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Behavioral and Immune Implications of Chronic Predator Exposure in Adolescent Mice

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A thesis in partial fulfillment of the requirements for the degree of Master of Science in Anatomy and Neurobiology at Virginia Commonwealth University

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Abstract

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Kimaya Ryan Council, M.S.

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Evidence suggests that toxic stressors introduced early in development have prolonged effects on neuronal function due, in part, to the maturation of the hypothalamic- pituitary-adrenal (HPA) axis during adolescence. Early life stress has been implicated as a driver of mood and anxiety disorders, like depression and post-traumatic stress disorder - the extent to which appears to be sex dependent. While it is known that early life stress results in several consequences in adulthood, the mechanisms by which these changes manifest are unclear. Stress-

induced changes in mood and behavior are often associated with alterations in inflammatory reactivity in both the brain and in the periphery. Previous work from our lab, and others, demonstrates that both male and female rats respond to chronic adolescent stress (CAS) but may differ in inflammatory markers within the brain and periphery and in the induction of negative affective-like behaviors. Inflammatory reactivity has been targeted as a means of identifying how these sex differences arise in studies of chronic stress in adults. Circulating concentrations of inflammatory cytokines have not been directly employed as predictors of behavioral outcomes of stress exposure in adolescence but may be a useful tool in uncovering mechanisms that protect or predispose an organism from the effects of chronic stress.

To further assess immunological and behavior deficits following chronic stress in adolescence, the current work used a model of chronic adolescent stress where male and female adolescent mice were exposed to a predator stress for 15 consecutive days. In late adolescence, these mice were treated with an acute inflammatory challenge with lipopolysaccharide (LPS) to elicit an inflammatory response. We predicted that chronic, predatory stress experienced during adolescence would induce negative anxiety-like behaviors and alter circulating proinflammatory levels. Furthermore, we expected females to be more susceptible to the effects of adolescent stress than males. We observed that, chronic, predatory stress during adolescence increased anxiety-like behaviors in males and females, but did not alter social behaviors during late adolescence. Predatory stress also impacted circulating levels of TNFα, but no sex differences in LPS-induced cytokine concentrations were apparent.

Introduction

Major Depressive Disorder (MDD), Post-traumatic Stress Disorder (PTSD) and General Anxiety Disorder (GAD) all involve changes in affect, behavior, and in some cases, changes in cognition and memory. The National Institute of Mental Health estimates the prevalence of MDD to be approximately 6.7% within a given year among American adults. Comparably, the prevalence of PTSD and GAD in America are 4.3 and 3.7%, respectively. Chronic stress is a major risk factor for vulnerability to these diseases. Stress, experienced daily, is a natural part of life and protective mechanisms are in place to combat its effects. However, when perceived stress increases in intensity and duration, the systematic changes that occur as a result can promote development of mood and anxiety disorders and can contribute to cardiovascular and neurological dysfunctions (Gouin, 2011; Juster et al., 2010; Schneiderman et al., 2004). Stress is associated with elevated blood pressure and fat accumulation, and changes in sleeping and eating patterns, all of which can contribute to stress related pathologies (McEwen, 2008; Guilliams & Edwards, 2010). Together, stress related disorders in the United States cost more than $42 billion per year, and 1 in 4 Americans in the workplace suffer from stress related mental health issues (Kalia, 2002). The prevalence of stress-induced disorders makes exploring the implications of recurring stress exposure necessary for a better understanding of how physiological mechanisms of the stress response transform and promote pathological conditions.

While the consequences of chronic stress can be negative, chronic stress exposure during periods of developmental maturation can be particularly damaging. Adolescence is a period of endocrine and neuronal refinement making it a period sensitive to the effects of chronic stress (Spear, 2000). For example, brain regions significantly affected by prolonged stress exposure in

adulthood, like the hippocampus, are still maturing during adolescence (Romeo, 2013; Geidd & Rapoport, 2010). Stress exposure during this time is a risk factor for the development of major depression in adulthood (Andersen & Teicher, 2008). Moreover, adolescence is a period in which sex divergences in mood and behavior begin and persist throughout adulthood. This is of particular importance considering sex differences evident in disorders like depression. Taken together, the sex-specific consequences of chronic early life stress is of clinical relevance and the rapid development of brain and hormonal networks during adolescence make it a period of significant vulnerability to chronic stress.

Inflammation has emerged as a key variable in the mediation of stress effects on somatic and mental health. Chronic stress can drive inflammation while chronic adolescent stress can lead to long-lasting alterations in the inflammatory response. Prior exposure to stress induces a priming effect such that the immune system may respond to an inflammatory challenge with heightened reactivity (Perry and Holmes, 2014; Dilger and Johnson, 2008). These changes in inflammation and immune imbalance have been linked to depression, cardiovascular disease and type 2 diabetes. (Dantzer et al., 2008, Liu et al., 2017). These stress-induced physiological consequences are evident in multiple systems – the extent of which can vary depending on the nature of the stress and the duration of the stressor. While these consequences are evident in males and females, females appear particularly vulnerable to chronic stress exposure. This is evident in the prevalence of depression and PTSD being significantly higher in women compared to men (Kessler, 1993; Kessler, 2003; Altemus et al., 2014). These sex differences begin to emerge in adolescence as a result of the large shift in endocrine signaling during this developmental period (McCormick & Matthews, 2007; Romeo, 2013). Despite the higher rates evident in women, research has largely focused on adult males. Furthermore, the mechanism by

which developmental stress leads to immune dysfunction and subsequent physiological deficits remains unclear. This work addresses this gap in the literature by assessing the extent to which chronic stress during development alters markers of peripheral immune activity differentially in males and females and how it relates to behavioral outcomes.

Biology of the Stress Response

The stress response is characterized by physiological changes and alterations across several systems that serve to protect the organism from impending threats against homeostasis (Motzer and Hertig, 2004). The stress response is composed of activation of both the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. The "fight or flight" response, mediated by the SNS, involves physiological and behavioral changes that function to increase available energy substrates and blood flow to the brain and muscles in the periphery (Cannon, 1940). During this "fight or flight", the release of catecholamines causes a rise in cardiac output, respiration and an increase in arousal. This component of the stress response is designed to promote survival and prepare the organism to combat or escape the potential threat (Cannon, 1940).

The second branch of the stress response involves activation of the HPA axis and the release of glucocorticoids to promote more long-term changes in response to stress. Following stimuli deemed stressful, neurons of the central nervous system increase secretion of corticotrophin releasing hormone (CRH), which in turn results in release of glucocorticoids from the adrenal cortex (Motzer and Hertig, 2004). Glucocorticoids play a regulatory role in the actions of the HPA axis through negative feedback mechanisms that function in maintaining homeostasis (Padgette and Glaser, 2003). Other physiological aspects of glucocorticoids involve

carbohydrate metabolism, fluid balance, and immunosuppression (Munck et al., 1984). The inflammatory actions of glucocorticoids are of particular interest as they play a role in the regulation of several inflammatory mediators (Barnes, 1998). Actions of glucocorticoids are mediated through their receptors which act within the nucleus of cells as transcription factors; glucocorticoid receptors then induce changes in gene expression which in turn result in a cascade of functional changes throughout the body (Munck et al., 1984).

Due to the sensitive nature of glucocorticoids and the many systems they can impact, imbalances in glucocorticoid metabolism and HPA axis regulation may result in maladaptive behaviors and physiology. Allostasis is the term designated for the neural and immune processes by which the body maintains homeostasis. (McEwen, 2000). Activation of these processes, over frequent or extended periods of time, can leave an organism vulnerable to both internal wear and tear and external influences. "Allostatic overload" refers to the outcomes related to significant stress exposure and/or prolonged exposure without sufficient management (McEwen, 2007). The stress response is a sensitive balance between protection and damage, this overload can encourage maladaptive modifications to several systems and influence both behavior and immune outcomes.

As stress exposure shifts from acute to chronic, measures responsible for protecting an organism from internal and external influences are altered which results in a shift toward pathology instead of normal physiology. Behavioral consequences of chronic stress may be more pronounced compared to those associated with acute exposure. Data compiled from a large survey showed chronic stressors were more related to depression symptoms, compared to acute stressors (McGonagle & Kessler, 1990). These data showed increased chronic stress exposure correlating with depression symptoms in both men and women. Differences in the effects of

acute stress versus chronic stress are also apparent in the immune system. Acute stress is known to enhance immune function by increasing lymphocyte trafficking to important areas, whereas chronic stress is more associated with immunosuppression (Dhabar, 2006; Dhabar, 2008). Chronic stress has the potential to disrupt HPA activity and function. Increases and decreases in HPA activity, both of which can be a result of chronic stress, can promote dysregulation of hormone secretion, metabolic malfunctions and accelerate disease progression (Miller et al., 2007; Chrousos, 2009).

Cytokine imbalances have been correlated with above mentioned consequences of chronic HPA activation. Three cytokines often of interest in the literature are Interleukin-1 (IL-1B), Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα). IL-6 is a primary stimulator of acute phase proteins and acts predominantly as an anti-inflammatory cytokine, but also has proinflammatory characteristics (Gabay and Kushner.,1999). IL-6 participates in initiation of glucocorticoid synthesis and in downregulation of both IL-1 and TNFα (Opal and DePalo 2000). Increases in IL-6 are associated with cardiovascular disease, chronic stress, depression and aging (Kiecolt-Glaser et al., 2003; Cohen et al., 2012). Downregulated by IL-6, both TNFα, and IL-1B are considered to be proinflammatory cytokines and can also induce gene expression of other proinflammatory cytokines (Dinarello, 1991). IL-1B is produced by cells of the innate immune system and mainly functions in responding to infection, playing a role in regulation of inflammatory cell recruitment and inducing sickness behavior (Gabay et al., 2010; Dantzer, 2009). Similarly, TNFα also participates in regulation of the immune response following infection, inducing fever and inflammation, and at higher concentrations, shock (Dantzer 2009; Dinarello 1991). As stress exposure transforms from acute to chronic, differences in inflammatory patterns as they relate to these cytokines can be harmful. Such differences have

been implicated in Alzheimer's Disease, major depression, and cardiovascular disease (Elenkov & Chrousos, 1999; Chrousos, 2009).

Stress and the Peripheral Immune System

Mediators released during chronic stress exposure have been shown to suppress components of both the innate and acquired immune system. Decreases in cytotoxic T cells and natural killer cells have been reported following severe stress exposure; an inverse relationship between the activity and function of phagocytes and lymphocytes and release of norepinephrine has also been observed across studies (Reiche et al., 2004). Interestingly, the decrease in activity of blood leukocytes following stress has been attributed to redistribution and not cell loss with leukocytes being trafficked from the blood to other areas in the body (Dhabhar, 2002). In addition to immune cell populations, chronic stress also induces changes in both inflammatory and anti-inflammatory cytokines in the periphery. (Gouin et al., 2011). Cytokines produced in the periphery are mediators of numerous physiological processes and imbalances in inflammation that may result from stress contribute to its negative impacts on the immune system.

Other alterations in the immune system in the periphery as a result of stress are evident in wound healing and the immune response following vaccine administration. The early stages of wound healing involve the actions of various cytokines and chronic stress has been identified as impacting these processes (Goin and Kiecolt-Glaser, 2011). Apparent in both human (Broadbent et al., 2003) and animal models (Padgett et al., 1998; Gallucci et al., 2000), stress exposure alters and may delay healing. Comparable immune effects of chronic stress are demonstrated following administration of vaccines as well. Altered antibody responses have been observed following

administration of an influenza vaccine to persons exposed to chronic stress. Lower levels of virus-induced IL-2 and IL-1B have been reported in caregivers of spouses with dementia compared to control counter parts. (Kiecolt-Glaser et al., 1996). Taken together, stress can disrupt or alter several processes within the peripheral immune system which may lead to increased susceptibility to infections and promote the progression of disease.

Stress and the Central Immune System

Chronic stress also has implications for the brain. In the central nervous system, microglia, the resident immune cells, function in the regulation of homeostatic processes within the brain, particularly in the cases of infection and disease (Hanisch and Kettenmann, 2007). Furthermore, microglia function in the release of cytokines following activation from stimuli including stress or an immune challenge, like the endotoxin Lipopolysaccharide (LPS). Upon activation, microglia undergo a morphological change, display phagocytotic activity and increase production of inflammatory cytokines (Frank et al., 2007). Increased microglial activation can cause altered levels of inflammatory cytokines in the brain (Liu et al., 2017) and prolonged microglial activation, as in the case of chronic stress, can contribute to its consequences (Stein et al., 2017; Espinosa-Olivia et al., 2011; Frank et al., 2007).

Changes in the expression of cytokines in brain regions that actively participate in the stress response, have been reported in rodent models following acute stress exposure (Blandino et al., Nguyen et al., 1998, Audet et al., 2011). However, effects vary depending on the region of interest and mode of chronic stress. For example, decreases in expression of IL-6 in the hypothalamus were reported following both single and repeated restraint (Miyahara et al., 2000). Comparably, within this same study, IL-6 levels significantly increased following one-time

restraint, but decreased following repeated restraint in the midbrain of adult male rats (Miyahara et al., 2000). Variations in cytokine expression have also been reported in mice following chronic stress. Mice subjected to chronic psychosocial stress exhibited attenuated expression levels of both IL-1B and TNFα in the hippocampus (Bartolomucci et al., 2003); whereas, mice subjected to a chronic mild stress procedure exhibited increased expression of IL-6 in the hypothalamus (Mormede et al., 2002). Bidirectional communication between the central and peripheral immune systems, in addition to cytokine differences in either, imply that other mechanisms are in place to regulate the above-mentioned changes that result from chronic stress. Cytokines have a variety of functions in the central nervous system that in turn influence both the central and peripheral immune system. Their roles in behavior, mood, stress and cognition have been proposed throughout the literature (Maier, 2003; Dantzer, 2009; Dunn et al., 2005). Thus, alterations in cytokine expression in the brain could significantly impact a variety of functions.

Stress, Inflammation, and Depression

Alterations in cytokine concentrations resulting from chronic stress can lead to imbalances in inflammation and anti-inflammation, which can contribute to stress-related diseases (Liu et al., 2017). Among the diseases that may in part result from stress is depression. Depression and anxiety, among other disorders, are linked to prolonged stress exposure; however, this relationship has not yet been fully deciphered. Exposure to stressful life experiences is higher among people with a history of depression than among those without such a history (Infrasca, 2003; Kessler, 1997). Changes in behavior, like increases in anxiety-like behaviors have been reported in animal studies following stress exposure (Kiank et al., 2006; Belzung et al., 2001), and in addition, stress exposure has resulted in changes in circulating

levels of inflammatory cytokines like IL-6 and IL-10 (Voorhees et al., 2013; Himmerich et al.,2013).

In one study, elevated levels of IL-6 were reported following acute stress exposure in mice that later displayed a profile consistent with being susceptible to stress (Hodes et al., 2014). This same group concluded IL-6 to be a strong predictor of behavioral outcomes associated with social stress exposure as elevated IL-6 levels were also reported in persons with treatment-resistant MDD (Hodes et al., 2014). Elevated peripheral cytokine levels in patients with depression and anxiety disorders have been reported elsewhere (Maes et al 1995; Alesci et al., 2005). In some cases, behavioral distinctions and immune alterations following stress coincide. Although behavioral outcomes related to stress and inflammation have been discussed throughout the literature, further investigation is necessary to determine how the three interact and influence each other.

Mediators of stress induced behaviors and those associated with depression include the above mentioned proinflammatory cytokines and others like TNFα and IL-1B (Tuglu et al., 2003; Thomas et al., 2005). Due to size and hydrophobic characteristics, most pro-inflammatory cytokines released in the periphery are incapable of crossing the blood-brain barrier (Dantzer, 2009). Several means of immune-brain communication have been identified, however. Afferent nerves of the vagus nerve are activated following episodes of visceral inflammation, Toll-like receptors in circumventricular organs produce proinflammatory cytokines in response to the presence of pathogens and cytokine transport systems are also in place as a means of giving the brain an idea of the level of inflammation existing in the periphery (Dantzer, 2009; Maier, 2003). Stress-induced changes in inflammation in the periphery may translate to differences in neuroinflammation. Following inflammation in peripheral tissues, through use of these

pathways, locally produced cytokines in turn promote changes in the central nervous system that then influence behavior.

Stress during Adolescent Development

Behavioral and immune consequences have been associated with chronic stress exposure in adolescence. For example, early life stress in adolescence results in the induction of anxiety and depressive-like behaviors. Barnum and colleagues reported an increase in anxiety-like behaviors and a suppressed neuroinflammatory response following an acute endotoxin challenge in adolescent mice with a history of chronic stress (Barnum et al., 2012). The sensitivity of the adolescent period may contribute to the robust effects of chronic stress exposure during this time.

Adolescence is a developmental period associated with neural plasticity and rapid development in brain regions responsible for emotional processing, regulation of behavior, memory and decision making. (Steinberg, 2005). Rapid development and maturation of the HPA axis, during adolescence contribute to the sensitivity of the period. Differences in HPA reactivity and hormonal levels, in adolescence, as compared to adulthood, are evident and may be responsible for the long-term implications associated with chronic stress exposure in adolescence (Romeo, 2013). For example, anxiety-like behaviors in adulthood were reported in male and female rats following chronic social stress in adolescence (McCormick and Matthews., 2007) indicating long term behavioral effects of chronic early life stress. Chronic stress exposure in adolescence may also leave an organism at risk to the effects of stress exposure later in life. Stress exposure increased anxiety-like behavior in adult rats; however, these behaviors were more pronounced following the same stress exposure in rats with a history of adolescent stress (Avital and Richter Levin, 2005). Another group reported similar findings following a chronic

restraint paradigm in both adolescent and adult mice, and in addition, reported deficits in cognition and memory in adolescent mice only (Lander et al., 2017). The processes by which chronic adolescent stress can lead to changes in cognition, behavior and immune function have not yet fully been made clear. Some effects of chronic adolescent stress, however, have been elucidated in studies mentioned above and other rodent studies and these affects appear to be more distinct compared to consequences of chronic stress exposure in adulthood.

Importance of Sex as a Biological Variable

In addition, the consequences of stress related behavioral alterations following chronic stressor exposure are also mediated by sex. A survey study compiled of data from three different countries reported a higher prevalence of depression among women beginning around age 14 and this trend persisted into early adulthood (Wade et al., 2002). Women also have a higher lifetime prevalence of generalized anxiety disorder and post-traumatic stress disorder than men (Altemus et al., 2014). How these sex differences in prevalence of affective disorders among men and women come about has yet to be fully understood. According to the National Comorbidity Survey, women reported higher instances of major depressive episodes in a twelve-month period compared to men; however, analysis based on the data in this study also concluded this higher prevalence was due to a higher risk of onset (Kessler et al., 1993). In another study, examining the link between stressful life events and onset of Major Depressive Disorder (MDD), women reported a greater frequency of stressful life events as compared to men prior to the onset of MDD (Harkness et al., 2010). Overall, a higher frequency of stressful life events has been reported in persons with a history of depression than those without such history (Kessler, 1993).

Sex differences in mood and behavior begin to emerge during adolescent ages 11-13 (Angold et al., 1998). These sex differences may be due in part by the interactions between the HPA axis and hypothalamic-pituitary-gonadal (HPG) axis that occur during adolescence, also marking the sensitivity of the developmental period. Bourke and Neigh reported sex differences in behavior following a chronic, mixed modality stress paradigm in both adult and adolescent rats. Female rats in both groups displayed an increase in depression-like and anxiety-like behaviors whereas male counterparts did not exhibit any changes in behavior following the stress paradigm (Bourke and Neigh, 2011). Using the same chronic stress paradigm, sex differences in hippocampal gene expression were reported. In this study, chronic adolescent stress resulted in an increased inflammatory response to LPS in the hippocampus in male rats only, as indicative by proinflammatory cytokine levels (Pyter et al., 2013). Here, behavioral implications of chronic adolescent stress were not considered and therefore cannot be ruled out as being associated with these sex-dependent inflammatory differences.

Recently, Bekbhat and colleagues assessed whether the sex-dependent neuroimmune consequences of chronic adolescent stress in rats paralleled those in the periphery (Bekbhat et al., 2019). This group reported increases in hippocampal IL-1B gene expression in adult females only following CAS; however, female rats did not exhibit differences in peripheral levels of IL-1B. Chronic adolescent stress did, however, have an effect on immune activity in the periphery of male rats. Sex differences in immune function in both the central and peripheral immune systems resulting from chronic adolescent stress imply sex dependent mechanisms in place that function to regulate HPA activity and outcomes related to chronic stress. Taken together, similarities and differences among these studies call for further investigation of the sex differences in behavior and immune function that may arise in adulthood following chronic

adolescent stress. Nonetheless, they place emphasis on the sexually dimorphic nature of the induction of anxiety and depression like behaviors and the related inflammatory changes that can result from chronic stress exposure in adolescence.

Critical Gaps Addressed in this Thesis Work

Indirect mechanisms are responsible for the manner in which stress-induced changes in inflammatory responsiveness relate to behavior. These behaviors, dictated by the central nervous system, imply a signal cascade linking peripheral alterations to neural consequences. This is reflected in the increases in neuroinflammation and induction of sickness behavior following peripheral administration of LPS or recombinant cytokines (Dantzer, 2009; Perry and Holmes, 2014; Norden et al., 2015). An underlying signaling cascade may be responsible for the differences in inflammatory reactivity that are driving neuroinflammation. Communication between the periphery and central immune systems appears to be an implicit mechanism by which chronic stress and inflammation influence behavior.

Although studies examining the behavioral effects of chronic adolescent stress have been gaining attention, additional investigation is necessary to identify measures of predicting stress resilience and susceptibility. Increasing evidence supports the relationship between chronic stress, the immune system and how they can influence behavior, though, the complexity of this interconnection has yet to be fully assessed (Miller, 1998; Reiche et al., 2004; Dantzer, 2009). Altered inflammatory consequences following a history of chronic stress are of particular interest as it has been reported that patients with depression also exhibit differences in inflammation (Dowlati et al., 2010; Maes et al., 1995). Inflammation is further implicated in animal models of chronic stress (Munhoz et al., 2006;) and depression (Kubera et al., 1998; Song and Wang,

2011; Felger and Lotrich, 2013). Identifying alterations in stress-induced cytokine release in relation to any changes in behavior may aid in understanding the mechanisms by which chronic adolescent stress may drive behavioral deficits. Furthermore, by determining if sex-dependent cytokine signaling ensues following stress we may establish how physiological differences among males and females may protect or induce vulnerabilities to stress. Sex dependent differences in behavior and immune function in adolescence have been identified (Bourke and Neigh; Pyter et al., 2013), and use of circulating cytokine levels as a metric for predicting behavioral profiles associated with adolescent stress has been considered in rats (Bekbhat et al., 2019). Therefore, in the current study, we examined the effects of chronic adolescent stress on the peripheral immune response in mice and the potential for behavioral outcomes to serve as accurate predictors of peripheral cytokine levels following an acute endotoxin challenge.

Much work from our lab has focused on the mechanisms underlying the effects of chronic adolescent stress in male and female rats. By using a mouse model to determine the impact of chronic adolescent stress on neuroinflammation, we can lay the groundwork for future studies to use genetic modifications specific to mouse lines that will allow us to characterize the molecular and genetic mechanisms underlying stress-induced alterations to the brain and behavior. As stress-induced inflammation is known to create an environment that promotes neuronal dysfunction (Perry, 2004), studying the effect of chronic adolescent stress in mice may elucidate pathways that mediate the development of both mood disorders and neurodegenerative diseases, such as Alzheimer's disease. Furthermore, evaluating the behavioral and immunological effects of long-term stress exposure during adolescence will contribute to what is known about the sensitivity of the adolescent period, sex differences that may arise during this period, and how stress-induced changes in inflammation contribute to maladaptive conditions.

Alterations in affective-like behaviors that result from chronic early life stress are evident in several studies. Adolescent male mice subjected to chronic social defeat stress exhibited reduced sucrose intake and increases in social avoidance (Iniguez et al., 2014). Chronic social isolation stress in adolescent mice induced anxiogenic behavior and hyperlocomotion in open field and novel object recognition assessments (Lander et al., 2017). Additionally, in close relation to this current study, mice subjected to predatory stress exhibited increased anxiety and depressive-like behaviors indicative by reduced sucrose consumption and increased marble burying.

Variations in peripheral cytokine levels have also been reported following chronic stress, similar animal models of depression and in human studies of depression. Elevations in IL-6 following acute psychological stress (Lemay et al., 1990) and chronic restraint stress (Himmerich et al., 2013; Voorhees et al., 2013) have been detected. In addition, peripheral levels of IL-6 have been characterized as being a strong predictor of susceptibility to social stress and consistently elevated in adults with MDD (Hodes et al, 2014) and in adolescents with MDD (Gabbay et al., 2009). Consistent in the literature, acute and chronic stress induces IL-1B expression in the brain (Nguyen et al., 1998; Deak et al., 2005). Furthermore, alterations in IL-1B in the brain and in the periphery have been found to contribute to the development of depression states in human and animal models (Levine et al., 1999; Thomas et al., 2005). Elevations in peripheral IL-1B following chronic restraint stress in rats (Mekaouche at al., 1994) and chronic variable stress in adolescent rats (Bekbhat et al., 2019) have also been reported. Similarly, increased serum levels of TNFα have been observed in patients with MDD (Tuglu et al., 2003) and contribute to the pathological development of depression (Litchblau et al., 2013). Data from a study following a four-week period of chronic mild stress in rats also indicated a negative correlation between

TNFα levels in the periphery and sucrose intake, suggesting the stress paradigm resulted in related behavioral and immune implications. (Grippo et al., 2005). Altered peripheral cytokine levels accompanied by behavioral implications were also apparent following chronic social defeat stress in mice. This stress paradigm resulted in increases in circulating IL-6 and TNFα and increases in anxiety-like behaviors (Reader et al., 2015).

We propose that chronic adolescent stress will result in an increase in anxiety-like behaviors and a negative impact on social behavior. The predator stress paradigm used in the current study was chosen to induce chronic stress as it is an ethologically relevant stressor and has been shown to induce anxiety-like and depression-like behaviors in adult male mice (Burgado et al., 2014). In addition, as a relationship between stress, inflammation and behavior is apparent, we propose that behavioral outcomes resulting from predatory stress will correlate with increases in circulating IL-6, IL-1β and TNFα, following an acute inflammatory challenge. Work within this thesis will lead to a broader understanding of behavioral and immune implications of chronic stress exposure in adolescence and the underlying mechanisms related to stress and its consequences across biological systems.

Materials and Methods

Rationale for Experimentation

To assess the extent to which chronic adolescent stress alters the inflammatory response in the periphery, a group of mice were subjected to Predatory stress for fifteen consecutive days. A group of 32 mice were divided into two groups: control and stress with equal numbers of males and females in each group. Following the predatory stress paradigm, the behavior of the mice was assessed. Following behavioral assessments, mice were injected with Lipopolysaccharide, then euthanized two hours later. Peripheral levels of TNFα, IL-1B and IL-6 were determined following stress exposure on the 15th day of the paradigm and post-behavior in late adolescence following an acute inflammatory challenge of LPS. An experimental timeline can be found in Figure 1.

1. Animal Husbandry

C57BL/6 mice (Male n=16, Female n=16) (Taconic) arrived post-natal day (PND) 22. Mice were pair housed in ventilated cages and maintained on a reverse 12:12h light:dark cycle in a temperature and humidity controlled environment in an AAALAC-approved facility. Mice were given *ad libitum* food and water throughout the experiment and were housed separately from rats. Mice were weighed every three days throughout the duration of the paradigm. Six male (>400 g) Long Evans rats obtained from Charles River (Charles River, Morrisville, NC) were used as the predatory animals. Rats were maintained on a 14:10 light:dark cycle and given *ad libitum* food and water throughout the experiment. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of Virginia Commonwealth University and in accordance with guidelines set forth by the National Institutes of Health.

2. Predatory Stress

Predatory stress was chosen as a stress paradigm for chronic adolescent stress (CAS) in mice because it was demonstrated that a 15 consecutive day predator stress paradigm is sufficient to produce changes in behavior and affective-like behavior in male mice. Female mice were added to this study because of the sex differences previously observed in the effects of early life stress in rats (Bourke & Neigh, 2011; Pyter et al., 2013).

The predator stress paradigm occurred for all mice PND 37-51. Prior to experimentation, mice designated in the stressed condition were single housed which is considered part of the stress experience in the experiment. Mice in the control condition remained pair housed during the study. Predatory stress involved placing a mouse inside a 5" diameter clear plastic hamster ball. The lid of the hamster ball was then secured with three pieces of tape, the last piece containing crushed rat chow. The hamster ball was then placed into the home cage of a large aggressive male Long Evans rat for 30 minutes. The hamster ball allowed the mice to be subjected to sight, sound and smell of the rat while prohibiting direct physical contact with the predator animal. Mice in the stressed condition were subjected to predatory stress for 30 minutes for 15 consecutive days. To avoid the possibility of habituation, the stressed mice were paired with a different rat for each session. It was made sure that no mouse was paired with the same rat more than three times. After each round, notes were made on the condition of the tape, number of fecal boli from the mouse and the behavior of the Long Evans rat. During this fifteen-day period, mice in the control group were exposed to daily handling and cage transport.

3. Behavioral Assessments

Following predatory stress, two behavioral tests, open field and social interaction were conducted to measure the extent to which mice subjected to predatory stress developed any anxiety-like behaviors or changes in social behavior compared to controls.

3.1 Open Field

All mice were habituated to the testing room, for 30 minutes per day for three days prior to the open field assessment. On testing day, mice were brought to the testing room and were able to acclimate for 30 minutes. Following this acclimatization period, mice were handled by the base of the tail and placed in the center of the arena **(**31.75cm x 31.75cm x 30.48cm). Prior to experimentation, testing groups were randomized. During each session, mice were allowed to roam freely about the arena for 10 minutes and then returned to their home cages. Movement within the field was recorded and scored using the Ethovision software (Nodlus, Ethovision XT). 70% ethanol was used to clean up each arena between rounds.

3.2 Social Interaction

All mice were habituated to the testing room during the above mentioned three days prior to the open field behavioral assessment. On testing day, mice were brought to the testing room and acclimated for 30 minutes. Two social interaction chamber apparatuses were utilized for this assessment. Animal ID, cup side and novel animal were all counterbalanced for the trial list. Following this rest period, mice were handled by the base of the tail and placed in a center chamber connected to two other domains. Mice habituated to this center chamber for five minutes then returned to their home cages. Doors to the other two adjacent domains remained closed during this time. Following habituation, the arena was cleaned with 70% ethanol. Next,

social interaction cups were placed in each of the other two domains. Cups were centered in front of the doors, and positioned so that the back of the cup was resting on the chamber wall. In one cup, a novel mouse of the same sex and similar age was placed, and the other cup remained empty. To begin the testing phase, doors to the two adjacent chambers were removed and the experimental mouse was placed back in the center chamber and allowed to move freely about all three domains for ten minutes. Movement and exploratory behavior was tracked and recorded using Ethovision (Nodlus, Ethovision XT) noting the time spent in either chamber.

4. Lipopolysaccharide (LPS) Administration

In order to determine the extent to which chronic stress altered the peripheral inflammatory response, an immune challenge was given to the mice. Following the predatory stress paradigm and behavioral assessments, mice were weighed and injected intraperitoneally with 7.5×10^5 EU/kg LPS from Escherichia coli O111:B4 (Sigma-Aldrich, L4391) suspended in saline using 1 mL syringes and 25G x 1" hypodermic needles. The LPS dosage within this study has been utilized in studies of chronic low-grade inflammation and is sufficient in producing a peripheral immune response (Frank-Cannon et al., 2008). Injections were carried out two hours prior to tissue collection for each animal based on peak inflammatory cytokine levels observed two hours following LPS administration in humans and mice (Copeland et al., 2005).

5. Tissue Collection and Processing

Blood was collected via retro-orbital eye bleeds 30 minutes following the predatory stress paradigm on the 15^{th} consecutive day. Mice were anaesthetized in a small chamber using isoflurane (99.9%/mL). Heparin-coated capillary tubes were used to collect the blood which was

then stored in 1mL EDTA coated tubes and placed on ice. Following collection, blood samples were centrifuged for 20 minutes at 1800g and 4°C whereby the plasma was aliquoted and stored at -80°C until time of assay. Two hours following LPS injections on PND 58, each mouse underwent rapid decapitation. Terminal trunk blood was collected, and plasma was aliquoted and stored until the time of assay in the same manner as the blood samples produced from eye bleeds. Brains were dissected out, submerged in a beaker full of 2-methyl butane, wrapped in aluminum foil and stored at 80°C until future use (not included in this thesis).

6. Cytokine Assessment

Note: Following the initial IL-6 cytokine assessment, high standard deviation among triplicates of the standard curve and large %CV range indicated the need for additional analysis of samples. Thus, samples were analyzed via a Meso Scale Discovery analysis. This analysis allowed for the inclusion of IL-1B and TNFα in the cytokine profile of samples. Following cytokine assessments, LPS-induced cytokine concentrations were all blindly represented, regardless of group. Nine animals consistently exhibited lower concentrations of cytokines in both assessments compared to those within their group as well as higher coefficients of variation among duplicates. Both variation in administration accuracy and in animal-specific responses to LPS could have contributed to these differences. These data were not statistical outliers within their groups, however, following agglomerative hierarchal clustering analysis, these data consistently appeared to cluster separately from all others. These nine included three male controls, two stressed males, two female controls and two stressed females. Data including these samples and excluding them, will both be presented within this thesis.

6.1 AlphaLISA

Peripheral IL-6 levels were assessed using a Mouse Interleukin 6 (mIL6) AlphaLISA kit (Product #AL504C) (Perkin Elmer, USA). The detection range for this assay was 1.4- 100,000 pg/mL. Prior to assay, mouse IL-6 analyte (0.3ug) was reconstituted with 100uL of ultrapure water and supplied 10X AlphaLISA Immunoassay buffer was diluted to 1X using ultrapure water. Terminal trunk blood samples were diluted (1:2) with cell culture grade FBS prior to the start of the assay as per the manufacturer's directions. Each assay involved preparation of an IL-6 standard curve with concentrations ranging from 1- 300,000 pg/mL. First, 5uL of each analyte standard dilution or sample was added to a 384 well plate. Next, 10uL of 5X Anti-Analyte Acceptor beads (10ug/mL final) was added to each sample well. The plate was covered with optic film and was placed on a shaker and samples incubated for 30 minutes at room temperature. Following this incubation period, 10uL of 5X Biotinylated Antibody Anti-analyte (1nM final) was added to each sample well, the plate was covered with optic film and allowed to incubate on a shaker for 60 minutes at room temperature. Following this incubation period, 25uL of 2X SA-Donor beads (40ug/mL final) was added to each sample well, the plate was then covered with optic film and aluminum foil and placed on a shaker for 30 minutes. Next, the 384 well plate was placed in a Synergy HTX multi-mode plate reader (BioTek Instruments, Inc., Winooski, VT, USA) and analyzed for IL-6 content. Assays were performed in duplicate and total assay volume was 50uL. Limit of detection of IL-6 in this assay was 60.91pg/mL and coefficients of variation ranged from 0.1-62%.

6.2 Meso Scale Discovery (MSD) Analysis

Remaining baseline and terminal samples were then analyzed using an MSD VPLEX Proinflammatory Panel 1 Mouse; IL-6, IL-1B, TNFα Kit (MesoScale Discovery, Maryland). This assessment involved analyzing samples for cytokine concentrations using a MESO QuickPlex SQ 120 system. Prior to the start of this assessment, similarly processed trunk blood samples, both LPS treated and non-treated, were used to test serial dilutions in order to determine the appropriate dilution for experimental. From this assessment, it was determined that a 1:20 dilution for LPS treated samples and a 1:2 dilution for baseline samples were appropriate. Due to limited sample volume, baseline samples were sorted based on sex, stress group, and affective-like behavior observed in the open field assessment. Baseline samples were then pooled together in groups of five or six leaving a sample number of one in each experimental group. Samples were diluted with provided diluent buffer, run in duplicate and processed by the Virginia Commonwealth University School of Nursing Core. The detection limit range was 0.0833-1430pg/ml for IL-1B, and the coefficients of variation fell below 41% prior to excluding outliers and 17% afterward. The detection limit range of TNFα was 0.0415- 629 pg/mL and all coefficient of variation fell below 5% for TNFα before and after exclusion of outliers. The detection limit range of IL-6 was 0.471- 4730pg/mL and the majority of samples were detected above this range. Coefficients of variance for IL-6 fell below 10% before and after exclusion of outliers which is attributed to the high concentration of IL-6 present in samples being outside the upper limit of the assay.

7.. Statistical Analysis

Behavioral and peripheral cytokine data were analyzed using GraphPad Prism version 8.0 for Mac OS X, (GraphPad Software, La Jolla California, USA). Data were assessed for normality and equal variance. Biological outliers were excluded based on agglomerative hierarchical analysis using the XLSTAT Excel program (Addinsoft, Inc., 2017). A one-way analysis of variance (ANOVA) was used to determine the effect of LPS on circulating cytokine levels. A three-way ANOVA was used to determine effects of stress, sex and day on adolescent weight gain. Two-way ANOVAs were used to determine the effects of chronic predator exposure on physiological, behavioral and immune endpoints. Factors for two-way ANOVAs were sex and stress. Effect sizes were calculated for all analyses using Cohen's *d* for ANOVA. When multiple comparisons were necessary, post-hoc analyses using Bonferroni's method were used to assess individual group differences. The alpha value was set to 0.05.

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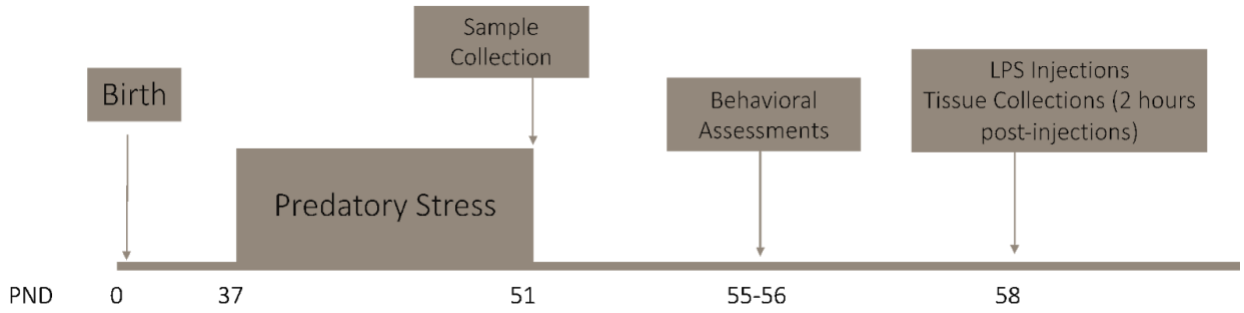

Sample Collection
Birth
Behavioral Assessments
LPS Injections
Tissue Collections (2 hours post-injections)
Predatory Stress
PND
0
37
51
55-56
58

Figure 1. Experimental timeline of predatory stress paradigm.

Results

Predatory Stress Observational Data

There was no effect of stress on adolescent body weight in male and female mice ($F(1, 28) = 0.006539$, $p = 0.9361$). A main effect of day was observed as males and females gained weight over time ($F(5.542, 155.2) = 8.339$, $p < 0.0001$). A main effect of sex was also observed following analysis as males weighed more than females ($F(1, 28) = 452.3$, $p < 0.0001$) (Figures 2A-B). The number of fecal boli for each mouse in the stressed condition following predatory stress was counted and recorded (Figure 3). There was a main effect of sex observed as female stressed mice produced more fecal boli during the paradigm compared to stressed males ($F(1, 14) = 19.45$, $p = 0.0006$). There was also an effect of time, as male and female mice decreased fecal production over time ($F(14, 196) = 11.29$, $p < 0.0001$).

Behavioral Assessments

Open Field

Predatory stress increased anxiety-like behaviors in both male and female mice in the open field test (Figures 4-5). Control mice spent a greater percent of their time in the center of the field compared to stressed mice ($F(1, 28) = 19.82$, $p = 0.0001$; Figure 4). There was no effect of sex in the percent time spent in the center of the field ($F(1, 28) = 0.6975$, $p = 0.4107$). A main effect of stress was observed as stressed mice traveled a greater total distance in the field compared to control counterparts ($F(1, 28) = 43.97$, $p < 0.0001$; Figure 5A). An interaction of stress and sex was observed ($F(1, 28) = 6.548$, $p = 0.0162$), post-hoc analysis indicated that the effect of stress was driven by females ($p < 0.0001$). Stressed mice also traveled at a greater average velocity in the field, compared to controls ($F(1, 28) = 43.97$, $p < 0.0001$; Figure 5b).

There was an interaction of stress and sex on average velocity ($F(1, 28) = 6.548$; $p=0.0162$) and post-hoc analysis indicated that the effect of stress on average velocity was driven by differences in females ($p < 0.0001$).

Social Interaction

There was no significant effect of sex ($F (1, 28) = 1.616$, $p = 0.2141$) or stress ($F (1, 28) = 2.680$, $p =0.1128$) on the percent time mice spent in the chamber with the novel mouse (Figure 6). These data indicate predatory stress had no effect on sociability. A main effect of sex was observed in the percent time mice spent in the middle chamber ($F (1, 28) = 4.621$, $p = 0.0404$; Figure 7A). Following the habituation period, male mice spent a greater percent of their time in the middle chamber compared to females. No effect of stress was observed ($F (1, 28) = 3.220$, $p = 0.0835$). A main effect of sex was observed in the percent time spent in the vacant chamber ($F (1, 28) = 8.565$, $p = 0.0067$; Figure 7B) as female mice spent a greater percent of their time in the vacant chamber compared to males. There was no effect of stress ($F (1, 28) = 0.6607$, $p = 0.4323$).

Cytokine Assessment

Baseline samples analyzed via AlphaLISA fell below the lower detection limit of 60.591pg/ml and were considered undetectable, therefore, no effects of stress or sex could be observed. Circulating peripheral levels of cytokines at baseline determined via MSD analysis are depicted in Table 1. A sample number of one due to volume constraints prevented further in-depth analysis of stress effects on circulating cytokine levels at baseline, For each cytokine, values found in Table 1 were averaged and designated as the mean of comparison for LPS-induced cytokine levels (data not shown here). LPS significantly increased circulating levels of

TNFα in stress males (p = 0.0005), female controls (p = 0.0019) and stress females (p =0.0001). The male control group did not significantly increase however, this group did exhibit a trend toward significant increase (p = 0.0738). For IL-1B, LPS significantly increased circulating LPS in male controls (p= 0.0004), stress males (p= 0.0004), female controls (p= 0.0004), and stress females (p<0.0001)

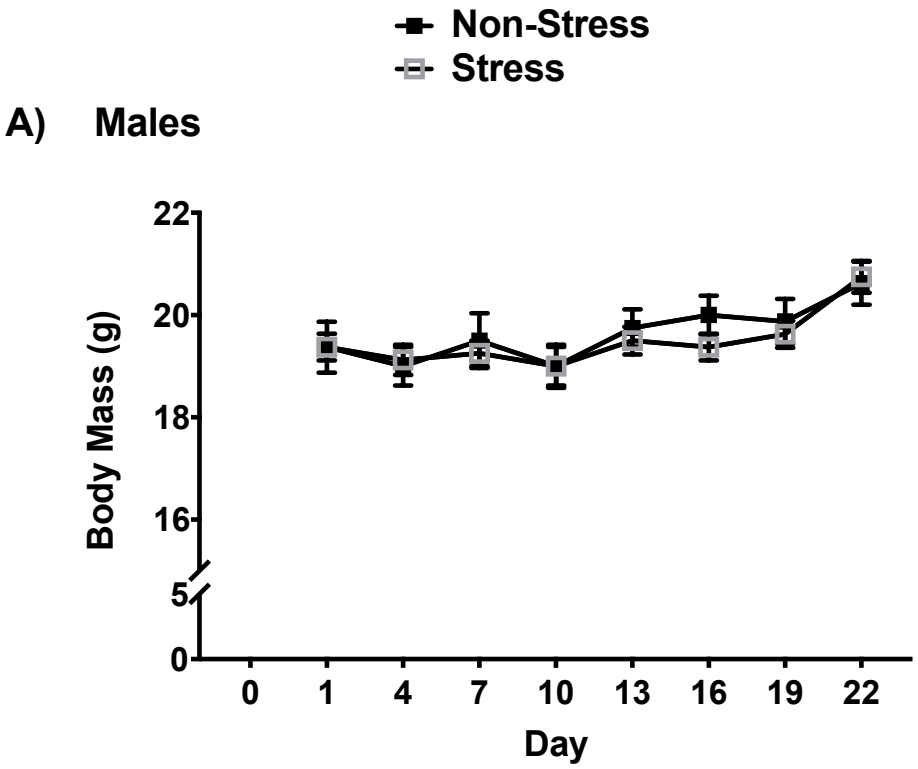
Circulating concentrations of IL-1B, IL-6 and TNFα following LPS administration were determined through use of a multiplex analysis system **(**Figures 8-11). Prior to the exclusion of biological outliers, predatory stress had no significant effect on circulating levels of IL-1B (F (1, 28) = 0.3334, p=0.5683, Figure 8A). Sex also did not impact circulating IL-1B (F (1, 28) = 0.9467, p= 0.3389). After excluding biological outliers from analysis, neither predatory stress (F (1, 19) = 0.07787; p = 0.7832; Figure 8B) nor sex (F (1, 19) = 0.4607; p = 0.5055) impacted IL-1B. Initially, neither predatory stress (F (1, 28) = 3.043; 0.0921) or sex (F (1, 28) = 2.673; p = 0.1132) had an impact on circulating level TNFα (Figure 9A). Upon exclusion of biological outliers, predatory stress did, however, have an effect on circulating levels of TNFα (F (1, 19) = 4.748, p= 0.0421; Figure 9B), but this impact was not sex dependent (F (1, 19) = 2.817, p= 0.1096).

LPS-induced levels of IL-6 fell well above the upper detection limit of the multiplex assay, 4,730 pg/mL (data not shown here). Comparably, this was not the case for peripheral IL-6 concentrations when analyzed via AlphaLISA. Neither predatory stress (F (1, 28) = 2.019; p = 0.1664) nor sex (F (1, 28) = 2.011; p = 0.1672) had an effect on circulating IL-6 (Figure 10A**)** Following exclusions, this assessment still indicated no significant differences of stress (F (1, 19) = 4.169; p = 0.0553) or sex (F (1, 19) = 1.644, p = 0.2153) on circulating IL-6 levels in these samples (Figure 10 B). Prior to exclusions, LPS-induced IL-1B (p = 0.9221), TNFα (p = 0.5752

and IL-6 (p=0.2796) levels did not correlate with anxiety-like behaviors indicated by percent time spent in the center of the open field assessment (data not shown here) and after excluding biological outliers, behavioral deficits did not correlate with LPS-induced IL-1B (p= 0.6915), TNFα (p = 0.2671) or IL-6 (p =0.3684) (Figure 11).

	IL-1B		TNFα		IL-6	
	Concentration (pg/mL)	%CV	Concentration (pg/mL)	%CV	Concentration (pg/mL)	%CV
Control Males	1.236	10.809	20.972	3.871	21.341	0.249
Stress Males	1.545	3.918	16.506	0.398	17.366	8.394
Control Females	1.857	6.177	23.018	4.430	13.178	6.504
Stress Females	0.654	27.188	15.227	0.610	12.846	4.746

Table 1: Circulating concentrations of cytokines at baseline following sample pool (n=1) and corresponding coefficients of variance.


Non-Stress
Stress
A) Males
Body Mass (g)
22
20
18
16
5
0
0 1 4 7 10 13 16 19 22
Day

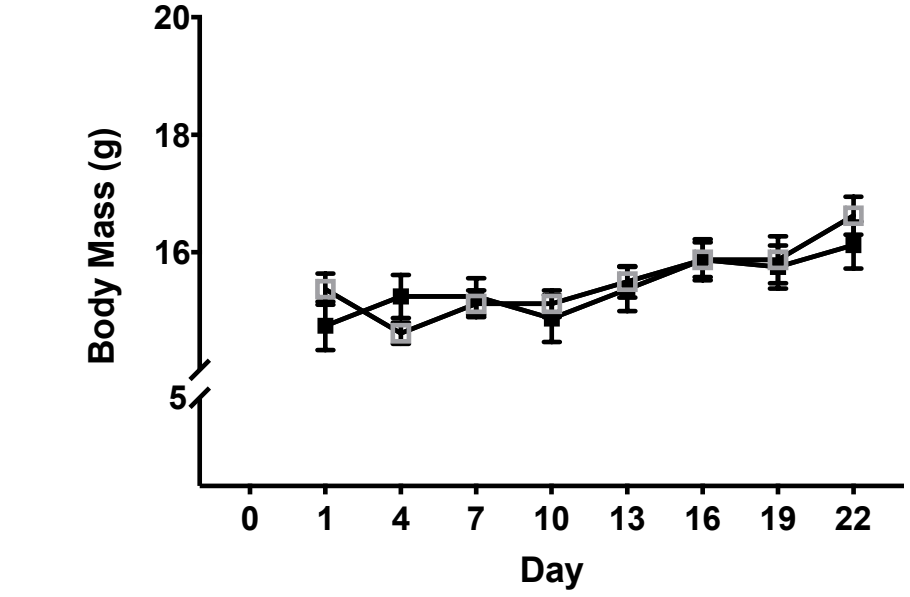

B) Females
Body Mass (g)
20
18
16
5
0 1 4 7 10 13 16 19 22
Day

Figure 2: Body mass of male and female mice. (A) Predatory stress did not impact body mass in males throughout the paradigm. (B) Predatory Stress did not impact body mass in females throughout the paradigm. Predator stress did not impact weight gain of male and female mice ($p = 0.9361$). A main effect of day was observed as males and females gained weight over time ($p < 0.0001$) and a main effect of sex was also observed following analysis as males weighed more than females ($p < 0.0001$) over time.

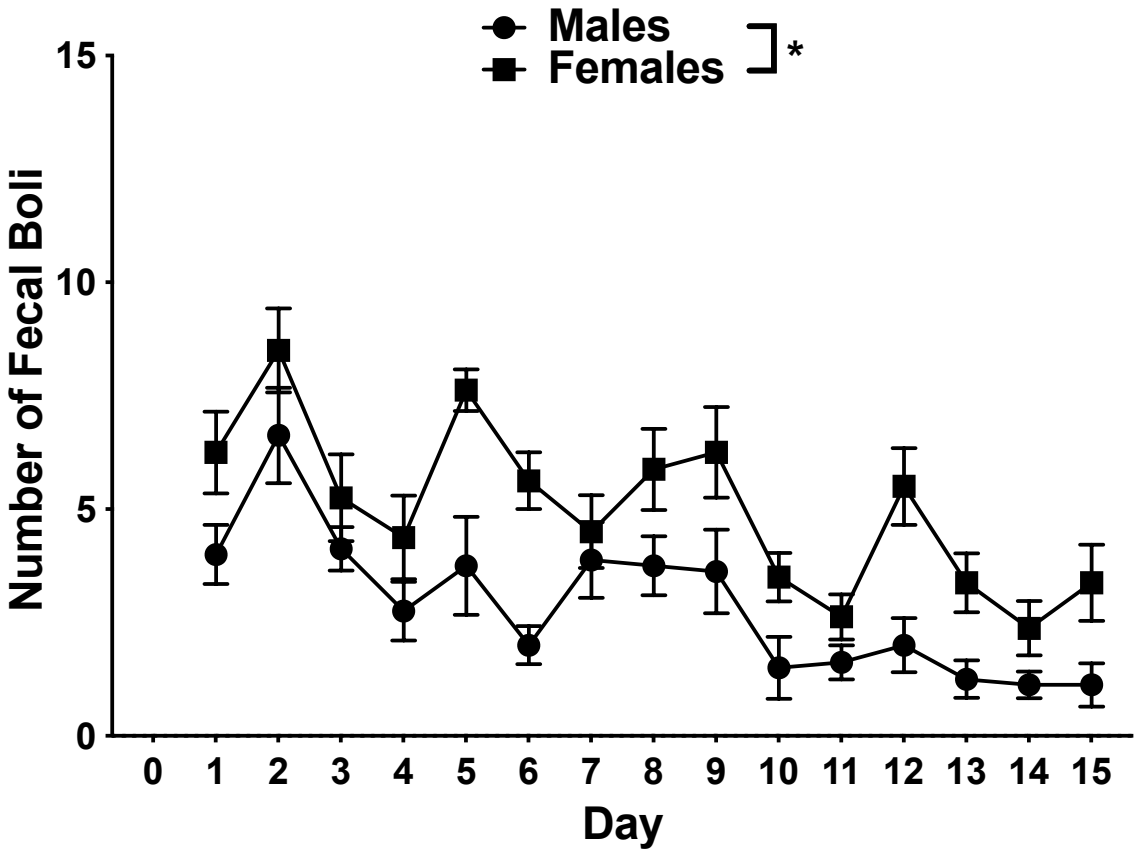

Males
Females
*
Number of Fecal Boli
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Day

Figure 3: Daily number of fecal boli observed during predator exposure in female and male mice. Female mice produced significantly more fecal boli during stress exposure throughout the paradigm compared to male mice ($p = 0.0006$). There was also a main effect of time in that fecal production decreased over time ($p < 0.0001$).

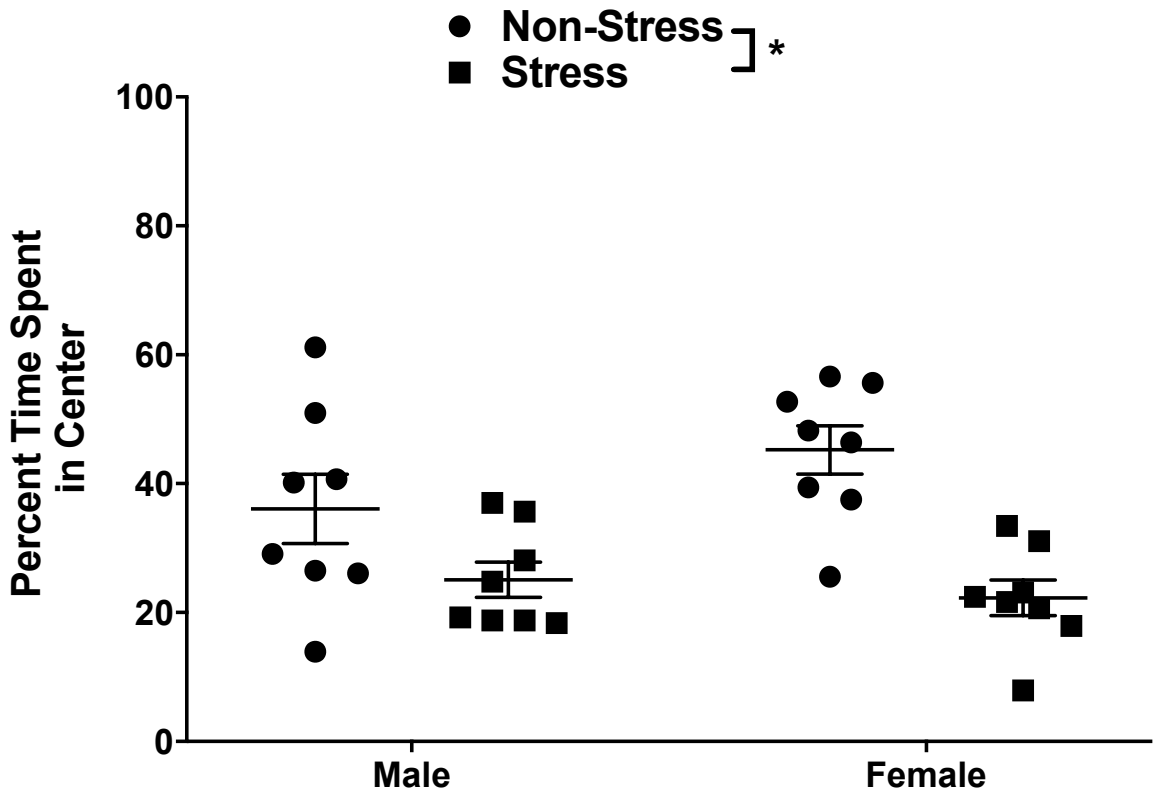
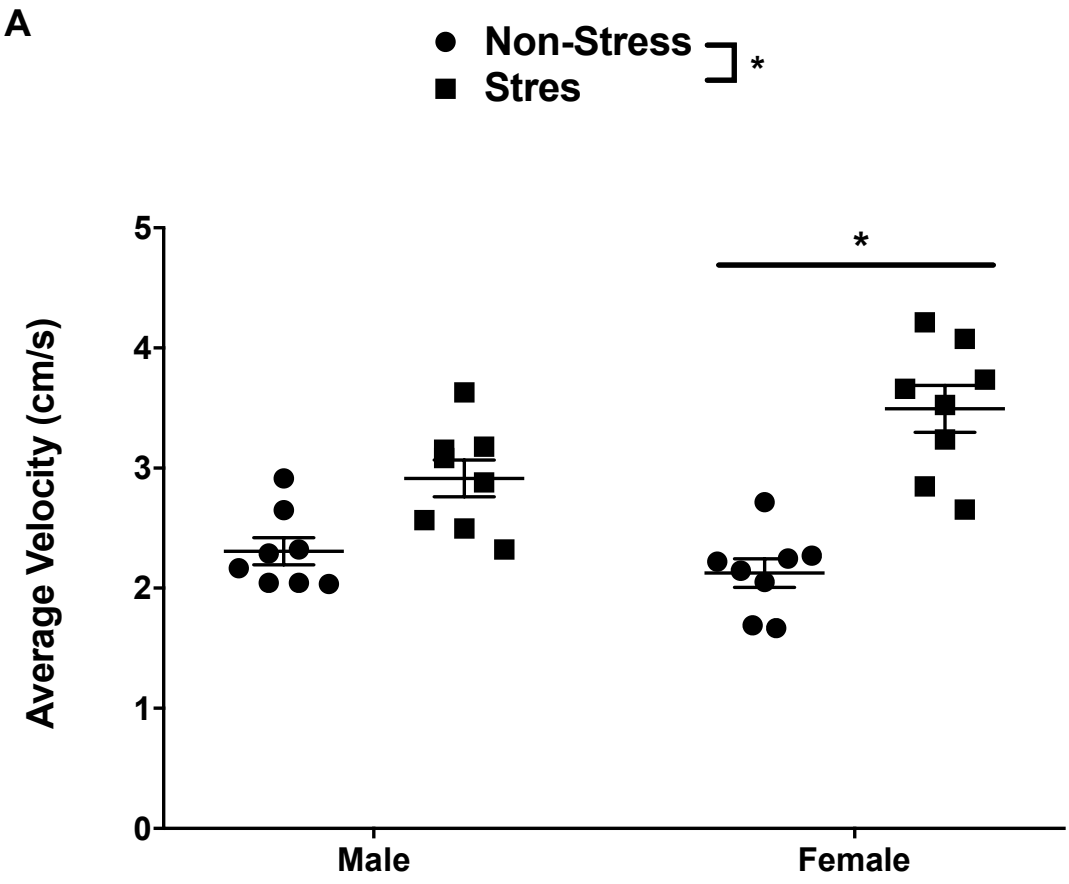

Non-Stress
Stress
*
Percent Time Spent in Center
100
80
60
40
20
0
Male
Female

Figure 4: Percent time spent in the center in the arena in the open field assessment. Control mice spent a greater percent of the time in the center of the field compared to those in the stressed group ($p = 0.0001$).


A
Non-Stress
Stres
*
Average Velocity (cm/s)
5
4
3
2
1
0
*
Male
Female

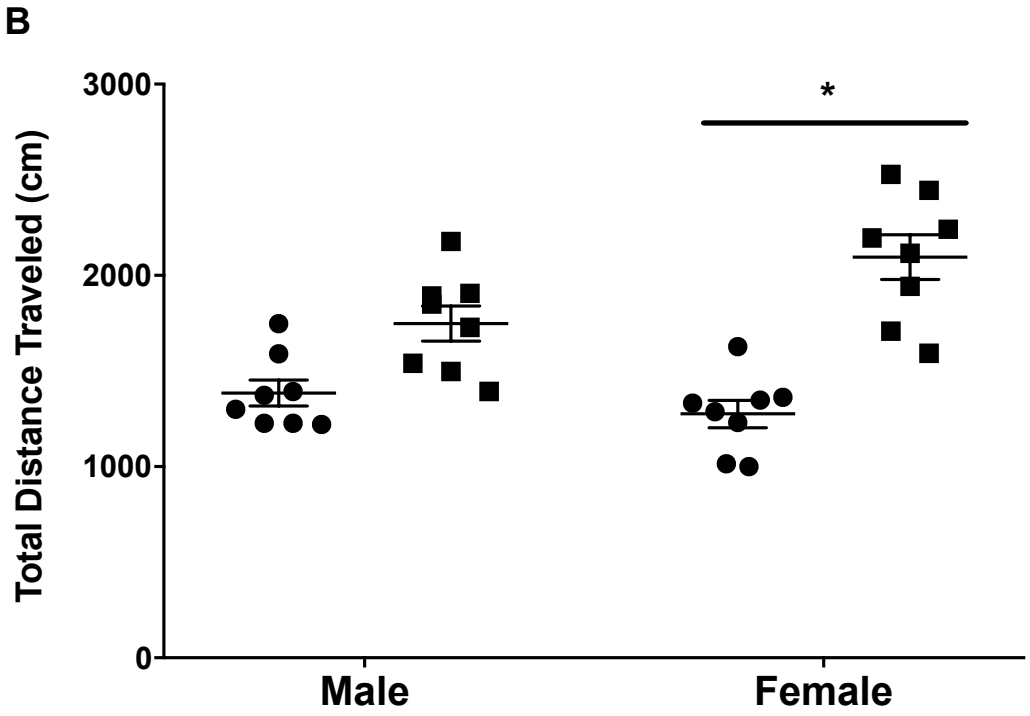

B
Total Distance Traveled (cm)
3000
2000
1000
0
*
Male
Female

Figure 5: Effects of predator stress on average velocity and total distance in the open field assessment. (A) Predatory stress resulted in an increase in average velocity ($p < 0.0001$) in stressed mice compared to controls and (B) an increase in total distance traveled ($p < 0.0001$) Post-hoc analysis indicated the effects of stress on locomotion were driven by females ($p<0.0001$).

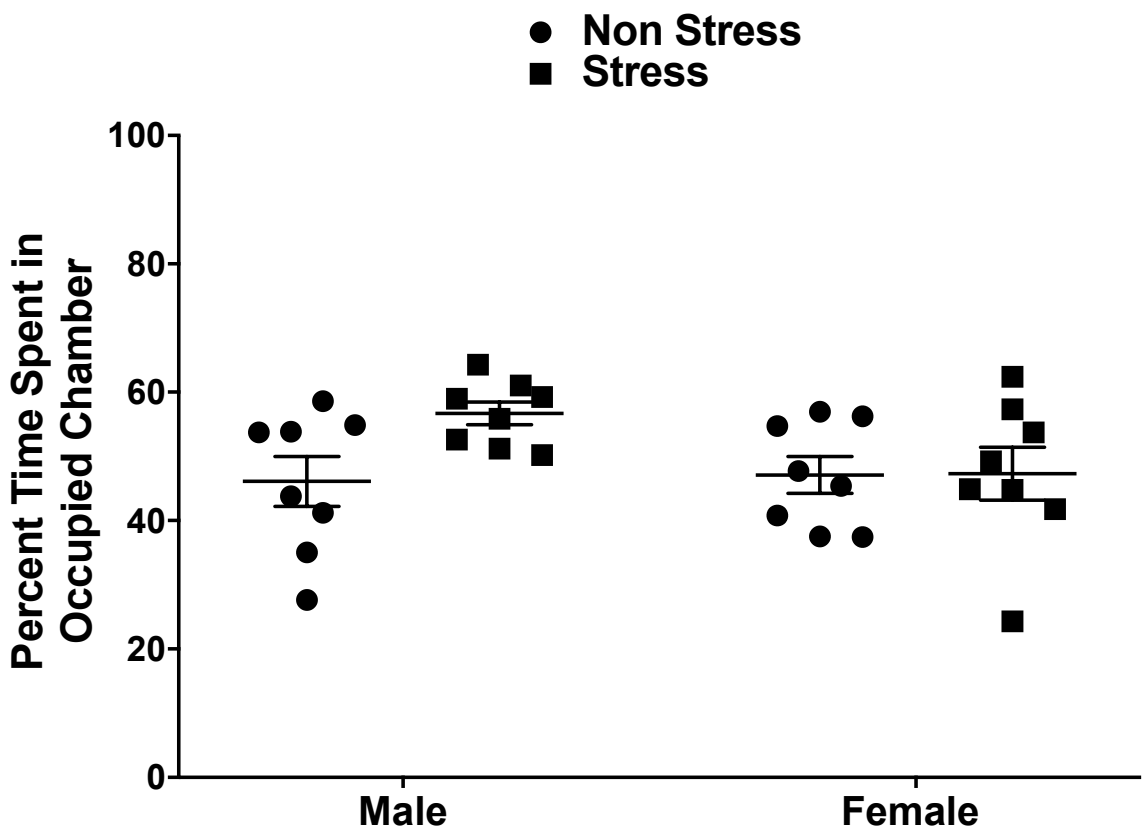
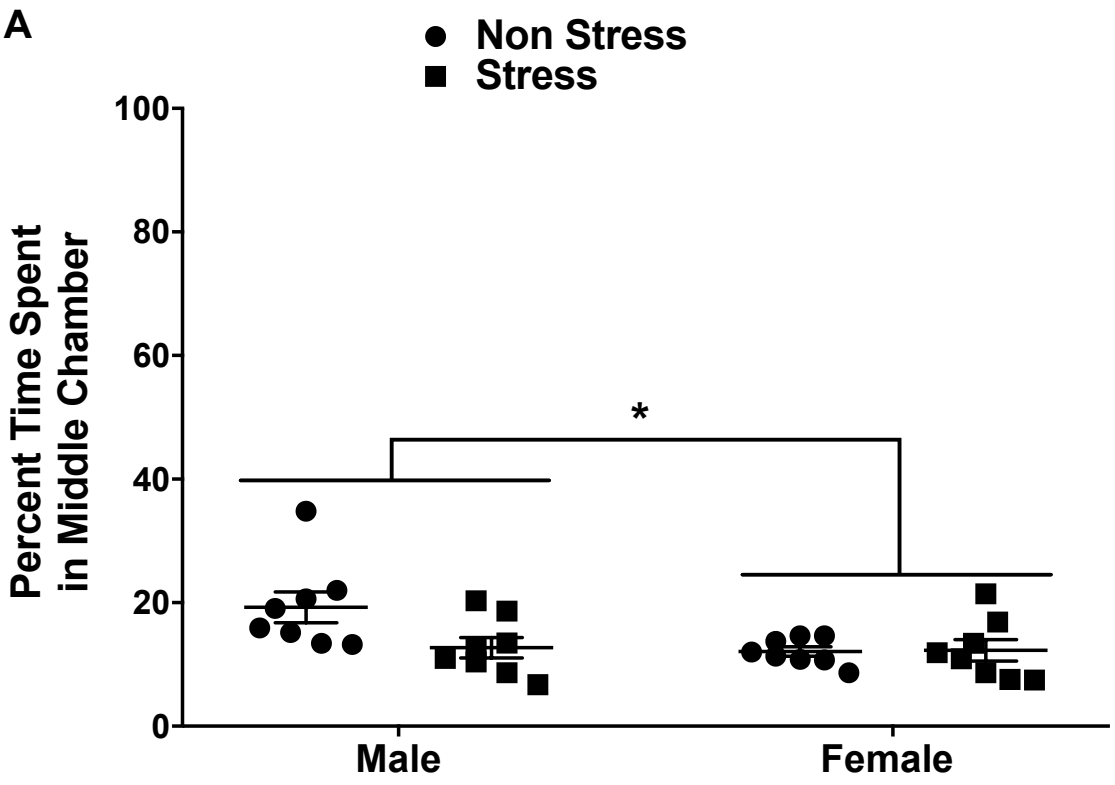

Non Stress
Stress
Percent Time Spent in Occupied Chamber
100
80
60
40
20
0
Male
Female

Figure 6: Percent time spent in the occupied chamber following the habituation period in the social interaction test. No significant differences in sex ($p = 0.2141$) or stress ($p = 0.1128$) were observed in the percent time mice spent in the chamber occupied by a novel mouse.


A
Non Stress
Stress
Percent Time Spent in Middle Chamber
100
80
60
40
20
0
*
Male
Female

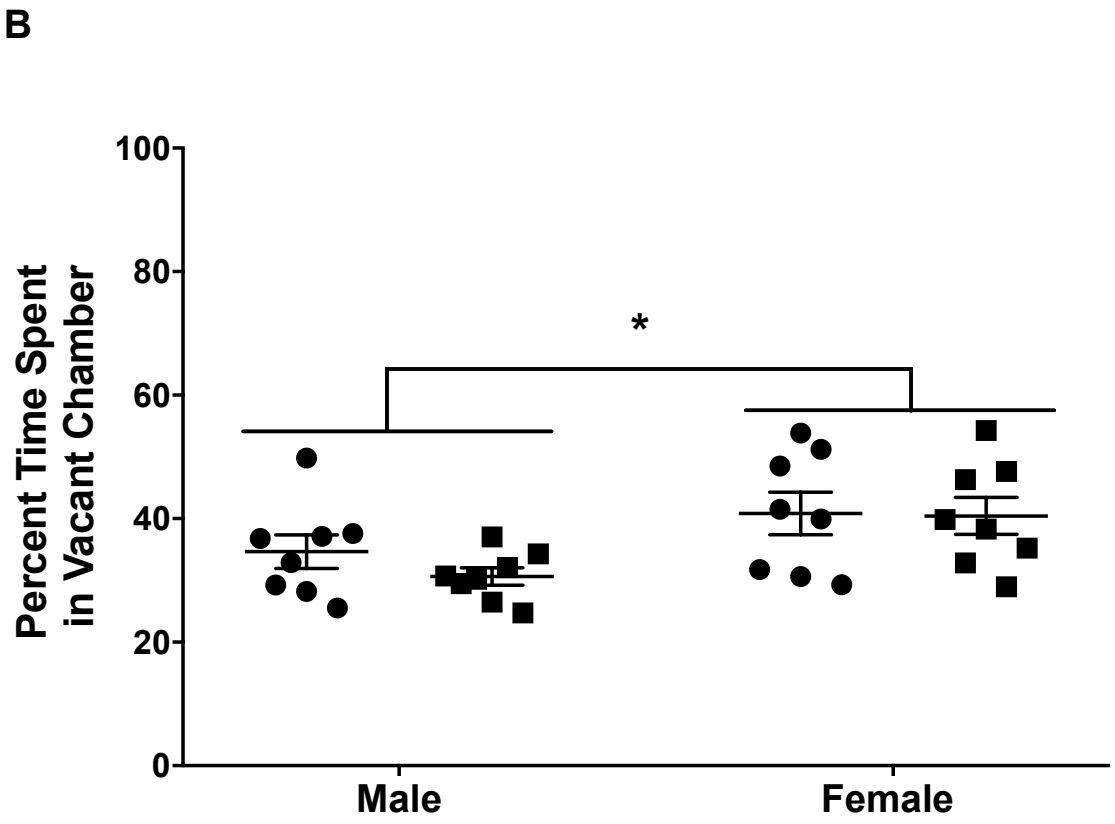
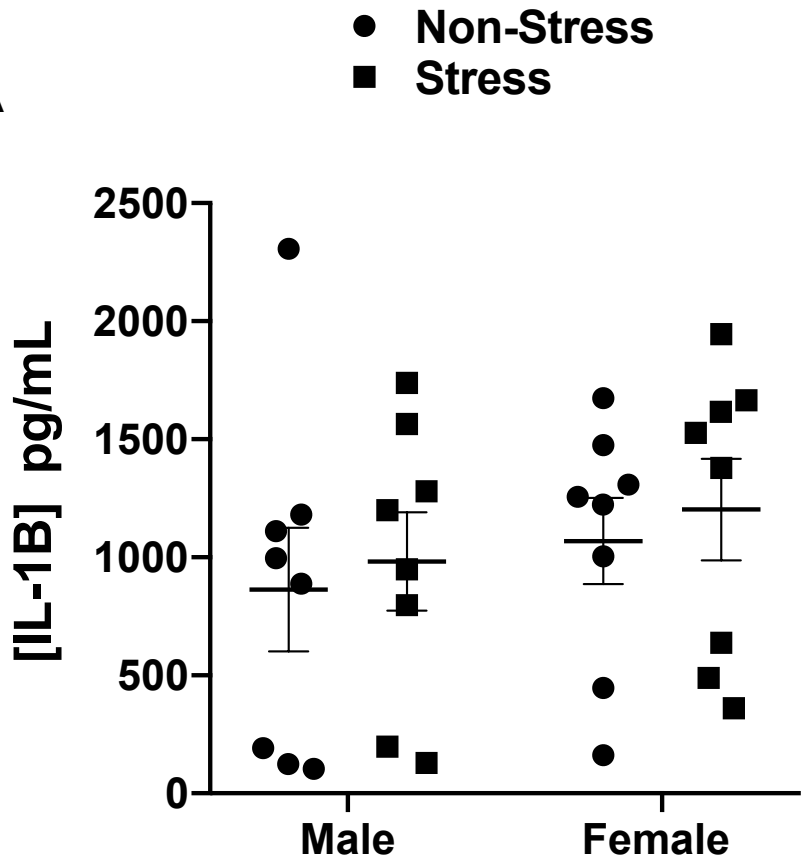

B
Percent Time Spent in Vacant Chamber
100
80
60
40
20
0
*
Male
Female

Figure 7: Percent time spent in the middle chamber and vacant chamber following the habituation period in the social interaction test. (A) Male mice spent a greater percentage of time in the middle chamber compared to control mice ($p = 0.0404$). No effect of stress was observed ($p=0.0835$). (B) Female mice spent a greater percent of their time in the vacant chamber compared to males ($p=0.0067$). There was no effect of stress ($p = 0.4323$).
$p = 0.0067$;

A


Non-Stress
Stress
[IL-1B] pg/mL
2500
2000
1500
1000
500
0
Male
Female

B

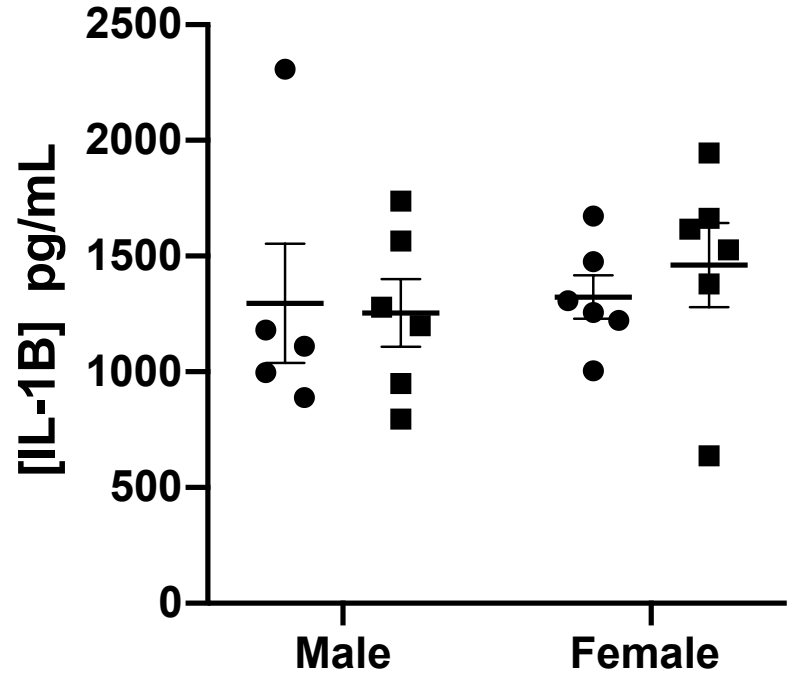
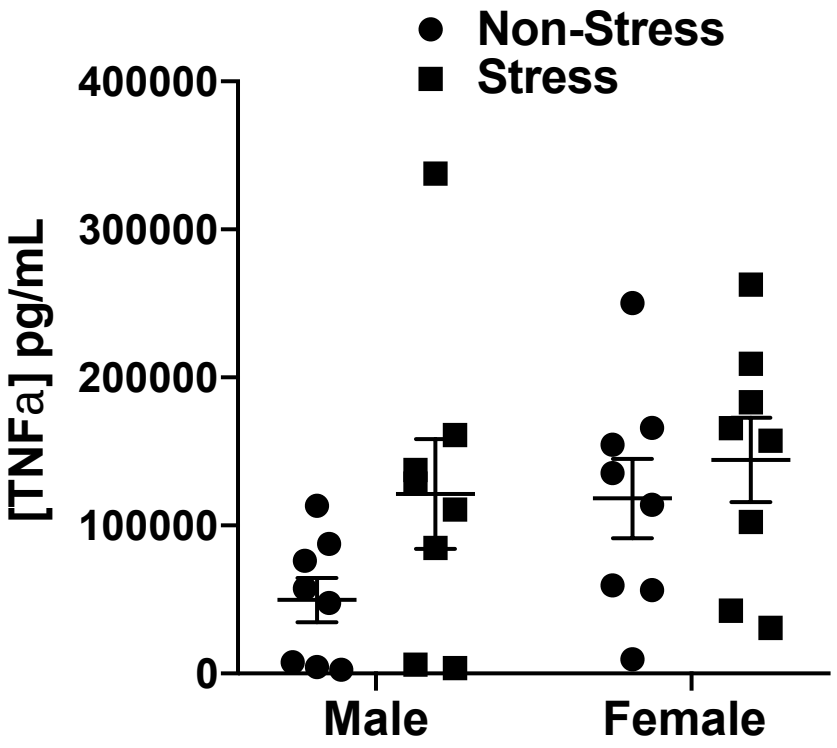

[IL-1B] pg/mL
2500
2000
1500
1000
500
0
Male
Female

Figure 8: LPS-induced levels of IL-1B in plasma before and after exclusion of biological outliers. After excluding biological outliers, neither predatory stress ($p = 0.7832$) nor sex ($p=0.5055$) impacted circulating IL-1B.

A


Non-Stress
Stress
400000
300000
200000
100000
0
[TNFa] pg/mL
Male
Female

B

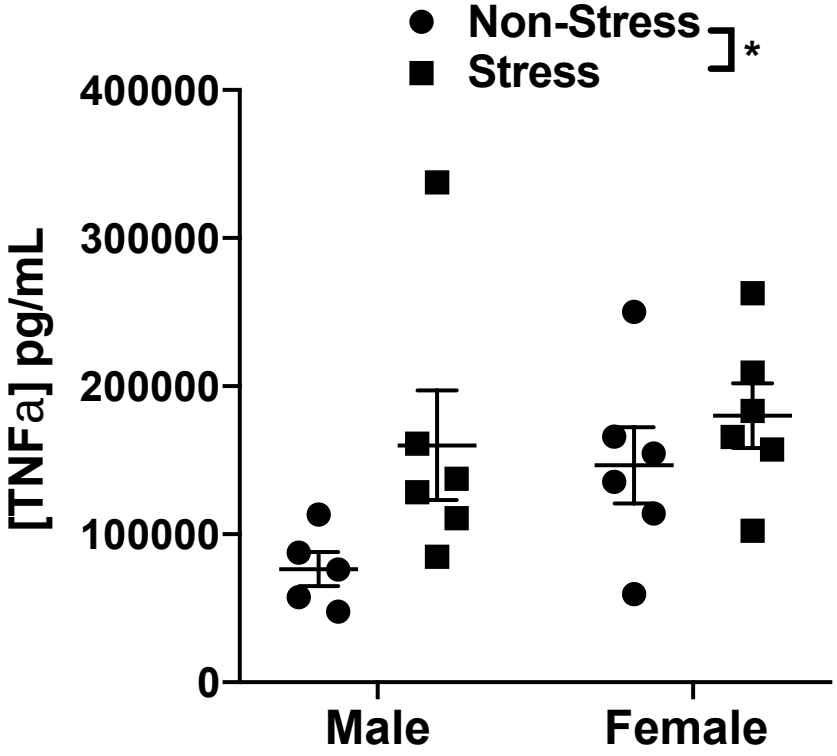

Non-Stress
Stress
*
400000
300000
200000
100000
0
[TNFa] pg/mL
Male
Female

Figure 9: LPS-induced levels of TNFα in plasma before (A) and after (B) exclusion of biological outliers. Upon exclusion**,** predatory stress significantly impacted circulating TNFα levels ($p = 0.0421$) and no differences of sex were observed ($p = 0.1096$).

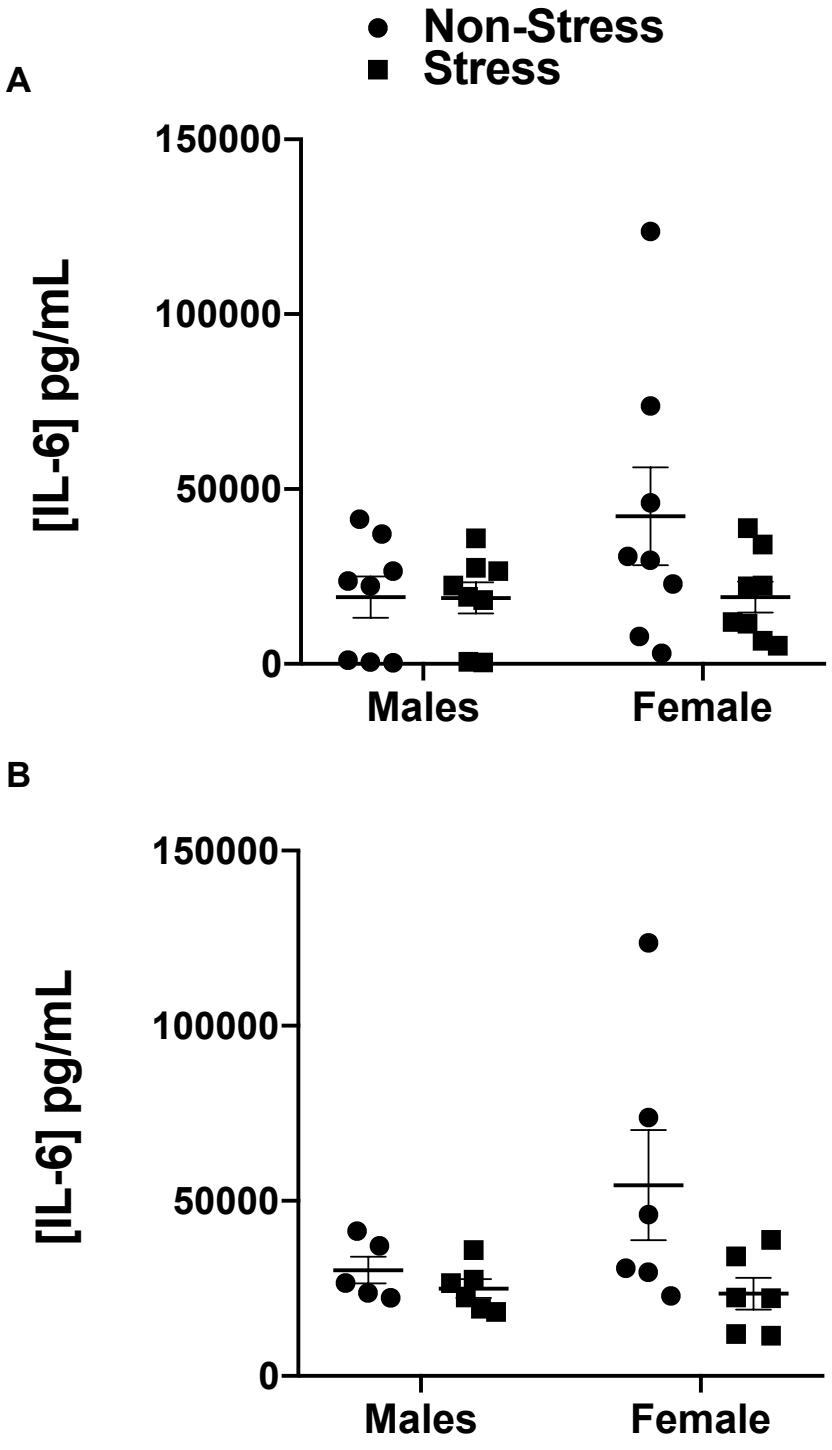

A
Non-Stress
Stress
150000
100000
50000
0
[IL-6] pg/mL
Males
Female
B
150000
100000
50000
0
[IL-6] pg/mL
Males
Female

Figure 10: LPS-induced circulating IL-6 levels before (A) and after (B) exclusion of biological outliers. Following exclusions, this assessment indicated no significant effects of stress ($p = 0.0553$) or sex ($p = 0.2153$) on circulating IL-6 levels.

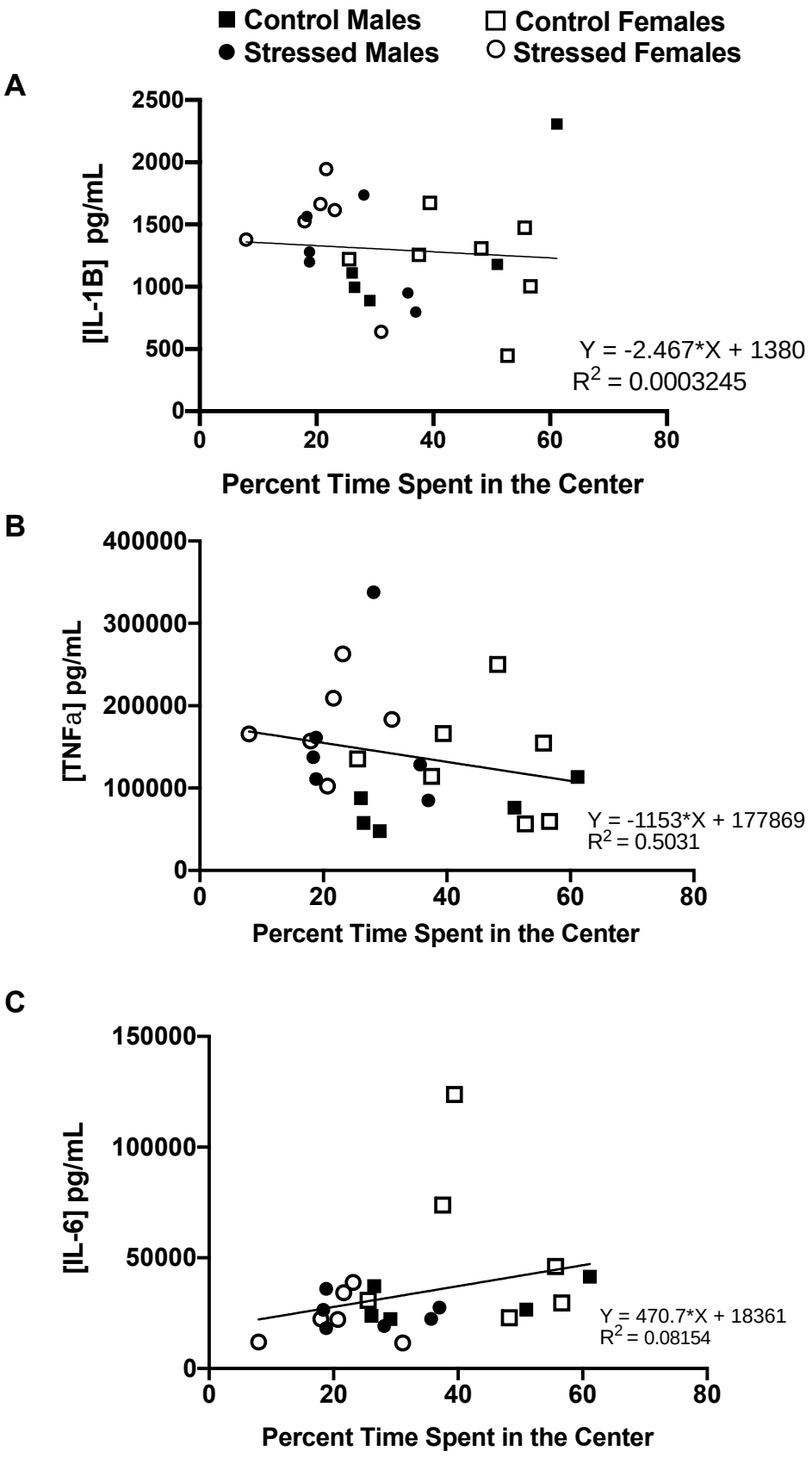

Control Males
Control Females
Stressed Males
Stressed Females
A
[IL-1B] pg/mL
2500
2000
1500
1000
500
0
0
20
40
60
80
Percent Time Spent in the Center
Y = -2.467*X + 1380
R2 = 0.0003245
B
[TNFa] pg/mL
400000
300000
200000
100000
0
Percent Time Spent in the Center
Y = -1153*X + 177869
R2 = 0.5031
C
[IL-6] pg/mL
150000
100000
50000
0
Percent Time Spent in the Center
Y = 470.7*X + 18361
R2 = 0.08154

Figure 11: LPS-induced levels of all cytokines compared to percent time spent in the center of the arena in the open field test after excluding biological outliers. After excluding biological outliers, behavioral deficits did not correlate with LPS-induced IL-1B ($p = 0.6915$), TNFα ($p = 0.2671$) or IL-6 ($p = 0.3684$).

Discussion

The work from this thesis indicates chronic adolescent stress induces anxiety-like behaviors and exacerbates inflammatory reactivity in males and females. Specifically, males and females exposed to predator stress showed anxiety-like behavior, hyperactivity in the open field test and enhanced TNFα post-LPS administration. Taken together, these findings suggest chronic stress exposure during a vulnerable period of development results in immediate behavioral deficits and differences in immune function.

Mice subjected to predator exposure exhibited decreases in percent time spent in the center of the open field indicating an increase in anxiety-like behavior. It is of important note that the induction of anxiety-like behavior was observed in both female and male mice. Females have been considered more vulnerable to the effects of chronic stress (Dalla et al., 2005;) however, the inclusion of females in this study resulted in no sex dependent differences in the percent time spent in the center of the arena. Nonetheless, the induction of anxiety-like behaviors following predator exposure has been reported elsewhere. Increases in anxiety-like behaviors have also been reported in adolescent mice following twelve days of chronic predator exposure (Batista-Guimaraes et al., 2016). Furthermore, previous work using a similar two-week paradigm of predator exposure in adult male mice also resulted in an increase in anxiety-like behavior (Burgado et al., 2014). Similarly, in the current study, fifteen days of predator exposure was sufficient in inducing behavioral deficits in male and female adolescent mice. Burgado and colleagues also reported increased palatable food consumption in the sucrose preference test Typically, decreased sucrose consumption is indicative of depressive-like behavior. Though depressive-like behaviors were not assessed in this thesis, future work should include other

behavioral assessments to determine the full extent to which predator exposure induces changes in behavior.

In addition to the induction of anxiety-like behavior, predatory stress impacted locomotor activity in that stressed mice exhibited increases in total distance traveled and average velocity compared to controls. Post-hoc analysis indicated that stress effects on locomotion were more pronounced in females. Similarly, in addition to increases in anxiety-like behaviors, four weeks of chronic stress exposure in adult male mice resulted in hyperactivity in an open field assessment (Strekalova et al., 2005). In the present study, females also produced a greater number of fecal boli during predator exposure compared to males. The amount of fecal boli produced during stress exposure is of interest due to alterations in colonic activity mediated by CRH, a factor previously mentioned as playing a role in primary activation of the HPA axis following stress exposure (Williams et al., 1987; Tache et al., 1993). These data indicate that predatory stress exposure induced changes in locomotion and that there were differences in colonic activity among male and females subjected to predator exposure. It cannot be determined, however, if the differences in colonic activity resulted of predatory stress as fecal boli was only assessed during predator exposure, excluding controls.

In the social interaction assessment, predatory stress had no significant effect on social preference or social behavior. No difference in the percent time spent in the occupied chamber was observed among male and female mice. These data are contrary to changes in social behavior reported elsewhere following other experimental models for chronic adolescent stress. Following a 3-week paradigm of social isolation, adolescent male mice displayed an increase in sociability as compared to controls (Lander et al., 2017). In addition, stress impacts on social behavior were also reported following a 10-day paradigm of social defeat in adolescent male

mice (Iniguez et al., 2014). In this study, mice exposed to the defeat stress exhibited an increase in social avoidance during a similar social interaction assessment. Differences in social behavior in these studies, compared to the current study, could have resulted from variations within the experimental models. Chronic social isolation stress resulted in an increase in sociability, whereas in the social defeat model, mice subjected to defeat avoided further contact with a novel mouse. The experimental model in the current study did involve social isolation, however, the additional predator-prey interaction could have negated expected changes in social behavior as mice would be expected to avoid predator interaction versus interaction with a mouse of similar sex and age.

Male and females differed in the percent time spent in the middle and vacant chambers. Male mice spent a larger percentage of their time in the middle chamber whereas female mice spent a larger percentage of their time in the vacant chamber. These data suggest male mice preferred to remain in a familiar environment versus a novel environment and, furthermore, female mice exhibited greater exploratory behavior. Differences in avoidance and exploratory behaviors among males and females have been documented in the literature (Frick and Gresack, 2003). In a study using a dual chamber apparatus, adult rats were familiarized to one chamber then allowed to explore either chamber (Russell, 1975). This study concluded that female rats exhibited a stronger preference for the novel environment compared to male counterparts which is in line with findings of the present study. Furthermore, avoidance of novel environments following traumatic stress exposure in rodents is also well documented in the literature. For example, adult male rats exposed to intense foot shock exhibited avoidance of a novel rat in a social interaction assessment and increased latency to enter a novel environment (Chen et al.,

2012). Although predatory stress did not result in social preference distinctions, sex differences in exploration were evident.

Peripheral levels of TNFα did not correlate with behavioral deficits in this study, but were increased as a result of predatory stress. Stress-induced increases of TNFα were also not sex-dependent. Previous findings indicate that chronic adolescent stress in rats did not affect circulating TNFα in adulthood (Bekhbat et al., 2019). In this study, male and female rats were exposed to chronic variable stress for 12 days then subjected to an acute challenge of LPS in adulthood. This study most closely mirrors that of the present study in that peripheral effects of chronic stress in adolescence were assessed, however, immune consequences were evaluated in adulthood and not in late adolescence. It is possible stress impacts on peripheral TNFα in adolescence dissipate by adulthood, but differences could also be attributed to use of mice instead of a rat model. In support of the findings of the present study, a chronic social defeat stress paradigm in male mice resulted in an increase of circulating TNFα (Reader et al., 2015). Furthermore, the induction of a depressive state as a result of increased peripheral levels of TNFα is well documented in literature (Tuglu et al., 2003; Litchblau et al., 2013; Dunn et al., 2005) though peripheral levels of TNFα in the current study did not directly correlate with behavioral deficits in adolescence.

In the present study, peripheral levels of IL-1B did not correlate with behavior deficits and were also not impacted by predatory stress. Previous work identifying the effects of chronic social disturbance stress in the periphery in adult mice also reported no effect of stress on circulating IL-1B (Gibb et al., 2008). Though the nature of the stressor and time of exposure differed from those in the present study, here, chronic stress did not impact IL-1B even in the presence of an acute inflammatory challenge. Conversely, male rats subjected to recurrent social

defeat exhibited an increase in peripheral IL-1B four hours following LPS administration (Carobrez et al., 2002). Differences among these studies could be attributed to the nature of the stressor, time following LPS administration, as well as the developmental period in which the stress exposure occurred. Unlike IL-1B, peripheral TNFα was impacted by predatory stress. Though these cytokines share similar functions in the immune response, IL-1B and TNFα operate under different kinetics. The lack of stress-induced IL-1B can be attributed to the time at which levels were assessed following LPS administration as time course in the periphery for these cytokines have varied in other studies (Johnson et al., 2002; Bekbhat et al., 2019; Kenma, 2005). The absence of stress impacts on IL-1B could also be attributed to the time of analysis; assessed in late adolescence, subjects could display stress-induced increases of IL-1B later in life, in adulthood.

Data from the both the AlphaLISA and MSD indicated predatory stress did not have an impact on circulating levels of IL-6, however caveats associated with either analysis method led to inconclusive findings on stress-induced IL-6 in the periphery. Aspects of the AlphaLISA cytokine assay that led to questions on the validity of the assessment include high standard deviation observed among triplicates of the standard curve. Any variability within the standard curve could negatively impact determined concentrations of unknowns. In addition, the lower detection limit of the assay was determined to be 60.591pg/mL whereas the lower sensitivity range of the assay as reported by Perkin Elmer was 1.4pg/mL. An approximate 10-fold difference in the lower limit could have significantly impacted analysis of the baseline samples which from this assessment, were considered undetectable. Other groups have reported undetected cytokine concentrations in the absence of a peripheral inflammatory challenge (Mormede et al., 2002; Deak et al., 2003) and this consistent with previous findings from our lab.

However, baseline samples analyzed via the MSD multiplex system fell within the range of detection. Furthermore, although multiplex systems are to compensate for expected differences in cytokine concentrations, an avenue of difficulty in the present study for this method of analysis was determining the appropriate dilution that could encompass all three cytokines within the detection range. Due to time constraints, a 1:20 dilution was employed which resulted in IL-1B and TNFα within the range of detection, but not IL-6.

It is of important note that these two assessments employed different methods of analysis. AlphaLISA assessments, utilize antibody/antigen interactions to quantify analyte concentrations based on chemiluminescence (Bielefeld-Sevigny, 2009). Advantages of AlphaLISA's over other methods include small sample volume requirements, minimal processing time and dynamic range of detection, making it an ideal analysis technique. On the contrary, MSD analysis utilizes electrochemiluminescent (ECL) readout to assess sample analyte content. ECL analysis is more advantageous over AlphaLISA because of its high signal to background ratio, large dynamic range and increased sensitivity (Kuhle et al., 2010; Leary et al., 2013). Future work in relation to the assessment of changes in immune reactivity include running additional dilution assessments prior to analysis and employing MSD analysis for both baseline and LPS-treated samples.

The work in this thesis demonstrates that the model of chronic adolescent stress in mice was sufficient in causing immediate changes in behavior and in the periphery. Predatory stress induced anxiety-like behaviors and impacted immune reactivity although these differences were not directly sex-specific. In this study, no neural endpoints were determined. Previous work has demonstrated that chronic adolescent stress alters both neuroinflammation (Pyter at al., 2013; Bekbhat et al., 2019) and behavior (Brydges et al., 2014; McCormick et al., 2008) and differences were sex-specific. Because behavior was altered, in the present study, as a result of

stress, it is possible that chronic adolescent stress also induced changes in immune reactivity in the brain. Furthermore, in the present study behavioral and immune implications of predatory stress were assessed in late adolescence. Persistent effects of stress on behavior (Chaby et al., 2015; Bourke and Neigh, 2011; Avital and Richter-Levin, 2005) and immune reactivity (Carobrez et al., 2002; Johnson et al., 2002). have been discussed throughout literature. Future work in relation to this thesis should include multiple time points of analysis such as a baseline prior to predator exposure, following predator exposure in late adolescence and in adulthood. These three points of analysis will serve to uncover the full extent at which chronic stress exposure in adolescence promotes differential effects over time. In addition, future work should also include neural endpoints to determine if chronic adolescent stress in mice alters immune reactivity in the brain, if these alterations parallel that in the periphery and how these differences contribute to alterations in behavior.

In this study, immediate effects of chronic stress exposure were apparent in adolescent mice. Predator exposure is an ethologically relevant stressor and caused behavioral and immune differences in both male and female mice. Behavioral deficits, however, did not correlate with differences in immune reactivity in this study, which could be attributed to the period of analysis. Persistent behavioral implications of chronic adolescent stress exposure have been discussed in the literature, with studies assessing consequences in adulthood rather than late adolescence (Sterlemann et al., 2008; Chaby et al, 2015). It is possible that behavioral deficits associated with adolescent stress exposure do correlate with differences in the periphery, but long after exposure, in adulthood. Unlike outcomes related to psychosocial stressors, the lack of sex-dependent differences as a result of chronic predator exposure suggest the salience of the stress paradigm is more suited in identifying consequences of chronic stress exposure in adolescence. It is possible

that social stress exposure is perceived differently among males and females, thus introducing another window of variability. As sex differences begin to arise in adolescence and persist through adulthood, the absence of sex differences in behavior or immune reactivity in the current study support outcomes being model-specific. Use of a predator/prey model lessens the possibility for variability in perceived stress among males and females. Thus, additional studies employing predatory stress, as described in this thesis, may be useful in identifying mechanisms related to how stress and inflammation relate to the induction of behavioral changes in mice.

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Vita

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