‘THE EFFECTS OF STRESS IN EARLY AND LATE ADOLESCENCE ON THE ANXIETY-LIKE, DEPRESSION-LIKE, AND SOCIAL BEHAVIOUR OF MALE AND FEMALE ADULT MICE’

by

Paula D. Torres Muñoz

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Abstract

The effects of adolescent stress on (particularly social) behaviour, has been scarcely studied despite possible increased neural sensitivity to stress due to the extensive neural development characterizing adolescence. Despite the continuous development of the brain and HPA axis throughout this stage, differential sensitivity to stress in sub-periods within adolescence have been scarcely explored. The effects of chronic psychological stress in early and late adolescence on anxiety-like, depression-like, and social behaviour in male and female mice were investigated. All stressed mice showed hyperactivity and increased anxiety-like and pro-social behaviours, and females showed anhedonia while males showed increased sucrose preference. Early adolescent stress also produced opposite changes in rearing in males and females. This study provides evidence that stress during adolescence leads to long-term effects on behaviour, that these effects can differ based on sex, and that certain aspects of behaviour can be differentially affected by stress in different sub-stages within adolescence.

List of Abbreviations Used

ACTH: Adrenocorticotropic Hormone

ADHD: Attention deficit hyperactivity disorder

ANOVA: Analysis of Variance

BDNF: Brain-derived neurotrophic factor

BAOT: Bed Nucleus of the Accessory Olfactory Tract

CAMKII: Calcium/calmodulin-dependent protein kinase II

CNS: Central Nervous System

CRH: Corticotropin-releasing Hormone

Crhr2: Corticotropin-releasing Hormone Receptor 2

CUMS: Chronic Unpredictable Mild Stress

CV: Coefficient of Variability

DRN: Dorsal Raphe Nucleus

EC: Early Control

EPM: Elevated Plus Maze

ERK: Extracellular signal-regulated Kinase

ES: Early Stress

FST: Forced Swim Test

GR: Glucocorticoid Receptors

HPA: Hypothalamus-pituitary-adrenal

LC: Late Control

LD: Light/Dark

LH: Learned Helplessness

LHb: Lateral Habenula

LS: Late Stress

MD: Major Depression

mPFC: Medial Prefrontal Cortex

MR: Mineralocorticoid Receptors

NAc: Nucleus Accumbens

N/OFQ: Nociceptin/Orphanin FQ

OF: Open Field

P: Post-natal Day

pCAMKII: Phospho-Calcium/ calmodulin-dependent protein kinase II

PFC: Prefrontal Cortex

PTSD: Post-traumatic Stress Disorder

PVN: Paraventricular Nucleus of the Hypothalamus

REM: Rapid Eye Movement

SAM: Sympathetic-adreno-medullar

TDS: Time-dependent Sensitization

VTA: Ventral Tegmental Area

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Chapter 1: Introduction

1.1. Overview of Stress

1.1.1 What is stress?

Stress is a physiological response meant to support survival by promoting adaptive responses to external factors that threaten the body's homeostasis (Godoy et al., 2018; Tottenham & Galván, 2016). The stress response prepares the body to react to stressors by either intensifying bodily functions, or reducing and even stopping them in order to utilize the energy for whichever response is needed. This response depends on a network of brain areas recognizing a stressor, an event that poses current or potential threat (Godoy et al., 2018; Joëls & Baram, 2009). Once an event is detected and identified as a threat the stress response is initiated, triggering a wide range of molecular, physiological, and behavioural events aimed at restoring homeostasis and promoting adaptation for similar stressors in the future. Stressors can be either acute, if they are experienced short-term, or chronic, if they are experienced recurrently or long-term. Acute stressors lead to rapid and temporary changes in neuronal and hormonal function that are eventually terminated (Joëls & Baram, 2009) . Chronic stressors, on the other hand, elicit long-term changes by altering gene expression and neuronal structure and function (Joëls & Baram, 2009). However, some of the temporary changes induced by acute stress can also lead to changes in gene expression. Therefore, despite their differences, both acute and chronic stress can have the potential to cause long-lasting effects (Joëls & Baram, 2009).

Stressors can also be categorized into physical or psychological stressors. Physical stressors are disturbances to the body's physiological integrity, such as an

infection (Joëls & Baram, 2009). Psychological stressors are stimuli that anticipate a potential threat based on innate cues, learning, and memory (McCormick et al., 2010), which include cues related to the presence of a predator. Each of these engage different brain structures and neuronal networks when triggering the respective stress response (Godoy et al., 2018). Physical stressors are mostly processed by brain structures in the brainstem and hypothalamus while psychological stressors mainly recruit brain areas involved in emotion processing, learning and memory, and cognition. However, these systems rarely function separately because most stressors have both physical and psychological components, even though one component usually dominates (Joëls & Baram, 2009). While both types of stressors remain relevant, physical stressors today are not as common as they have been historically, in the most developed nations. With the development of medicine, technology, housing and shelter, and food reserves, many of the physical stressors that were more relevant to our ancestors are no longer the most common stressors for individuals that have access to these developments (Nesse et al., 2007). In our current environment, psychological threats such as social and anticipatory stress are more prevalent.

Stress triggers both physical and cognitive responses, engaging the prosencephalic nuclei, prefrontal cortex (PFC), amygdala, hippocampus, paraventricular nucleus of the hypothalamus (PVN), ventral tegmental area (VTA) and nucleus accumbens (NAc; Andersen & Teicher, 2008; Godoy et al., 2018). Two distinct axes have been identified as part of the stress response: the sympathetic-adreno-medullar (SAM) axis and the hypothalamus-pituitary-adrenal (HPA) axis (Figure 1). The SAM axis is activated in the initial phase of the stress response and is aimed at the immediate promotion of survival to

a threat. Once a threat is identified, the hypothalamus signals to the medulla of the adrenal glands. The adrenal medulla then releases catecholamines into the bloodstream, which are distributed throughout the body. Through the secretion of catecholamines (epinephrine and norepinephrine) the SAM axis triggers rapid and short-lasting physiological responses in blood vessels, glands, visceral organs and smooth muscles leading to changes in heart and respiration rates, blood flow, and digestive activity (Joëls & Baram, 2009).

The HPA axis corresponds to the secondary phase of the stress response and is responsible for both short-term effects and amplified long-lasting changes that prepare the individual for similar stressors in the future. The HPA response begins when a stressor is detected as a result of the cognitive interpretation of stimuli by both limbic and cortical structures. If a stimulus is interpreted as a stressor, the hypothalamus is recruited. This results in the release of corticotropin releasing hormone (CRH) from the PVN. CRH then reaches the pituitary gland causing the production and release of adrenocorticotropic hormone (ACTH) into the hypophyseal portal system. When ACTH reaches the adrenal glands, glucocorticoids (mainly cortisol in humans and corticosterone in rodents) are produced and released into the bloodstream. Glucocorticoids then act on the brain by binding to glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (Koning et al., 2019). These two receptors exert complementary and sometimes opposite effects on the brain (Koning et al., 2019). GRs, which are activated only during the peak glucocorticoid levels of the circadian cycle and during stress due to their lower affinity, are expressed in most brain areas while MRs, which are activated at basal glucocorticoid levels due to their higher affinity, are expressed in selective brain regions such as the

hippocampus, amygdala, and prefrontal cortex (Koning et al., 2019). Through these receptors, glucocorticoids produce molecular, structural, and functional effects (de Kloet et al., 2008; Godoy et al., 2018; McCormick et al., 2010). The high level of glucocorticoids in the bloodstream is then detected by GRs in the pituitary and hypothalamus and by MRs in the hippocampus and the stress response is terminated (Koning et al., 2019). Through epigenetic mechanisms, this axis causes effects including changes in long term potentiation, synaptogenesis, dendritic structure, neurogenesis and cell death that can persist beyond the time of exposure to the stressor (McCormick et al., 2010). Through these changes, the HPA axis induces effects such as increases or decreases in stress reactivity, improved or impaired memory and learning, and changes in behaviour that shape the development of the central nervous system (CNS) in order to determine how the organism will respond to fluctuating environmental demands in the future (Andersen & Teicher, 2008; McCormick et al., 2010).

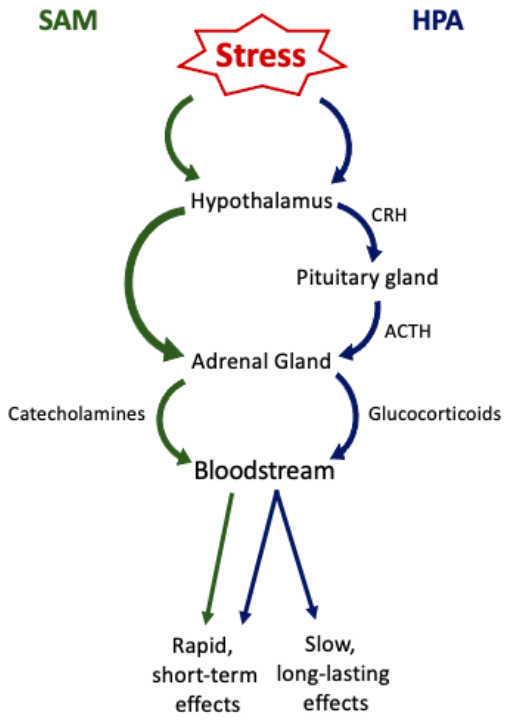

Figure 1. Activation of the sympathetic-adreno-medullary (SAM) axis (green) and hypothalamic-pituitary-adrenal (HPA) axis (blue) to stress leading to short-term and long-term effects in the body.

1.1.2 Effects of Stress

While the objective of the stress response is to maintain the body's wellbeing, it can lead to pathological states if the timing or duration of the response is inappropriate. For example, chronic stress leads to digestive (Gao et al., 2018), immune (Gao et al., 2018; Zhang et al., 2020), and metabolic (Zhang et al., 2020) dysregulation. Stress has also been linked to adverse neurological consequences. For example, an over-exposure to stress lasting for days or longer can lead to long-term structural and functional effects in the brain. These include both increased and decreased dendritic complexity and reduced synaptic transmission, neurogenesis, and synaptogenesis (Andersen & Teicher, 2008;

Godoy et al., 2018). The neurological effects of stress also include changes in the HPA axis such as increases or decreases in basal or stress-induced HPA activity (Sandi & Haller, 2015). These changes are achieved through alterations of gene expression in areas involved in HPA activity such as the hippocampus, PFC, and amygdala (de Kloet et al., 2005; Sandi & Haller, 2015).

The effects of stress on the brain are associated with predisposition to psychopathology, cognitive impairments, and learning deficits, as well as behavioural changes such as increased anxious behaviour, dysregulated affect, and impaired social interaction (Andersen & Teicher, 2008; Sandi & Haller, 2015; Tottenham & Galván, 2016). Consequently, prolonged stress exposure and dysfunction of the neural systems that respond to stress are linked to the development of several neuropsychiatric disorders including major depression (MD), anxiety disorders, post-traumatic stress disorder (PTSD), substance abuse, and epilepsy (Andersen & Teicher, 2008; Godoy et al., 2018; Saavedra-Rodríguez & Feig, 2013). In line with this association is evidence that a large number of patients with MD present increased concentrations of cortisol, an exaggerated cortisol response to ACTH, and adrenal and pituitary gland hyperplasia (Godoy et al., 2018). PTSD patients also present alterations of the HPA axis manifesting either as unusually high or low cortisol levels, increased GR sensitivity, enhanced negative HPA axis feedback, increased CRH levels, and reduced ACTH release in response to CRH administration (Godoy et al., 2018).

There is ample evidence that stress affects males and females differently (Adamec et al., 2006; An et al., 2011; Goel & Bale, 2008; Goodwill et al., 2019), which is consistent with the sex differences in the prevalence rates of many stress-related

neuropsychiatric disorders. For example, most anxiety disorders occur twice as frequently in females as in males and MD occurs up to three times more frequently in females (American Psychiatric Association, 2013). Male and female patients with the same disorders can also show different symptomatic patterns. For example, females with depression experience more anxiety, sleep and appetite disturbance, fatigue, and inability to focus while males experience more anhedonia and variations of mood and energy during the day (Andersen & Teicher, 2008). Given that stress affects each sex differently, any investigation of the effects of stress must address effects in both females and males. However, most of the existing literature on the effects of stress is currently focused on male subjects. Hormonal differences, particularly those emerging during puberty, are a possible contributor to the origin of these sex differences (Andersen & Teicher, 2008; Romeo et al., 2002). The relationship between stress and puberty will be discussed in later sections (see 1.2 Sensitivity to stress during adolescence). Another potential contributor to these sex differences is the differences in brain maturation between males and females; males overproduce synapses more than females and cortical grey matter density peaks earlier in females than in males (Andersen & Teicher, 2008).

In addition to sex, the timing of stress exposure can also influence the effects of stress. Early life stress can cause more persistent effects than stress experienced in adulthood and can lead to increased vulnerability to brain disorders (Godoy et al., 2018; Tottenham & Galván, 2016). The quality of the changes that result from stress exposure can also vary depending on the time of exposure. For example, although stress at several different points of early life has been shown to affect sociability in adulthood in rodents, prenatal (de Souza et al., 2013), neonatal (Wei et al., 2013), and peripubertal (Márquez et

al., 2013) stress have inhibited social interaction, while juvenile stress has either caused no effect (Workman et al., 2011) or increased social interaction (Shimozuru et al., 2008). These differences are partly due to the fact that the brain is differentially sensitive to stress at different periods of development. This can also explain the differential effects of stress on males and females. The differences in brain maturation and the differential exposure to hormones described above are likely connected, as exposure to hormones such as estrogen and testosterone regulate maturation trajectories (Andersen & Teicher, 2008). Therefore, the two factors can interact to result in the brains of males and females being more sensitive to stress at different timepoints, and each sex to be impacted differently by a stress exposure that was experienced at the same age. Sensitive periods will be discussed in detail in the following section (see 1.2 Sensitivity to stress during adolescence).

1.1.3 Animal Models of Stress

The precise effects of either acute or chronic stress experienced at particular stages of life are difficult to study in humans. This is because each individual has a unique history with particular combinations of acute, chronic, physical, and psychological stress at different timepoints. The ethical constraints of exposing humans to long periods of distress further prevent the investigation of stress-induced changes. These restrictions often do not allow for the establishment of direct causal relationships between stress and its potential effects, limiting the clinical literature to observational and correlational studies (Sandi & Haller, 2015). Moreover, the effects of stress on humans are molded by socioeconomic and educational factors, making the separation of such confounds from stress-induced changes difficult. In light of these issues, animal models

provide an opportunity to investigate the effects of stress with as few confounding factors as possible under controlled conditions. Due to the ease with which mice can be housed, handled, and bred and the genetic similarities they share with humans, mouse models present an advantage compared to other animal models like insect or primate models.

A variety of paradigms have been used to experimentally produce both physical and psychological stress in rodents. Common techniques for producing physical stress include painful stimuli (tail pinch, foot shock), physically restraining/inescapable conditions (tail suspension, forced swim, restraint in a small enclosure), changes in environment or living conditions (cage tilt, moist or soiled bedding, changes in temperature or humidity, changes to 24-hour light/dark cycle), deprivation (of either food or water), and exposure to stressful stimuli (noise, foreign odour; Antoniuk et al., 2019). Given that the field has moved away from exposures that can cause physical damage or pain, one of the most common protocols for production of physical stress is restraint stress. Restraint stress has resulted in impaired working memory in the Y-maze (Kim et al., 2018) and in the radial arm water maze (Grizzell et al., 2014), and impaired memory consolidation and retrieval in the novel object recognition test in male mice. These effects were associated with increased levels of extracellular signal-regulated kinase (ERK)1 and ERK2 phosphorylation in the PFC and increased plasma corticosterone (Kim et al., 2018). Restraint stress has also produced depression-like behaviours demonstrated by increased immobility in both the tail suspension and forced swim (FST) tests (Christiansen et al., 2011; Grizzell et al., 2014; K.-S. Kim & Han, 2006) and decreased sucrose preference (Zhu et al., 2019), and anxiety-like behaviour shown by increased locomotion and velocity in the open field and reduced entries and time spent in the open

arms of the elevated plus maze (EPM) in male mice (Grizzell et al., 2014; K.-S. Kim & Han, 2006). These changes were associated with GSK3 activity in the hippocampus and PFC (Grizzell et al., 2014), either up- or down- regulation of a wide range of genes (involved in energy metabolism, signal transduction, transcription, synaptic plasticity, and synaptic remodeling) in the hippocampus and amygdala (Kim & Han, 2006), and increased neuropeptide Y mRNA levels in the medial amygdala (Christiansen et al., 2011). In addition to anxiety- and depression-like behaviour, restraint has also increased aggression in male rats, which was associated with heightened corticosterone levels (Wood et al., 2003).

Multiple physical stressors have been combined in several ways to model different aspects of stress such as learned helplessness or sensitization. The learned helplessness (LH) paradigm originally resulted from observations that dogs previously exposed to physically inescapable shocks later failed to attempt to escape in situations in which escape was possible (Seligman, 1972). This was then extended to rodents and was shown to result in the same behavioural despair seen in dogs (Van Dijken et al., 1992). Due to the behavioural lack of motivation and physiological effects it elicited, LH was mainly used to study depression and PTSD (Schöner et al., 2017). Time-dependent sensitization (TDS) was developed to model the phenomenon of sensitization in which an initial exposure to intense stress causes an intensified response to later pharmacological stressors (Schöner et al., 2017). This has been studied in the context of antidepressant drugs and therapies, regarding how their effects progress over time (Antelman & Gershon, 1998). Thus, physical techniques were initially preferred in the production of rodent models of neuropsychiatric diseases.

Psychological stress paradigms are generally based on interaction (or lack of interaction) with conspecifics or predator species. Common techniques include manipulating the number of house-mates (crowded housing/isolation), social defeat, social instability, and predator stress (Antoniuk et al., 2019; Schöner et al., 2017). Social defeat is commonly used as a model of psychosocial stress because, although it does include the initial physical component of confrontation between conspecifics, most of the stress comes from protected exposure to the aggressor. While social defeat paradigms have been recently developed for female subjects, this form of stress has mainly been studied in males due to the higher prevalence of attack behaviour in males. Social defeat in both male and female mice has been reported to induce increased depression-like behaviour shown by reduced sucrose preference (Harris et al., 2018; Yu et al., 2011) and decreased exploration of a novel object (Newman et al., 2019), as well as social avoidance (Harris et al., 2018; Takahashi et al., 2017; Yu et al., 2011). Cognition has also been affected by social defeat, with impaired working memory in the T-maze and enhanced fear in fear conditioning tests in males (Yu et al., 2011). These changes were linked to increased activity in the PFC, cingulate cortex, hippocampus, amygdala and hypothalamic nuclei (Yu et al., 2011). In females, defeat impaired nesting behaviour, which was associated with elevated plasma corticosterone and increased activation of the medial amygdala, ventral lateral septum, ventromedial hypothalamus, and hypothalamic paraventricular nucleus. Defeat has also induced anxiety-like behaviors in females such as increased vigilance (Newman et al., 2019) and reduced time in the open arms of the EPM (Harris et al., 2018).

Social isolation has been reported to increase depression-like behaviour, shown by a decrease in motivation for sucrose consumption and social interaction in male rats (Van den Berg, 1999). This type of stress has also induced anxiety-like behaviour shown by decreased time spent in the centre of the open field in both adolescent and adult males (Lander et al., 2017). Furthermore, isolation led to increased exploration of both social and non-social novel stimuli in the novel object recognition test and three-chamber sociability tests, respectively, in adolescent, but not adult, male mice. Cognition was also affected, as isolation impaired reversal learning and extra-dimensional set shifting in the water T-maze. These changes were associated with increased expression of excitatory markers in the mPFC (Lander et al., 2017). Similar to social isolation, crowded housing has also increased anxiety-like behaviours in male mice shown by reduced exploration in the open field, decreased entries and time spent in the open arms of the EPM, and an exaggerated startle response. The effects were associated with increased mRNA expression of both the nociceptin/orphanin FQ (N/OFQ) precursor and receptor in the hippocampus (Reiss et al., 2007).

Predator stress is frequently used to model PTSD in rodents because it mimics discrete traumatic events and is generally limited to either the exposure to predator-related stimuli of one modality (odour or sound) rather than physical contact, or to short physical exposure (5-10 minutes) in order to maintain the physical safety of the animal (Schöner et al., 2017). Exposure to predator odour has resulted in increased anxiety-like behaviours in male rats such as freezing during exposure, decreased time in the open arms of the EPM, and increased mean startle amplitude (Cohen et al., 2006; Liang et al., 2014). This was associated with impaired connectivity between the amygdala and mPFC

(Liang et al., 2014) and a blunted HPA response to stress (Cohen et al., 2006). In addition to increased anxiety-like behaviour demonstrated by avoidance of odour-paired stimuli, predator odour stress has led to decreased weight gain, increased alcohol self-administration, higher alcohol responding, and hyperalgesia in male rats (Weera et al., 2020). These effects were associated with increased activation and CRF immunoreactivity in the central nucleus of the amygdala (Weera et al., 2020). Exposure to predator odour has also induced depression-like behaviours such as decreased activity in the open field (Y.-P. Wu et al., 2019).

Direct exposure to a predator produced impairment of hippocampal-based memory in the radial arm water maze in male and female rats, which was linked to elevated serum corticosterone levels (Park et al., 2008; Zoladz et al., 2012). In males, the impairment was also associated with differences in molecular markers of synaptic plasticity, including calcium/calmodulin-dependent protein kinase II (CaMKII) phosphorylation having increased in the basolateral amygdala but decreased in the mPFC and a blockage of the increases in the phospho-calcium/calmodulin-dependent protein kinase II (p-CaMKII), total CaMKII, and Brain-derived neurotrophic factor (BDNF) levels in the dorsal CA1 of the hippocampus necessary for learning (Zoladz et al., 2012). Predator exposure has also been reported to induce impaired sensorimotor gating in male rats, as shown by disrupted pre-pulse inhibition, which was associated with increased stimulation of CRH receptors (Bakshi et al., 2012). Additionally, predator exposure produced a sex-dependent increase in anxiety-like behaviour. Exposure induced decreased exploration in the open field test (Toth et al., 2016) and avoidance of the light area in the light/dark box in all stressed females but only in a subset of male mice in

which forebrain CRH over-expression was induced. These effects were associated with a lack of corticotropin-releasing hormone receptor 2 (Crhr2) expression changes in response to stress, while animals that showed no behavioural effects showed an increase in Crhr2 expression in the bed nucleus of the stria terminalis (Toth et al., 2016). However, another study found that both male and female mice showed decreased time in the light compartment after predator exposure along with increased startle amplitude and decreased time in the open arms of the EPM (Adamec et al., 2006). Other anxiety-like behaviours have also been reported, such as decreased time spent in a novel compartment, reduced activity in a free exploration test (Belzung et al., 2001), and increased risk assessment in the EPM (Adamec et al., 2004) in male mice. The increased anxiety in the free exploration test was associated with increased turnover rates of noradrenaline, dopamine and serotonin in the hippocampus, hypothalamus, and striatum (Belzung et al., 2001).

In addition to increased anxiety-like behaviour, shown by reduced open arm exploration in the EPM and reduced light area entries of the light/dark box, predator exposure resulted in social avoidance in male rats, shown by increased withdrawal from a social partner and reduced social interaction in a social interaction test (Blundell et al., 2005). Increased depression-like behaviours have also resulted from predator exposure in male mice, as demonstrated by decreases in spontaneous activity and locomotion in the open field and dark avoidance tests (Wu et al., 2019). These depression-like behaviours were linked to decreased hippocampal neurogenesis and serotonin levels (Wu et al., 2019).

While predator odour has successfully produced psychological stress and some associated behavioural effects, direct predator exposure has produced stronger effects when compared directly to odour exposure. Male and female mice exposed to a predator showed an enhanced peak startle amplitude (Adamec et al., 2006), increased time spent in the dark compartment of the light/dark box (Wu et al., 2019), decreased time spent in the open arms of the EPM, and decreased hippocampal neurogenesis (Wu et al., 2019) compared to unstressed controls but mice exposed to a predator odour did not (Adamec et al., 2006; Wu et al., 2019). Given the efficacy of actual exposure, modified predator stress models are being developed to increase the contact between predator and prey as well as the duration of exposure. The predator stress model used in this study is described in the methods section (see 2.2.1 Background).

Within the broader scope of the literature, physical and psychological stress have been shown to have some effects in common such as changes in HPA axis function and related systems including changes in the weights of the adrenal glands and thymus, increased basal corticosterone, decreased ACTH levels, flattened circadian corticosterone rhythm, altered adrenal sensitivity, and decreased expression of corticosteroid receptors in the hippocampus. Moreover, these changes have been associated with impaired cognitive performance and behavioural effects including social avoidance, submissive behaviour, aggression and increases in depression-like and anxiety-like behaviour (Kim et al., 2018; McCormick et al., 2010; Saavedra-Rodríguez & Feig, 2013; Schmidt et al., 2007; Schöner et al., 2017; Yu et al., 2011). However, within the scope of individual studies physical and psychological stress have produced different effects. For example, when female rats were exposed to either the physical stress of foot shocks or to the

psychological stress of witnessing the foot shocks from an adjacent compartment, the psychologically stressed rats showed a wider range of long-term effects, although the stress-induced increase in serum corticosterone was similar for both groups (Mousavi et al., 2019). Psychologically stressed rats showed a reduction in both anxiety-like and depression-like behaviours, with an increased proportion of time spent in the open arms of the EPM, and decreased time spent immobile in the FST. Psychological stress also resulted in spatial memory impairment in the Y maze and in object recognition memory impairment in the novel object recognition test. Neither of these effects were shown by the physically stressed group (Mousavi et al., 2019). In similar foot shock-witness paradigms, male rats that observed the foot shocks demonstrated enhanced rapid eye movement (REM) sleep with no changes in plasma corticosterone (Ranji et al., 2007) and increased body temperature (Endo & Shiraki, 2000) while rats that physically experienced the foot shocks showed inhibited REM and non-REM sleep with increased plasma corticosterone (Ranji et al., 2007) and unchanged body temperature (Endo & Shiraki, 2000). Similar results have been found in mice, with actual foot shock exposure, but not witnessing the foot shock, producing increased anxiety-like behaviours such as decreased locomotion in the open-field and reduced entries and time spent in the open arms of the EPM (Palermo-Neto et al., 2003). Overall, models of psychological stress produce a wider range of long-term behavioural effects and are most useful in translational research, as many of the most relevant stressors experienced by humans are psychological in nature.

Protocols for the induction of early-life stress are different from traditional physical and psychological stressors, as they involve individuals with different physical

and cognitive abilities than adults. Several of these techniques are based on disruptions in maternal interactions (early weaning, maternal separation). Maternal separation consists of separating the litter from the dam for at least 3 hours a day during the first weeks after birth (Sandi & Haller, 2015). Another common technique, early social deprivation, is similar to the traditional social isolation paradigm of psychological stress and consists of separating the pup from both the dam and the littermates in the weeks following birth (Sandi & Haller, 2015). These techniques have been used to study the consequences of childhood trauma, deprivation, or neglect in humans. Maternal separation in early life produced increased social and anxiety-like behaviour in female mice and disrupted locomotor and exploratory activity in male mice (Bondar et al., 2018). Females demonstrated increased time spent with a social target in a social interaction test and increased latency to leave the centre as well as decreased time spent in the open arms in the EPM (Bondar et al., 2018). On the other hand, males showed decreased locomotion and exploratory activity in the open field and social interaction tests (Bondar et al., 2018). Investigations in rats also revealed sex differences in the effects of early-life stress. Daily maternal separation in males induced higher baseline corticosterone with reduced activity in the open arms of the EPM and increased frequency of pinning behaviour in a social play behaviour test, while females did not show these behavioural effects (Lundberg et al., 2017).

Most of these paradigms can be used to produce either acute or chronic stress depending on the duration and frequency of exposure. Chronic unpredictable mild stress (CUMS) is a protocol for the induction of chronic stress and generally uses a combination of several physical and/or psychological stressors for an extended period of time (several

weeks). The individual stressors are administered in a random order to render the stress unpredictable and thus limit habituation to the paradigm. While acute individual exposures to the previously mentioned physical and psychological protocols have proven effective in some way, the specific effects of the stress tend to vary based on the paradigm used and across studies. CUMS, however, has been shown to be particularly reliable and effective as it mimics chronic stress exposure and combines various psychological and physical stress protocols (Antoniuk et al., 2019). Therefore, CUMS is one of the most common techniques used to induce stress and is frequently used to model depression in rodents. CUMS has been reported to induce anxiety-like behaviours such as increased time spent in the dark area of the light dark box and increased locomotion in the open field in both male and female mice, as well as increased locomotion in the EPM and light dark box in either males or females depending on the strain (Mineur et al., 2006). CUMS has also produced depression-like behaviours such as either increased immobility or increased time spent struggling, depending on the strain used, in male mice in the tail suspension test, reduced time spent struggling in both males and females in the forced swim test, and fur deterioration in males (Hei et al., 2019; Mineur et al., 2006). Similar results were found in male rats exposed to CUMS, which showed reduced weight gain and sucrose preference as well as decreased locomotion and time spent in the centre of the open field. Additionally, both male mice and male rats exposed to CUMS showed impaired spatial memory in the Morris water maze (Hei et al., 2019; Wu et al., 2017).

Overall, individual stress paradigms vary greatly. This allows stress researchers using these animal models to choose the best paradigm for the particular type of stress they intend to model to ensure its face validity (i.e. the type of stress produced in the

animal model must be similar to type of stress in humans that the paradigm intends to model).

1.2 Sensitivity to Stress during Adolescence

1.2.1 Sensitive Periods

The effects of stress vary depending on the time of exposure. For example, early social deprivation prior to weaning leads to a reduced HPA stress response (Rees et al., 2006) while post-weaning social isolation leads to an increased HPA axis stress response in adulthood (Toth et al., 2011). This has been proposed to result from the brain being more sensitive to stress during particular time periods. Windows of increased and rapid development in early life during which brain areas and systems are the most vulnerable to environmental influences are referred to as sensitive periods (Andersen & Teicher, 2008). During these periods, external influences are easily incorporated into the organization of neural circuits to determine responses to stimuli in adulthood (Romeo et al., 2002). If any of the brain areas involved in the stress response exhibit increased sensitivity during a particular time period, they are likely to be more susceptible to long-lasting effects of stress if stress is experienced during that time of increased sensitivity. Many factors including rates and trajectories of synaptic development, neurotrophic factor levels, rates of myelination, and connectivity can render brain structures sensitive. Given that both the CNS as a whole, and the HPA axis are still developing during early-life (McCormick et al., 2010), stress during this period can disrupt the development of both, especially brain structures essential to the stress response (Godoy et al., 2018) and lead to aberrant stress function.

Additionally, the factors that contribute to sensitivity mentioned above vary across different brain structures at different times, which likely leads to different brain areas having unique sensitive periods (Andersen & Teicher, 2008; Tottenham & Galván, 2016). One example of these factors is the rate of growth and development of particular brain structures. From age 10 to 19 in humans, the volume of the amygdala increases and the PFC grey matter volume decreases at faster rates compared to other periods of life (Andersen & Teicher, 2008). On the other hand, hippocampal volume decreases at a faster rate from age 6 to 13 than it does later in life (Andersen & Teicher, 2008). Therefore, while the amygdala and PFC are likely to be more sensitive from ages 10 to 19, this happens at ages 6 to 13 for the hippocampus

Certain brain areas can be especially sensitive to stress hormones, which can render them more susceptible to stress. For example the amygdala, PFC and ventral striatum are significantly modulated by cortisol and other stress hormones which makes these areas more sensitive to stress compared to other structures in the brain (Tottenham & Galván, 2016). Expression, density, distribution, and function of GR and MR, also vary across different parts of the brain (Godoy et al., 2018; McCormick et al., 2010) and can make them more or less sensitive to stress. For example, GR expression peaks in adolescence in the dorsal PFC while it peaks during adulthood in the CA1 region of the hippocampus (Andersen & Teicher, 2008). This could lead to higher sensitivity to stress in the dorsal PFC during adolescence and in the CA1 in adulthood. Additionally, receptor distribution could cause similar differences in stress sensitivity, as GR distribution is widespread throughout the brain while MR distribution is restricted to limbic structures (hippocampus, amygdala, and PFC). Because GR affinity for glucocorticoids is almost

ten-fold lower than for MR, MR are occupied at basal glucocorticoid levels, while GR activate at stress levels. Receptor type, affinity, localization and density of glucocorticoid receptors act together with cell type and physiological context to result in a wide range of effects on the brain (de Kloet et al., 2005; Koning et al., 2019; Meijer et al., 2019) and thus different times of stress sensitivity in different structures.

The idea of unique sensitive periods for different brain areas is supported by reports of stress exposure at different ages affecting distinct brain areas in different ways. For example, it has been found in both humans and rodents that adolescent stress reduced the size of the PFC more than early life stress, but reduced the size of the hippocampus less than early life stress (Andersen & Teicher, 2008). This would indicate that the PFC is more sensitive to stress during adolescence while the hippocampus is more sensitive at earlier ages. These times of likely higher sensitivity coincide with the periods of accelerated development in grey matter volumes mentioned above.

1.2.2 Adolescence

The prenatal and neonatal periods of life have long been known as sensitive periods due to the influence from the mother through the placenta and the importance of maternal interaction (Sandi & Haller, 2015), as well as the heightened neural development that characterizes both periods (Romeo et al., 2002). However adolescence, which has not been as thoroughly studied as a sensitive period, is also a time of extensive neural development (Blakemore, 2008). Given that cognition and behavioural responses to the environment differ significantly between young and adult individuals (Romeo et al., 2002), it follows that in the transition from youth to adulthood a wide range of changes must take place. This is evident in humans, as adolescents tend to be more prone

to risk-taking and sensation-seeking behaviours as well as gregariousness compared to adults (Dayan et al., 2010). The rodent literature also shows marked differences between adolescents and adults. Adolescents have shown higher levels of exploration and general activity and were more likely to divert their focus from task-relevant cues than adults (Sturman et al., 2010). Adolescents also showed higher cocaine-induced and novelty-induced increases in locomotion (Marin & Planeta, 2004), either increased (Stone & Quartermain, 1997) or decreased (Doremus et al., 2004; Genn et al., 2003) stress-induced anxiety-like behaviour in the in the EPM, increased stress-induced time spent in social interaction (Genn et al., 2003), and lower immobility in the forced swim test (Martínez-Mota et al., 2011) compared to adults. Adolescent mice show a higher physiological reactivity to stress than adult mice (Brust et al., 2015). Corticosterone release in response to acute stress is higher and more prolonged during pre-pubescent adolescence compared to adulthood (McCormick et al., 2010), and adolescents show decreased weight gain and decreased habituation of the corticosterone response to stress compared to adults (Doremus-Fitzwater et al., 2009). This is also true in humans, with heightened stress levels and reactivity to stress in adolescents compared to children and adults (Tottenham & Galván, 2016).

These differences indicate that the brain structures that regulate such responses and behaviours are still immature and developing during adolescence, resulting in differences once adulthood is reached. Importantly, the differences reported in HPA response to acute stress between adolescents and adults are reduced when stress occurs during sexually-mature adolescence rather than pre-pubescent adolescence (McCormick et al., 2010). This illustrates the development that occurs during adolescence regarding

systems involved in the stress response. Independently of the mechanisms underlying these differences, it follows that if reactivity to stress is higher during adolescence, then stress experienced during this time will have more potent effects than similar stressors experienced during adulthood. Nonetheless, it is important to consider the physiological underpinnings of these differences, as adolescence may provide a developmental period during which stress-induced effects are intensified. Combined with the increased plasticity and constant change occurring in the brain, this likely results in a unique window of sensitivity to stress.

The development of responses to stress can be traced back to physiological developments within the brain and the stress response system. Adolescence comprises significant hormonal changes, increased sensitivity to gonadal and adrenal hormones, anatomical and functional reorganization of the brain and also presents heightened rates of brain development, particularly of brain areas involved in emotion processing and higher cognitive function (Andersen & Teicher, 2008; Brust et al., 2015; McCormick et al., 2010; Tottenham & Galván, 2016). For example in humans the PFC does not reach its adult volume until the second decade of life and continuously develops during adolescence (Andersen & Teicher, 2008). Similarly, substructures of the amygdala undergo permanent structural changes during this time (Romeo et al., 2002). Furthermore, as mentioned above, the volumes of the amygdala and PFC change at an increased rate in adolescence, the PFC is most affected by stress experienced in adolescence, and GR expression peaks in adolescence in the dorsal PFC. Connections between the amygdala and the PFC, which regulate affective responses to stress, also continue to develop well into adolescence and, given that they are highly sensitive to

HPA axis hormones, they are susceptible to stressful environments (Tottenham & Galván, 2016). Neurotransmission properties act together with connectivity to make the brain sensitive to stressful contextual information. The NAc, which is heavily involved in reward processing through its dopaminergic connections with the VTA, receives inputs from the cortex, which is implicated in the processing of contextual stimuli and enhancement of stress responsiveness. During adolescence, dopaminergic activity is higher in the cortex and lower in the NAc than in other time periods (Andersen & Teicher, 2008). As connections between the NAc and the cortex continue to develop throughout adolescence, the PFC increasingly modulates NAc activity. Together, these factors may lead to enhanced responsivity to stress and increased sensitivity to context (Andersen & Teicher, 2008) molding future behavioural and physiological responses to stress. Corticosterone-binding globulin, a transport protein for glucocorticoids, and CRH-responsive cells in the pituitary gland both increase their concentration during adolescence (McCormick et al., 2010). Additionally, GR expression in the cortex peaks during adolescence and corticosterone response to acute stress is enhanced and prolonged during this time (Andersen & Teicher, 2008).

Overall, the brain is more plastic during adolescence to allow for optimization of behaviour for the future, as opposed to adulthood, when there is higher physiological and behavioural stability throughout (Brust et al., 2015; Tottenham & Galván, 2016). Furthermore, it has been shown that environmental enrichment during adolescence is able to reverse negative consequences of early-life stress (McCormick et al., 2010). This corroborates the idea that adolescence is a time during which the brain can be more easily

molded by the environment. Thus, environmental stimuli during adolescence can lead to not only beneficial remodeling of the brain but also to long-term detrimental changes.

1.2.3 Puberty

The differences in reactivity to stress between adulthood and earlier stages have been proposed to result from changes stemming from sexual maturation (Brust et al., 2015; Romeo et al., 2002; Stone & Quartermain, 1997). This is consistent with evidence from clinical research, as puberty in both men and women is associated with problematic changes in behaviour, increased anxiety and depression, and increased risk for self-harm (Romeo, 2017). For women, the onset of depression, which is strongly linked to stress, tends to coincide temporally with the onset of menarche. One of the main changes that come with puberty in both humans and rodents is the increase of sex hormones such as testosterone and estrogen. Sex hormones have a significant influence on neurogenesis and neuronal survival, synaptogenesis, receptor expression, neuronal excitability and neurotransmitter synthesis (Blakemore, 2008). Puberty also leads to the activation of hormones that regulate development of the PFC, amygdala, and hypothalamus (Andersen & Teicher, 2008), and triggers neuroanatomical changes in the frontal and parietal cortices in humans (Blakemore, 2008). This is supported by evidence in rodents. Estrogen has been shown to suppress overproduction of neurons in the PFC of females while testosterone has been proposed to stimulate dendritic pruning in the amygdala of males. These factors have been suggested to contribute to sex differences observed in the effects of stress and the onset, prevalence, and symptomatology of mental disorders. Importantly for stress, many of the brain areas affected by puberty play a major role in the stress response. Additionally, human HPA axis reactivity increases after puberty

(Tottenham & Galván, 2016). In sum, these factors show that puberty leads to significant changes in hormonal and neural function, particularly in brain structures associated with stress. This, together with HPA axis changes during this period, can render the brain sensitive to stress.

While puberty-related hormones are one possible contributor to the behavioural differences between juveniles and adults, it has been proposed that they do not completely explain the differences (Romeo et al., 2002). Early experimental exposure to these hormones before natural puberty does not result in rodents performing sexually mature behaviours (Romeo et al., 2002), suggesting that non-hormonal factors, perhaps maturation of certain neural circuits or structures, are necessary in addition to hormones for sexually mature behaviours to emerge. This not only corroborates that the brain undergoes significant changes from the pre-pubertal period to the post-pubertal period, but also points to likely differences in stress-related brain systems between pre- and post-pubertal periods. Regardless of the mechanisms behind these differences, if sexual maturity does lead to a change in the response to stress, then the effects of stress could differ even within adolescence. In other words, the pre-pubescent stage (early adolescence) and the sexually mature stage (late adolescence) could be characterized by different reactivity to stress, and stress exposure at each stage could lead to different effects. So far, the literature comparing the effects of stress during different stages of adolescence is scarce and the difference between early adolescent stress and late adolescent stress has not been directly investigated.

In mice, puberty is considered to be the period during which the individual becomes capable of reproduction (Sisk & Zehr, 2005), a process that begins in early

adolescence (Brust et al., 2015). In female mice, the vagina opens approximately on day 26 (Brust et al., 2015) and in males, spermatogenesis begins at approximately day 21 (Krishnamurthy et al., 2001). Spermatids begin to elongate around day 28 and are completely developed by day 35 (Krishnamurthy et al., 2001). Testosterone rises in males and sex differences in body weight appear around day 30 (Jean-Faucher et al., 1978). By mid-adolescence, the process is complete (Brust et al., 2015). Females begin ovulating and successful mating begins to occur on day 36 (Jean-Faucher et al., 1978; Silver, 2001). Testicular testosterone content reaches its maximum at day 40 and then decreases continuously until day 60 (Jean-Faucher et al., 1978). Based on these sexual developmental milestones, puberty occurs roughly during early and mid-adolescence, and is completed by late adolescence in laboratory mice. However, adolescence encompasses much more than the physiological changes leading to sexual maturity, as discussed above.

1.2.4 Adolescent animal models

Adolescent stress is difficult to study in humans because stress limited to this period is rare and opportunities to study it are scarce (Tottenham & Galván, 2016). Given that the period of stress exposure in laboratory animals can be precisely controlled, animal models present a unique opportunity to study the effects of adolescence-limited stress and mice are especially advantageous for the reasons previously mentioned. Although it is clear that adolescence is the time of transition from early life to adulthood, the exact ages marking adolescence in mice have varied among research groups and disciplines. Consequently, researchers in the past have generally used mice of age ranges

anywhere from post-natal day (P) 22 to P60 to study adolescence (Brust et al., 2015; McCormick et al., 2010). This seems to have partly contributed to the varying results across studies and research groups resulting in contradicting information in the literature. Some studies have reported long-term effects of adolescent stress such as elevated corticosterone along with decreased GR and MR expression in the hippocampus for up to a year after stress exposure (Schmidt et al., 2007). Adolescent stress has also been reported to cause long term behavioural effects such as increased anxiety behaviour and impaired spatial memory during adulthood, depression-like behaviours and social avoidance (Adamec et al., 2006; Iñiguez et al., 2014; Saavedra-Rodríguez & Feig, 2013; Schmidt et al., 2007; Sterlemann et al., 2009). However, researchers have also reported a lack of long-term effects of adolescent physical and psychological stress on anxiety-like, depression-like or social behaviour, and plasma ACTH levels or corticosterone concentrations (McCormick et al., 2010; Pyter et al., 2013). More recently, however, several authors have come to divide adolescence into 3 periods: pre-pubescent or early adolescence (P21-P34), mid-adolescence (P34 – P46), and sexually-mature or late adolescence (P46 – P59), based on the timing of behavioural and neurological changes characteristic of adolescence (Brust et al., 2015; McCormick et al., 2010; Spear, 2000a, 2000b). The exact contribution of this undefined timeline to the lack of consistency in the literature remains unknown, as not many research groups have yet begun to utilize the three-stage timeline to investigate stress. This defined timeline will hopefully lead to more consistent results in the adolescent stress literature and will be used in the study described here.

1.3 Stress and neuropsychiatric disease-related behaviour

1.3.1 Effects of stress on anxiety-like behaviour

Anxiety and mood disorders are one of the most common psychiatric disorders in the United States (Kessler & Wang, 2008) and the most common in Canada (McRae et al., 2016). The main symptom of these disorders is excessive fear and anxiety, and it is persistent and interferes with many daily functions (American Psychiatric Association, 2013). While it is the main symptom of anxiety disorders, anxiety as a symptom is also present in other neuropsychological disorders such as schizophrenia, and PTSD (American Psychiatric Association, 2013). Rodent models are commonly used to investigate the causes of anxiety, as well as possible treatment methods. Behavioural paradigms based on avoidance of potentially aversive stimuli include the elevated plus maze (EPM), open field (OF) test, light/dark (LD) test, and are used to assess anxiety levels in response to stimuli or experimental manipulations. As stress exposure in humans is strongly linked to the development of anxiety disorders (Andersen & Teicher, 2008; Godoy et al., 2018; Saavedra-Rodríguez & Feig, 2013), many studies have examined the effects of stress exposure on the anxiety-like behaviour of animal models. As mentioned above (1.1.3. Animal Models of Stress), stress exposure has been reported to cause long-term increases in anxiety like behaviour (McCormick et al., 2010; Saavedra-Rodríguez & Feig, 2013; Schmidt et al., 2007) which is likely linked to the long-term effects also reported on HPA axis function (Schmidt et al., 2007).

1.3.2 Effects of stress on depression-like behaviour

The category of depressive disorders encompasses several conditions including major depressive disorder, persistent depressive disorder, and premenstrual dysphoric disorder. However depression as a symptom refers to feelings of sadness, emptiness, or irritability that significantly affect daily function (American Psychiatric Association, 2013). Similar to anxiety, depression has been well studied using animal models. Behavioural tests based on motivation such as the tail suspension test, forced swim test (FST), and sucrose preference test, as well as coat state, have been commonly used to assess the presence of depression-like behaviours. Given that, similarly to anxiety disorders, depression has been linked to stress exposure (Andersen & Teicher, 2008), the effects of stress on depression-like behaviour have also been studied in depth.

As mentioned in a previous section (1.1.3. Animal Models of Stress), exposure to stress in adulthood has been reported to result in increased depression-like behaviours in rodents. These increased depression-like behaviours are linked to HPA axis dysregulation (similar to anxiety-like behaviour) as well as to abnormal synaptic development in the PFC and hippocampus (Hei et al., 2019). Dendritic remodeling in the hippocampus and amygdala found in rodents in response to chronic stress or high levels of glucocorticoids are similar to abnormalities found in depressed patients (McCormick et al., 2010). Other brain structures implicated in depression are the VTA and NAc, which regulate reward processing, and areas that communicate with the NAc such as the hypothalamus and amygdala. Abnormalities in these regions such as reductions in dopamine neurotransmission have been found in patients with depression. Specifically, this has been proposed to be linked to the anhedonia, loss of appetite, and social withdrawal

components of depression (Andersen & Teicher, 2008). Many studies assessing depression-like behaviours also characterize anxiety-like behaviour (Hei et al., 2019; Mineur et al., 2006), perhaps due to the high rate of comorbidity between anxiety disorders and depressive disorders (McCormick et al., 2010; Zbozinek et al., 2012). The increase in depression-like behaviours is often accompanied by changes in anxiety-like behaviour in tests such as the OF and EPM, although the direction of these changes varies across different studies (Hei et al., 2019; Mineur et al., 2006).

1.3.3 Effects of stress on social behaviour

Social behaviour refers to interaction or communication between conspecifics (Chen & Hong, 2018). Correct processing of social stimuli and in turn adequate social behavioural responses significantly affect the success of an individual. When adequate social behaviour is not present, many aspects of life can be impacted. This is evident in some neurodevelopmental and schizophrenia spectrum disorders, social anxiety, depression, and PTSD (American Psychiatric Association, 2013; Sandi & Haller, 2015). In these neuropsychiatric disorders, social dysfunction symptoms such as reductions in social motivation and increased social avoidance, impaired social cognition, social anxiety, increased aggression, and otherwise altered social behaviour are major contributors to the detrimental impacts of mental illness on daily functioning (Sandi & Haller, 2015).

Because this type of behaviour is integral for healthy living, and is often impacted by disease, there has been much interest for the mechanisms underlying it. Human social behaviour is complex due to factors like theory of mind, which allows individuals the

ability to attribute mental states to others and to predict their intentions and motivations (Blakemore, 2008). Although this added complexity is not present in animal social interaction, social behaviour is essential to all social species. The laboratory mouse (*Mus musculus*) is a social species and while social behaviours such as mating, and parenting are more obviously essential to survival in mice, aggression, scent marking, communal nesting, and reciprocal social interaction are also important at the individual and species level (Silverman et al., 2010). Not only does social behaviour have an essential role among mice but this species also shows a clear similarity to humans regarding brain areas involved in social behaviour (involvement of the PFC, thalamus, hippocampus, amygdala, hypothalamus and periaqueductal grey; Sandi & Haller, 2015). This combined with the convenience of laboratory mice has resulted in this species being commonly used to study the underpinnings of social behaviour and the effects of experimental manipulation on social behaviour. Thus, behavioural paradigms have been developed to assess different components of social behaviour in mouse models. The olfactory habituation / dishabituation test is used to assess social olfaction and the three-chamber test is used to measure both sociability (social motivation) and social memory. Tests of free social interaction assess the overall quality of interactions with conspecifics. The resident-intruder paradigm measures aggressive behaviour and tests based on the reaction to distressed conspecifics are used to assess empathic behaviour. Social competition tests are used to investigate the formation of social hierarchies and other social dynamics.

Like anxiety- and depression-like behaviour, impaired social behaviour has been linked to stress exposure, as many stress-related neuropsychiatric disorders present social impairments and stress-induced changes (both excess and deficits) in glucocorticoid

levels have been proposed to underlie social impairment (Sandi & Haller, 2015). Indeed, brain areas that are sensitive to stress such as the amygdala, PFC, hippocampus, and the mesolimbic pathway, are generally abnormal in individuals that exhibit impaired social behaviour (Sandi & Haller, 2015). Thus, although they are scarce in comparison to other behaviours, a number of studies have investigated how stress in adulthood affects social behaviour. In sum, acute stress in adulthood has been reported to result in social withdrawal and increased aggression in male mice (de Almeida, 2002; Takahashi et al., 2012), while chronic stress during adulthood results in reduced social motivation and interaction, and increased aggression in adult male rats (van der Kooij, Fantin, Kraev, et al., 2014; van der Kooij, Fantin, Rejmak, et al., 2014; Wood et al., 2003). Additionally, social isolation stress during adolescence has also been shown to decrease social exploration in male rats (Van den Berg, 1999) and maternal separation stress in early life has been reported to impair social recognition in male mice (Franklin et al., 2011). Such changes in social behaviour are often accompanied by changes in anxiety- and depression-like behaviours. For example, chronic stress has been shown to especially result in reduced social motivation and interaction in anxious animals (Castro et al., 2012).

Despite these reports, the effects of stress on the brain structures that underlie social behaviour have only recently begun to be directly investigated (Sandi & Haller, 2015). The dorsal raphe nucleus (DRN), lateral habenula (LHb), VTA and NAc (along with structures from which the NAc receives input such as the cortex, hypothalamus, and amygdala), regulate the processing of natural rewards such as social interaction. Congruently, deficits in the social reward system have been implicated in autism

spectrum disorder (Chen & Hong, 2018) and abnormalities in regions that provide input to the NAc including reductions in dopamine neurotransmission have been found in patients with depression. Specifically, this has been proposed to be linked to anhedonia, loss of appetite, and social withdrawal (Andersen & Teicher, 2008). Furthermore, areas involved in the perception of biological motion as well as those necessary for the sensory recognition of conspecifics such as the medial amygdala are essential to the perception and processing of incoming social stimuli (Chen & Hong, 2018) and therefore have a major influence on social behavior (Chen & Hong, 2018). These areas all undergo significant development during adolescence to allow for the programming of future social behaviour. These changes are evident in humans, as areas associated with face processing in the PFC show an increase in activity between childhood and adolescence, which reflects the excess production of untuned (less efficient) synapses, followed by a decrease in activity between adolescence and adulthood, which reflects the synaptic pruning that happens during adolescence that finetunes function and renders it more efficient (Blakemore, 2008). Accordingly, face recognition performance steadily increases from childhood until adolescence and decreases from adolescence until adulthood (Blakemore, 2008). It has been proposed that this pruning and finetuning of synapses is carried out in an input dependent manner (Blakemore, 2008). If this development is dependent on environmental influence, it provides an opportunity for stress to leave long-lasting or even permanent effects on these areas and thus deficient social behaviour.

Appropriate social behaviour requires not only normal development of the relevant brain areas but also successful social learning (Sandi & Haller, 2015), much of which happens during adolescence (Blakemore, 2008; Tottenham & Galván, 2016). The

increased neural plasticity during adolescence provides an opportunity for learning about the environment in order to better prepare for the future (Andersen & Teicher, 2008; Brust et al., 2015; McCormick et al., 2010; Tottenham & Galván, 2016). While this time of increased plasticity allows for useful learning of the social environment, it also provides a window of vulnerability to stress. Further, the HPA axis is particularly sensitive to social stress (Tottenham & Galván, 2016) which may render social behaviour significantly affected during this time. Indeed, as mentioned above, stress induced changes in glucocorticoid levels are thought to underlie changes in social behaviour. Moreover, direct exposure to glucocorticoids leads to effects on social behaviour that are similar to the effects of stress exposure (Sandi & Haller, 2015).

It has been proposed that an individual's behavioural response to stimuli is affected by internal states, which are a result of the combination of factors unique to the individual that modulate the behavioural output based on sensorimotor information (Anderson, 2016; Chen & Hong, 2018). Unique internal states therefore result in different individuals responding differently to the same sensory cues. Chen and Hong (2018) state that past experience is essential to this internal state. The integration of experiences into the internal state of the individual likely happens during periods of learning such as adolescence, a time of extensive social learning. Furthermore, internal states are heavily impacted by neurotransmitter function, sex hormones, and hypothalamic projections to the amygdala (Anderson, 2016; Canteras et al., 1994) and thalamic projections to amygdala and PFC, all of which undergo substantial development during adolescence. Consequently, external influences such as stress during times of learning and

development can heavily impact the internal state and thus determine behavioural output in response to incoming social information from the environment later in life.

Consistent with the idea that adolescent stress affects social behaviour differently than stress in other time periods, a review by Sandi & Haller (2015) identified 4 distinct social behavioural profiles emerging from stress at different timepoints. Prenatal stress led to general asociality, including reduced social motivation, interaction, and even aggression. Maternal separation during the neonatal period led to general behavioural withdrawal, including decreased social motivation and interaction, but also led to increased aggression. Acute stress in adulthood led to a transient increase in agonistic behaviour (i.e., behaviours related to fighting). On the other hand, stress during the juvenile period by either early subjugation, peripubertal stressors, or post weaning social isolation, led to antisociality (aggressive behaviours outside of species-typical norms), which is not present in any other behaviour profile. Consequently, the effects of adolescence-restricted stress directly on social behaviour should be characterized, as it can be different to the effects of stress at any other timepoint.

1.4 The Present Study

Prolonged exposure to stress, particularly during sensitive periods of development, can lead to adverse effects on health. Long-term structural and functional neurological changes resulting from stress exposure can lead to behavioural and cognitive impairments as well as predisposition to psychopathology. While the prenatal and neonatal periods of life have been extensively investigated as periods of sensitivity to stress, adolescence has not been studied as thoroughly in this context despite the

significant developmental changes that come with it. Therefore, one of the main objectives of this study is to determine the effects of stress during adolescence on adult behaviour. Furthermore, there is evidence indicating that reactivity to stress varies among different periods within adolescence and that maturational changes take place throughout adolescence that affect behaviour. This suggests that stress exposure during different periods within adolescence can lead to distinct long-term effects. Therefore, this study aims to determine whether early- and late-adolescent stress cause different effects on behaviour. While changes in anxiety- and depression-like behaviour in response to stress have been studied in depth, social behaviour has not been explored to the same extent in this context. This study therefore aims to assess the effects of adolescent stress not only on anxiety- and depression- like but also social behavior, including exploration of social odours and same-sex conspecifics, and parental behaviour provided by dams. Lastly, stress known to affect males and females differently and the maturational events that take place during adolescence differ between the sexes. However, much of the existing research on the effects of stress has only included male subjects. Thus, this study aims to investigate sex differences in the effects of adolescent stress. The results of this study will provide insight into the overlap between stress, behaviour, sex, and adolescent neural sensitivity, all of which have been of interest in the neuroscience literature, but which have scarcely been studied together.

Chapter 2: Methods

2.1 Animals

2.1.1 Mice

The subjects of this study were 42 male and 46 female C57/Bl6j mice bred in-house from 10 male and 20 female mice of the same strain obtained from Jackson Laboratories (Bar Harbor, ME). Breeding success ranged from 50% to 75% (50% to 75% successful breedings out of all breedings attempted) throughout the 4 rounds of breeding that were needed to produce all 4 cohorts of mice used. An average of 6.33 pups were obtained per breeding pair. All mice were weaned on post-natal day (p) 21 and housed in same-sex pairs or groups of 3 in standard clear plastic cages (18.75 x 28 x 12.5 cm) lined with woodchip bedding and covered with a wire-mesh cage top and a filtertop. Mice were kept in a colony room under a reversed 12:12 h light: dark cycle with lights off from 9:30 – 21:30. The colony room was maintained at a temperature of approximately 22ºC and 22% humidity. All cages contained filtered water and food ad libitum and 1 or 2 small black PVC tubes for environmental enrichment. All procedures were performed in accordance with the Canadian Council on Animal Care guidelines and were approved by the Dalhousie University Committee on Laboratory Animals (Protocol #19-017).

2.1.2 Groups

Mice were group-housed in same-sex cages post-weaning and each cage was randomly assigned to one of four groups. Out of the total 88 subjects, 19 were assigned to the early stress (ES) group, 19 to the early control (EC) group, 24 to the late stress (LS) group and 26 to the late control (LC) group (Figure 2). The number of litters (number of

successful breedings) contributing to each group is shown in Table 1. Subjects in the early stress group underwent the stress procedure during pre-pubescent (early) adolescence from p22 to p34 and subjects in the late stress group underwent the procedure during sexually mature (late) adolescence from p48 to p60. Mice in the early group underwent behavioural testing from p70 to p100 and mice in the late group were tested from p96 to p126 in order to test mice 36 days after stress in both the early and late stress groups.

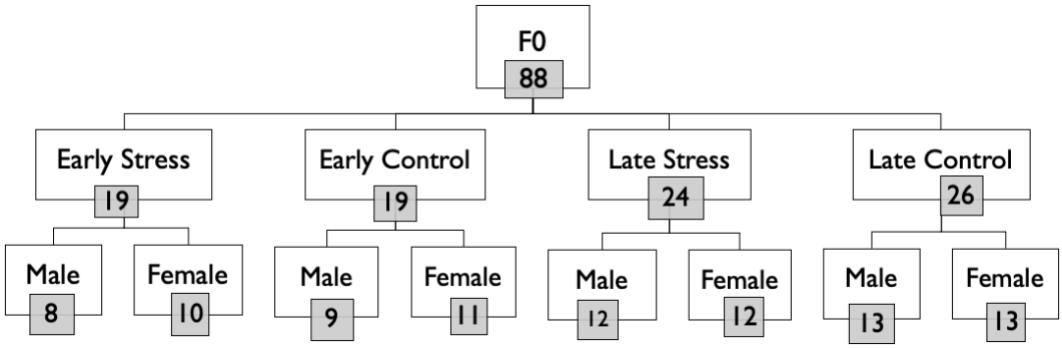

Figure 2. Number of subjects per age, stress treatment and sex.

Table 1.
Number of litters contributing to each experimental group per sex. ECF=Early control females, ECM=Early control males, ESF=Early stress females, ESM=Early stress males, LCF= Late control females, LCM=Late control males, LSF=Late stress females, LSM=Late stress males.

Group	ECF	ECM	ESF	ESM	LCF	LCM	LSF	LSM
Litters	4	4	6	4	7	7	7	8

2.1.3 Rats

Eight male Long Evans retired breeder rats obtained from Charles River Laboratories were used in this study. The rats were housed individually in a separate colony room under the same light cycle previously mentioned for the mice in clear plastic cages (45.75 x 25.5 x 20.25 cm) lined with woodchip bedding and covered with a wire-

mesh cage top and a filtertop. All cages contained filtered water and food ad libitum and a large black polymer tube for enrichment.

2.2 Predator stress

2.2.1 Background

The stress paradigm used in this study follows a procedure adapted from the predator stress procedure published by Barnum et al. (2012). Barnum and colleagues (2012) adapted the procedure described by (Yang et al., 2004), which used an apparatus consisting of a home chamber connected by a tunnel to an exposure cage (Figure 3). The mice were able to escape to the home enclosure, or explore the exposure cage, in which a rat was introduced and separated from the rest of the apparatus by a wire mesh barrier (Yang et al., 2004). Barnum et al. (2012) adapted this procedure to maximize prey-predator interaction by introducing the mice into a clear plastic hamster ball, which was then introduced to a rat cage (Figure 4). This allowed not only visual, olfactory, and auditory predator exposure, but also allowed the rat to physically manipulate the hamster ball without incurring harm to the mice. Using this setup, Barnum et al (2012) produced long-term behavioural effects including increased anxiety and depressive behaviour and physiological effects including changes in inflammatory response to immune challenge and increased plasma corticosterone (Barnum et al., 2012). The procedure used in this study is most similar to the latter study and consists of introducing the mice into a small enclosure, which is then placed inside a large cage with a rat (Figure 5). However instead of a hamster ball, this study utilized a small clear 17.75 x 17.75 x 14 cm Plexiglas box which allowed the same level of predator-prey contact, but improved protection for the mice due to the stronger material.

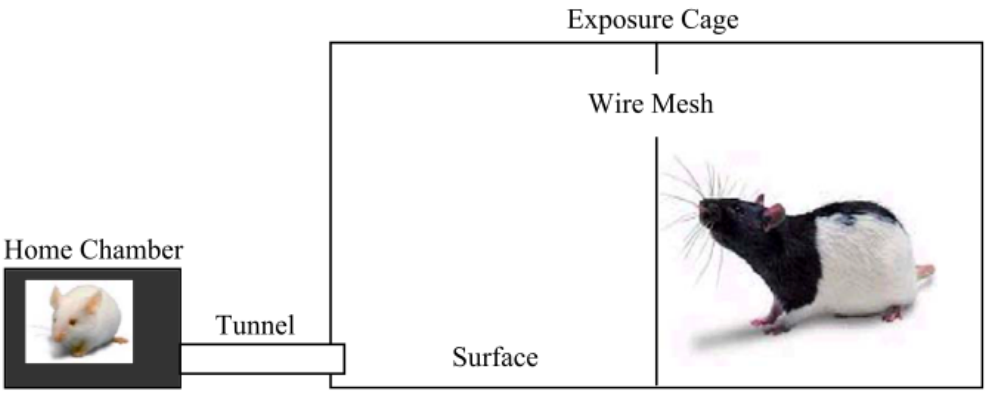

Figure 3. Predator stress set-up from Yang et al. (2004).

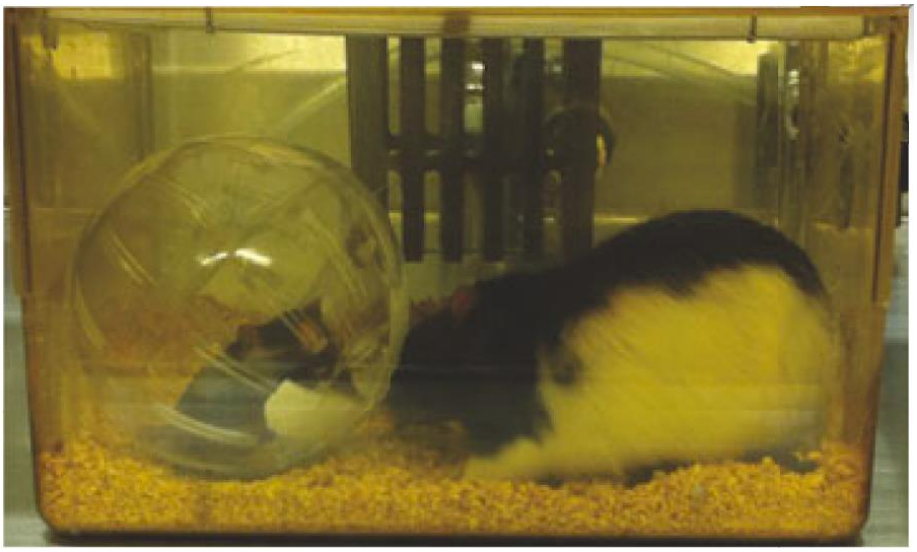

Figure 4. Predator stress set-up from Barnum et al., (2012).

2.2.2 Stimuli

Four male Long Evans rats were used as stimuli for this stress procedure. Environmental enrichment tubes and food were removed from the rat cages 30 minutes prior to being used as stimuli for the stress procedure to increase the rat's aggressiveness. They were transported from the colony room to the testing room in their home cages. The rat cages were covered by a dark cloth while being transported to the testing room to minimize disturbance to the rats.

2.2.3 Procedure

The stress procedure was performed in a dark room illuminated only by red light. At the time of testing, subjects were placed inside a 17.75 x 17.75 x 14 cm clear Plexiglas box, which was subsequently placed inside the stimulus rat's home cage for 30 minutes. After the 30 minutes of stress, the Plexiglas boxes containing the mice were removed from the rat cages. The mice were then removed from the box and placed back into their testing cages, while the number of fecal boli in each box was counted as an indicator of stress levels. The number of fecal boli was recorded by the experimenters and the mice were returned to the colony room and returned to their home cages. As 4 rats were used per cohort of mice in this study, 4 mice underwent the stress procedure at once. The plexiglass boxes were cleaned with water and Sparkleen (Fisherbrand, Pittsburgh, PA, USA) between uses. After all mice had been tested for the day, the rats were transported back to the colony room, and water, food and enrichment were returned to the cages.

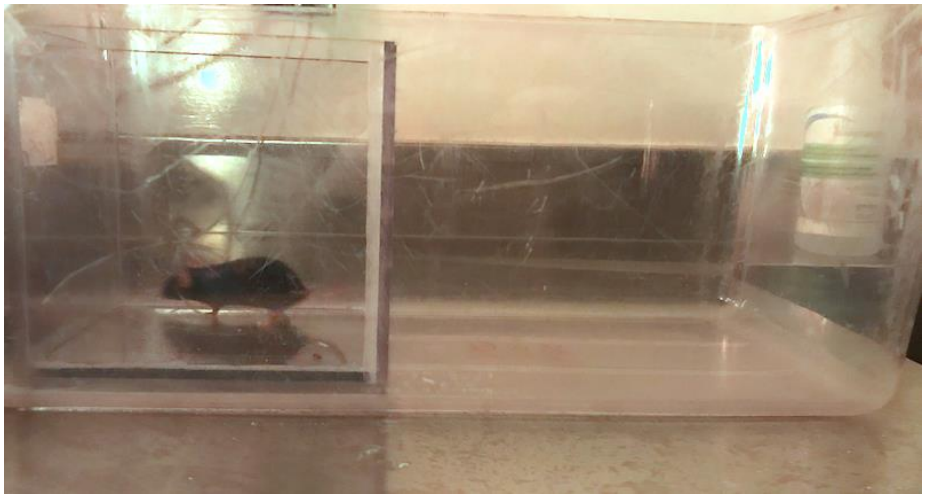

Figure 5. Predator stress set-up for this study.

2.2.4 Stress-related measures

As defecation has been reported to indicate the emotional response of mice to aversive stimuli and has been associated with anxiety-like behaviour (Carola et al., 2002; Lister, 1990), the number of fecal pellets produced during each predator stress session was recorded for each mouse throughout the stress period. Additionally, given that water and food consumption have been reported to be affected by stress (Dalooei et al., 2016; Stone & Quartermain, 1997), water and food consumption were measured for both stress and control mice daily from 5 days before the start of the stress period to 5 days after the end of the stress period (for a total of 22 days) to investigate the acute and chronic effects of stress on food and water consumption. This was done by weighing food pellets and water bottles in each cage and dividing each day's weight change from the previous day by the number of mice in the cage, to obtain an approximation of individual consumption. Because mice in the early group were not weaned until the day before stress started, their consumption prior to this day could not be separated from the dam's. Moreover, the mice were not separated into groups until weaning. Therefore, measurement of food and water consumption started on the first day of stress for ES and EC mice.

Fecal samples were collected from each mouse on the day prior to the beginning of the stress period as well as days 1, 6 and 12 of stress for analysis of fecal glucocorticoid levels as an indicator of stress levels. As glucocorticoids take approximately 4h-10h to be excreted in rodent feces (Touma et al., 2004), samples were collected approximately 6h after the stress procedure, as this would be reflective of the levels of circulating glucocorticoids at the time of stress. Samples were collected individually by placing each mouse on an empty cage covered by a wire-mesh top. As

they were produced by the mouse, the fecal pellets were allowed to fall through the wires of the wire top into the empty cage below. Fecal pellets were then collected into 1.5ml microcentrifuge tubes (Fisherbrand, Pittsburgh, PA) and stored in a freezer at -80 °C until the time of analysis. Prior to extraction, samples were shaken and dried in an oven at 60°C for 3 hours. Samples were then pulverized with a pestle and 2mg of sample were transferred to clean microcentrifuge tubes. After this, 200 µl of 80% HPLC-grade methanol were added to each microcentrifuge tube and samples were shaken at high speed for 30 minutes, and then centrifuged at 2500 x g for 20 minutes. Then, 10 µl of supernatant were transferred to clean microcentrifuge tubes and used for analysis. Samples were analyzed in duplicate for glucocorticoid content using a Cayman corticosterone ELISA kit (Cayman Chemical, Michigan, USA), according to the manufacturer's instructions. This essay detects corticosterone as a main target, as well as related molecules such as 11-deoxycorticosterone, prednisolone, 11-dehydrocorticostone, cortisol, progesterone, aldosterone, 17α-hydroxyprogesterone, 11-deoxycortisol, androstenedione, testosterone, and pregnenolone, to a lesser extent. Only a subset of the samples collected were used in an exploratory analysis of the glucocorticoid levels of the stressed and control mice. Data points with a Coefficient of variability (CV) over 50% were excluded from analysis.

2.3 Behavioural Testing

2.3.1 Overview

All mice underwent a behavioural test battery in order to assess their social, anxiety-like and depression-like behaviour starting at 36 days after the last day of the

stress procedure. The mice were given 4 days to rest between each test and the tests were performed in order of aversiveness from least aversive to most aversive, in the order in which they are described below. All testing was performed during the dark phase of the light: dark cycle and, except for the light/dark and sucrose preference tests, all tests were performed only under red light from a lamp placed above the testing area and were recorded using the 3.0 Biobserve Viewer Software (Biobserve GmbH, Bonn, Germany) for later analysis. All videos were scored manually by two different scorers that were blind to treatment. Due to the length and scale of this project, four cohorts of mice were bred, stressed, and tested, and each measure collected was scored by different pairs of scorers for each cohort. Therefore, no measure of inter-scorer reliability was calculated. However, inter-scorer differences were calculated for each measure. For scores that were more than one standard deviation from the mean difference, the video was re-scored by a third scorer and the two closest scores were averaged to produce the final score to be used for analysis.

2.3.2 Olfactory Habituation and Dishabituation

2.3.2.1 Background

This test was used to assess social olfaction and was performed as described by Yang and Crawley (2010). In the olfactory habituation / dishabituation paradigm a series of odours are presented sequentially, and the time spent investigating each odour is measured. Each odour is presented three times in order to assess the progression of investigation time across presentations of the same odour, as well as the change in investigation time between different odours. Habituation is defined as the progressive decline in investigation time across the 3 presentations of the same odour. Dishabituation

is defined as the increase in investigation time from the last presentation of an odour to the first presentation of the next odour. Normal habituation and dishabituation indicate that the abilities to detect and remember odours and to discriminate between different odours are present. Additionally, based on the natural preference of mice for social over non-social stimuli, the difference in investigation time between these different types of stimuli can be used to assess social motivation. A marked increase in general investigation time of social odour compared to non-social odours, as well as an increased dishabituation in response to the first presentation of a social odour compared to the first presentation of a non-social odour, indicate normal social preference.

2.3.2.2 Stimuli

The stimuli used for this test were plain water (control), almond odour (non-social odour 1), banana odour (non-social odour 2) , and the odour of the soiled cage of a group of unfamiliar sex-matched mice (social odour). All odours were prepared at the beginning of each testing day, while the first mouse was habituating. For the non-social odours, 1:100 dilutions were prepared in 15 mL conical centrifuge tubes from imitation almond and banana extracts (La Cie McCormick Canada) and distilled water. Non-Social odour cues were prepared a maximum of 20 minutes before use by immersing 15 cm wood-handled cotton swabs (Puritan Medical Products Company, Guildford, MN, USA) into the centrifuge tube containing the dilution and were stored in small airtight plastic bags until use. Social odour cues were prepared by directly swiping the swabs across the base of the soiled home-cages and were also stored in a small plastic bag prior to use. The mice used to provide the social odours were not used as stimuli in any other test.

2.3.2.3 Procedure

This test was performed on p70 for the early stress and control groups and p96 for the late stress and control group. Only one mouse was tested at a time. Prior to testing, mice were placed in a clean plastic cage lined with wood-chip bedding with a dry cotton swab for 30 minutes to habituate to the testing cage and to the presence of the swab. After the habituation period, the dry swab was removed, and the mouse was transported to the testing room in the same cage. The mice were presented with 4 different odours, for 3 trials each, as follows: 3 trials of no odour (distilled water control), 3 trials of almond scent solution, 3 trials of banana scent solution, and 3 trials of the social odour. All odour cues were presented by inserting the corresponding cotton swab in between the metal wires of the wire-mesh cage tops and held in place by a binder clip with the cotton tip at approximately 5 cm from the base of the cage (Figure 6). Each odour was presented for 2 minutes, during which the experimenter left the room and prepared a new cage-top with the next odour. Experimenters ensured that the swab was introduced to the wire top from the wooden end to prevent odour contamination on the cage tops. After the presentation of all 12 odour cues, the mice were returned to the colony room and to their home cage.

Figure 6. Setup of the olfactory habituation / dishabituation test.

2.3.2.4 Analysis

Videos of each 2-minute trial were scored for time spent sniffing the swab. Sniffing was defined as the mouse's nose orienting towards the cotton swab at a maximum distance of 2 cm from the tip. Habituation was calculated by subtracting the sniffing time corresponding to the last presentation of an odour cue from the sniffing time corresponding to the first presentation of the same odour. Dishabituation was calculated by subtracting the sniffing time corresponding to the first presentation of an odour cue from the last presentation of the previous odour. Habituation and dishabituation were analyzed using a repeated measures Analysis of Variance (ANOVA) with odour as the within-subjects factor and sex and stress treatment as between-subjects factors with Jamovi version 1.6 (Sydney, Australia). Outliers more than two standard deviations from the mean for each group were excluded from analysis.

2.3.3 Open Field Test

2.3.3.1 Background

This test was used to assess anxiety-like behaviour. The open field test is based on the natural tendency of mice to avoid exposed and unprotected areas (Carola et al., 2002). The amount of time spent in the unprotected area (the centre of the open field) compared to more protected areas (the corners of the open field) is used as an indicator of anxiety level. The overall level of activity and locomotion can also be measured using this test and are generally used as contextual information for the interpretation of the anxiety results obtained. The open field also elicits behaviours indicative of anxiety, such as increased grooming, and of exploration, such as rearing, which can be concurrently observed as additional measures.

2.3.3.2 Procedure

The test was performed on day p75 for the early stress and control groups and p101 for the late stress and control groups. Prior to testing, each mouse was transferred to the testing room in a clean cage as described above. The open field was a square apparatus (71 x 71 x 39 cm) made from grey expanded PVC (Figure 7). Each mouse was placed in the centre of the arena and allowed to explore freely for 30 minutes. The arena was cleaned with water and Sparkleen (Fisherbrand, Pittsburgh, PA, USA) between test subjects. After each trial, the mouse was returned to the colony room and to its home cage.

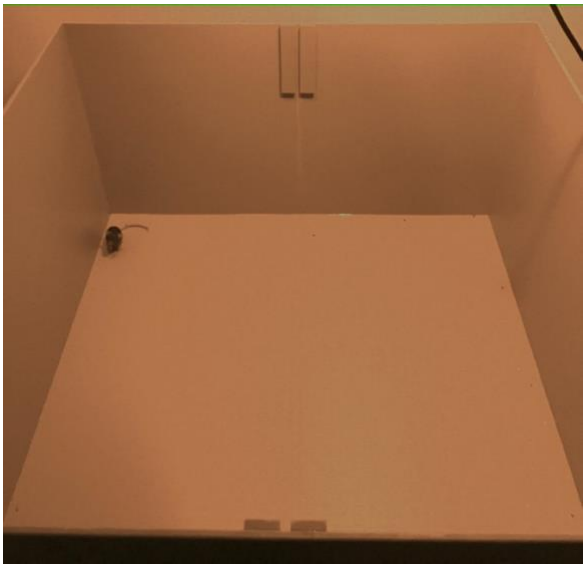

Figure 7. Setup of the open field test.

2.3.3.3 Analysis

All trials were recorded, and the movement of the mice was tracked with the Biobserve Viewer Software (Biobserve GmbH, Bonn, Germany). The number of fecal boli produced during the test was recorded by the experimenter as an indicator of stress levels after each trial before the arena was cleaned and the next trial started. The number of rears and duration of grooming were scored from video. The time spent in the centre in the arena and total distance travelled by each mouse were obtained from the tracking software. All measures were analyzed using a three-way ANOVA with age, sex, and stress treatment as factors with Jamovi version 1.6 (Sydney, Australia). Outliers more than two standard deviations from the mean for each group were excluded from analysis. Overall, only a small number of mice produced fecal boli. Thus, the number of fecal boli was not analyzed for this test.

2.3.4 Three-Chamber Sociability Test

2.3.4.1 Background

This test was used to assess sociability (preference for a social stimulus over a non-social stimulus; Nadler et al., 2004). In the protocol described by Nadler and colleagues (2004), the subject is placed in the centre of the apparatus, a rectangular arena with dividers creating 3 separate chambers and openings in the dividers to allow movement between chambers. The mouse is then presented with a wire cage containing an unknown conspecific (social stimulus) and an empty wire cage on opposite sides of the apparatus, and allowed to explore freely, having the choice to explore either object presented. In this study, the wire cage opposite to the social stimulus contained a novel object (non-social stimulus), instead of the cage being empty. Instead of a partitioned apparatus, the test was performed in clear plastic cage (45.75 x 25.5 x 20.25 cm) with no dividers. This modified apparatus forced the subject to choose one side of the arena and prevented the mouse from staying in the middle chamber and avoiding any interaction with the stimuli. Based on the natural tendency of the mouse to prefer a social stimulus over a non-social one, increased time spent investigating the conspecific indicates normal social behaviour. On the other hand, a lack of preference for the social stimuli indicates abnormally low sociability.

2.3.4.2 Procedure

This test was performed on day p80 for the early stress and control groups and p106 for the late stress and control groups. Prior to testing, each mouse was transferred from its home cage to clean cage and transferred to the testing room as described above.

The arena contained 2 Galaxy Pencil & Utility Cups (Galaxy Cup; Spectrum Diversified Designs Inc., Streetsboro, OH, USA), one on each side approximately 3cm from the walls, containing the stimuli. A 500 mL HDPE bottle (Nalge Nunc International Corporation, Rochester, NY, USA) filled with water was placed on top of each Galaxy cup to prevent the mice from climbing on top of the cup. Each mouse was placed in the centre of the arena with empty galaxy cups for 10 minutes to habituate to the arena (Figure 8). The mice were then removed from the arena and returned to the clean cage for approximately 2 minutes while the stimuli were placed under the galaxy cups. A plastic toy was placed under one of the cups and used as a novel object while an unfamiliar, sex-matched, stimulus mouse was placed under the other cup and used as social stimulus (Figure 9). The placement of the stimuli was counterbalanced across trials. After the stimuli were placed under the cups, the mouse was reintroduced to the centre of the arena and allowed to explore freely for another 10 minutes. The arena was cleaned with water and Sparkleen (Fisherbrand, Pittsburgh, PA, USA) between test subjects. Both the habituation and testing periods were recorded on video.

Figure 8. Setup for the Three-Chamber habituation trial.

Figure 9. Setup for the Three-Chamber testing trial.

2.3.4.3 Analysis

The time spent investigating each stimulus was scored from video. The mice were considered to be investigating the stimuli if their nose was oriented toward the Galaxy cup or if they were in direct contact with it. Social preference ratios were calculated by dividing the time spent investigating the social stimulus by the total time spent investigating both stimuli each trial. The time spent investigating each empty cup during the habituation trial were also calculated to indicate any difference in baseline exploration. A repeated measures ANOVA with stimulus as the within-subjects factor and age, sex and stress treatment as between-subjects factors was used to analyze the times spent investigating each cup in the habituation phase and testing phase and a three-way ANOVA with age, sex and stress treatment as factors was used to analyze the social preference ratio in the testing phase with Jamovi version 1.6 (Sydney, Australia). Outliers more than two standard deviations from the mean for each group were excluded from analysis.

2.3.5 Light / Dark Box

2.3.5.1 Background

This test was used to assess anxiety-like behavior. The light/dark test is based on the natural avoidance in mice of bright open spaces (Bourin & Hascoët, 2003). The mouse is placed in the apparatus and given the opportunity to explore either a brightly lit area or a dark covered compartment. Decreased time spent in the light area in proportion to the total time spent in the apparatus indicated increased anxiety and vice versa. A decrease in transitions between the two areas also indicates increased anxiety. Similarly to the open field test, this test elicits behaviours such as rearing and grooming that can be used to interpret the main findings.

2.3.5.2 Procedure

This test was performed on day p85 for the early stress and control groups and p111 for the late stress and control groups. The open field arena was transformed by placing a grey expanded PVC divider in the center of the arena to form 2 identical compartments. A removable box was placed in each of the 2 compartments to form the dark area of the apparatus with a small opening to allow the mice entry into the area (Figure 10). This apparatus allowed 2 mice to be tested at a time. Prior to testing the mice were transported to the testing room in a clean cage as described above. The test was performed in dim white light and was recorded on video. One mouse was introduced to the light area on each of the 2 compartments of the arena and allowed to explore freely for 10 minutes. After the 10 minutes, both mice were removed from the arena and returned to the colony room in their respective clean cages and subsequently returned to their home cages. The number of fecal boli in each compartment was recorded by the

experimenter and the arena was cleaned before the next mice were introduced with water and Sparkleen (Fisherbrand, Pittsburgh, PA, USA).

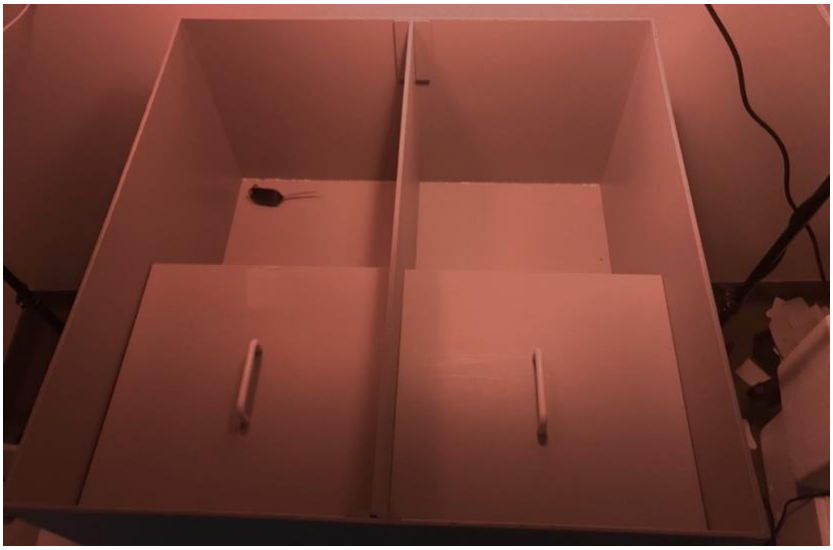

Figure 10. Light Dark box set-up.

2.3.5.3 Analysis

Time spent in the light area of the box, the frequency of entries into the light area, and the number of rears in the light area were scored from video. Time in the dark area of the box was not scored because it is dependent on the amount of time spent in the light area and time in the light was sufficient to assess anxiety-like behaviour. A three-way ANOVA with age, sex and stress treatment as factors was used with Jamovi version 1.6 (Sydney, Australia) to analyze all measures. Outliers more than two standard deviations from the mean for each group were excluded from analysis. Overall, only a small number of mice produced fecal boli. Thus, the number of fecal boli was not analyzed for this test.

2.3.6 Elevated Plus Maze

2.3.6.1 Background

This test was used to assess anxiety-like behavior. Like the open field and light/dark tests, the elevated plus maze is based on rodents' aversion to open spaces. The mice are placed in the centre of a plus-shaped maze and given the opportunity to explore 4 arms, 2 of which are only platforms with no walls covering them, and 2 of which have walls and are closed (Pellow et al., 1985). Decreased entries into and time spent in the open arms of the maze relative to the total time spent in the apparatus indicate increased anxiety (Pellow et al., 1985). Additionally, increased exploratory/risk assessment behaviour such as, stretch attends, and head dips indicate reduced anxiety.

2.3.6.2 Procedure

This test was performed on day p90 for the early stress and control groups and p116 for the late stress and control groups. The apparatus was made of 4 arms made from grey expanded PVC and was placed on a platform (83 cm tall). The 4 arms (33 in long each) formed the shape of a plus sign and 2 opposing arms were closed with walls (19 cm tall) made from the same material while the other 2 opposing arms remained open (Figure 11). This test was performed under red light and all trials were recorded on video. Each mouse was placed into the centre of the apparatus and allowed to explore freely for 5 minutes before being returned to the colony room in the clean cage and returned to its home cage. The arena was cleaned with water and Sparkleen (Fisherbrand, Pittsburgh, PA, USA) between test subjects.

2.3.6.3 Analysis

After the mice were removed from the apparatus, the number of fecal boli was recorded by the experimenter and the arena was cleaned before the next mouse was introduced. The time spent in the open and closed arms, number of rears, number of head dips and number of stretch attends were scored from video. A ratio of time spent in the open arms relative to the time spent in the closed arms was calculated by dividing the time spent in the open arms by the time spent in the closed arms of the maze for each mouse. Given that rearing was only displayed in the closed arms of the maze, the percent frequency of rearing in the closed arms of the maze was calculated for each mouse by dividing the number of rears by the amount of time spent in the closed arms. The data were analyzed with Jamovi version 1.6 (Sydney, Australia) using a three-way ANOVA with age, sex, and stress treatment as factors. Outliers more than two standard deviations from the mean for each group were excluded from analysis. Overall, only a small number of mice produced fecal boli. Thus, the number of fecal boli was not analyzed for this test.

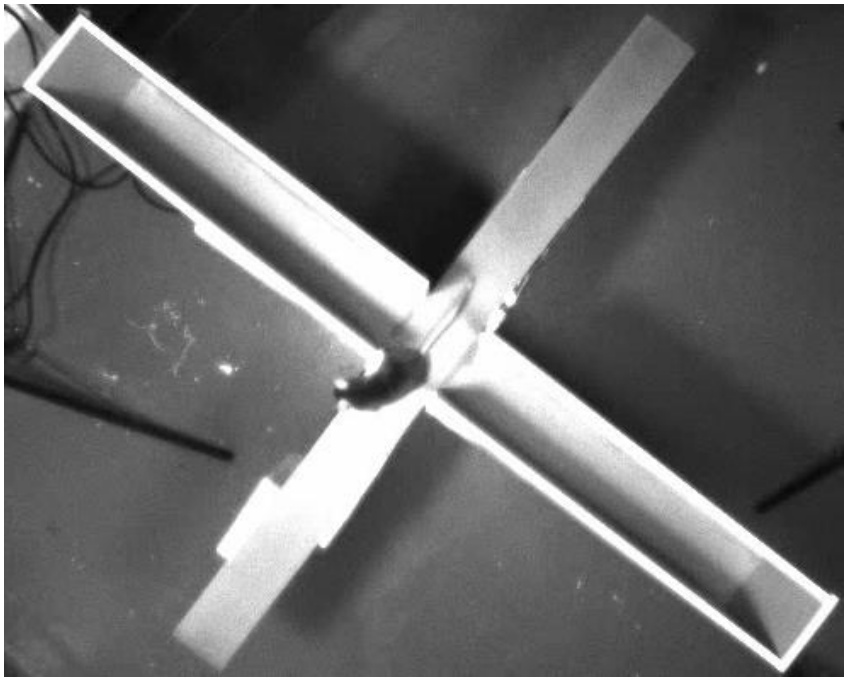

Figure 11. Elevated Plus Maze set-up.

2.3.7 Forced Swim Test

2.3.7.1 Background

This test was used to assess depression-like behavior. The forced swim test is commonly used to assess the efficacy of drugs and treatments targeting depression (Can et al., 2011). The subject is placed in an inescapable container with room temperature water for a short period of time and the time spent actively swimming or attempting to escape (mobile) is recorded as well as the amount of time spent floating or immobile. An increased time spent immobile is interpreted as behavioural despair or helplessness (Can et al., 2011). Therefore, increased immobility time is considered as an increase in depressive behaviour and vice versa. The forced swim test is a popular tool in the study of depression as it a stressful test for the animals. Exposure to stress is thought to be a trigger for depression in humans and depression is thought to result from the inability to cope with stress. Furthermore, performance in this test that is indicative of depressive-like states is often accompanied by other depressive-like symptoms such as anhedonia and changes in food consumption and sleep (Yankelevitch-Yahav et al., 2015). However, some authors have advocated for a different interpretation of results from the forced swim test. Molendijk & de Kloet (2015) suggest that immobility in this test might be a measure of adaptation rather than depression, as it has been reported that rats that are more active in the test tend to sink more than those which remain immobile for longer periods of time (Molendijk & de Kloet, 2015). Additional indicators of depression-like

behaviour such as the sucrose preference test and assessment of the fur coat can help interpret results from the forced swim test.

2.3.7.2 Procedure

The test was performed on day p95 for the early stress and control groups and p121 for the late stress and control groups. Three to four mice were tested at once in individual beakers placed side by side. The animals being tested were identified by placing a piece of paper with the corresponding animal number below the respective beakers. Clear 500ml glass beakers were filled to the 300ml mark with 24°C tap water (Figure 12). Each mouse was slowly introduced into the beaker and allowed to float or swim for 5 minutes. The mice were then removed from the beaker, placed in a cage lined with paper towel and allowed to dry from approximately 5 minutes before being returned to their home cage. All beakers were drained and cleaned with water and Sparkleen (Fisherbrand, Pittsburgh, PA, USA) before being reused.

Figure 12. Forced Swim Test set-up.

2.3.7.3 Analysis

Each trial was recorded, and the amount of time spent floating was scored from video. The time spent floating was analyzed with Jamovi version 1.6 (Sydney, Australia) using a three-way ANOVA with age, sex, and stress treatment as factors. Outliers more than two standard deviations from the mean for each group were excluded from analysis.

2.3.8 Sucrose Preference

2.3.8.1 Background

This test was used to assess depression-like behavior (Liu et al., 2018). The sucrose preference test assesses the presence of anhedonia, the inability to experience pleasure, which is a major symptom of depression. This test consists of giving the subject access to both their regular drinking water and to a sucrose solution. The consumption of both the water and the sucrose solution are recorded and a ratio of preference for sucrose over water is calculated. Based on the natural rewarding quality of sucrose, a ratio indicating preference for sucrose is considered to be normal behaviour, while a decreased preference or no preference for sucrose is considered a sign of anhedonia indicative of a depressive-like state. The two-bottle choice paradigm has been considered a reliable measure of taste preference for decades in nutritional research and its application in the context of depression research has proved reliable as rodents have been shown to prefer sucrose over water in this test and a wide range of animal models of depression have shown decreased or absent sucrose preference.

2.3.8.2 Procedure

This test was performed on days p100 to 102 for the early stress and control groups and p126 to 128 for the late stress and control groups. Mice were single housed for 2 days and 2 water bottles were placed in the new home cage, one containing regular filtered tap water and the other containing 2% Sucrose solution made using the same water (Figure 13). The position of the bottles was counterbalanced across subjects. Both bottles were weighed before being introduced in the cage and after 48 hours of the test. After the 48h all mice were group-housed with their previous littermates and monitored to ensure that no injurious aggression occurred.

Figure 13. Two-bottle set-up for the sucrose preference test.

2.3.8.3 Analysis

Sucrose preference ratios for the 48h of testing were calculated by dividing sucrose consumption by the total sucrose and water consumption. The sucrose preference

ratios were analyzed with Jamovi version 1.6 (Sydney, Australia) using a three-way ANOVA with age, sex, and stress treatment as factors. Outliers more than two standard deviations from the mean for each group were excluded from analysis.

2.3.9 Maternal care and offspring body weight

After the conclusion of behavioural testing, group-matched males and females were bred and maternal care provided by dams was observed from days p0 to p7. Maternal care was observed by an experimenter and manually recorded twice a day, at random times of the dark portion of the light: dark cycle. Observation times were randomized to obtain a complete overview of maternal behaviours throughout the dark period of the light: dark cycle. Although observation times were random, all cages observed on a given day were observed at the same time for each observation session. Observations consisted of recording the activity of the dam every minute for 30 minutes. The behaviours scored were grouped into maternal and non-maternal behaviours. Maternal behaviour included nursing, a combination of nursing with licking and grooming of the pups, and nest building. Non-maternal behaviours included feeding or drinking water, self-grooming, and no contact with pups. Additionally, pups were weighed at p0, p7, p14, and p21 to assess the impact of maternal care on pup physical development.

Chapter 3: Results

3.1 Immediate response during the stress treatment

Fecal samples were collected before and at different timepoints of the stress period and their corticosterone content was analyzed using an ELISA. Because the means and standard deviations of the corticosterone content were similar between the males and females of each group (Table 2), the data is shown pooled across sex in Figure 14. Nonetheless, it is important to note that most data points that stood out as being higher than the rest corresponded to females. Overall, data for the day prior to stress (day 0) was slightly higher compared to the rest of the days for both the early and late group, although the difference in the late group seems to be driven by a single high data point in the early stress female group (Figure 15). Furthermore, the mean corticosterone content for the control groups are slightly higher than the stress groups for days 0 [control (M =48.44, SD =86.03), stress (M=37.51, SD = 24.99)] and 1 [control (M =30.86, SD = 22.33), stress (M =27.00, SD =16.17)], while the mean for the stress groups is slightly higher than the control groups for days 6 [control (M =22.60, SD = 22.94), stress (M = 36.34, SD = 33.48)] and 12 [control (M = 20.98, SD = 19.41), stress (M = 39.20, SD = 31.01); Figure 14].

Table 2.

Mean, standard deviation (SD) and number of data points (N) for the fecal corticosterone content (pg/mg) per group for the day prior to stress (0), the first day of stress (1), midway through the stress period (6), and the last day of stress (12).

Group		Day 0	Day 1	Day 6	Day 12
Early Control Female	Mean	34.71	14.00	4.48	5.29
	SD	18.53	7.27	0.59	2.68
	N	4	3	2	3
Early Stress Female	Mean	24.81	23.17	12.18	41.69
	SD	10.99	15.45	12.39	43.63
	N	4	4	5	4
Early Control Male	Mean	35.13	33.18	8.25	6.32
	SD	22.09	18.56	0.01	1.51
	N	4	4	2	4
Early Stress Male	Mean	38.52	14.26	20.84	13.53
	SD	13.78	4.12	14.83	
	N	3	2	2	1
Late Control Female	Mean	82.32	40.40	31.23	32.35
	SD	154.71	38.56	36.07	25.41
	N	6	4	5	5
Late Stress Female	Mean	33.67	20.90	55.30	37.69
	SD	26.50	1.31	45.71	13.95
	N	5	2	3	4
Late Control Male	Mean	29.43	31.65	26.98	30.78
	SD	15.11	6.86	5.09	13.27
	N	5	4	5	5
Late Stress Male	Mean	54.32	40.28	55.33	44.64
	SD	36.35	18.06	33.20	38.46
	N	4	4	5	4

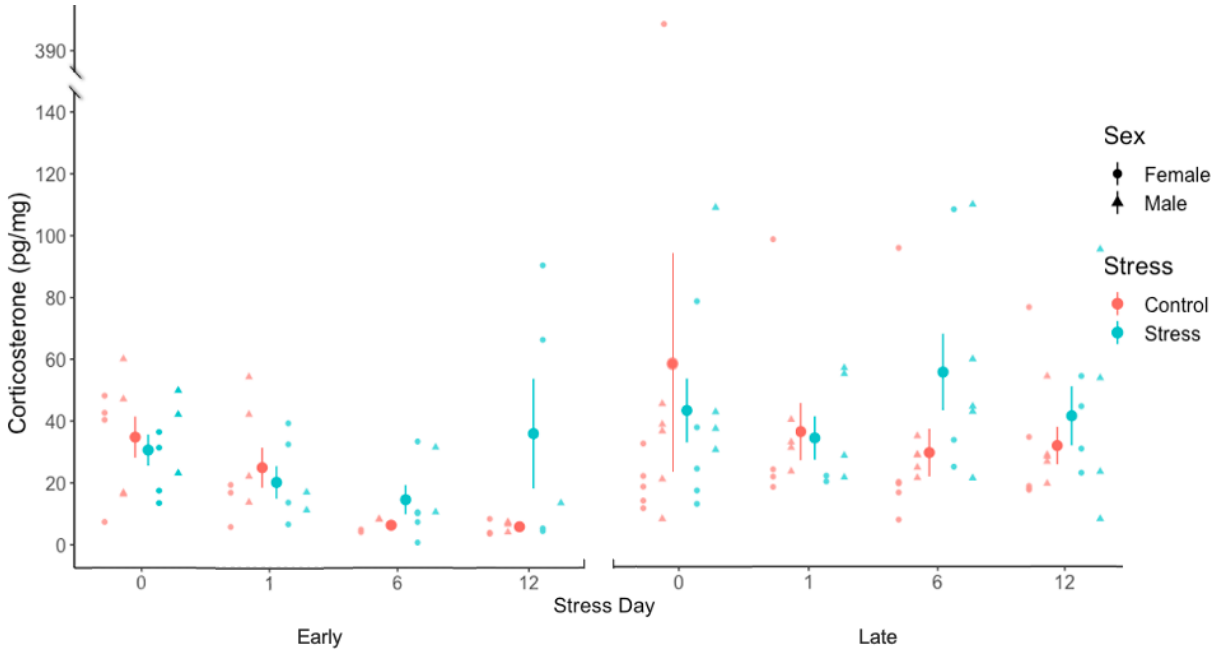

Figure 14. Fecal corticosterone content (pg/mg) collapsed across sex for the day prior to stress (0), the first day of stress (1), midway through the stress period (6), and the last day of stress (12). Large points indicate group means. Error bars indicate standard deviation. Small points indicate individual data points for each mouse included in the analysis. Individual data points are grouped by sex (females in the first column and males in the second column) and the shape of the points indicates sex.

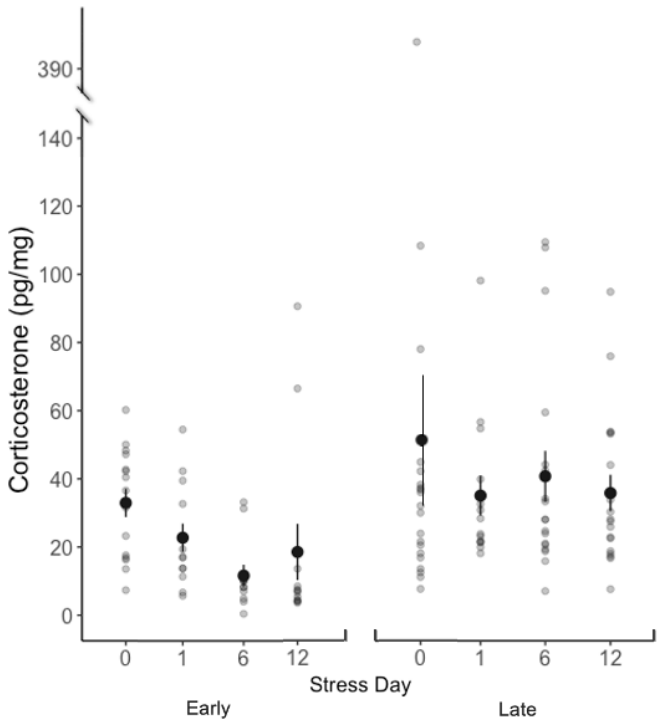

Figure 15. Fecal corticosterone content (pg/mg) for the day prior to stress (0), the first day of stress (1), midway through the stress period (6), and the last day of stress (12) per age group.). Large points indicate group means. Error bars indicate standard deviation. Small points indicate individual data points for each mouse included in the analysis.

The water (Table 3) and food (Table 4) consumption immediately before, during, and immediately after stress were analyzed using 95% bootstrapped confidence intervals. For the early group, stress animals [males (13.23±3.27g), females (12.06±4.34 g)] consumed a higher daily average of water compared to control animals [males (6.00±0.14 g), females (6.10±0.37 g)] during the stress period (Figure 16B). There were no differences in water consumption between stress conditions or males and females after the stress period (Figure 16C). For the late group, there were no differences in water consumption before (Figure 16A) or after (Figure 16C) the stress period. Similar to the early group, late stress animals [males (15.32±4.11 g), females (12.49±1.16 g)] consumed more water

compared to control animals [males (8.70±1.29 g), females (8.33±1.01 g)] during the stress period (Figure 16B). There were no differences in food consumption before, during or after the stress period for either the early or late group (Figure 17).

Table 3.

Bootstrapped 95% confidence intervals for the average water consumption per day (g) before, during, and after predator stress.

Intake (g)	ECF	ESF	ECM	ESM	LCF	LSF	LCM	LSM
Before Stress					[5.85, 7.96]	[4.41, 6.59]	[6.34, 8.11]	[6.24, 7.87]
During Stress	[5.57,6.44]	[8.21, 16.53]	[5.87, 6.15]	[10.60, 16.63]	[7.61, 9.02]	[11.58, 13.68]	[7.56, 9.70]	[10.99, 18.61]
After Stress	[5.94, 6.62]	[4.76, 6.62]	[6.57, 7.51]	[5.56, 8.01]	[6.45, 7.41]	[4.42, 6.51]	[6.19, 8.83]	[4.81, 6.91]

Table 4.

Bootstrapped 95% confidence intervals for the average food consumption per day (g) before, during, and after predator stress.

Intake (g)	ECF	ESF	ECM	ESM	LCF	LSF	LCM	LSM
Before Stress					[5.85, 7.96]	[4.41, 6.59]	[6.34, 8.11]	[6.24, 7.87]
During Stress	[5.57,6.44]	[8.21, 16.53]	[5.87, 6.15]	[10.60, 16.63]	[7.61, 9.02]	[11.58, 13.68]	[7.56, 9.70]	[10.99, 18.61]
After Stress	[5.94, 6.62]	[4.76, 6.62]	[6.57, 7.51]	[5.56, 8.01]	[6.45, 7.41]	[4.42, 6.51]	[6.19, 8.83]	[4.81, 6.91]

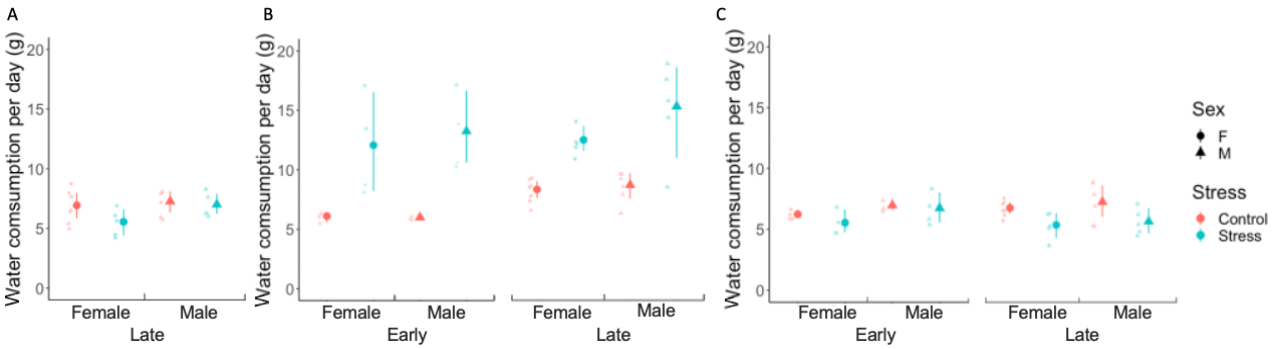

Figure 16. Average water consumption for each cage (normalized for the number of mice in each cage) per day before (A), during (B), and after (C) the stress period. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse cage included in the analysis.

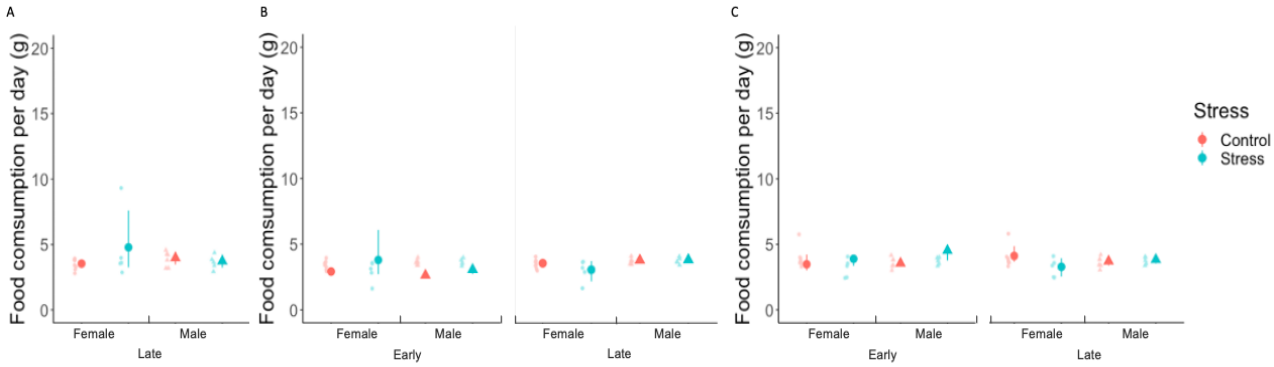

Figure 17. Average food consumption for each cage per day during (A) and after (B) the stress period for the early group and before (C), during (D), and after (e) the stress period for the late group. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse cage included in the analysis.

A repeated measures ANOVA with stress day as the within-subjects factor and age and sex as between-subjects factors was used to analyze the number of fecal boli produced during each predator stress exposure. Outliers beyond two standard deviations

from the group mean were excluded from analysis. There was a significant main effect of day ($F_{(11, 275)}$= 2.70, p=0.003, η^2p=0.097; Figure 18A), with more fecal pellets on day 6 (6.27±2.69) compared to day 10 (5.71±2.82) according to pairwise comparisons with Bonferroni correction ($t_{(25)}$=3.887, p=0.044), but no other differences between days. There was a significant day x sex interaction ($F_{(11, 275)}$=2.11, p=0.020, η^2p=0.078; Figure 18B), with females producing more fecal pellets on day 7 (7.77±2.88) than on day 10 (4.95±2.33) according to pairwise comparisons with Bonferroni correction ($t_{(25)}$=4.593, p=0.029), but no differences between days and no differences in males. There was also a main effect of age ($F_{(1, 25)}$ =21.902, p< .001, η^2p=0.467; Figure 18C), with mice stressed in early adolescence (7.42±1.17) producing more fecal pellets than mice stressed in late adolescence (5.32±1.01) according to pairwise comparisons with Bonferroni correction ($t_{(25)}$=-4.68, p<0.001). There was no significant main effect of sex ($F_{(1, 25)}$ =0.664, p=0.423), or other interactions [day x age($F_{(11, 275)}$=1.12, p=0.344), day x age x sex ($F_{(11, 275)}$ = 1.07, p=0.386), age x sex ($F_{(1, 25)}$ =0.008, p=0.929)] on the number of fecal pellets (Figure 19).

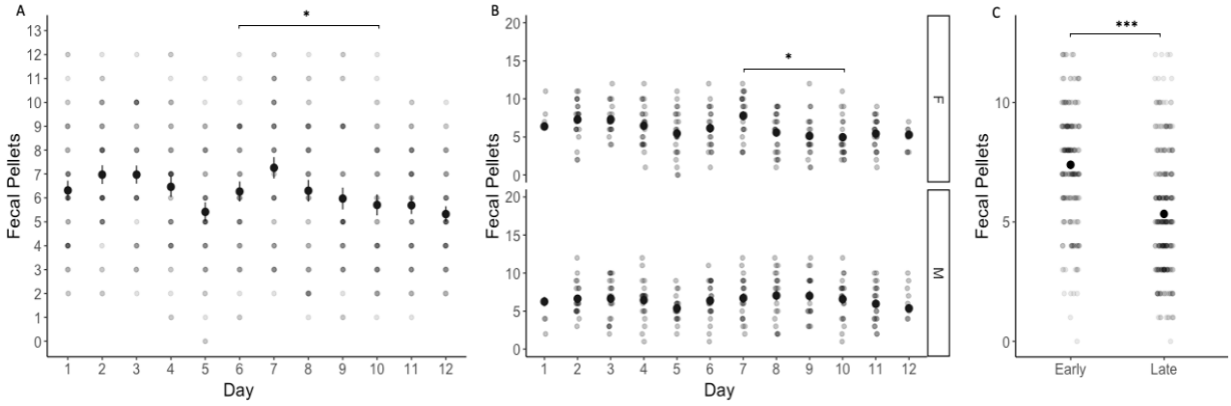

Figure 18. (A) Main effect of stress day, (B) day x sex interaction and (C) main effect of age on the number of fecal pellets produced by stressed mice during each 30-minute predator stress session. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each stressed mouse included in the analysis.

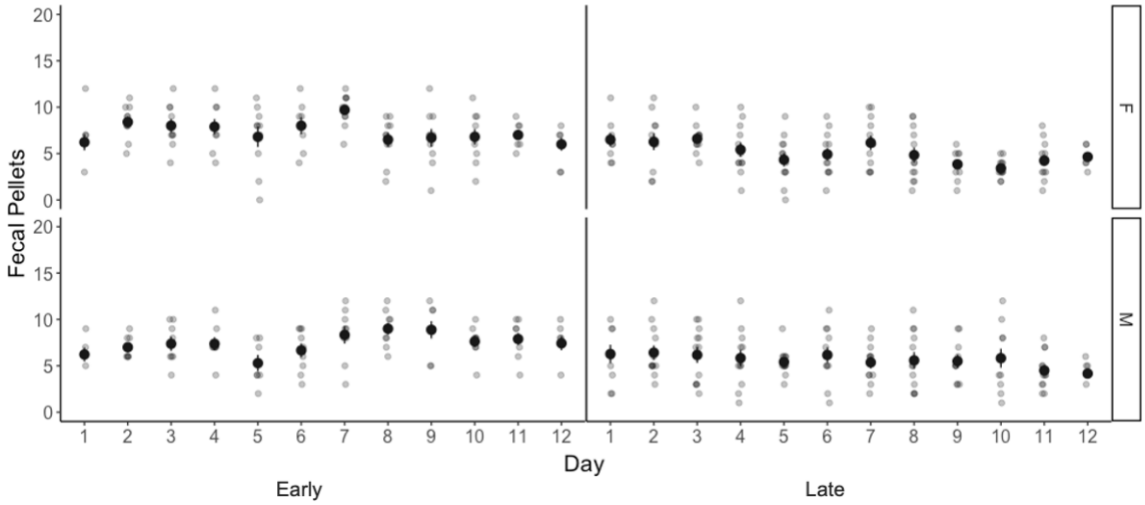

Figure 19. Number of fecal pellets produced by stressed mice during each 30-minute predator stress session for each age group. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

3.2 Olfactory Habituation and Dishabituation

All groups showed the expected habituation (decrease in sniffing time along subsequent presentations of the same stimulus) and dishabituation (increase in sniffing time when transitioning to the first presentation of a new odour; Figure 20).

Repeated measures ANOVA with odour as the within-subjects factor and sex, age, and stress treatment as between-subjects factors were used to analyze habituation (Figure 21) and dishabituation to each odour (Figure 22). There was a main effect of odour ($F_{(3,198)}$=65.059, p<0.001, η^2p=0.496) on habituation (Figure 23A). Pairwise comparisons with Bonferroni correction indicated that habituation remained similar between water (-4.74±6.71 s) and almond (-6.47±8.31 s; $t_{(80)}$=1.72, p=0.285) and then decreased with each subsequent odour [Ban (-17.4±16.4 s; $t_{(80)}$=5.40, p<0.001), Soc(-33.7±23.1 s; $t_{(80)}$=5.92, p<0.001). This effect was modulated by an odour x age interaction ($F_{(3,198)}$=4.469, p=0.005, η^2p=0.4960.063; Figure 23B). Pairwise comparisons with Bonferroni correction indicated that mice in early group habituated less to the social odour than to the banana odour ($t_{(80)}$=5.444, p<0.001), but mice in the late group did not ($t_{(80)}$=2.753, p=0.204). There was also a main effect of sex ($F_{(1,66)}$=4.008, p=0.049, η^2p=0.057; Figure 23C), with females (-13.4±8.34 s) habituating more than males (-18.4±10.8 s). There were no other significant main effects [age ($F_{(1, 66)}$=2.694, p=0.105), stress ($F_{(1, 66)}$=0.274, p=0.602)] or interactions [odour x stress ($F_{(3, 198)=}$0.111, p=0.953), odour x sex ($F_{(3,198)=}$2.362, p=0.073), age x stress ($F_{(1,66)}$=0.897, p=0.347), age x sex ($F_{(1,66)}$=0.015, p=0.902), stress x sex ($F_{(1,66)}$=0.007, p=0.931), age x stress x sex ($F_{(1,66)}$=0.905, p=0.345), odour x age x stress ($F_{(3,198)}$=0.421, p=0.738), odour x age x sex ($F_{(3,198)}$=0.1.311, p=0.272), odour x stress x sex ($F_{(3,198)}$=0.574, p=0.632), odour x age x stress x sex ($F_{(3,198)}$=0.035, p=0.991)] for habituation.

Similarly, there was a main effect of odour ($F_{(2,140)}$=92.369, < .001) on dishabituation (Figure 24A). Pairwise comparisons with Bonferroni correction indicated that dishabituation increased with each subsequent odour [Alm(4.58±7.20 s),

Ban(17.3±15.2 s), ($t_{(70)}$=-6.73, p<0.001), Soc (39.9±24.7 s), ($t_{(70)}$=-7.52, p<0.001)]. There was also a significant odour x age interaction ($F_{(2,140)}$=4.615, p=0.011, (Figure 24B), with increased dishabituation only to the social odour in the early group (46.2±23.4 s) compared to the late group (35.0±24.8 s), but no age differences for the non-social odours. There were no other significant main effects [age ($F_{(1,70)}$=1.670, p=0.200), sex($F_{(1,70)}$=2.725, p=0.103), stress ($F_{(1,70)}$=0.034, p=0.853)] or interactions [age x stress ($F_{(1,70)}$=2.913, p=0.092), age x sex ($F_{(1,70)}$=0.913, p=0.342), stress x sex ($F_{(1,70)}$=0.77, p=0.352), age x stress x sex ($F_{(1,70)}$=0.137, p=0.290), odour x stress ($F_{(2,140)}$=0.280, p=0.756), odour x sex ($F_{(2,140)}$=2.263, p=0.108), odour x age x stress ($F_{(2,140)}$=0.711, p=0.493), odour x age x sex ($F_{(2,140)}$=0.895, p=0.411), odour x stress x sex ($F_{(2,140)}$=2.749, p=0.067), odour x age x stress x sex ($F_{(2,140)}$=0.152, p=0.859)].

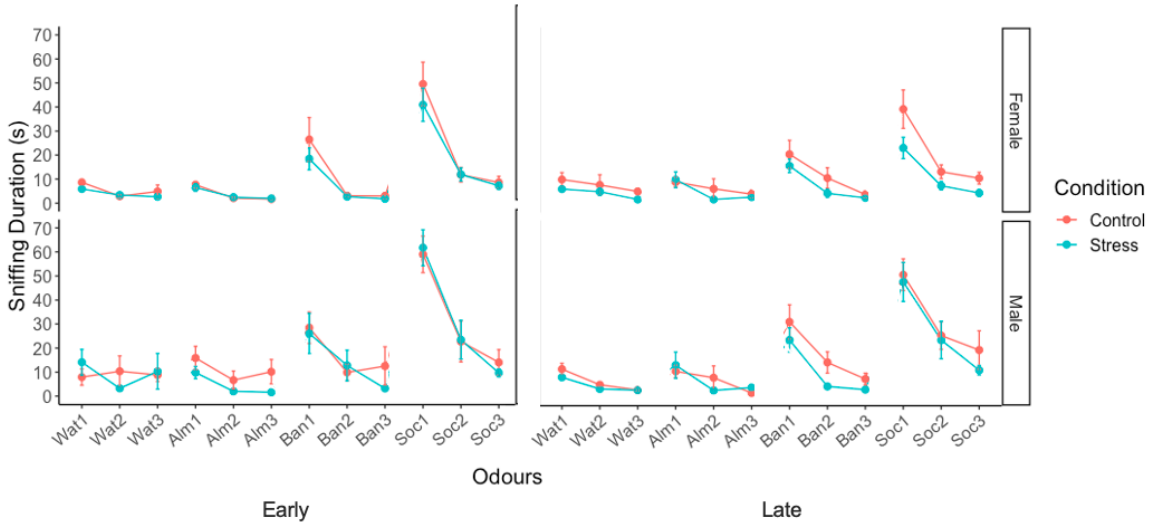

Figure 20. Sniffing durations (s) during the olfactory habituation / dishabituation test. Large points indicate group means. Error bars indicate standard error of the mean. Connecting lines indicate the progression of time spent sniffing the swab for the three presentations of each odour.

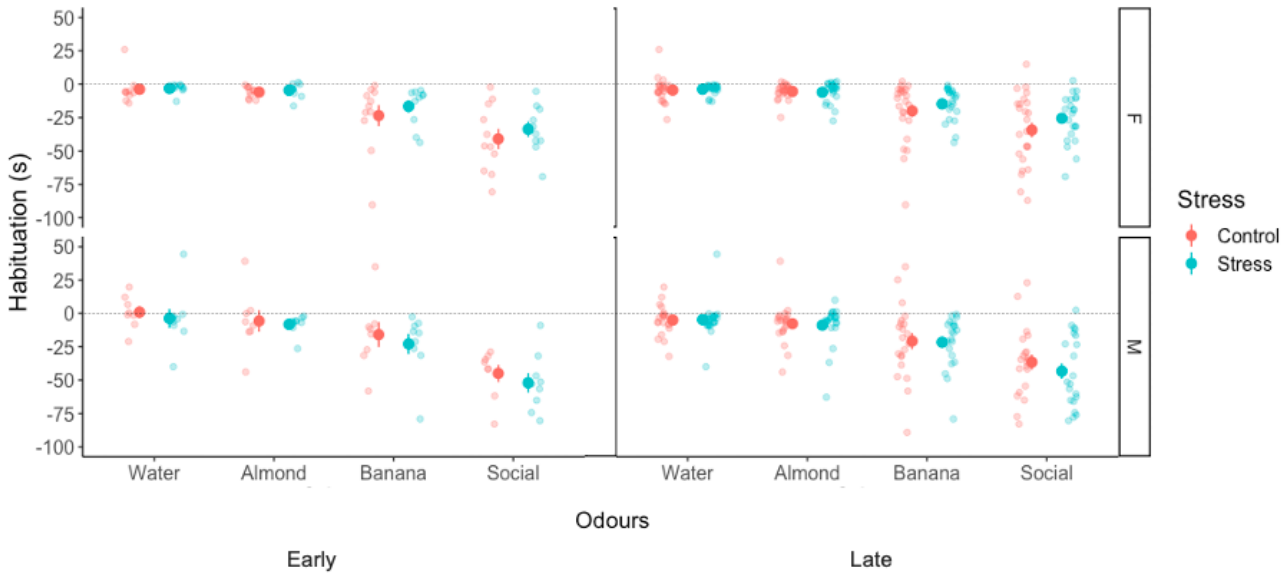

Figure 21. Habituation (difference in sniffing time from the first to the last presentation of each odour) for each of the four odours. Zero (shown by dashed lines) indicates no change in sniffing time between odour presentations. Points above zero indicate increased habituation and points below zero indicate decreased habituation to the odours. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

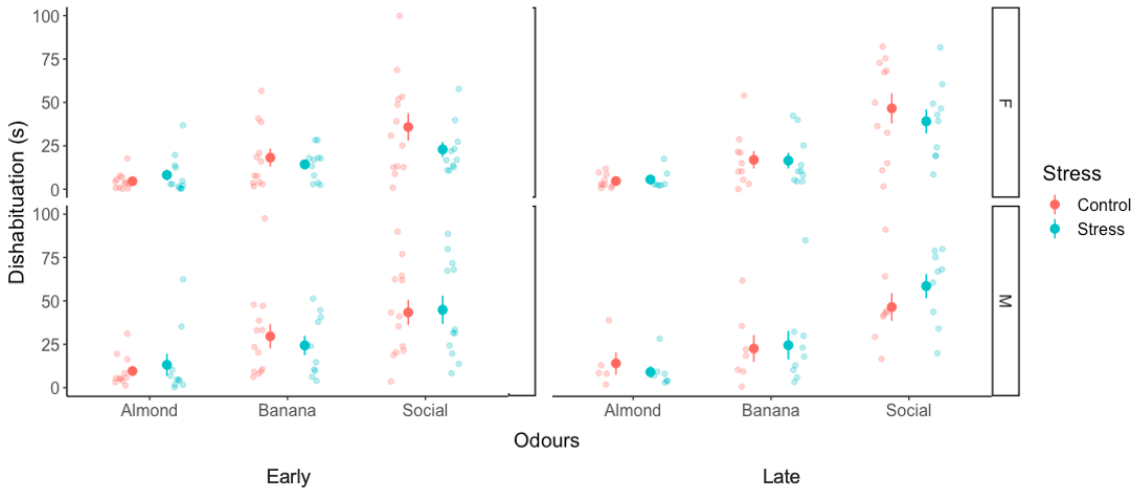

Figure 22. Dishabituation (sniffing time difference between the first presentation of each odour and the last presentation of the previous odour) for the almond, banana, and social odours. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

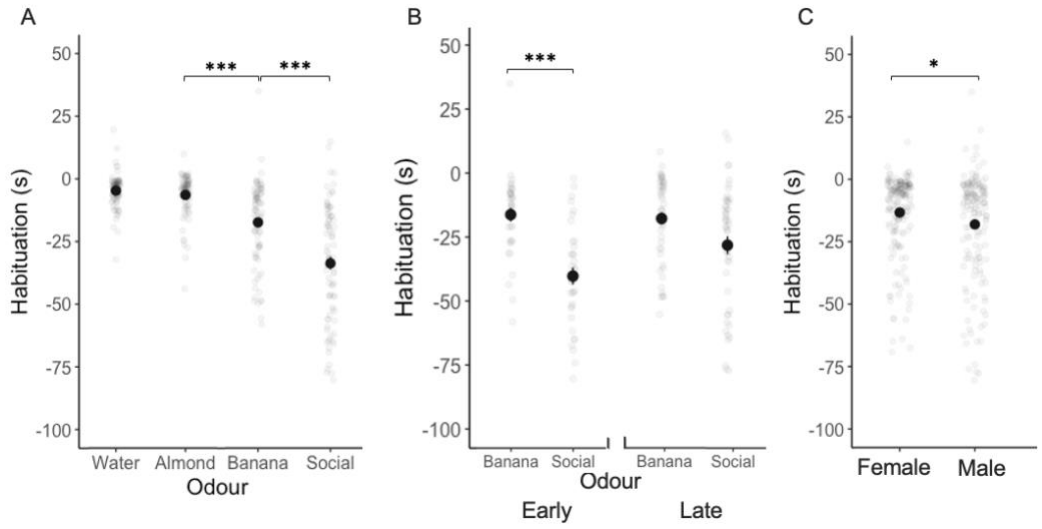

Figure 23. (A) Main effect of odour, (B) odour x age interaction, and main effect of sex on habituation. *p<0.05. **p<0.01.***p<0.001. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

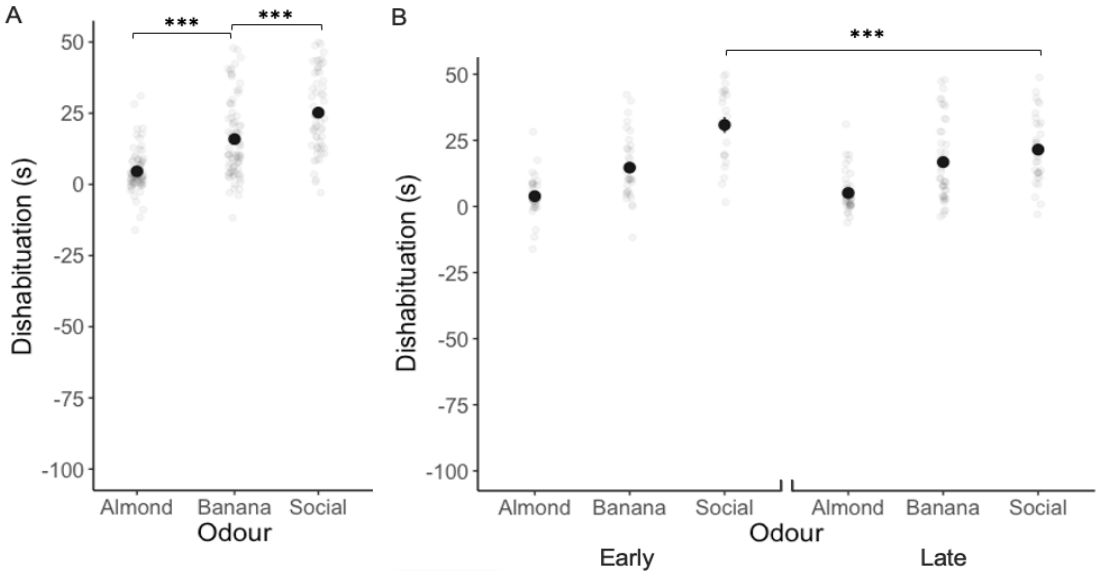

Figure 24. A) Main effect of odour and B) odour x age interaction on dishabituation. ***p<0.001. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

3.3 Open Field Test

A three-way ANOVA with age, sex and stress treatment was used to analyze all measures collected in the open field. There were no significant main effects [age ($F_{(1,78)}$=0.358, p=0.551), stress ($F_{(1,78)}$=2.755, p=0.101), or sex ($F_{(1,78)}$= 0.541, p=0.464)] or interactions [age x stress ($F_{(1,78)}$= 3.618, p=0.061), age x sex ($F_{(1,78)}$= 0.332, p=0.566), stress x sex ($F_{(1,78)}$= 1.797, 0.184), age x stress x sex ($F_{(1,78)}$= 0.698, p=0.406)] for the time spent in the centre of the open field (Figure 25).

There was a significant main effect of stress ($F_{(1,78)}$=9.033, p=0.004, η^2p=0.104) on distance travelled, with stress animals (13746±2526 cm) travelling a longer distance than control animals (12278±2315 cm; Figure 26A) according to pairwise comparisons with Bonferroni correction ($t_{(78)}$=3.01, p=0.004). There was also a main effect of sex

($F_{(1,78)}$= 4.476, p=0.038, η^2p=0.054) on the distance travelled, with females (13519±2258 cm) travelling a longer distance than males (12415±2682 cm; Figure 26B), according to pairwise comparisons with Bonferroni correction ($t_{(78)}$=2.12, p=0.038). There were no other significant main effects [age ($F_{(1,78)}$= 0.137, p=0.712], or interactions [age x stress ($F_{(1,78)}$= 0.0324, p=0.858), age x sex ($F_{(1,78)}$= 0.2654, p=0.608), stress x sex ($F_{(1,78)}$= 0.920, p=0.340), age x stress x sex ($F_{(1,78)}$= 2.3817, p=0.127) on the distance travelled (\Figure 27).

There was a significant main effect of sex ($F_{(1,78)}$=5.644, p=0.020, η^2p=0.067) on the frequency of rearing, with females (166±31.1) rearing less than males (180±38) according to pairwise comparisons with Bonferroni correction ($t_{(78)}$=-2.38, p=0.020; Figure 28A). This effect was driven by an age x stress x sex interaction ($F_{(1,78)}$= 5.432, p=0.022, η^2p=0.065; Figure 28B), with early control females (152±28.8) rearing less than early control males (211±21.4; $t_{(78)}$=-3.90, p=0.006) but no sex differences in the stress animals of the early group ($t_{(78)}$=-0.037, p=1.000) or in either stress treatment in the late group [control ($t_{(78)}$=-0.005, p=1.000), stress ($t_{(78)}$=-0.565, p=1.000)], according to pairwise comparisons with Bonferroni correction (Figure 26B). There were no other significant main effects [age ($F_{(1,78)}$= 0.742, p=0.392), stress ($F_{(1,78)}$= 3.644, p=00.060)] or interactions [age x stress (F(1,78)= 0.339, p=0.562), age x sex ($F_{(1,80)}$= 3.305, p=0.073), stress x sex ($F_{(1,78)}$= 3.182, p=0.078)].

There were no significant main effects [stress ($F_{(1,78)}$=2.751, p=0.101), age ($F_{(1,78)}$= 2.952, p=0.090), sex ($F_{(1,78)}$=0.320, p=0.573)] or interactions [age x stress ($F_{(1,78)}$=0.033, p=0.855), age x sex ($F_{(1,78)}$=0.353, p=0.554), stress x sex ($F_{(1,78)}$= 2.688,

p=0.105), age x stress x sex ($F_{(1,78)}$= 0.113, p=0.737)] on the time spent grooming in the open field (Figure 29).

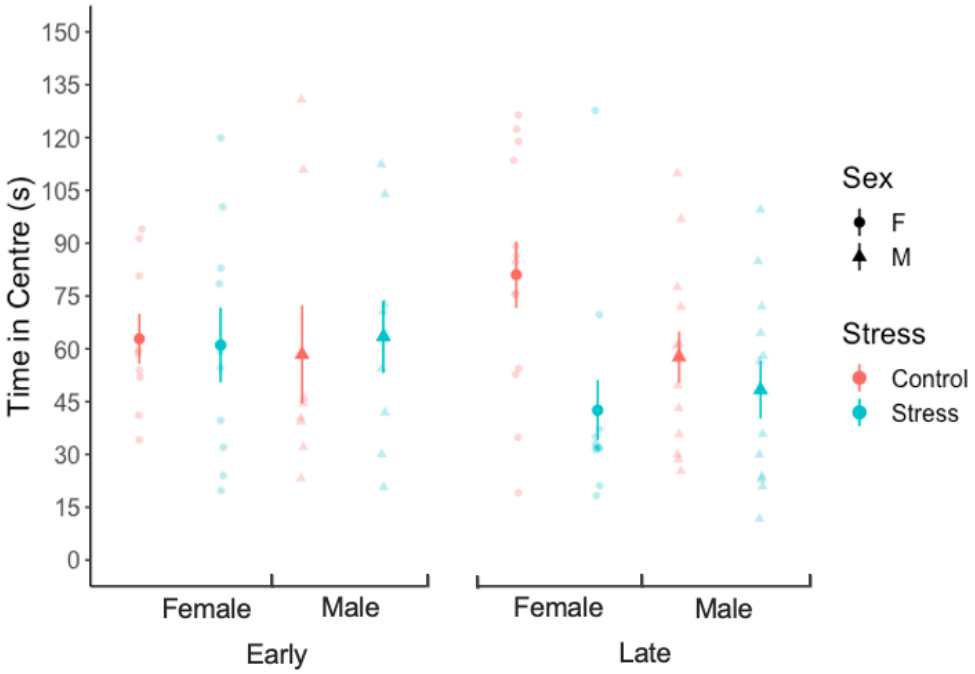

Figure 25. Time (s) spent in the centre of the open field. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

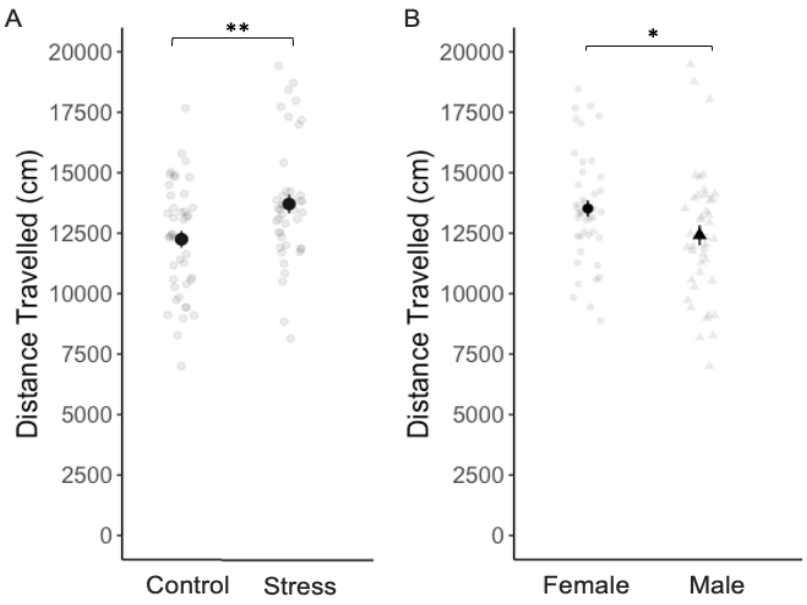

Figure 26. Main effects of stress (A) and sex (B) on the total distance travelled (cm) during the open field test. **p<0.01, *p<0.05. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

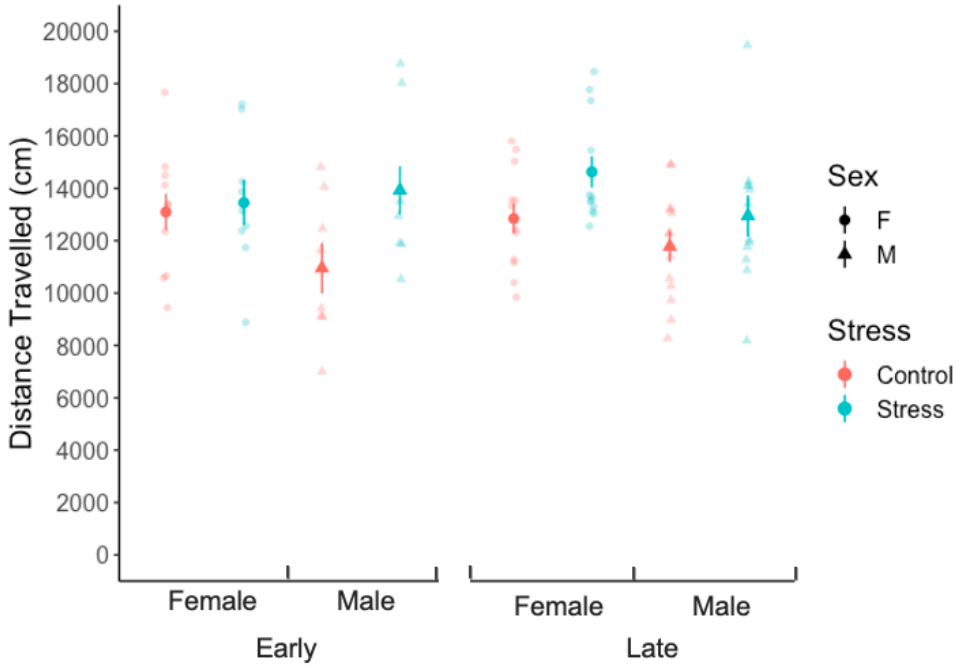

Figure 27. Total distance travelled (cm) during the open field test. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

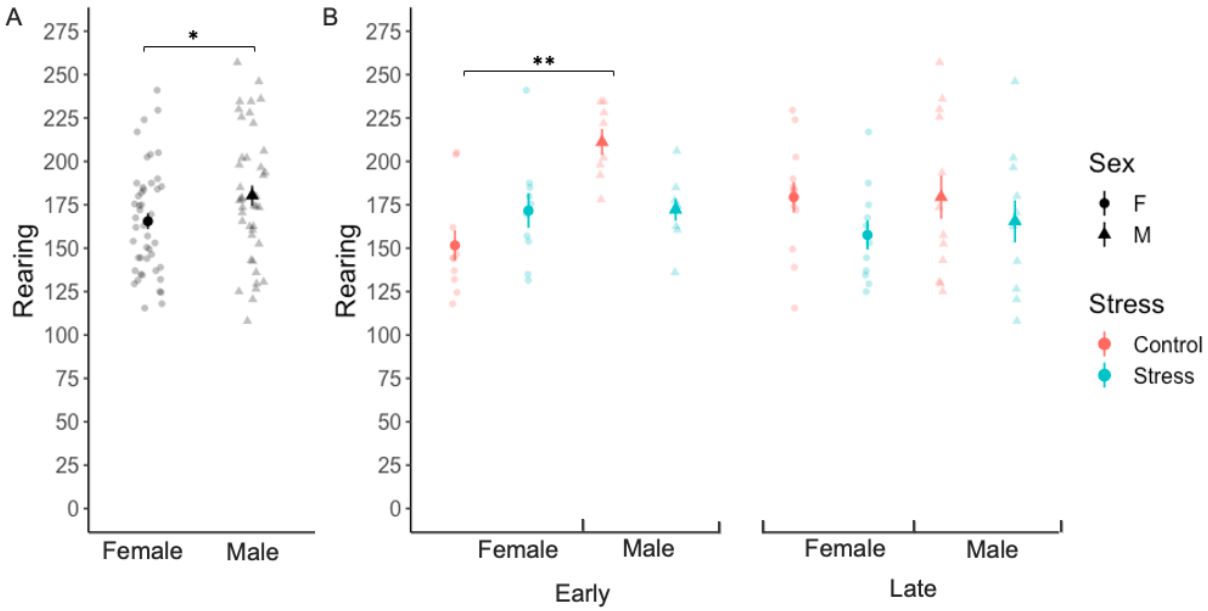

Figure 28. A) Main effect of sex and B) age x stress x sex interaction on the frequency of rearing during the open field test. *p<0.05. **p<0.01. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

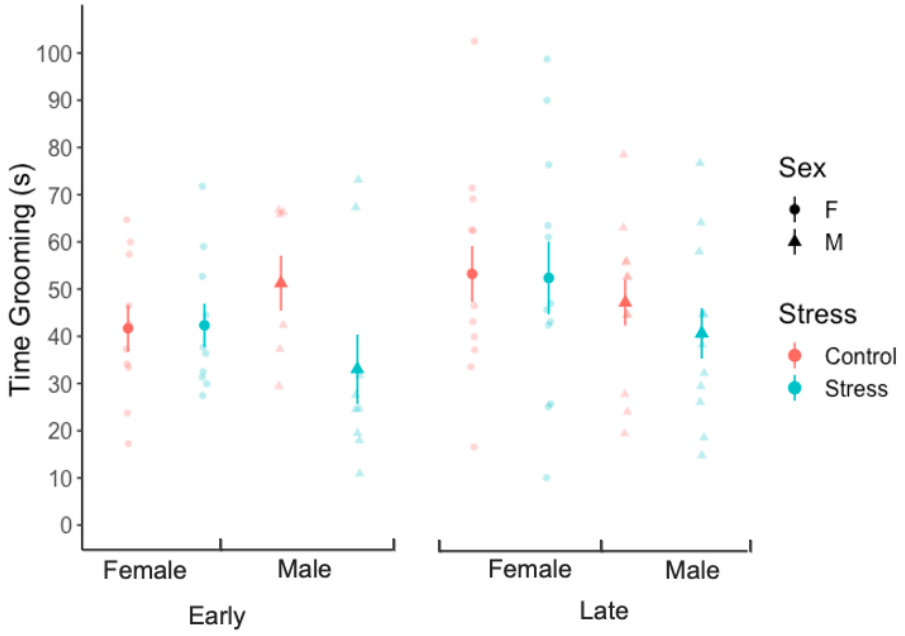

Figure 29. Time spent grooming (s) during the open field test. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

3.4 Three-Chamber Sociability Test

A repeated measures ANOVA with stimulus as the within-subjects factor and age, sex and stress treatment as between-subjects factors was used to analyze the times spent investigating each cup in the testing phase (Figure 30). There were main effects of stimulus ($F_{(1,75)}$=270.937, p< .001, η^2p=0.783) and sex ($F_{(1,75)}$=5.078, p=0.027,

η^2p=0.063). Pairwise comparisons with Bonferroni correction indicated that investigation time was higher for the social stimulus (98.9±34.4 s) compared to the non-social stimulus (42.10±15.20 s; $t_{(75)}$=16.5, p<0.001) and higher for males (76.6±23.9 s) than for females (65.6±22.7; $t_{(75)}$=-2.25, p=0.027 s). These effects were modulated by a stimulus x sex interaction ($F_{(1,75)}$=9.297, p=0.003, η^2p=0.110), with males investigating the social stimulus (111±27.5 s), but not the novel object [males (41.8±14.8), females (42.4±15.7)], more than females (88.9±36.7; $t_{(75)}$=-2.88, p=0.031; Figure 31). There was also a significant main effect of age ($F_{(1,75)}$=8.320, p=0.005, η^2p=0.100). Pairwise comparisons with Bonferroni correction indicated that younger mice (78.2±21.9 s) investigated more than older mice (65.3±23.9 s; $t_{(75)}$=-2.88, p=0.005;Figure 32A). This effect was modulated by a stimulus x age interaction ($F_{(1,75)}$=4.837, p=0.031, η^2p=0.061), with investigation of the social stimulus, but not the novel object [early (44.9±12.6 s), late (40.0±16.7 s); $t_{(75)}$=-1.35, p=1.000], being higher in younger mice (110±32.2 s) compared to older mice (90.6±34.0; $t_{(75)}$=-2.84, p=0.035 s), according to pairwise comparisons with Bonferroni correction (Figure 32B). There were no other significant main effects [stress ($F_{(1,75)}$=0.004, p=0.945)] or interactions [age x stress ($F_{(1,75)}$=0.114, p=0.736), age x sex ($F_{(1,75)}$=0.029, p=0.864), stress x sex ($F_{(1,75)}$=3.326, p=0.072), age x stress x sex ($F_{(1,75)}$=0.294, p=0.589), stimulus x stress ($F_{(1,75)}$=1.082, p=0.301), stimulus x age x stress ($F_{(1,75)}$=0.606, p=0.439), stimulus x age x sex ($F_{(1,75)}$=5.32e^{-4}, p=0.982), stimulus x stress x sex ($F_{(1,75)}$=0.107, p=0.743), stimulus x age x stress x sex ($F_{(1,75)}$=0.053, p=0.818)].

A three-way ANOVA with age, sex and stress treatment as factors was used to analyze the social preference ratio in the testing phase (Figure 33). There were main effects of stress ($F_{(1,79)}$=6.817, p=0.011, η^2p=0.079), with stress (0.72±0.08 s) animals

showing higher social preference than controls (0.67±0.08 s; $t_{(79)}$=2.61, p=0.011; Figure 34A) and sex ($F_{(1,79)}$=7.293, p=0.008, η^2p=0.085), with males (0.72±0.08 s) showing higher social preference than females (0.66±0.09 s; $t_{(79)}$=-2.70, p=0.008; Figure 34B), according to pairwise comparisons with Bonferroni correction. There was no significant main effect of age ($F_{(1,79)}$=0.270, p=0.598) or interactions [age x stress ($F_{(1,79)}$=1.497, p=0.225), age x sex ($F_{(1,79)}$=0.020, p=0.887), stress x sex ($F_{(1,79)}$=596, p=0.442), age x stress x sex ($F_{(1,79)}$=0.330, p=567).

To account for the possibility that any effect in testing was the result of a side preference, we performed repeated measures ANOVA with side as the within-subjects factor and age, sex, and stress treatment as between-subjects factors to analyze the time spent investigating each cup during the habituation phase of the test (Figure 35). There were differences in overall exploration of both cups, but no side preferences. There was a significant stress x sex interaction ($F_{(1,73)}$=4.687, p=0.034, η^2p=0.060), with control males (49.2±6.36 s) investigating both cups more than control females (43.6±10.1 s; $t_{(73)}$=-2.379, p=0.020), but no sex difference in the stress group [males (46.1±10.9 s), females (48.3±8.70 s); $t_{(73)}$=0.720, p=0.474;Figure 36], according to pairwise comparisons. There were no significant main effects of side ($F_{(1,73)}$=0.097, p=0.756), age ($F_{(1,73)}$=3.91e^{-4}, p=0.984), stress ($F_{(1,73)}$=0.125, p=0.734), or sex ($F_{(1,73)}$=1.262, p=0.265), or any other significant interactions [age x stress ($F_{(1,73)}$=0.163, p=0.687), age x sex ($F_{(1,73)}$=0.014, p=0.906), age x stress x sex ($F_{(1,73)}$=2.233, p=0.139), side x age ($F_{(1,73)}$=1.138, p=0.289), side x stress ($F_{(1,73)}$=0.727, p=0.396), side x sex ($F_{(1,73)}$=0.035, p=0.851), side x age x stress ($F_{(1,73)}$=1.068, p=0.305), side x age x sex ($F_{(1,73)}$=0.408, p=0.525), side x stress x

sex ($F_{(1,73)}$=0.409, p=0.524), side x age x stress x sex ($F_{(1,73)}$=0.560, p=0.457)] on the time spent investigating each cup during the habituation phase.

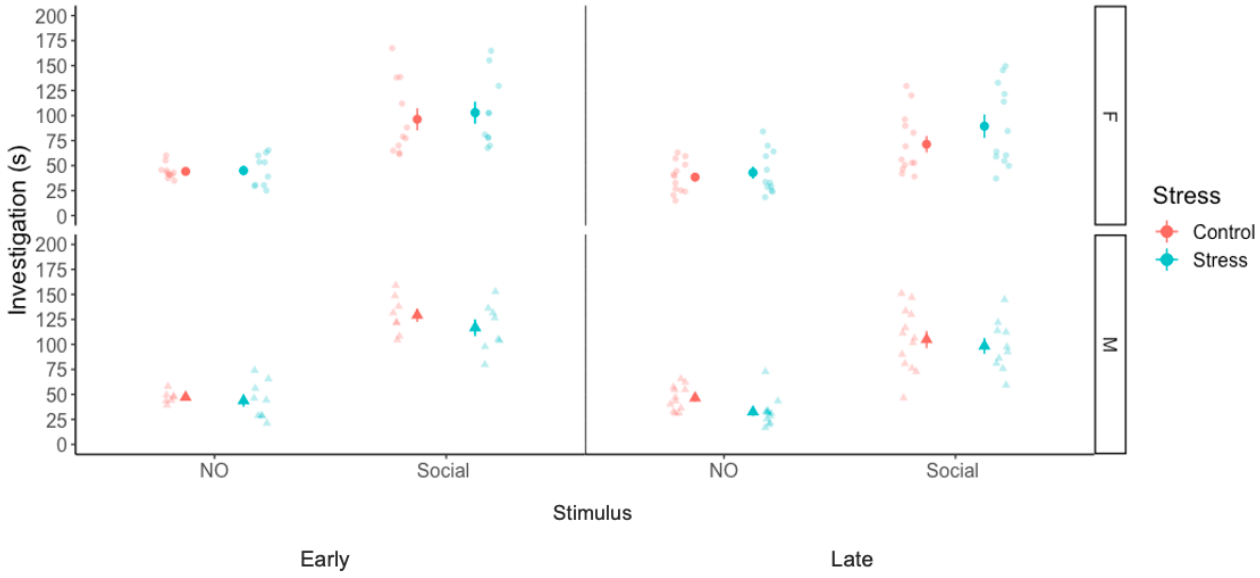

Figure 30. Time (s) spent investigating (sniffing and in contact with) the social stimulus and novel object during the testing phase of the three-chamber sociability test. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

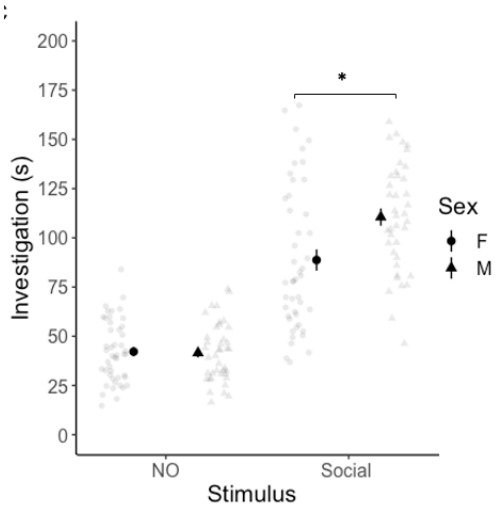

Figure 31. Sex x stimulus interaction on the general time (s) spent investigating (sniffing and in contact with) both stimuli during the testing phase of the three-chamber test. *p<0.05. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

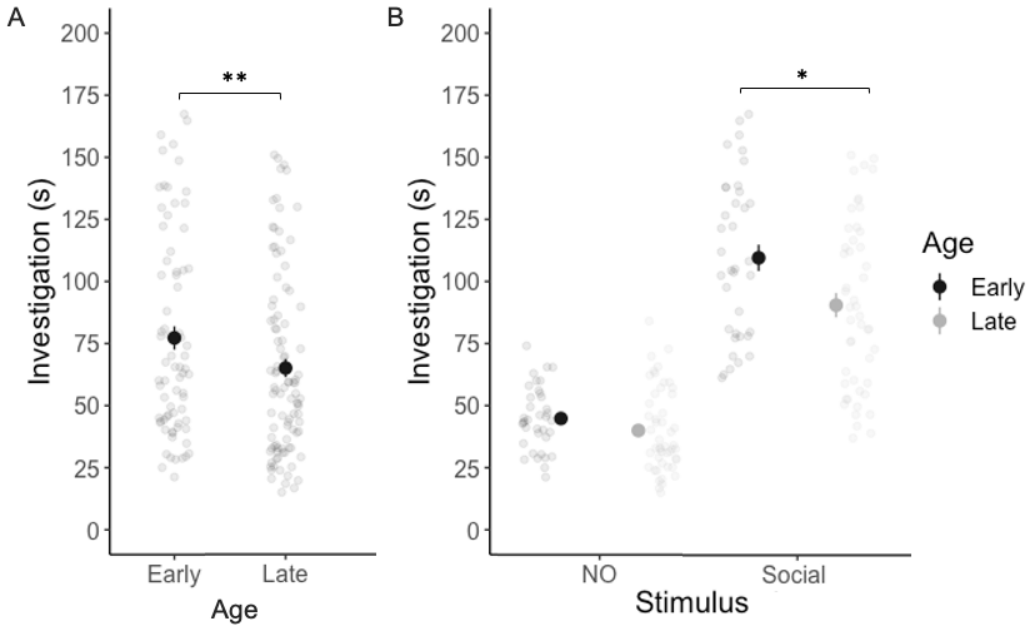

Figure 32. A) Main effect of age and B) age x stimulus interaction on the general time (s) spent investigating (sniffing and in contact with) both stimuli during the testing phase of the three-chamber test. *p<0.05. **p<0.01. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

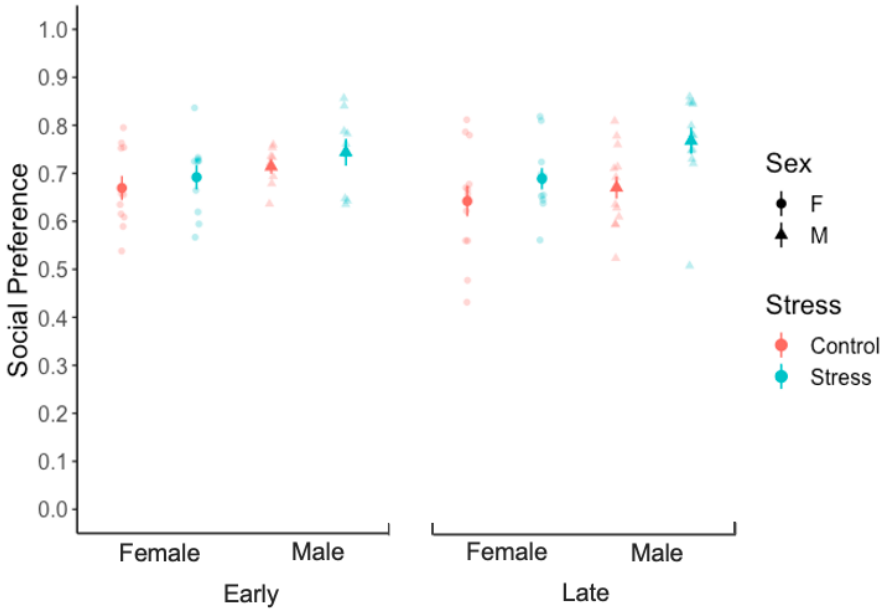

Figure 33. Social preference ratios (time spent investigating the social stimulus relative to the time spent investigating both stimuli) for the testing phase of the three-chamber sociability test. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

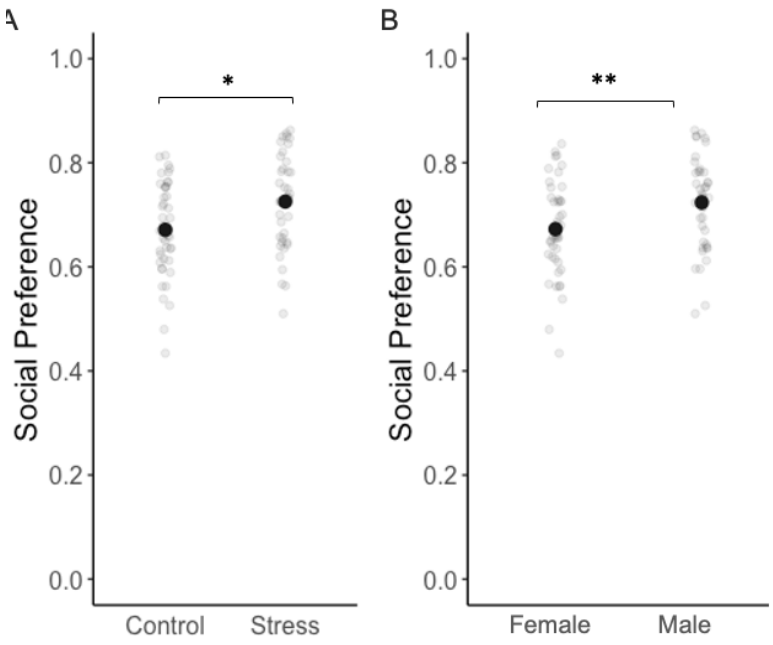

Figure 34. A) Main effects of A) stress and B) sex on social preference ratios (time spent investigating the social stimulus relative to the time spent investigating both stimuli) in the testing phase of the three-chamber test. *p<0.05. **p<0.01. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

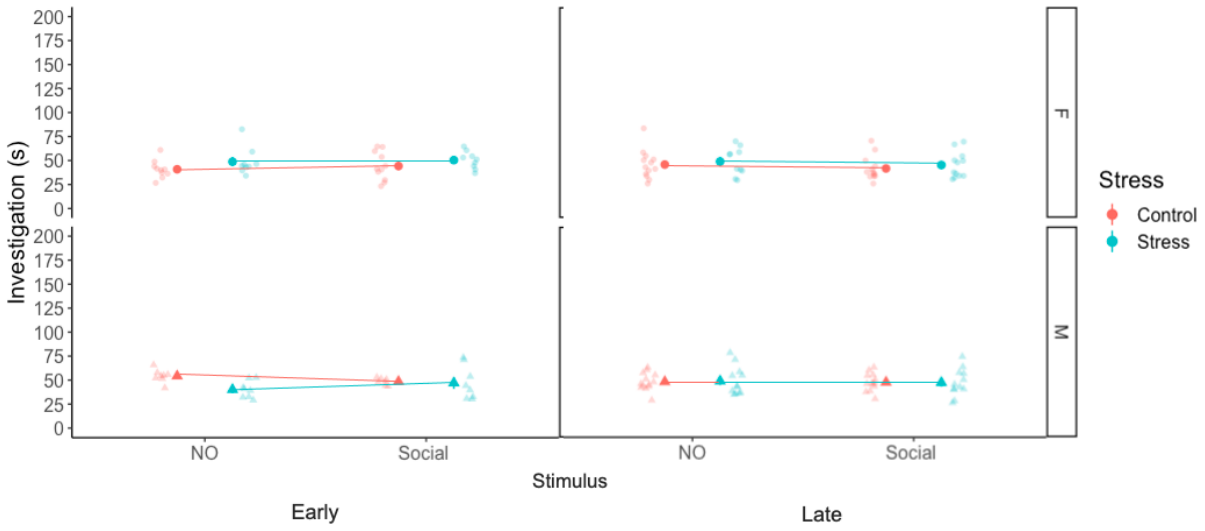

Figure 35. Time (s) spent investigating (sniffing or in contact with) each empty cup during the habituation phase of the three-chamber sociability test. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

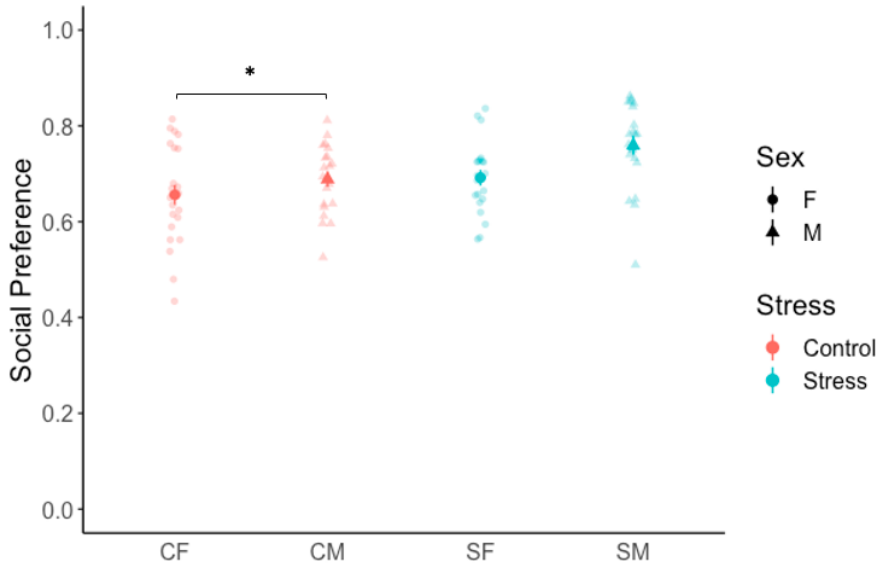

Figure 36. Sex x stress interaction on the general time (s) spent investigating (sniffing and in contact with) both stimuli during the habituation phase of the three-chamber sociability test. *$p<0.05$. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

3.5 Light / Dark Box

A three-way ANOVA with age, sex and stress treatment was used to analyze all measures collected in the light / dark box. There were no significant main effects or interactions on the time spent in the light area [age ($F_{(1,75)}$=0.66, p=0.0418), stress ($F_{(1,75)}$= 0.134, p=.714), sex ($F_{(1,80)}$=1.714, p=0.194), age x stress ($F_{(1,75)}$=0.438, p=0.510), age x sex ($F_{(1,75)}$= 0.085, p=0.771), stress x sex ($F_{(1,75)}$=0.007, p=0.932), age x stress x sex ($F_{(1,75)}$=0.425, p=0.516); Figure 37], frequency of entries into the light area [age ($F_{(1,79)}$=1.793, p=0.184), stress ($F_{(1,79)}$=0.338, p=0.562), sex ($F_{(1,79)}$=2.777, p=0.100), age x stress ($F_{(1,79)}$=0.877, p=0.352), age x sex ($F_{(1,79)}$=0.028, p=0.866), stress x sex ($F_{(1,79)}$=2.916, p=0.092), age x stress x sex ($F_{(1,79)=}$2.947, p=0.090);Figure 38], or frequency of rearing [age ($F_{(1,80)}$=0.283, p=0.596), stress ($F_{(1,78)}$=0.191, p=0.663), sex ($F_{(1,80)}$=1.076, p=0.303), age x stress ($F_{(1,78)}$=0.399, p=0.529), age x sex ($F_{(1,78)}$=0.114, p=0.736), stress x sex ($F_{(1,78)}$=0.243, p=0.623), age x stress x sex ($F_{(1,78)}$=0.001, p=0.971); Figure 39].

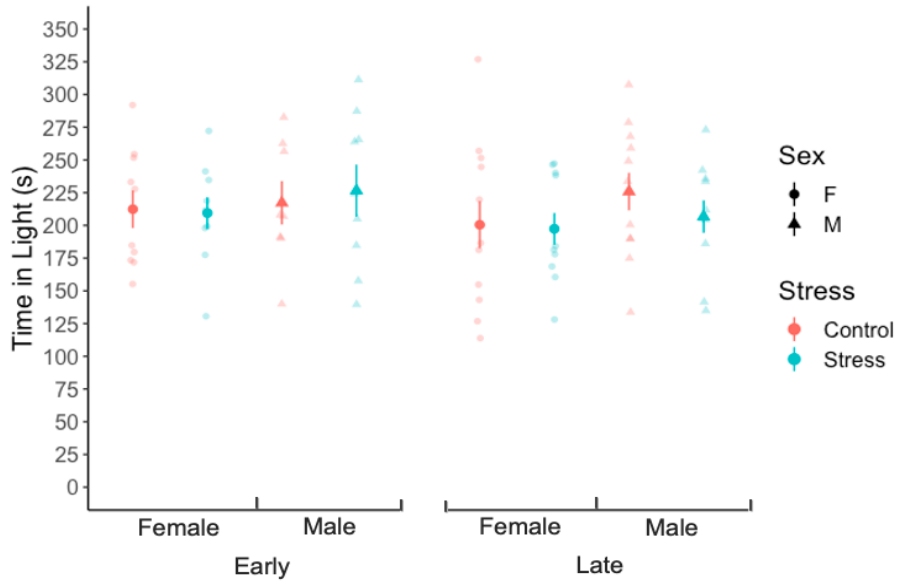

Figure 37. Time (s) spent in the light area of the light/dark box. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

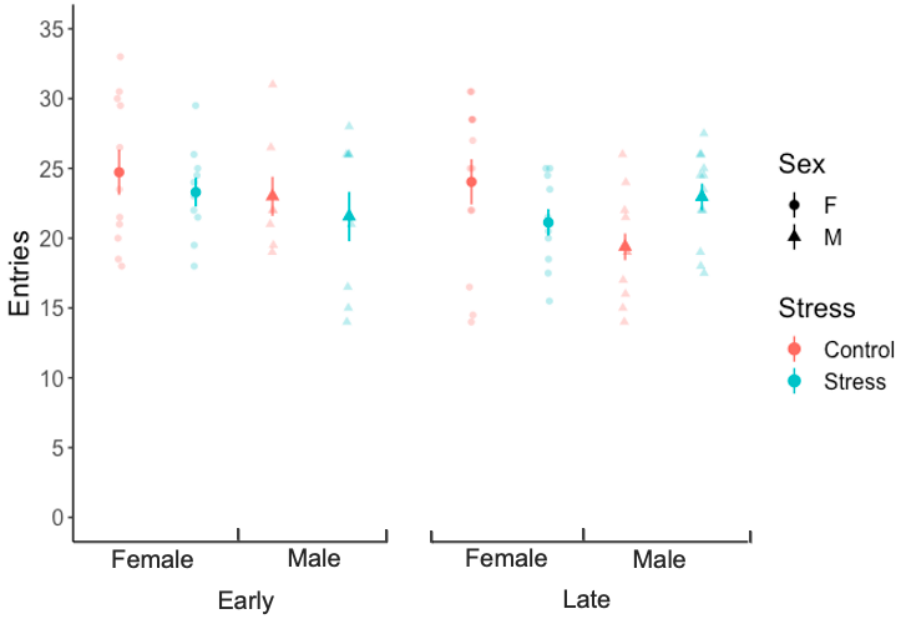

Figure 38. Frequency of entries into the light area of the light/dark box. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

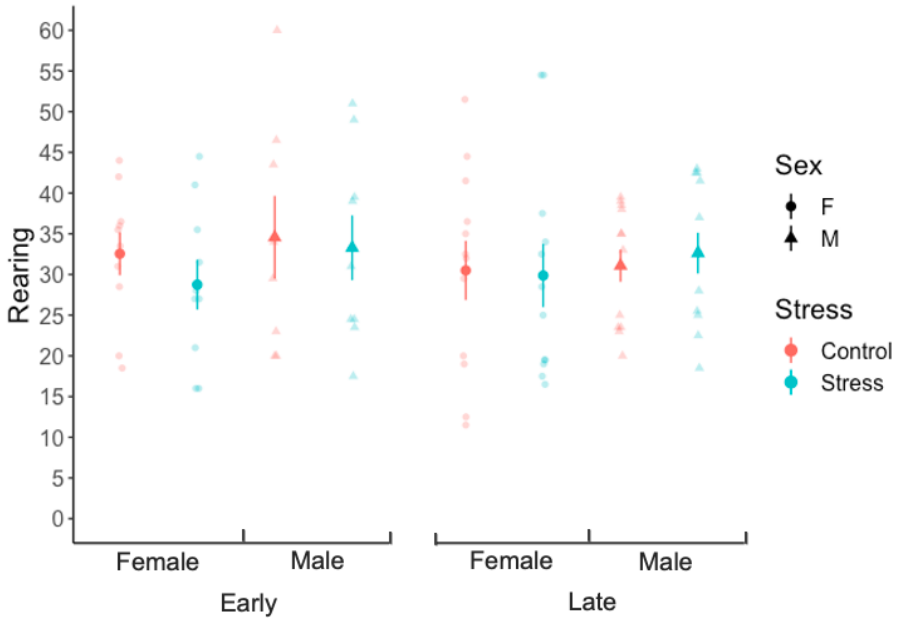

Figure 39. Frequency of rears in the light area during the light/dark box test. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

3.6 Elevated Plus Maze

A three-way ANOVA with age, sex and stress treatment was used to analyze all measures collected in the elevated plus maze. There were no significant main effects of age ($F_{(1,76)}$=0.593, p=0.444), stress ($F_{(1,76)}$=2.802, p=0.098), sex ($F_{(1,80)}$=2.715, p=0.104)

or interactions [age x sex ($F_{(1,76)}$=0.411, p=0.523), stress x sex ($F_{(1,76)}$=0.921, p=0.340), age x stress x sex ($F_{(1,76)}$=2.164, p=0.145)] on the time spent in the open arms (Figure 40). For the time spent in the closed arms, there were main effects of stress ($F_{(1,77)}$=18.347, p< 0.001, η^2p=0.192), with stress animals (193±22 s) spending more time in the closed arms than controls (170±23.3 s; $t_{(77)}$=4.28, p<0.001; Figure 41A), and sex ($F_{(1,77)}$=7.548, p=0.007, η^2p=0.089), with females (187±26.7 s) spending more time in the closed arms than males (173±26.4 s; $t_{(77)}$=2.75, p=0.007; Figure 41B), according to pairwise comparisons with Bonferroni correction. There was no significant main effect of age ($F_{(1,77)}$=0.417, p=0.520) or interactions [age x stress ($F_{(1,77)}$=1.577, p=0.213), age x sex (F(1,77)=1.254, p=0.266), stress x sex ($F_{(1,77)}$=0.002, p=0.958), age x stress x sex ($F_{(1,77)}$=1.378, p=0.244)] on the time spent in the closed arms of the maze (Figure 42).

There was a significant main effect of stress on the open / closed arm time ratio ($F_{(1,77)}$=6.314, p=0.014). Pairwise comparisons with Bonferroni correction indicated that stress (0.177±0.116 s) mice spent less time in the open arms relative to the closed arms than controls (0.241±0.119 s; $t_{(79)}$=-2.51, p=0.014;Figure 43A). There were no other significant main effects [age ($F_{(1,77)}$=0.077, p=0.781), sex ($F_{(1,77)}$=3.317, p=0.072)] or interactions [age x stress ($F_{(1,77)}$=0.017, p=0.896), age x sex ($F_{(1,77)}$=2.507, p=0.117), stress x sex ($F_{(1,77)}$=0.328, p=0.568), age x stress x sex ($F_{(1,77)}$=2.974, p=0.089); Figure 43B].

There were no significant main effects of age ($F_{(1,79)}$=0.062, p=0.985), stress ($F_{(1,79)}$= 2.258, p=0.137), or sex ($F_{(1,79)}$= 1.742, p=0.191) on the percent frequency of rears in the closed arms of the maze. However, there were a significant stress x sex interaction ($F_{(1,79)}$= 5.000, p=0.028, η^2p=0.060). Pairwise comparisons indicated that

stress males (0.10±0.03) reared more than control males (0.08±0.04; $t_{(79)}$=2.579, p=0.012), but there was no difference between stress females (0.08±0.03) and control (0.086±0.031) females ($t_{(77)}$=-0.532, p=0.596; Figure 44A). This effect was driven by an age x stress x sex interaction ($F_{(1,79)}$= 17.581, p< 0.001, η^2p=0.182). Pairwise comparisons with Bonferroni correct revealed that early stress males (0.126±0.024) reared more than early control males (0.09±0.03; $t_{(79)}$=3.929, p=0.007), but there was no difference between early stress females (0.063±0.038) and early control females (0.099±0.026; $t_{(79)}$=-2.130, p=1.000; Figure 44B) in the early group or any differences between stress treatments in the late group. There were no other significant interactions [age x stress ($F_{(1,79)}$=0.408, p=0.525), age x sex ($F_{(1,79)}$=0.018, p=0.892)] for this measure.

All mice performed similar number of stretch attends. There were no significant main effects [age ($F_{(1,77)=}$1.010, p=0.318), stress ($F_{(1,77)=}$1.744, p=0.190), sex ($F_{(1,77)}$=0.007, 0.932)] or interactions [age x stress ($F_{(1,77)}$=0.822, p=0.367), age x sex ($F_{(1,77)}$=0.018, p=0.891), stress x sex ($F_{(1,77)}$=0.059, p=0.808), age x stress x sex ($F_{(1,77)}$=0.060, p=0.806);Figure 45]. However, for head dips, there was a significant main effect of stress ($F_{(1,79)}$=8.562, p=0.004, η^2p=0.098), with stress animals (12.9±6.21) showing fewer head dips than controls (16.9±5.69), according to pairwise comparisons with Bonferroni correction ($t_{(79)}$=-2.93, p=0.004; Figure 46A). There were no other significant main effects [age ($F_{(1,79)}$=0.026, p=0.871), sex ($F_{(1,79)}$=0.801, p=0.374)] or interactions [age x stress ($F_{(1,79)}$=0.765, p=0.384), age x sex ($F_{(1,79)}$=0.534, p=0.467), stress x sex ($F_{(1,79)}$=0.490, p=0.486), age x stress x sex ($F_{(1,79)}$=0.257, 0.614); Figure 46B].

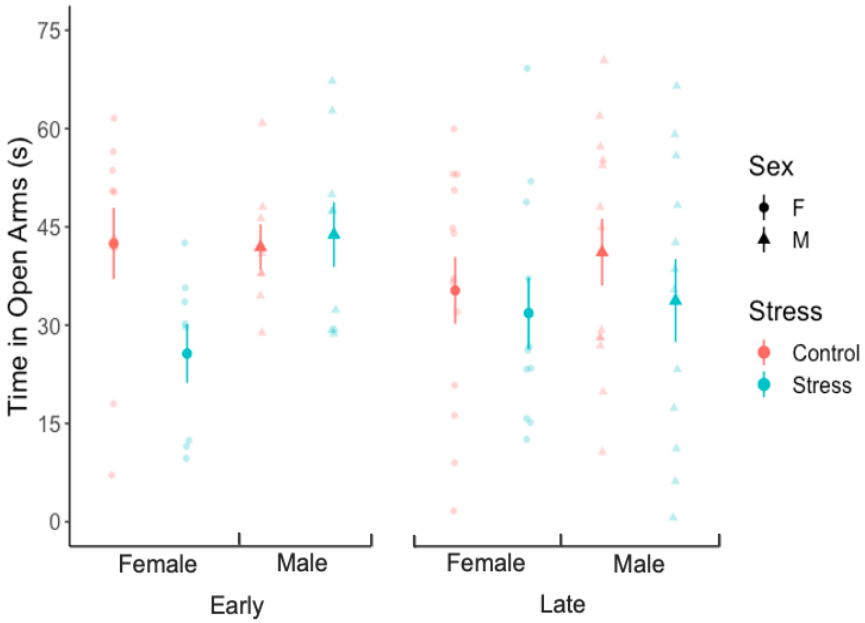

Figure 40. Time spent in the open arms (s) of the elevated plus maze. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

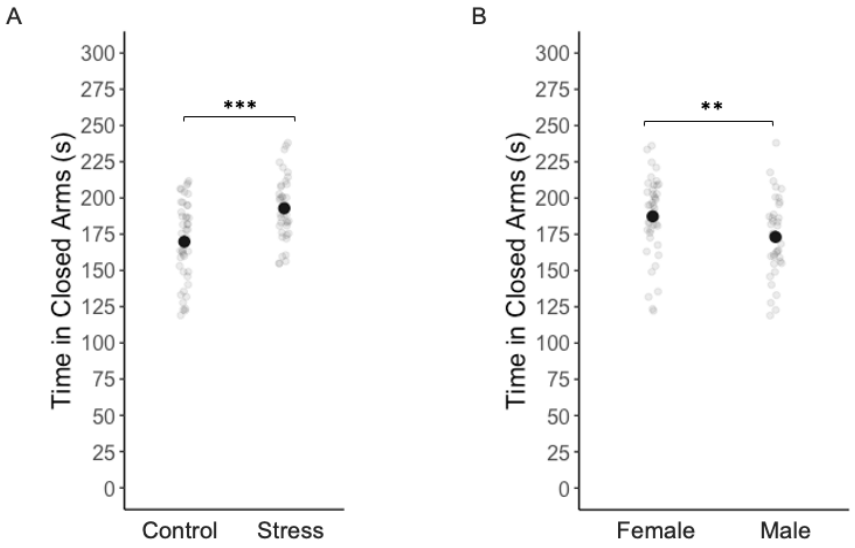

Figure 41. Main effects of stress (A) and sex (B) on the time spent (s) in the closed arms of the elevated plus maze. ***p<0.001, **p<0.01. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

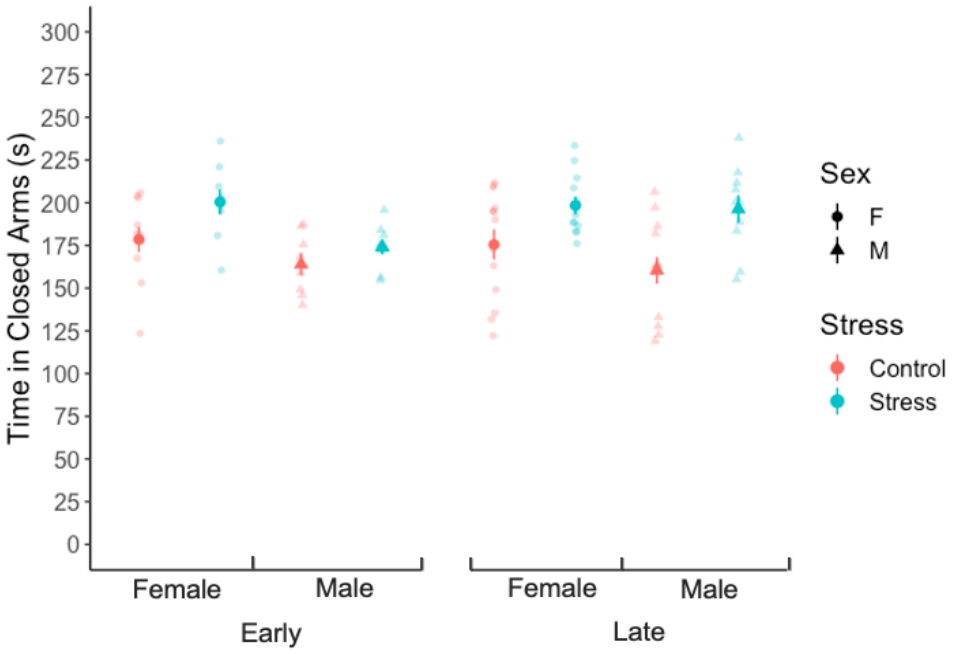

Figure 42. Time spent in the closed arms (s) of the elevated plus maze. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

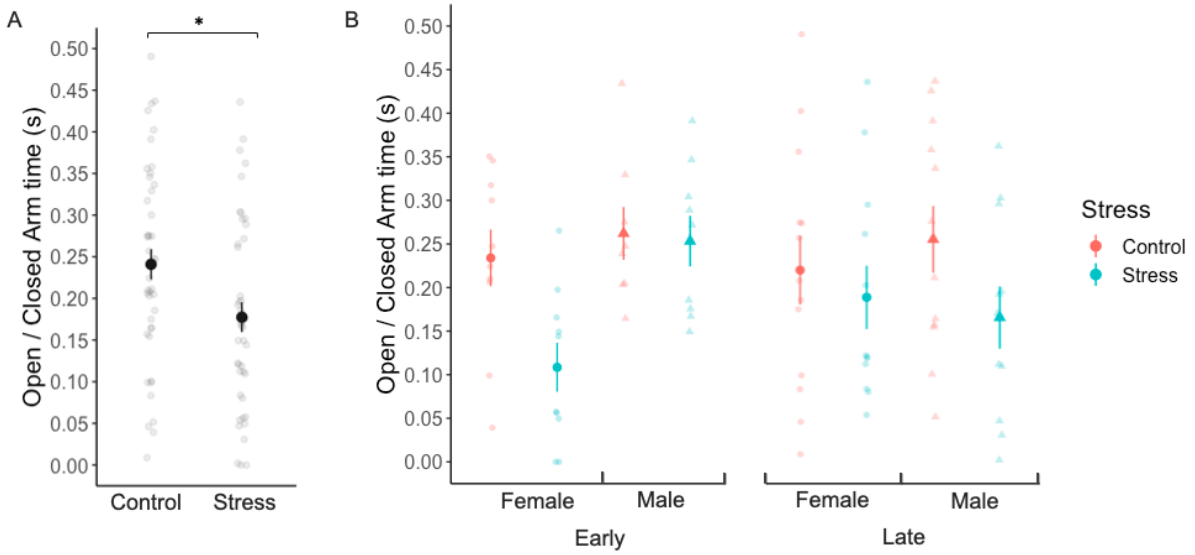

Figure 43. A) Main effect of stress treatment on B) ratios of time spent in the open arms relative to the closed arms (s) of the elevated plus maze. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

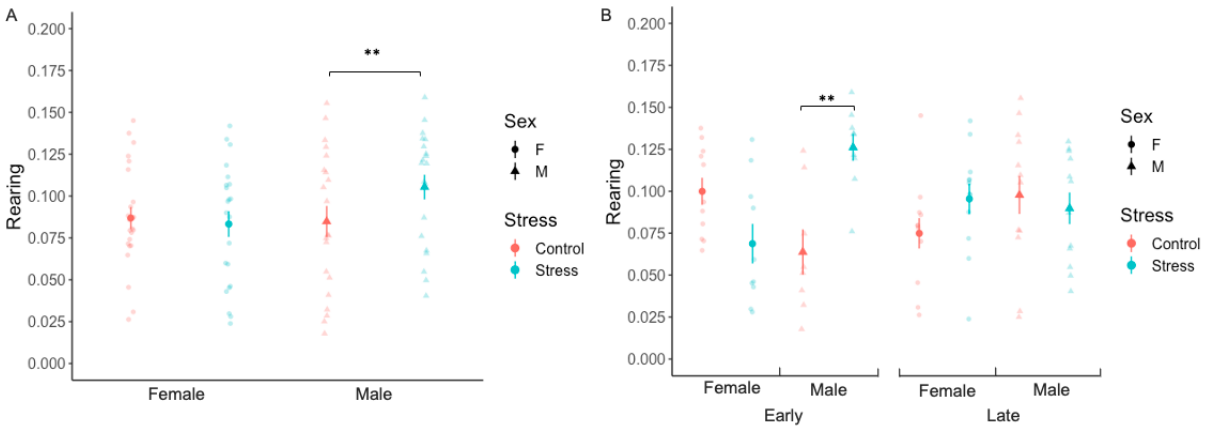

Figure 44. A) stress x sex and B) age x stress x sex interactions on rearing frequency in the closed arms of the elevated plus maze test. **p<0.01. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

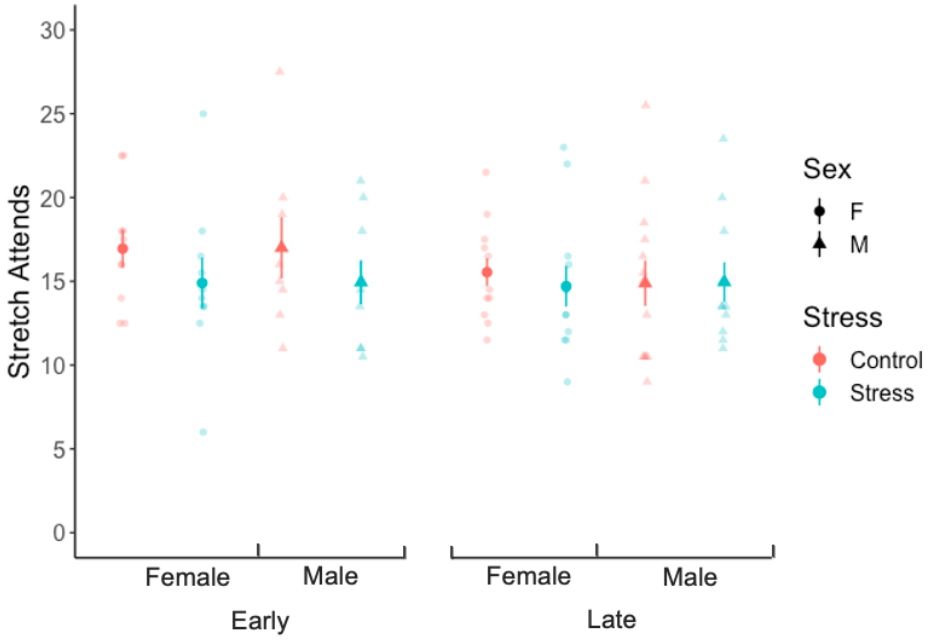

Figure 45. Frequency of stretch attends during the elevated plus maze test. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

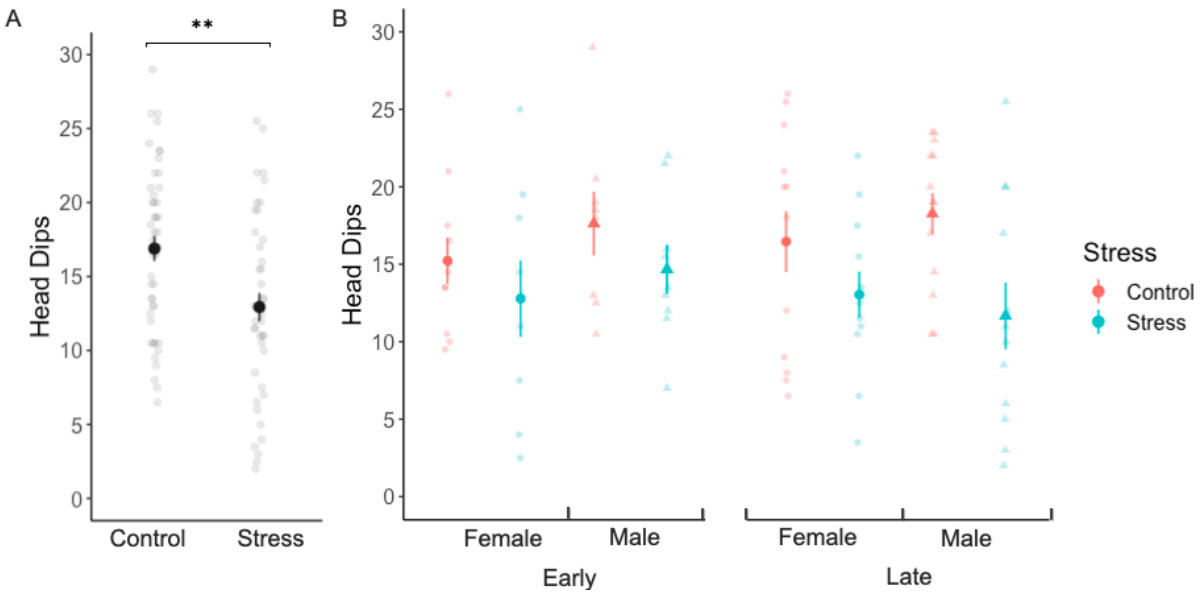

Figure 46. A) Main effect of stress on the B) frequency of head dips during the elevated plus maze test. **p<0.001. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

3.7 Forced Swim Test

A three-way ANOVA with age, sex, and stress treatment as factors was used to analyze the time spent floating during the forced swim test. There were no significant effects of either stress treatment ($F_{(1,78)}$=0.079, p=0.779), sex ($F_{(1,78)}$=0.9.38e-7, p=0.999) or age ($F_{(1,78)}$=2.234, p=0.139) on the time spent floating during the forced swim test (Figure 47). There were also no significant interactions [age x stress ($F_{(1,78)}$=0.592, p=0.444), age x sex ($F_{(1,78)}$= 2.110, p=0.150), stress x sex ($F_{(1,78)}$= 0.336, p=0.564), age x stress x sex ($F_{(1,78)}$= 1.846, p=0.178)].

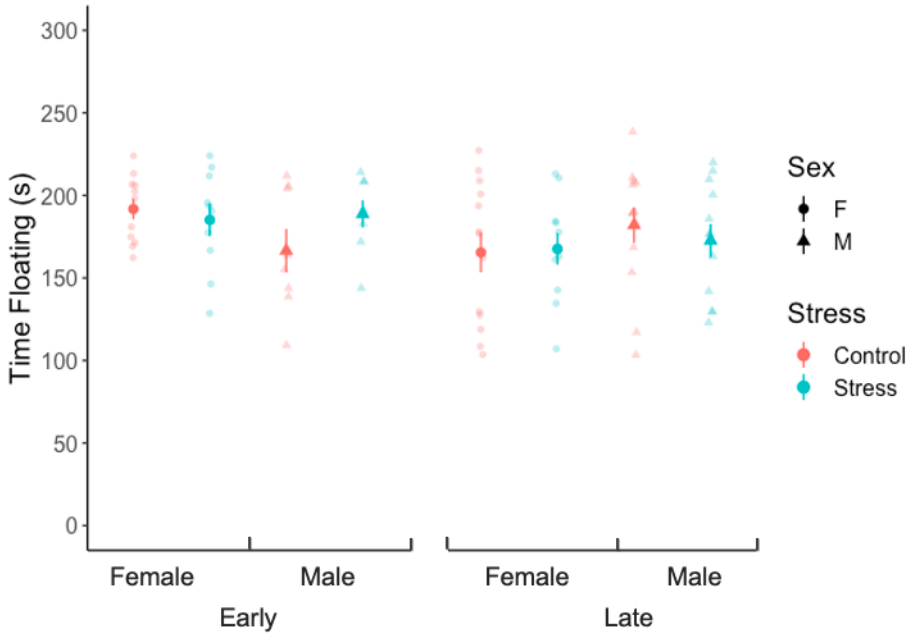

Figure 47. Time spent floating (s) during the forced swim test. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

3.8 Sucrose Preference

A three-way ANOVA with age, sex, and stress treatment as factors was used to analyze sucrose preference (Figure 48). There were no main effects of age ($F_{(1,78)}$= 0.053, p=0.818) or stress ($F_{(1,78)}$=0.043, p=0.835) treatment. However, there was a significant main effect of sex ($F_{(1,78)}$= 4.436, p=0.038, η^2p=0.054), with increased preference in females (0.752±0.102) compared to males (0.714±0.096; Figure 49A), according to pairwise comparisons with Bonferroni correction (t=2.11, p=0.038). This effect was modulated by a stress x sex interaction ($F_{(1,78)}$=6.320, p=0.014, η^2p=0.075; Figure 49B). Pairwise comparisons with Bonferroni correction indicated that while control females

(0.778±0.11) showed increased sucrose preference compared to control males (0.694±0.082; $t_{(78)}$=3.281, p=0.009), there was no sex difference in the stress group [males (0.734±0.107), females (0.724±0.084); $t_{(78)}$=-0.287, p=1.000]. There were no other significant interactions [age x stress ($F_{(1,78)}$=0.109, p=0.742), age x sex ($F_{(1,78)}$=3.639, p=0.060), age x stress x sex ($F_{(1,78)}$=1.959, p=0.166)] for this measure.

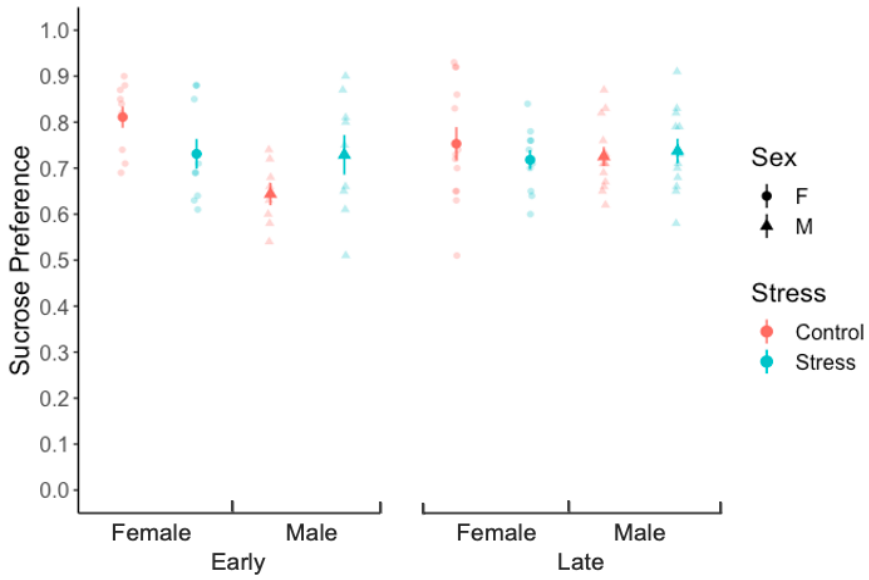

Figure 48. Sucrose preference ratios (consumption of sucrose solution relative to the total consumption of both sucrose solution and water). Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

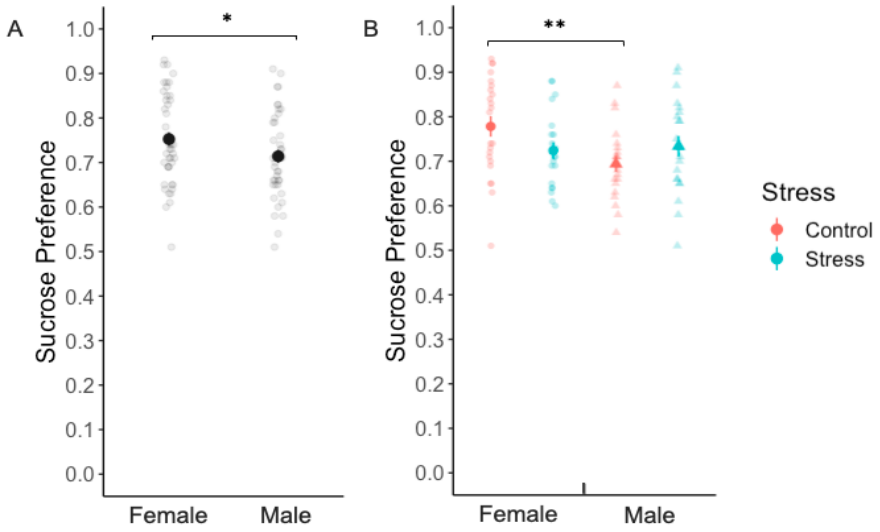

Figure 49. A) Main effect of sex and B) sex x stress interaction on sucrose preference ratios ratios (consumption of sucrose solution relative to the total consumption of both sucrose solution and water). *p<0.05. **p<0.01. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each mouse included in the analysis.

3.9 Maternal care and offspring body weight

The total litter size and number of male and female pups per litter were analyzed using a two-way ANOVA with age and stress treatment of the parents as factors. There were no significant main effects or interactions on the total litter size [age ($F_{(1,12)}$=6.32e$^{-4,}$ p=0.980), stress age ($F_{(1,12)}$=0.107, p=0.749), age x stress age ($F_{(1,12)}$=1.518, p=0.241)], number of males per litter [age ($F_{(1,12)}$=0.285, p=0.603), stress ($F_{(1,12)}$=0.425, p=0.527), age x stress ($F_{(1,12)}$=1.268, p=0.282)] or number of females per litter [age ($F_{(1,12)}$=0.323, p=0.580), stress ($F_{(1,12)}$=1.096, p=0.316, ($F_{(1,12)}$=0.108, p=0.748); Table 5]. The average

frequency of maternal and non-maternal behaviours per day (Table 6) were analyzed using two-way ANOVAs with age and stress treatment as factors. There were no main effects for either maternal [age ($F_{(1,12)}$=4.49, p=0.055), stress ($F_{(1,12)}$=0.06, p=0.806)] or non-maternal behaviors [age ($F_{(1,12)}$=4.441, p=0.057), stress ($F_{(1,12)}$=0.940)]. However, there was a significant age x stress interaction in both maternal ($F_{(1,12)}$=13.685, p=0.003, η^2p=0.533; Figure 50A) and non-maternal care ($F_{(1,12)}$=14.19, p=0.003, η^2p=0.542; Figure 50B), with opposite effects of stress in each age group. Stress decreased maternal behaviour [control (39.27±2.92 g), stress (31.75±2.15 g)] and increased non-maternal behaviour [control (20.72±2.92 g), stress (28.25±2.15 g)] in dams stressed in early adolescence but increased maternal behaviour [control (36.02±5.19 g), stress (43.3±2.12 g)] and decreased [control (23.97±5.19 g), stress (16.75±2.12 g)] non-maternal behaviour in dams stressed in late adolescence.

A repeated measures ANOVA with day as the within-subjects factor and age, sex, and stress treatment as the between-subjects factors was used to analyze pup body weight from birth to 21 days of age. There was a main effect of day ($F_{(3,48)}$=256.99, p<0.001, η^2p=0.941), with weight increasing from birth (1.35±0.166 g) to day 7 (3.65±0.887 g; $t_{(16)}$=-10.29, p<0.001), from day 7 to 14 (5.89±0.801 g; $t_{(16)}$=-8.41, p<0.001), and from day 14 to 21 (7.07±1.20 g; $t_{(16)}$=-7.12, p<0.001; Figure 51A), according to pairwise comparisons with Bonferroni correction. This effect was modulated by day x sex ($F_{(3,48)}$=3.04, p=0.0.038, η^2p =0.160; Figure 51B) and day x sex x age ($F_{(3,48)}$=2.96, p=0.042, η^2p=0.156; Figure 51C) interactions. Pairwise comparisons with Bonferroni correction indicated that weight increased for males from birth (1.30±0.123 g) to day 7 (3.52±0.541 g; $t_{(16)}$=-6.980, p<0.001), day 7 to 14 (5.71±7.11 g; $t_{(16)}$=-5.651, p=0.001),

and day 14 to 21(7.9±1.11 g; $t_{(16)}$=-8.887, p<0.001), but females did not increase their weight from day 14 (6.05±0.867 g) to 21 (6.88±1.28 g; $t_{(16)}$=-1.040, p=1.000), despite showing similar increases to males from birth (1.33±0.202 g) to day 7 (3.76±1.13 g; $t_{(16)}$=-7.579, p<0.001) and day 7 to 14 ($t_{(16)}$=-6.249, p<0.001). Furthermore, pairwise comparisons with Bonferroni correction indicated that, while females from younger parents did increase their weight from day 14 (5.96±1.04 g) to 21 (6.92±1.03 g; $t_{(16)}$=-6.802, p<0.001), females from older parents did not [day 14 (6.19±0.576 g), day 21 (6.82±1.70 g); $t_{(16)}$=2.749, p=1.000]. There were no other significant main effects [sex ($F_{(1,16)}$=0.448, p=0.513), age ($F_{(1,16)}$=0.118, p=0.735), stress ($F_{(1,16)}$=0.014, p=0.905)] or interactions [sex x age ($F_{(1,16)}$=0.7687, p=0.393), sex x stress ($F_{(1,16)}$=1.278, p=0.275), age x stress ($F_{(1,16)}$=2.001, p=0.176), sex x age x stress (F(1,16)=0.003, p=0.955), day x age ($F_{(3,48)}$=1.05, p=0.379), day x stress ($F_{(3,48)}$=1.97, p=0.132), day x sex x stress ($F_{(3,48)}$=0.1.06, p=0.375), day x age x stress ($F_{(3,48)}$=2.19, p=0.102), day x sex x age x stress ($F_{(3,48)}$=1.02, p=0.390) for pup body weights (Figure 52).

Table 5.

Number of pups (total and per sex) for each experimental group of dams.

Group	Dam	Litter size	Male pups	Female pups
Early Control (5/5)	61	6	2	4
	62	5	2	3
	63	2	0	2
	199	3	1	2
	200	5	3	2
Mean		4.2	1.6	2.6
Early Stress (4/7)	22	6	1	5
	23	9	3	6
	174	6	4	2
	197	2	0	2
Mean		5.75	2	3.75
Late Control (5/10)	15	5	1	4
	43	6	6	0
	38	7	3	4
	45	4	2	2
	156	5	3	2
Mean		5.4	3	2.4
Late Stress (2/10)	186	5	1	4
	152	4	2	2
Mean		4.5	1.5	3

Note. The number of litters obtained relative to the number of pairs bred per experimental group is shown as a fraction under each group name in the first column ("Group").

Table 6.

Average frequency for each maternal and non-maternal behaviour observed per day and aggregated averages for maternal and non-maternal behaviour per group. NB=Nest building, NLG=Nursing & licking and grooming, NCP= No contact with pups, FD=Feeding/drinking, SG=Self-grooming.

Group	Maternal				Non-maternal			
	Nursing	NB	NLG	Total	NCP	FD	SG	Total
Early Control	30.83	0.25	7.23	39.27	9.82	11.33	0.53	20.72
Early Stress	30.68	0.23	5.36	31.75	11.63	11.61	0.50	28.25
Late Control	32.51	0.35	4.14	36.02	11.96	10.71	0.33	23.97
Late Stress	25.56	0.40	13.03	43.25	6.65	13.62	0.71	16.75

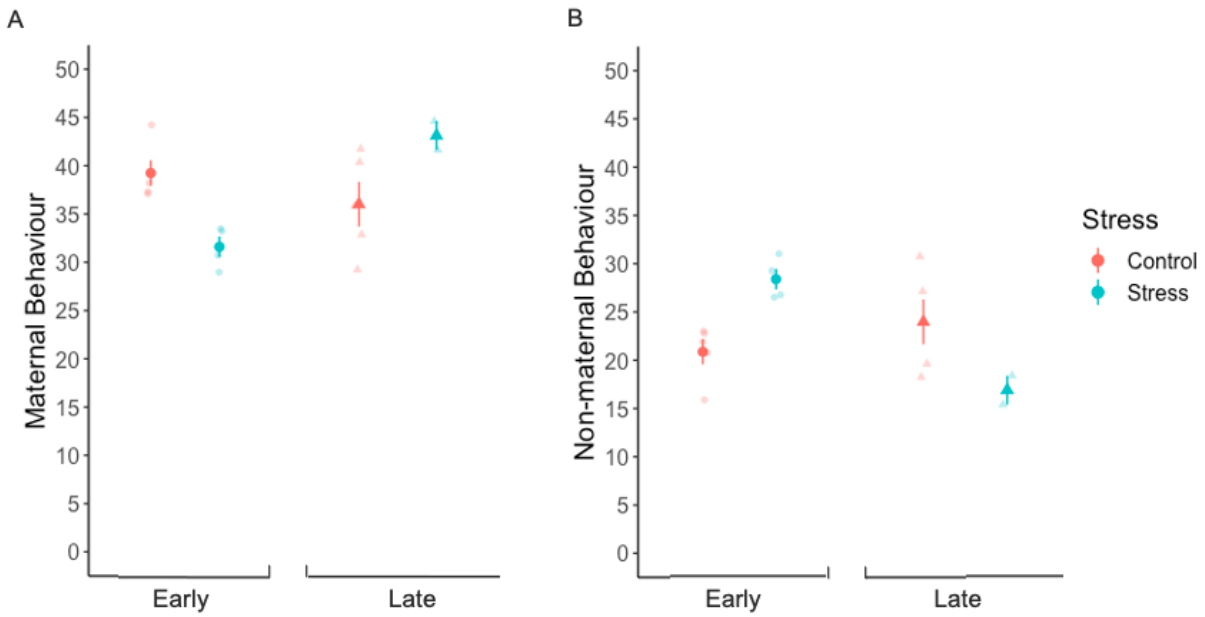

Figure 50. Significant age x stress interaction on the average daily frequency of maternal (A) and non-maternal (B) behaviours provided by dams to second-generation pups from birth to p7. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each dam included in the analysis.

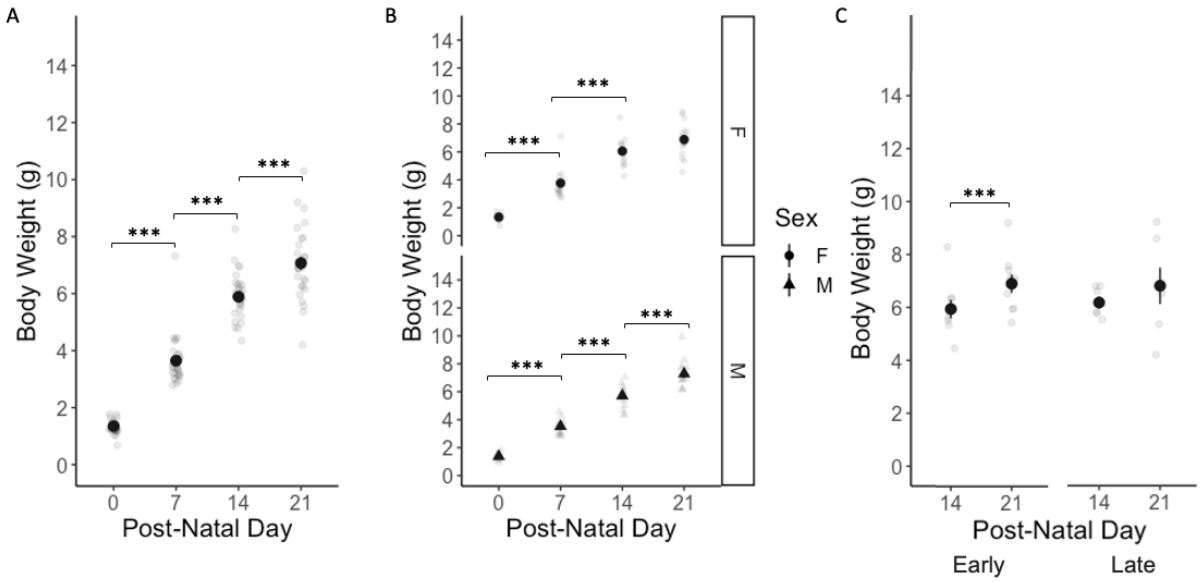

Figure 51. (A) Main effect of post-natal day, (B) sex x day interaction, and (C) sex x day x age interaction on body weight of second-generation pups. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each litter included in the analysis.

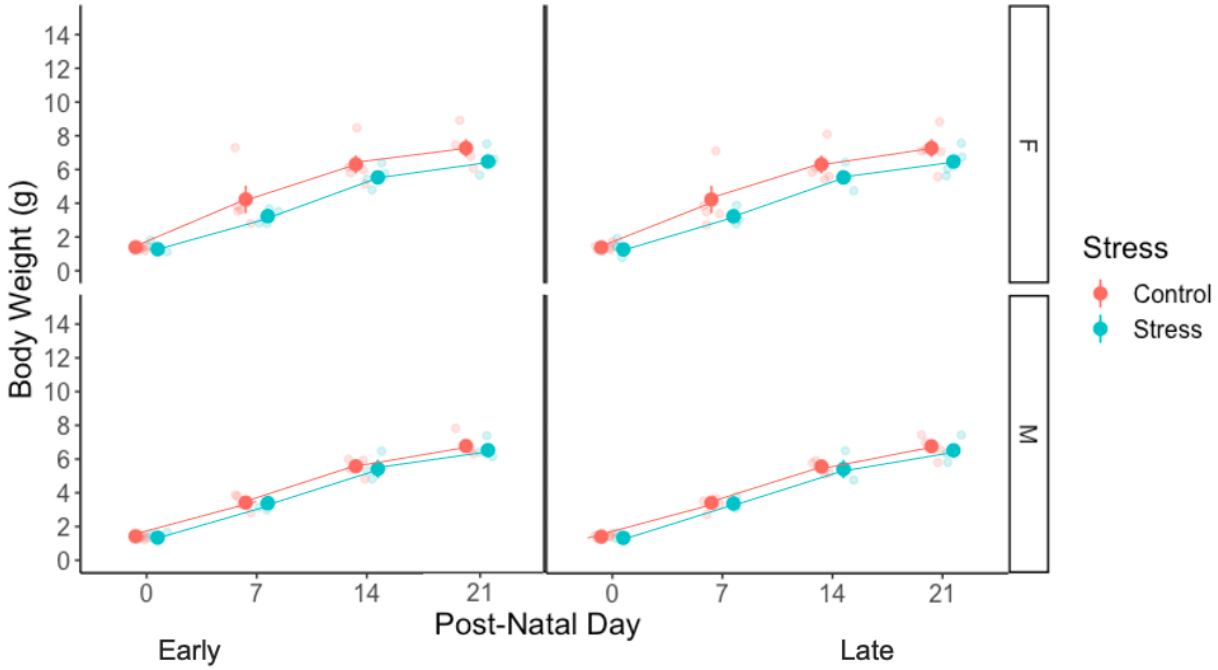

Figure 52. Body weights for days p0, p7, p14, and p21 of second-generation pups. Large points indicate group means. Error bars indicate standard error of the mean. Small points indicate individual data points for each litter included in the analysis.

Chapter 4: Discussion

Chronic predator stress during adolescence affected some anxiety-like, depression-like, and social behaviours in adulthood similarly across sexes and ages, which was expected given the extent of development changes occurring in the brain during this time. Nevertheless, some anxiety-like and social behaviours were affected differently depending on sex and the age of exposure, specifically rearing in the open field and elevated plus maze, and maternal care. Sex differences in the effects of stress and manifestations of these effects have been well reported in the literature. Thus, sex differences were expected in at least some of the measures collected. Furthermore, given the extensive development of brain structures, connectivity, and the HPA axis and the hormonal and behavioural differences across adolescence, some differences between the effects of early adolescence and late adolescence were also expected.

4.1 Response to predator stress

While food consumption was not affected by predator stress, there was a difference in water consumption between stressed mice and controls. In both age groups, stressed mice consumed more water during the 12 days of stress, but not before or after the stress period. Polydipsia has been reported in response to both physical and psychological stress (Dalooei et al., 2016; Goto et al., 2014), and this could indicate that our predator stress procedure similarly produced this effect. However, we cannot rule out that increased cage manipulation of stressed animals during the stressed period contributed to this finding. While controls remained undisturbed in their home cages, mice undergoing the stress procedure were routinely removed and returned to their cage,

which required additional manipulation of the water bottles in stress cages and could have resulted in water leakage during this period.

Although fecal samples were collected from all mice for corticosterone analysis, only a subset of samples were analyzed in this initial exploratory experiment. Corticosterone content was similar between males and females, but some data points that stood out as being higher than the rest were females. Statistical analysis of a larger data set could reveal a sex difference with females showing higher corticosterone levels than males, which would be consistent with the evidence that females have higher baseline and stress-induced corticosterone levels compared to males (Kalil et al., 2013).

Data for the day prior to stress (day 0) was slightly higher overall compared to the rest of the days for both age groups. This could be due to the time of sample collection. Although efforts were made to collect the fecal samples at similar points during the day, due to the constraints of the experimental design fecal samples were generally collected earlier in the dark cycle on the day prior to stress and later in the dark cycle on days on which mice were stressed, and fecal corticosterone has been reported to peak during the first third of the dark cycle and subsequently decrease throughout the dark cycle in both male and female mice (Touma et al., 2004). However, the difference in the late group seemed to be driven by a single high data point in the late control female group. After eliminating the outlier animal, corticosterone content is lower on the day prior to stress than on the first day of stress for the late control female group. Thus, it is more likely that the difference is only present in the early group and that it is due to increased stress from weaning, as mice stressed in early adolescence were weaned on the day prior to the start of the stress period.

Moreover, the mean corticosterone content for the control groups was slightly higher than for the stress groups for the day prior to stress and the first day of stress, while it was slightly higher for the stress groups midway through the stress period and on the last day of stress. This could indicate that there was no immediate reaction to the acute stress of the first day, but there was a progressive accumulation of stress that became apparent in corticosterone levels by the middle of the stress period. This would indicate that the rotation of stimulus rats that was used in this study effectively prevented habituation of the mice to the paradigm. The possibility of stress having affected corticosterone levels is supported by the changes found in water consumption and in behaviour. The effects of stress found in the behavioural test battery will be described in the following sections. As only a subset of samples were analyzed, it is possible that statistical analysis of all samples collected for all subjects from this experiment could confirm the potential effects of stress on corticosterone levels described here.

Despite the number of fecal pellets produced during stress sessions being higher overall for day 6 compared to day 10, and higher for day 7 compared to day 10 only for female mice, there was no distinguishable pattern of change across the stress period. However, there was a clear age difference, with mice in the early group producing more fecal pellets than mice in the late group across the multiple days of predator stress. As defecation in mice indicates emotionality in response to aversive stimuli (Carola et al., 2002; Lister, 1990), this result indicates higher immediate reactivity to early adolescent stress compared to late adolescent stress, which is consistent with the increased sensitivity of mice stressed in early adolescence in some measures collected in the

behavioral test battery, and with the increased susceptibility shown in subsequent maternal behaviours. These effects will be described in the following sections.

4.2 Social behaviours

The olfactory habituation/dishabituation and three-chamber sociability tests were used to assess social behaviour. In the olfactory habituation/dishabituation test, the odour presented had a significant effect on habituation and dishabituation in both age groups, with the social odour being the most salient of the four. This suggests that all mice showed a capacity to discriminate the different odours from each other and that all mice showed a similar preference for the social odour over the non-social odours. Sex significantly affected habituation, with females showing increased habituation to the odours compared to males. This could indicate superior olfactory memory in females compared to males leading them to habituate more rapidly than males to familiar odours. While this is a possibility, it would contradict the more commonly reported sex difference in olfactory memory in which males outperform females (Mihalick et al., 2000). Nevertheless, females have also been reported to either perform better or worse than males in olfactory tasks depending on the intensity of the odour (Roddick et al., 2016). Therefore, females might have outperformed males in terms of olfactory memory at the odour concentration used here. The increased habituation could also indicate overall decreased exploratory interest in the females leading to increased habituation after the initial novel exposure, and decreased exploration in females compared to males has been previously reported in mice (An et al., 2011; Bondar et al., 2018; Gioiosa et al., 2007). However, the increased habituation was not accompanied by decreased dishabituation,

which would also result from decreased exploration more generally in females. Therefore, it is more likely that this result is due to superior olfactory memory in females.

Dishabituation to the social odour, but not for the non-social odours, was higher for the mice of the early group, indicating increased social interest in the younger mice. This is consistent with evidence of younger mice exhibiting decreased anxiety and increased risk taking and reward seeking behaviours compared to older mice (Shoji et al., 2016). There were no significant main effects of stress or interactions between stress and other factors in the olfactory habituation / dishabituation test. It is possible that the social stimuli were not salient enough to cause a differential response, as there was no direct contact with the conspecific. It has been shown that different modes of exposure to social stimuli are distinct at the neuronal level (Contestabile et al., 2021). When exposed to a conspecific, mice exhibited increased firing of dopaminergic neurons in the VTA, a brain area involved in reward processing, but this was not observed when mice were exposed to a social odour (Contestabile et al., 2021).

Evidence that the modality of the stimulus is important for identifying stress-induced differences in social response was provided by findings in the three-chamber test, where stress did seem to differentially impact social exploration. All mice investigated the social stimulus more than the novel object, suggesting that the social stimulus was appetitive, but adolescent stress further increased social preference ratios in the three-chamber test. This contradicts the literature regarding the effects of stress, which reported that stress in adulthood (van der Kooij, Fantin, Kraev, et al., 2014; van der Kooij, Fantin, Rejmak, et al., 2014; Wood et al., 2003) and adolescence (Van den Berg, 1999) results in decreased sociability. However, stress-induced increases in

sociability have also been reported (Bondar et al., 2018; Borrow et al., 2018; Shimozuru et al., 2008) and it has been proposed that mice may attempt to increase social interaction in order to buffer their anxiety in aversive situations (Sandi & Haller, 2015). Therefore, our findings of increased social preference in stressed mice might be a result of increased anxiety.

Similar to the olfactory habituation/dishabituation, the three-chamber test also demonstrated a significant sex difference driven by the social stimulus, with males investigating the social stimulus more than females, but no sex difference for the novel object. This effect is consistent with the decreased investigation of odours in females found in the olfactory habituation / dishabituation test. However, while this sex difference was present for both social and non-social stimuli in the olfactory test, it was not present in the investigation of the non-social stimulus in the three-chamber test, suggesting that some quality of the social stimulus was specifically attractive to males but not females. This is consistent with the findings of Karlsson et al. (2015) that intact male mice investigate a novel conspecific more than gonadectomized males as well as intact and gonadectomized females do (Karlsson et al., 2015). The authors concluded that males investigate social stimuli more than females and that this depends on levels of circulating hormones released from the testis (Karlsson et al., 2015). Furthermore, the stimulus mice used for this test were sexually experienced and sexual experience has been reported to change hormonal factors such as the abundance of androgen receptors in the medial pre-optic area (Swaney et al., 2012), which is involved in sexual and aggressive behaviour (Cunningham et al., 2021). Experimental mice may have been receiving aggressive visual, acoustic, or olfactory cues from the sexually experienced males. Age also affected

investigation time, with younger mice investigating the social stimulus more than older mice. This is consistent with the increased dishabituation to the social odour in the younger mice found in the olfactory test.

In the three-chamber test, there was also a sex difference, with social preference being higher for males, which is to be expected given the higher investigation of the social stimulus in male mice. In the habituation phase of the test, there were no significant main effects of cup side or interactions of side with other factors, indicating that the stress-induced increase in social preference was not due to a side preference. Stress did eliminate a sex difference, as the difference present in the control group with control males investigating the stimuli more than control females, was not present in the stress group. Given that this effect was present for both stimuli, it is likely that the stress-induced increase in sociability found in the testing phase was not the result of this interaction. As the increased exploration in males in the three-chamber test contradicts the possibility of increased exploration in females found in the olfactory habituation/dishabituation test, it is more likely that the latter sex differences was due to differences in olfactory memory rather than overall exploration.

The stress-induced elimination of the sex difference could indicate masculinization of females resulting from stress in adolescence, as time investigating the stimuli increased for stressed females to a level similar to control males. Although the literature on stress-induced masculinization of females is limited, masculinization of ano-genital distance has been previously reported in female mice (Zielinski et al., 1991) as well as parental care for pups similar to that observed for control males (Del Cerro et al., 2015) as a result of pre-natal stress. The masculinization of maternal behaviour was

linked to an increase in the number of neurons in the bed nucleus of the accessory olfactory tract (BAOT) in females to a level similar to control males, along with a reduction of estradiol levels in stressed females compared to control females (Del Cerro et al., 2015). Such changes have been proposed to be mediated by ACTH release during the stress response (Del Cerro et al., 2015), as ACTH has been reported to stimulate adrenal progesterone (Feder & Ruf, 1969) and androgens (including dehydroepiandrosterone, dehydroepiandrosterone sulphate, and androstenedione; Odell & Parker, 1984) secretion. Pre-natal stress has also been reported to result in feminization of males shown as increased lordosis (Dahlöf et al., 1977) and demasculinization of glutamate-related protein levels (mGlu receptors, ionotropic glutamate receptors, and glutamate transporters) in the hippocampus and PFC (Verhaeghe et al., 2021). Stress in adulthood has also been linked to the elimination of sex differences (Wright et al., 2017). While control females showed higher levels of *Dnmt1* in the central amygdala compared to males, social defeat in adulthood resulted in the elimination of this sex difference in stressed animals (Wright et al., 2017), suggesting that stress resulted in changes in DNA methylation patterns in the central amygdala that led to stressed males and females being more similar to each other than controls. As, similar to the prenatal period, adolescence is a time of significant development of hormonal systems, neurotransmitter systems, and the epigenome, stress during this time could have altered neurotransmission, protein expression, epigenetic markers, and amounts and/or proportions of sex hormones in both male and female mice, particularly in stress-associated areas such as the amygdala, hippocampus, and PFC.

Stress decreased maternal behavior relative to non-maternal behaviour for dams stressed in early adolescence but increased it for dams stressed in late adolescence. This was not a result of differences in litter size or sex composition of litters between experimental groups, as there were no differences in any of these factors based on parental age or stress treatment. Although the immediate effects of early-life stress on maternal behaviour of the mothers of stressed mice has been well studied, the effects of stress on the subsequent maternal care provided by stressed females to their pups have not been investigated. However, given that other aspects of social behaviours have been affected by stress, it is not surprising that maternal behaviour was also affected. Nevertheless, whether this difference in maternal care resulted from stress-induced effects in the mothers, or from differences in the offspring of stressed animals transmitted from the parents, is not known at this stage. Parental stress did not affect the body weights of second-generation pups despite the differences in maternal care, which is consistent with a lack of effects of parental stress on pup body weight previously reported (Franklin et al., 2010). There was a difference in female pup body weight based on the age of the parents, as females from younger parents increased their weight from post-natal day 14 to 21, but females from older parents did not. This seems to be a result of females from older dams growing at an increased rate, as females from dams of both age groups were the same weigh at weaning but females from older dams reached this weight by post-natal day 14. Overall, these results indicate that stress in early adolescence led to impaired maternal care while stress in late adolescence resulted in a possibly compensatory increase in maternal care, but neither led to differences in pup body weight.

4.3 Depression behaviour

The forced swim test and the sucrose preference test were used to assess depression-like behaviours in response to stress. No effects of stress were found in either age group in the forced swim test. Time spent floating in the forced swim test has been suggested to measure either helplessness (Can et al., 2011) or the ability to adapt to stressful situations (Molendijk & de Kloet, 2015), both aspects that could be related to depression in humans. While this was not affected by our stress treatment, there was an effect of stress in the sucrose preference test, indicating that at least one depression-like behavior is altered in stressed mice.

This effect was different in males and females, as stress decreased sucrose preference in females but increased it in males resulting in the elimination of a sex difference that was present in the control animals in which control females showed higher sucrose preference than males. This is similar to the interaction found in the habituation phase of the three-chamber test, where a sex difference in investigation that was present in the control group was eliminated by stress. Therefore, the results from the sucrose preference test support the possibility of feminization of males and/or masculinization of females resulting from stress in early adolescence. Stress affecting males and females differently is consistent with the literature, as stress has been reported to reduce sucrose preference in females while it remained unchanged in males (Goodwill, et al., 2019), but the increased sucrose preference found in males here is surprising. Nevertheless, stress has been reported to increase sucrose preference in some rare cases (Dess, 1992; Sachs et al., 2014). Similar to the reports of rodents seeking social interaction to alleviate their

anxiety, it has been proposed that certain flavours such as those of milk, sugars, or fats, serve to buffer stress by triggering opioid release (Blass et al., 1989; Dess, 1992). There were no interactions between age and stress treatment in either test, suggesting that both early and late adolescent stress affected mice in a similar manner. Overall, these results suggest that, during both periods of adolescence, stress affects anhedonia in an opposite manner in male and female mice.

4.4 Anxiety behaviour

The open field, light dark box, and elevated plus maze tests were used to assess anxiety-like behaviours. There were no changes in anxiety-related measures in the open field. However, stressed mice did display hyperactivity, a behaviour previously reported in rodents exposed to isolation stress. Rodents exposed to social isolation show increased time in movement in the open field (Reinwald et al., 2018), increased locomotion in a novel environment (Niwa et al., 2013) and increased object exploration in a sociability test (Lander et al., 2017). Hyperactivity has been linked to abnormal dopaminergic activity, which is common in disorders such as schizophrenia, attention deficit disorder and drug addiction (Niwa et al., 2013; Zhuang et al., 2001). Moreover, increases in walking, overall arm visits in the elevated plus maze, transitions in the light dark box and distance travelled in the open field have been reported in response to foot-shock and CUMS (Füzesi et al., 2016; Mineur et al., 2006). There was also a sex difference, with females travelling longer distances than males, which is consistent with previous reports of females being more sensitive to acute stress than males (An et al., 2011; Goel & Bale, 2008). First-time exposure to the novel open field apparatus likely posed some

aversiveness that might have affected females more strongly than males, which manifested in hyperactivity as females compared to males.

In adult mice stressed during early adolescence, stress affected males and females differently, eliminating a sex difference present in the control group. While control females reared less than control males, early-adolescent stress increased rearing for females and decreased it for males, resulting in similar rearing frequencies for stressed males and females. Similar to the stress-induced elimination of sex differences in control mice related to both investigation in the three-chamber test and sucrose preference, the results found here could also indicate feminization of males and/or masculinization of females resulting from stress in early adolescence.

In the light/dark box, there were no differences based on sex or stress treatment in either age group, which is contrary to reports of reduced time spent in the light area after stress (Adamec et al., 2006; Samad et al., 2018; Samad & Saleem, 2018). This might be a result of testing conditions not producing the level of aversiveness necessary to elicit an anxiety-like response in the mice, as the light/dark box is known to be sensitive to factors such as lighting and structure of the apparatus resulting in mixed reports in the literature (Bourin & Hascoët, 2003). However, most studies that have previously reported behaviour in this test having been affected by stress performed the test immediately or soon after acute stress and in mouse strains other than C57Bl6 or in rats, while we used the test over a month after stress in C57Bl6 mice, suggesting that the behaviour in the light dark box of the animals that we used might be less sensitive to chronic stress compared to the animals used in previous studies. Furthermore, the percentage of time spent in the light area here for all mice (33% of total test time) is similar to what has been

previously reported in unstressed rodents previously (approximately 30 to 40 %; (Adamec et al., 2006; Kulesskaya & Voikar, 2014; Mineur et al., 2006; Rock et al., 2017; Samad et al., 2018; Samad & Saleem, 2018). This suggests that testing conditions were similar to that of other laboratories, including those that found reduced time in the light area in response to stress. Thus, these results indicate that the anxiety-like behaviour of our mice in the light/dark box was unaffected by the chronic psychological stress procedure used in this study. Mineur et al. (2006) reported a similar outcome in which the behaviour of C57Bl6 mice in the light dark box was unaffected by CUMS. Nonetheless, we did find effects of stress on anxiety-like behaviour in the elevated plus maze.

In the elevated plus maze, stressed mice spent reduced time in the open arms relative to the closed arms of the maze, indicating that stress in adolescence increased anxiety levels in these mice. This was likely a result of the increased time in the closed arms of the maze in stressed mice. Stressed mice also showed decreased head dips compared to controls, which also indicates an anxiety-like state. Decreased time in the open arms relative to the closed arms and decreased frequency of head dips in the elevated plus maze are commonly reported in the literature in response to chronic stress (Blundell et al., 2005; Cohen et al., 2006; Doremus-Fitzwater et al., 2009; Harris et al., 2018; K.-S. Kim & Han, 2006; Liang et al., 2014; Reiss et al., 2007; Schmidt et al., 2007). Females also spent more time in the closed arms than males, corroborating the sex difference in anxiety found in the open field test and suggesting that females have higher baseline anxiety levels than males.

Although the increased time in the closed arms in stressed mice was not accompanied by decreased time in the open arms, this can be explained by differences in

time spent on the centre platform, which was not recorded here. If all mice spent similar amounts of time in the open arms, but controls split the remaining time of the test between the centre platform and the closed arms while stressed mice spent the rest of the test in the closed arms only, this would result in increased time in the closed arms for stressed mice but no differences in time spent in the open arms. Decreased time in the centre would also reflect increased anxiety in the stressed mice, as the centre platform offers less protection than the closed arms. Accordingly, time in the centre platform of the elevated plus maze has been reported to correlate with impulsivity and anxiety levels in rodents (Albani et al., 2015; Rico et al., 2017).

Stress increased rearing in the closed arms only in males of the early group, which contradicts the decrease in rearing in the same mice in the open field test but is consistent with the overall hyperactivity in stressed mice. The relatively protected area of the closed arm likely allowed increased risk assessment in a safe space for these stressed mice. In the open field, mice were unprotected and the aversive nature of the arena likely inhibited rearing behaviour. Alternatively, the differences in rearing between tests could be due to differences in overall aversiveness between the open field and the elevated plus maze, as anxiety could be reflected by opposite changes in rearing depending on the levels of stress induced by the tests. It has been proposed that grooming follows an inverse u-shaped curve, increasing in situations of moderate aversiveness and decreasing in response to extreme aversiveness (Fernández-Teruel & Estanislau, 2016). The results here could indicate a similar u-shaped curve for rearing leading to different results in differently aversive tests.

Overall, stress led to reductions in head dips and time in the open arms relative to the closed arms of the elevated plus maze, all of which indicate increased anxiety in stressed mice, and hyperactivity in the open field. Early-adolescent stress also affected rearing in the early group but led to different pattens in each sex in both the open field and the elevated plus maze. In the open field, rearing increased in females and decreased it in males, resulting in the elimination of the sex difference present in the control group. In the elevated plus maze, stress increased rearing in males but did not affect it in females. The differences in rearing between the two tests might be due to differences in the overall aversiveness and rearing locations in each test. Despite these differences, the effects of stress on rearing frequency were consistently found to be different in males and females and were only present in the early group.

Chapter 5: Conclusion

Chronic predator stress during either early or late adolescence can have a persistent impact on behavioural responses in adulthood. Stress in both periods of adolescence led to a reduction in both head dips and time in the open arms relative to the closed arms of the elevated plus maze, both of which indicate increased anxiety in stressed mice. Stressed mice also showed hyperactivity in the open field. This could reflect the frequent comorbidity seen in humans between neuropsychiatric disorders that include hyperactivity as a symptom, such as attention deficit hyperactivity disorder (ADHD) and schizophrenia, and anxiety disorders (American Psychiatric Association, 2013).

Stress in both periods of adolescence led to an increase in social preference in both male and female mice. Given that this result does not support the commonly reported decrease in sociability resulting from stress, it is likely to be an indicator of increased anxiety in stressed mice and an attempt to buffer this anxiety through increased social interaction. This possibility is supported by the increased anxiety-like behaviour resulting from stress found in the elevated plus maze.

While the effects of stress on these aspects of anxiety and social behaviour did not differ between sexes or age groups, stress-induced changes in rearing behaviour were dependent on both factors. In the elevated plus maze, stress increased rearing in males but did not affect rearing in females. Meanwhile, in the open field, stress led to increased rearing in females but decreased rearing in males. Both of these effects were specific to mice that experienced stress in early adolescence.

While they were not age-dependent, results from the three-chamber and sucrose preference tests also differed between males and females. Stress did not affect the helplessness or coping aspect of depression-like behaviour in the forced swim test, but it did affect anhedonia in the sucrose preference test. Control females showed higher sucrose preference than control males, but stress decreased sucrose preference in females and increased it in males resulting in the elimination of the sex difference. Similarly, in the habituation phase of the three-chamber test, control males investigated the empty cups more than control females, but stress decreased exploration in males and increased it in females resulting in the elimination of the sex differences. This is consistent with the results of the open field, with rearing decreasing in males and increasing in females. While the decreased sucrose preference in females was expected as it is a common result of chronic stress, the preference increase in males is surprising as chronic stress rarely increases sucrose preference. The increased sucrose preference in males may indicate increased anxiety rather than a change in depression-like behaviour. I suggest that while females showed susceptibility to the effects of stress on anhedonia, males showed an attempt to cope with the increased anxiety suggested by the anxiety tests.

These results indicate that our predator stress procedure in both early and late adolescence produced increased social and anxiety-like behaviour as well as hyperactivity in both male and female mice, and anhedonia in females but did not induce depressive-like helplessness. Furthermore, the findings demonstrate that adolescent stress does affect male and female mice differently, and early adolescent mice may be more sensitive than late adolescent mice, at least in some aspects of anxiety-like behaviour. Thus, this procedure can be an effective method to model the effects of chronic

psychological stress, as well as the sex and age differences seen in humans exposed to stress. This adds to the existing evidence that stress in adolescence leads to long-lasting changes in behaviour, that the brain can be more sensitive to stress earlier in life in relation to exploratory/risk assessment behaviour, and that males and females are affected differently by stress in relation to exploratory/risk assessment behaviour and anhedonia.

These results demonstrate the importance of investigating adolescence both as a whole, and with different defined sub-periods within adolescence, as potential sensitive periods of development, particularly in the context of stress. This is significant for researchers investigating stress as it is essential that they additionally take into account the sex of the control and experimental animals. Historically, it has been common in health research to hold males as the standard for diagnosis of many illnesses, including those of the brain (Shansky & Murphy, 2021). Although the prevalence of many neuropsychiatric disorders is higher in women (American Psychiatric Association, 2013), men and women present different symptoms of the same illnesses (Andersen & Teicher, 2008), and research from animal models, including our study, has shown that males and females react differently to stress (Adamec et al., 2006), the majority of stress research has only included male subjects (Beery & Zucker, 2011). While the inclusion of females in biomedical research has improved in the last decade, only about half of studies in 2019 included both male and female subjects (Woitowich et al., 2020). Thus, there is still a need to improve the inclusion of females in research of the effects of stress.

The results here are also important to adolescent clinical research, particularly related to pre-pubescent adolescents, as they might be especially susceptible to the development of neuropsychiatric diseases as a result of stress exposure. Furthermore,

adolescence might provide a window of opportunity for intervention, leading to potentially improved outcomes. These results are also significant for the descendants of individuals who have experienced stress in adolescence, as maternal care was affected by stress in both early and late adolescence in this study. Maternal early-life trauma has been reported to predict post-partum depression (Choi et al., 2017), which in turn predicts maternal bonding problems and negative impacts on physical growth (Choi et al., 2017) and emotional and behavioural problems in childhood (Bor et al., 2003), which can then lead to increased risk for psychopathology. Thus, early intervention might prevent adverse effects of stress not only in the stressed individuals, but also in a second generation.

Some limitations of this study should be noted. Although the Plexiglas enclosure in which mice were exposed to the rats allowed for the exchange of visual, auditory, and olfactory stimuli between the animals, it did not allow for physical manipulation of the mouse. While Barnum et al. (2012) used plastic hamster balls that could be physically manipulated by rats, these hamster balls did not prove to be strong enough to resist manipulation and biting from the rats in this study, and thus posed physical danger to the mice. Therefore, we opted for the use of more resistant Plexiglas enclosures. Perhaps an enclosure that can be manipulated similarly to the hamster balls, but made or a more resistant material, can produce stronger effects and model psychological stress more closely. Furthermore, possible confounding effects of the behaviour of individual rats on the levels of stress produced in the mice were not accounted for here. Future studies could benefit from recording the interactions between mice and predators during exposure, which would provide additional insight into individual differences in the

stressed mice. Finally, the time of fecal sample collection could be improved. Fecal samples for corticosterone analysis were collected 6 hours after stress for all mice given that corticosterone is metabolized into the feces from 4 to 10 hours depending on the time of day (Touma et al., 2004) and the stress procedure was done at varying times of the day to reduce the predictability of the stress. Although a standardized time lag of 6 hours after stress for all mice allowed for more efficient timing of daily stress sessions and fecal sample collection, it might not have resulted in the fecal samples obtained being the most reflective of peak corticosterone levels during stress, as this would vary between 4 hours if the stress was experienced at the start of the dark cycle and 10 hours if the stress was experienced at the beginning of the light cycle. Different collection times depending on the timing of stress for each day could yield more accurate analysis of corticosterone levels as a result of stress. Furthermore, depending on the qualities of the particular stressor, the corticosterone reaction to stress can be stronger if the stressor is experienced during the light or dark phase of the light cycle. Future studies could determine the time of peak corticosterone reactivity to stress specifically for this stress paradigm and adapt the timing of stress accordingly to see the effects of stress on corticosterone levels more clearly (Retana-Márquez et al., 2003). It is also important to note that the high coefficients of variation in the ELISA performed (up to 50%) limits the reliability of these exploratory results and that while corticosterone is the main target of the assay, other steroids such as progesterone, aldosterone, testosterone, and estradiol can be detected. Despite these limitations, our small dataset of fecal samples suggested the possibility of no initial effect of the acute stress of the first day of predator stress on corticosterone levels along with an accumulation of stress by the midpoint of the stress

period through to the final day of stress reflected in increased corticosterone in stressed mice compared to controls. More studies are needed that investigate the progression of stress-induced changes in corticosterone levels throughout a chronic psychological stress treatment. Such studies would provide detailed insight into the progression on the HPA axis response to chronic stress.

Given that the majority of stressors that adolescents face are psychological, it is essential that the effects of psychological stress during this period of development continue to be investigated. Moreover, more studies that include both male and female subjects and that investigate different sub-periods within adolescence are needed. Additional studies that investigate the effects of stress on the maternal care provided by stressed mothers are also needed as this has not been explored in depth in rodents. Whether the effects of stress in adolescence on maternal care found in this study resulted from stress-induced changes in social behaviour in the dams, or from different demands from pups born of stressed parents stemming from the transmission of stress-induced changes through genetic mechanisms, remains unclear. Future studies could clarify this with cross-fostering experiments after pups from stressed parents are born. This could help differentiate between effects transmitted through behaviour and through genetic mechanisms, as well as between maternal care differences caused by the mothers and differences caused by demands from the offspring. Regardless of whether the differences in maternal care were produced by stressed mothers or by their offspring, both possibilities indicate that the offspring can be affected by the stress experienced by the parents. Future studies should assess anxiety-like, depression-like, and social behaviour in a second generation of mice to determine whether the effects of stress found in the first

generation are transmitted to offspring. It has been shown that the effects caused by early-life stress in a first generation can be transmitted to a third generation even if the second generation does not seem to be affected, meaning that stress-induced behavioural changes can be transmitted to offspring without being outwardly shown (Franklin et al., 2010, 2011). Therefore, behaviours that seemed to be unaffected by adolescent stress in the stressed generation could be affected in subsequent second and third generations, leading to different behavioural patterns in different generations. Given that any effects found in the offspring of stressed animals could be transmitted not only through maternal care but also via the germline by either the stressed fathers (Franklin et al., 2010, 2011), or both the stressed mothers and fathers (Saavedra-Rodríguez & Feig, 2013; Weiss et al., 2011), future studies should also investigate the molecular processes underlying transmission of the effects of adolescent stress and whether they differ between early-adolescent and late adolescent stress.

Although changes in sociability and maternal care were observed in stressed mice these are only a limited portion of the wide range of social behaviours that could be affected by stress, and while the research on the effects of adolescent stress on social behaviour is limited, the range of social behaviours that have been studied in this context is even more narrow. Even though clinical research has linked stress-related mental illness to impairments in several aspects of social behaviour (including social withdrawal, aggressive behaviour, social anxiety, and social avoidance; American Psychiatric Association, 2013) the many constraints posed by clinical research have rendered the direct investigation of how stress affects specific aspects of social behaviour very difficult. Therefore, future studies using animal models should investigate other more

complex social behaviours such as juvenile play, empathy, and adult social interaction with unfamiliar conspecifics and with cage-mates to gain more insight into how adolescent stress affects different aspects of social behaviour.

Given the evidence of differential effects of stress at different timepoints of adolescence, future studies should also investigate the neural developmental changes underlying differences between the effects of early and late adolescent stress. Areas involved in the stress response as well as emotional and cognitive regulation and learning and memory like the amygdala, PFC, and hippocampus (Andersen & Teicher, 2008; Godoy et al., 2018; Joëls & Baram, 2009; Sandi & Haller, 2015) undergo important structural changes throughout adolescence, and thus are likely affected differently depending on the developmental state of each brain structure at the time of stress exposure. For example, cortical grey matter density peaks at around 12 years old in adolescents, and this is followed by a period of significant pruning of synapses and receptors that occurs throughout the rest of adolescence until the early 20s, meaning that cortical synaptic development occurs throughout adolescence until adulthood. Thus, the developmental state of the cortex in early adolescence is likely very different from late adolescence. Furthermore, subcortical changes have been shown to occur earlier in development than cortical changes (Andersen, 2003). Thus, adolescents who have experienced trauma at 11 to 13 years of age have shown maximal reductions of hippocampal volume while adolescents who experienced trauma at 14 to 16 years exhibit maximal reductions of frontal cortex volume (Andersen & Teicher, 2008), indicating specific windows of vulnerability to stress for each brain area. Moreover, there is an increased exposure to gonadal hormones during puberty that directs the development of

the PFC, amygdala, and hypothalamus (Andersen & Teicher, 2008), likely leading to significant changes in development between pre-pubertal and post-pubertal adolescence. Consequently, the differential effects of stress at different points of adolescence is likely reflected in different changes in the structural and functional development of these brain areas. Investigation of the state of the adult brain after either early-adolescent or late-adolescent stress would elucidate the neurological changes underlying behavioral effects such as those found in this study.

Finally, the results found for stimuli investigation during the habituation phase of the three-chamber test, sucrose preference, and rearing in the open field suggested the possibility of stress-induced feminization of males and/or masculinization of females. This phenomenon has mostly been studied in relation to the effects of pre-natal stress on sexual and reproductive behaviours but has not been as thoroughly investigated in relation to the behavioural effects of adolescent stress. Like the pre-natal stage, adolescence is characterized by significant neural programming and hormonal exposure, which could lead to feminization and or/masculinization of behaviour, hormonal systems and expression of proteins and epigenetic markers. This is important for adolescent clinical research, as stressful life events in utero have been linked to masculinization of ano-genital distance in female infants (Barrett et al., 2013) and masculinized play behaviour in girls similarly to reports in the rodent literature. This suggests that the pre-natal stress-induced disruption of sexually dimorphic characteristics reported in rodents can also be present in humans and adolescent stress could have a similar effect. Future studies investigating the effects of adolescent stress on hormonal systems in adulthood could help elucidate whether stress in adolescence can produce masculinization of

females and feminization of males and what molecular and hormonal mechanisms potentially underlie these changes.

By adding to this line of research, new insights can be gained into the consequences of stress in adolescence in males and females to better inform researchers using animal models and to establish times of increased risk for neuropsychiatric disease and of optimal intervention leading to improved results.

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