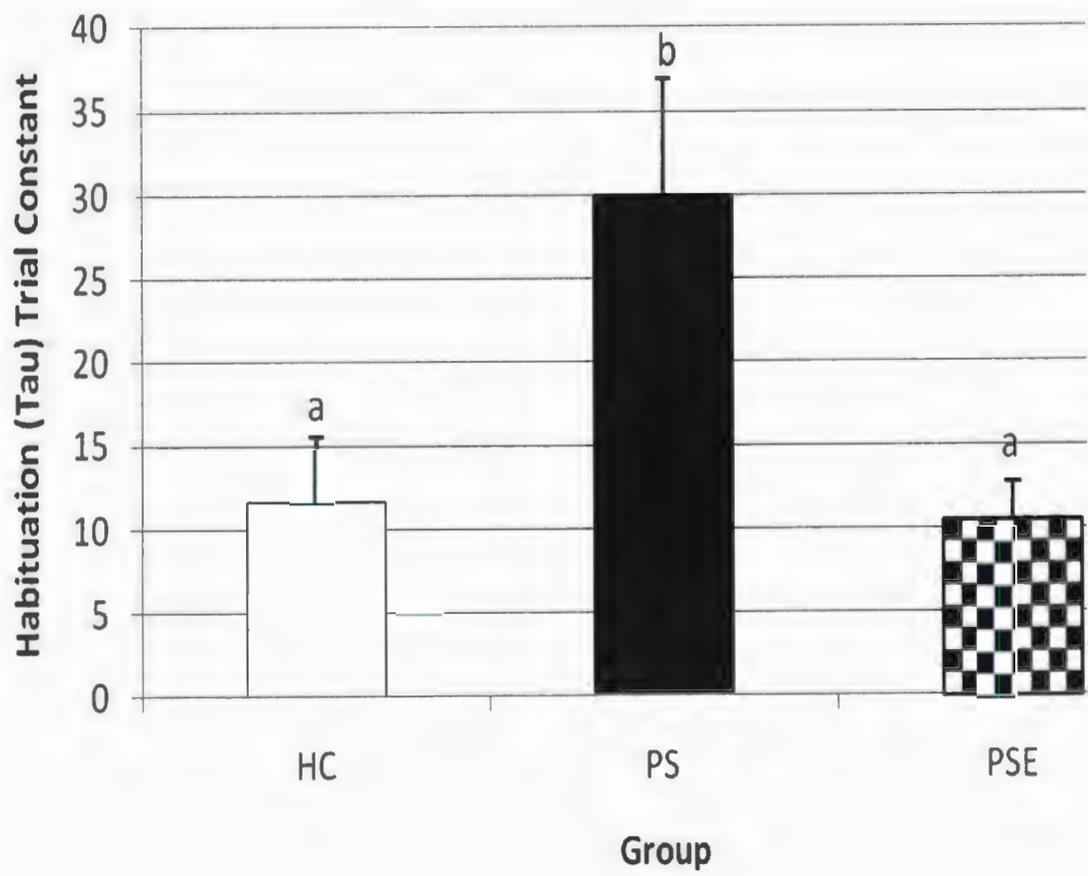


Figure 3

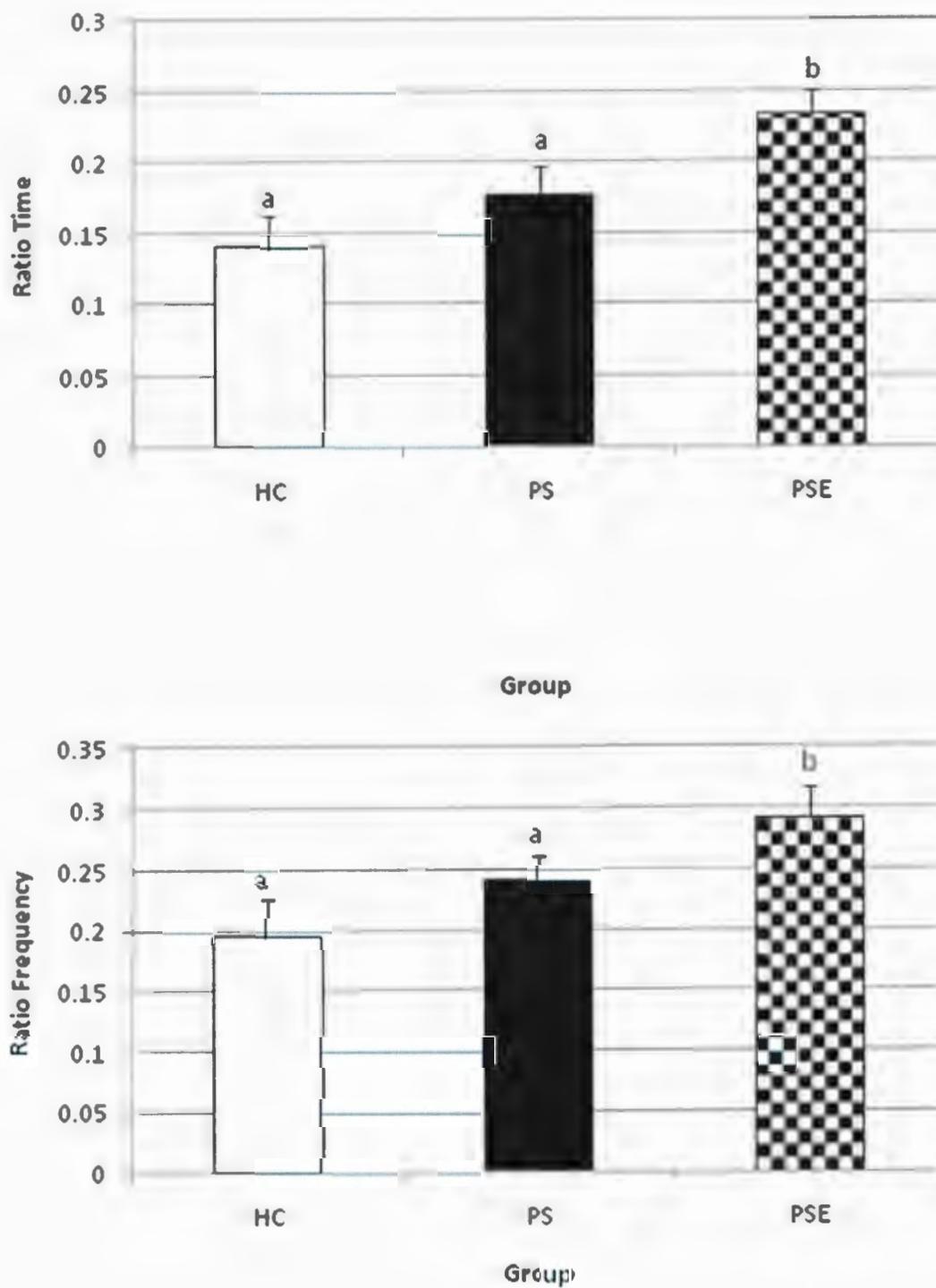


Figure 4

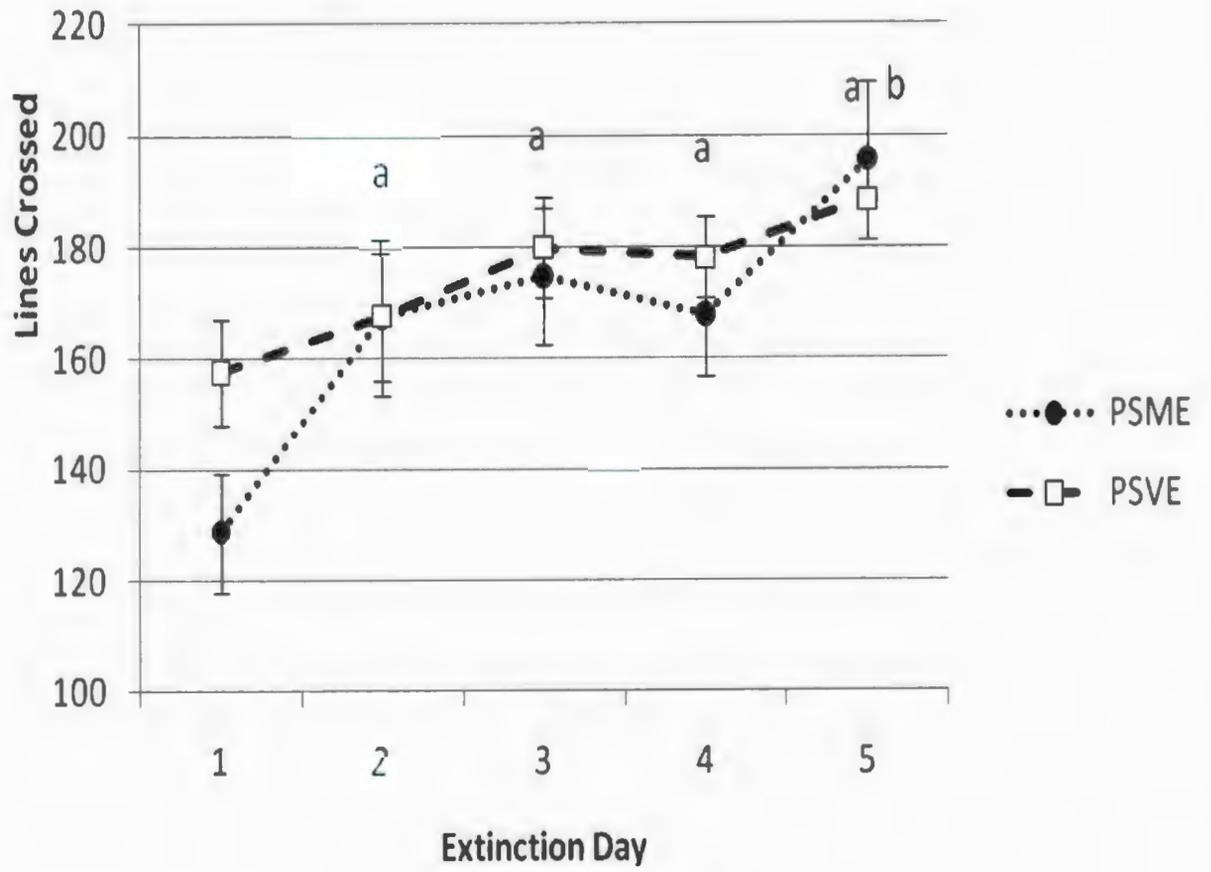


Figure 5

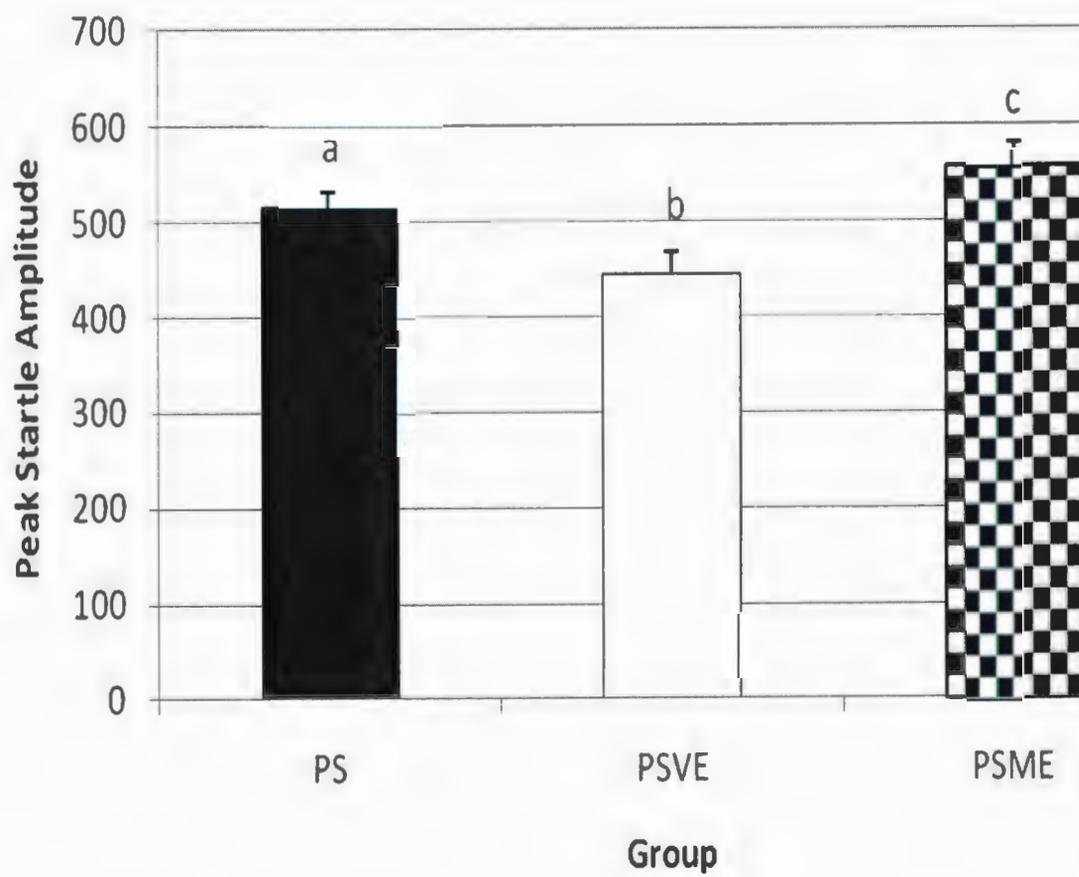


Figure 6

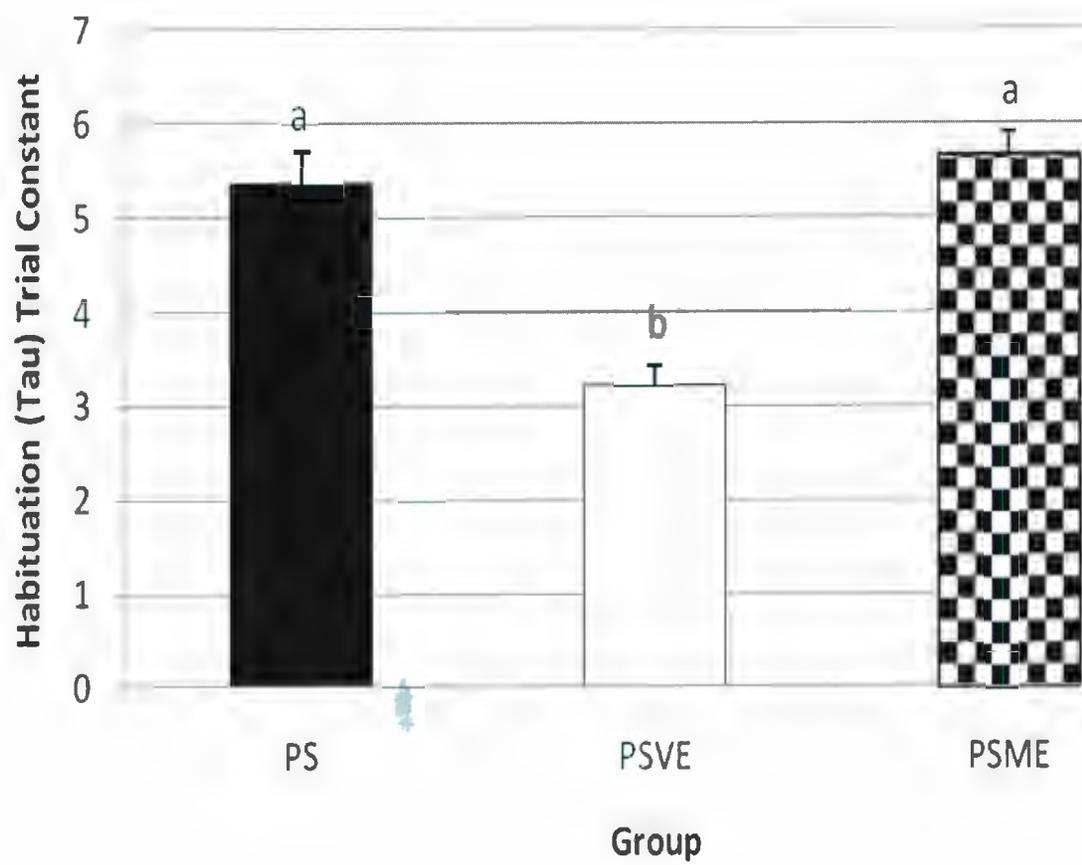


Figure 7

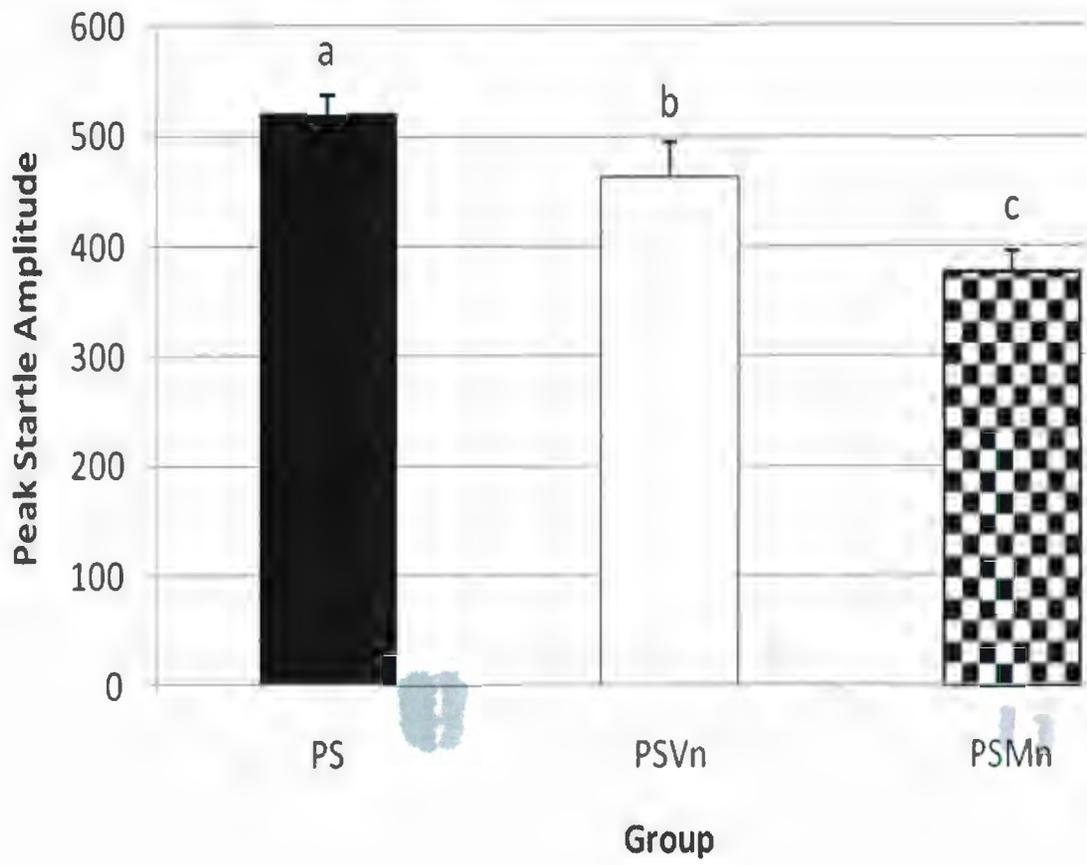


Figure 8

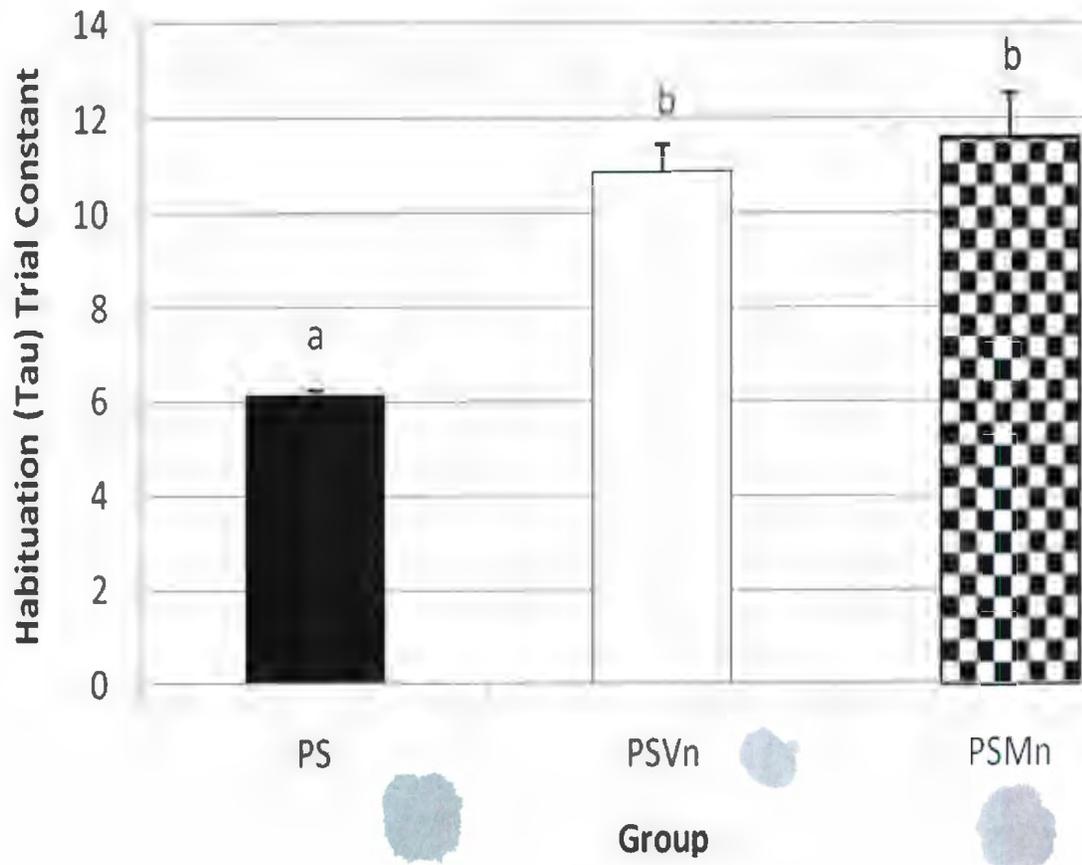


Figure 9





