Sexually dimorphic effects of prenatal stress on physical growth and stress-related behaviors in prepubertal mouse offspring

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Abstract

Several factors can modulate the link between fetal disruptions and later-life illnesses. The main objective of this thesis was to determine, in a mouse model, the impact of prenatal stressor timing and offspring sex on prepubertal metabolic and mental health outcomes. C57BL/6 dams in the first or second trimester of pregnancy experienced a restraint stressor or were left undisturbed. Pups were weighed daily until postnatal day (PND) 21, at which time fat distribution was measured. Anxiety- and depressive-like behaviors were tested on PND19-20 in open field, elevated plus maze, splash and tail suspension tests. Second trimester stressed males gained more weight and had increased fat deposits surrounding the kidneys. Although anxiety- and depressive-like behaviors were not apparent in prenatally stressed offspring of either sex, females stressed *in utero* exhibited a hyperactive phenotype. This work is the first to show sex- and trimester-specific consequences of early pregnancy stressors in prepubertal offspring.

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List of Abbreviations

%	Percent
°C	Degrees Celsius
11β-HSD2	11β-Hydroxysteroid Dehydrogenase Type 2
1TRI	First Trimester Stressor
2TRI	Second Trimester Stressor
ANOVA	Analysis of Variance
BMI	Body Mass Index
CCAC	Canadian Council on Animal Care
cm	Centimeter
CON	Non-Stressed Control
E	Embryonic Day
g	Grams
GR	Glucocorticoid Receptor
h	Hour
HPA	Hypothalamic-Pituitary-Adrenal
Inc.	Incorporated
LPS	Lipopolysaccharide
lux	Level of Illuminance, Luminous Flux Per Unit Area
min	Minutes
MR	Mineralocorticoid Receptor
p-value	Probability-value
PND	Postnatal Day
Poly I:C	Polyinosinic:Polycytidylic Acid
s	Seconds
S. E. M.	Standard Error of the Mean
x	By
X	Times

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1. Introduction

There is significant evidence that insults experienced during sensitive developmental periods can have lasting effects on an individual's physical and mental health (O'Donnell and Meaney, 2016; Padmanabhan et al., 2016). Specifically, fetal development is a particularly vulnerable time where environmental disturbances, including stressors, can have considerable negative consequences for the rapidly developing fetus (Kim et al., 2015). The "fetal programming hypothesis" suggests that challenges such as malnutrition, stressor exposure, alcohol or drug use, or illness experienced by a mother while she is pregnant can permanently alter the trajectory of biological system development in the fetus (Seckl and Holmes, 2007). Changes to the fetal nervous system, immune system, and hypothalamic-pituitary-adrenal (HPA) axis in particular, as well as to the newborn microbiota-gut-brain axis, can persist in the maturing offspring and lead to an increased risk of developing physical and mental illnesses later in life (Brown et al., 2009; Entringer et al., 2009; Gur et al., 2017, 2019). For instance, stress experienced during pregnancy has been shown to alter offspring physical growth and metabolic function throughout postnatal development (Borsonelo et al., 2011; Cao-Lei et al., 2015), and to predispose offspring to developing obesity, diabetes or other metabolic disorders over the lifespan (Virk et al., 2010, 2012; Hohwü et al., 2014). Additionally, gestational stress has been linked to an increased risk of the offspring developing neuropsychiatric diseases later in life, including depression, anxiety and schizophrenia (Khashan et al., 2008; Kingsbury et al., 2016; Herbison et al., 2017), which are often comorbid with metabolic disorders (Dunbar et al., 2008; Carroll et al., 2009; Huang et al., 2018).

To better understand the diverse consequences of stressors experienced during fetal development (referred to as prenatal stressors) on offspring metabolic and neuropsychiatric health and to provide a more comprehensive picture of these outcomes, more research is needed into important factors such as the timing of fetal stressor exposure and the sex of the offspring. These elements are vital considerations due to the sex bias which exists in the prevalence of the aforementioned metabolic and mental diseases (Wild et al., 2004; Steel et al., 2014; Chooi et al., 2019), and the oscillating sensitivity of stress-related biological systems that occurs over the course of fetal development (Semple et al., 2013). Most previous research has focused on studying the effects of prenatal stressors during the third trimester of pregnancy in male offspring, creating a knowledge gap regarding the potential outcomes of stressors experienced during the early phases of fetal development and, importantly, female offspring responses. Additionally, due to the interplay of sex and stress hormones during development and especially during puberty, another important aspect to consider is the age of onset of the metabolic and neuropsychiatric outcomes stemming from prenatal stressor experiences, which to date remains unclear. This thesis thus aims to provide insight into these understudied areas of the prenatal stress research field. The main objective of this thesis is to determine, in a mouse model, whether and how prenatal stressor timing and offspring sex may interact to cause distinct changes to offspring health before puberty. Physical growth and distribution of body fat, as well as anxiety- and depressive-like behavioral outcomes were examined in male and female offspring born to mothers stressed during early pregnancy. Stressor exposure during the first or second trimester of gestation were compared to a non-stressed control condition, and

metabolic and behavioral outcomes were studied in offspring at a prepubertal age in order to investigate whether the stress-related perturbations could manifest in sexually immature offspring.

1.1 Offspring health outcomes may be modulated by prenatal stressor timing

The development of organs and biological systems in the growing fetus is a complex and dynamic process such that the vulnerability of these systems to environmental insults changes over the course of pregnancy (Rice and Barone Jr., 2000; Semple et al., 2013). Proper functioning of the HPA axis, the nervous system and the inflammatory immune system are strongly linked to optimal physical and psychiatric outcomes (Ellman et al., 2008; Monk et al., 2008; Dowlati et al., 2010; Keller et al., 2017). Stressor exposure during critical maturational periods where any of these biological pathways are being established can thus significantly alter their developmental trajectories and subsequently impact offspring metabolic and mental health (Vallée et al., 1997; Rondó et al., 2003; Ellman et al., 2008; Virk et al., 2012; Grigoryan and Segal, 2013; Wu et al., 2018). Throughout gestation, the maternal HPA axis provides optimal levels of glucocorticoids to the growing fetus to ensure proper organ development (Harris and Seckl, 2011). Maternal stress hormones that transverse the placental barrier and enter fetal circulation bind to glucocorticoid and mineralocorticoid receptors (GRs and MRs), which act as transcription factors to change the expression of many genes, including those which control growth, metabolism, and brain maturation (Harris and Seckl, 2011). Importantly, the expression of GRs and MRs in the fetoplacental unit changes over the course of pregnancy. Levels of these receptors increase during the third trimester, potentially in response to elevated amounts of maternal corticosterone reaching the fetus

as required for the glucocorticoid-dependent developmental mechanisms which occur late in pregnancy (Brown et al., 1996; Diaz et al., 1998; Speirs et al., 2004). The fetal HPA axis does not begin maturing until the third trimester (third gestational week) of rodent pregnancy (Dupouy et al., 1975; Pintar and Lugo, 1987; Baram and Lerner, 1991), with the fetal adrenal gland beginning to produce low amounts of corticosterone on approximately embryonic day [E] 16 (Wood and Walker, 2015). Thus, any abnormal stimulation of the maternal or fetal HPA axis during *in utero* development (e.g., due to stressor exposure) can have significant consequences for maturing organ systems (Buss et al., 2012; Graham et al., 2019).

Under baseline conditions, approximately 10-20 percent (%) of maternal glucocorticoids enter fetal circulation (Murphy et al., 1974; Montano et al., 1991; Benediktsson et al., 1997) due to the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) regulating their passage through the placenta (Holmes et al., 2006; Harris and Seckl, 2011). Importantly, prenatal stressors can downregulate 11β-HSD2 in a trimester- and sex-specific fashion to result in more glucocorticoids reaching the fetal compartment of the placenta (Mairesse et al., 2007a; Pankevich et al., 2009; Mina et al., 2015), which may modulate GR and MR expression *in utero* and into adulthood (Sloboda et al., 2008; Cuffe et al., 2017). As these receptors have been implicated in the pathogenesis of stress-related disorders, including depression and anxiety (Pariante and Miller, 2001; Medina et al., 2013; Arnett et al., 2015), hyperstimulation and/or modulation of their expression by glucocorticoids may have significant consequences for the development and function of stress-sensitive brain regions to impact later-life behavior and mental health. In mice, the placenta becomes fully mature around E10.5

(Woods et al., 2018), and prior to this timepoint 11β-HSD2 may be absent or only present at very low levels the uterus and/or the embryonic trophoblast (Zhu et al., 2019; Zheng et al., 2020). Thus, the developing embryo may be particularly vulnerable to the effects of stressors experienced during the early stages of pregnancy due to incomplete placental protection from maternal glucocorticoid surges. Additionally, rapid cellular divisions occur during the first trimester (E0.5-7.5) of mouse pregnancy which culminate in the establishment of the primordial brain and spinal cord (known as the neural tube) on E7.5 (Chen et al., 2017; DeSesso and Williams, 2018). *In utero* disruptions during this period can therefore have dramatic consequences for the organization of the entire central nervous system. However, very few studies have investigated the *in utero* neurodevelopmental, epigenetic, and behavioral consequences of prenatal stressors administered during the early stages of pregnancy. Considering the increased plasticity of fetal systems during this period, more research is needed into the physical and psychological effects of gestational challenges during this sensitive time.

1.2 Offspring sex and age considerations contributing to the effects of prenatal stressors

Offspring sex is another important factor susceptible to mediating the health outcomes stemming from gestational challenges, as sex differences exist in the prevalence of many physical and mental illnesses that have been linked to prenatal stressors. Regarding metabolic diseases, women are more likely than men to suffer from obesity (Chooi et al., 2019) while males are more often diagnosed with type 1 and type 2 diabetes (Wild et al., 2004). Interestingly, after age 60 the prevalence of diabetes becomes higher in women, suggesting a possible role of menopausal hormonal changes

in mediating disease risk and onset (Wild et al., 2004). Females are also twice as likely to be diagnosed with anxiety and/or depression than males (Kessler et al., 1994; Steel et al., 2014), raising the question of whether sex hormones may play a role in these mental illnesses (Soares and Zitek, 2008). Early symptoms of both anxiety or depressive disorders may emerge in adolescence (Last et al., 1996; Luby et al., 2002; Bittner et al., 2007). Interestingly, however, whereas the increased prevalence of anxiety disorders in females seems to be present prior to the onset of puberty (Epkins, 2002; Canino et al., 2004; Beesdo et al., 2009; Orgilés et al., 2012), the incidence of depression prior to puberty is either the same between boys and girls (Kashani et al., 1983) or higher in males (Anderson et al., 1987). Female rates of depression rise following puberty onset, leading to the higher rates of this illness in adult women compared to men (Cohen et al., 1993; Angold et al., 1998). Unlike anxiety, for which factors other than sex hormones appear to underlie higher rates of the disorder in females, this suggests that the maturation of the female reproductive system and associated hormones (e.g., estrogen and progesterone) could interact with other risk factors to result in post-pubertal depressive symptoms that are more pronounced in females (Cyranowski et al., 2000; Hyde et al., 2008).

It has been reported that males and females may respond differently to gestational challenges, which may have important consequences for the prevention and/or treatment of associated physical and mental health issues. For instance, social defeat stress administered to rats during the third trimester of pregnancy resulted in reduced body weights, higher fasting triglyceride levels, and altered expression of lipid metabolism genes in the liver and muscle tissues of adult male offspring, while these measures in

females remained unaltered (Brunton et al., 2013). In humans, male, but not female, adults whose mothers had experienced severe stressful life events during early pregnancy had higher rates of depression and anxiety symptoms (Herbison et al., 2017). Importantly, there is evidence that sex differences in health outcomes elicited by gestational stressors may manifest prior to puberty, thus preceding the sex hormone surge associated with this period. For example, maternal stress during the second and third trimester of pregnancy was associated with reduced adiposity and increased interleukin-6 (a pro-inflammatory immune signalling molecule) methylation in children aged 4-6 years, and interestingly, these associations were stronger in girls than in boys (Wu et al., 2018). In rodents, sex differences in anxiety- and depressive-like behaviors have been reported in prenatally stressed offspring during juvenility (Rayen et al., 2011; Dang et al., 2018; Iturra-Mena et al., 2018), however there is a lack of agreement as to whether males or females show stronger phenotypes. Very few human studies have investigated the physical and mental health outcomes of prenatal stressors in children, and experiments done in juvenile and adolescent rodents have failed to report whether puberty status was confirmed. Thus, it remains unclear whether and how physical changes as well as anxiety and depressive symptoms associated with gestational stressors are impacted by sexual maturation. The question of age of onset of these stress-induced metabolic and mental health alterations is crucial for determining whether there may be critical windows for administering interventions for prevention. The present study therefore studied the physical growth and anxiety- and depressive-like behavioral effects of prenatal stress in mouse offspring at the prepubertal stage, to investigate whether there would be sex differences prior to gonadal hormone surges.

1.3 Evidence for sex- and trimester-specific metabolic outcomes of prenatal stressors

Findings from both human and animal studies have indicated that male and female growth trajectories may be differentially affected by stressful exposures *in utero*, and that stressors experienced in different trimesters of gestation can produce varied metabolic outcomes. In humans, stressful encounters during the second or third, but not first, trimester of pregnancy was associated with low birth weight (Rondó et al., 2003; Rice et al., 2010) and decreased body fat mass in childhood, with girls showing larger adiposity reductions than their male counterparts (Wu et al., 2018), suggesting that these outcomes may be specific to later timings of stressor exposure and that females may be more vulnerable. In contrast, early pregnancy stressors increased adiposity and body mass index (BMI) in young children (Dancause et al., 2015), further indicating distinct offspring growth consequences of different stressor timings. Prenatally stressed teenagers (Project Ice Storm; Cao-Lei et al., 2015; Liu et al., 2016) and adults (Entringer et al., 2008; Hohwü et al., 2014) also showed elevated BMI and body fat mass changes, and young adults who experienced gestational stressors were more likely to be diagnosed with diabetes, with the second trimester found to be the most vulnerable time for increasing risk of type 2 (Virk et al., 2012), but not type 1 (Virk et al., 2010), later-life diabetes development. In addition to metabolic outcomes, young teenagers born to mothers who were in their third trimester of pregnancy during the Quebec Ice Storm had an increased incidence of disordered eating (St-Hilaire et al., 2015). This indicates that gestational stressors not only have long-lasting consequences for offspring growth trajectory but may also predispose an individual to developing metabolic and/or eating disorders later in life, in a trimester-specific fashion. Although this research reveals

metabolic and physical growth outcomes in both children and adults exposed to prenatal stressors, sex and stressor timing influences have only been investigated by a handful of human studies. As will be described in the following paragraphs, animal research allows easier manipulation of these variables to provide additional insight on the potential impact of these factors on prenatally stressed offspring outcomes.

Rodent studies of the metabolic effects of prenatal stressors have produced variable results, even when the same stressor procedure and/or the same pregnancy trimester was used. In rats, a physical restraint stressor (45 minute [min], 3 times [X]/day) administered during the third trimester of pregnancy was reported to reduce (Vallée et al., 1996; Borsonelo et al., 2011; van den Hove et al., 2014; Zhang et al., 2017), increase (Pereira-Figueiredo et al., 2014a; Iturra-Mena et al., 2018), or have no effect (Bowman et al., 2004; Lesage et al., 2004; D'mello and Liu, 2006; Rayen et al., 2011; Amugongo and Hlusko, 2013; Sun et al., 2013a; Boulle et al., 2016; Oosterhof et al., 2016) on offspring growth during the first four weeks of life or in adulthood. Although there were slight differences in the embryonic days during which the stressor was administered, the strain of rats used and the use of a predictable versus a random stressor schedule are more likely to have contributed to these differences (Fride and Weinstock, 1984; Stöhr et al., 1998). Such inconsistencies have also been observed when stressors were applied during the first or second trimester of pregnancy. For example, dexamethasone injections (a synthetic glucocorticoid) during the second trimester resulted in heavier male and female rat offspring from postnatal weeks 3 to 8 (Dahlgren et al., 2001), or had no effect on male rat offspring weights at birth, on postnatal day (PND) 21, or at 12 weeks of age (Jeje and Raji, 2017). Furthermore, first or second

trimester restraint or variable stressors did not alter the growth of male or female rodent offspring from birth to 4 months of age (D'mello and Liu, 2006; Mueller and Bale, 2006; Amugongo and Hlusko, 2013), but variable stress during the first trimester resulted in lighter male and female mouse offspring at the later timepoint of 5-6 months (Pankevich et al., 2009). The contradictory results of these studies indicate that the influence of prenatal stressors on long-term developmental trajectories may be dependent on the strain/species of rodents, the type and/or nature of stressors used, and the timepoint which outcomes are measured. Finally, sex differences in the physical growth outcomes of prenatal stressors have been reported (Smith and Waddell, 2000; Mychasiuk et al., 2011; Brunton et al., 2013; Pereira-Figueiredo et al., 2014b; Dang et al., 2018), but with inconsistencies regarding whether males or females are more sensitive. Adding to these discrepancies, many studies only included male offspring (Fonseca et al., 2002; D'mello and Liu, 2006; Zhang et al., 2017; Castelli et al., 2020) or pooled male and female weights together (Hauser et al., 2006; Mueller and Bale, 2006), thus precluding any conclusions regarding sex differences from being made.

In contrast to weight variations, no differences in fasted plasma levels of insulin, leptin, or triglycerides have been reported in adult offspring that had received dexamethasone injections, restraint, or variable stressors during the first or second trimester of pregnancy (Dahlgren et al., 2001; D'mello and Liu, 2006; Pankevich et al., 2009). Adult female mice exposed to first trimester variable stress showed higher fasted plasma levels of glucose, but their male counterparts were unaltered (Mueller and Bale, 2006). In line with the view that the timing of prenatal stress may influence offspring metabolic outcomes, increased caloric intake was exhibited by male and female mice

exposed to first trimester variable stress (Pankevich et al., 2009), while second trimester shock stress did not alter the food or water intake of male offspring (females not measured) (Golub et al., 2016). Distribution patterns of body fat pads were also changed in a sex-dependent way in adult rodent offspring exposed to prenatal stressors, with first trimester variable stress reducing the total body fat of male mice only (Pankevich et al., 2009), but second trimester dexamethasone increasing fat mass at the retroperitoneal (male and female) and perigonadal (female only) locations (Dahlgren et al., 2001). In contrast, restraint, social defeat, and variable stressors experienced during the third trimester of pregnancy have been shown to consistently alter outcomes such as glucose tolerance, plasma leptin, and food intake in juvenile and adult animals (Vallée et al., 1997; Lesage et al., 2004; Brunton et al., 2013; Paternain et al., 2013), suggesting that late gestational challenges could have stronger metabolic effects than earlier exposure timings. Offspring exposed to stress during the third trimester also showed an increased susceptibility to the negative effects of hypercaloric diets including exaggerated food intake, increased body fat, and more pronounced metabolic changes (Tamashiro et al., 2009; Paternain et al., 2012, 2013). Furthermore, third trimester prenatal stress may increase an offspring's risk of developing eating disorders such as binge eating, with females being particularly vulnerable to these effects (Schroeder et al., 2017).

In summary, there is considerable evidence that gestational stressors can have lasting impacts on offspring growth and metabolism, and these changes may be exacerbated when an individual consumes obesogenic diets. This is important as it implies that prenatal stressor experiences may change the developmental trajectories of metabolic and stress-response systems and make individuals more susceptible to later-life

challenges. However, neither human nor animal studies have fully explored how the factors of prenatal stressor timing and sex may mediate offspring outcomes, and previous rodent research lacks the procedural standardization needed to draw definite conclusions of the long-term effects of gestational stressors on offspring physical development.

1.4 Prenatal stressors can increase offspring risk of anxiety and depression

Mental illnesses such as anxiety and depression are known as stress-related disorders, due to the fact that stressful experiences over the lifespan may increase their risk of onset and relapse (Kendler et al., 1999; Francis et al., 2012; Kinderman et al., 2013; Herbison et al., 2017). Stressors encountered early in life, including in the period prior to birth, are especially relevant in this regard, as individuals who have been exposed to stress *in utero* show increased depressive and anxiety symptoms in adulthood (Betts et al., 2015; Herbison et al., 2017). Importantly, sex- and trimester-specific differences have been reported in humans regarding the adult mental health outcomes of prenatal stressors, with depression and anxiety rates being highest in adult male offspring exposed to stressors in early pregnancy (Watson et al., 1999; Kleinhaus et al., 2013; Herbison et al., 2017). For obvious ethical reasons, there are a limited number of human studies that have investigated the trimester-specific mental health outcomes of gestational stressors. The variable of stressor timing can be more easily manipulated in animal models to study anxiety- and depressive-like behaviors in rodent offspring.

1.4.1 Tests to measure anxiety- and depressive-like behaviors in rodents

The mental health consequences of stressors can be modelled in rodents through well-validated tests of anxiety- and depressive-like behaviors. Anxiety-like behaviors are

most often assessed in the open field, elevated plus maze, and light-dark tests. These tests take advantage of the conflict between a rodent's instinctual fear of well-lit open spaces and their desire to explore novel environments (Lezak et al., 2017). Animals that spend more time in the "safer" dark and enclosed areas of the test arenas are said to display higher levels of anxiety-like behavior. Depressive-like behaviors are typically measured in the forced swim, tail suspension, sucrose preference, and splash tests. The forced swim and tail suspension tests involve exposing an animal to a stressful environment where no escape is possible. Rodents with depressive-like phenotypes will move less (e.g., reduced escape attempts), which is reflective of behavioral despair (Porsolt et al., 1978; Cryan et al., 2005). Reduced sugar water consumption in the sucrose preference test, and less grooming in the splash test, will also be exhibited by animals with a depressive-like phenotype (Overstreet, 2012; Kalueff et al., 2016). Treating animals with validated anxiolytics or antidepressants limits or reverses these anxiety- and depressive-like behaviors, confirming the tests are valid models of these phenotypes (Bailey and Crawley, 2009; Overstreet, 2012).

1.4.2 Prenatally stressed adult rodents show anxiety- and depressive-like phenotypes

Adult rodents exposed to stressors *in utero* exhibit behavioral changes that resemble the anxiety and depression symptoms displayed by prenatally stressed adult humans. Most of the rodent research on the behavioral effects of *in utero* stressors have manipulated pregnant animals during the third trimester. Exposing pregnant rodents to restraint stress (45 min, 3X/day) during the third trimester increased anxiety- (Sun et al., 2013b; Lee et al., 2016; Lussier and Stevens, 2016; Cao et al., 2018) and depressive-like (Tamura et al., 2011; Szczesny et al., 2014; Cao et al., 2018) behaviors in adult offspring

of both sexes, although no effect of prenatal stress during this trimester on these behaviors has also been reported (Bowman et al., 2004; Poltyrev and Weinstock, 2004; Poltyrev et al., 2005; Boulle et al., 2015, 2016). Antidepressants administered during juvenility or adulthood reversed these effects, but unfortunately only male offspring were included in these studies (Morley-Fletcher et al., 2004; Szymańska et al., 2009; Trojan et al., 2019).

There is some evidence for sex differences in the depressive- and anxiety-like behaviors of adult offspring stressed during the third trimester, but this research is inconsistent regarding which sex shows a stronger phenotype, likely due to differences in the rodent strains, stressor procedures, and behavioral tests used and, importantly, in view of the fact that most studies have not included females. For example, when adult Sprague-Dawley rats were stressed in the third trimester, 2 hours (h) of daily restraint increased forced swim immobility, reflective of a depressive-like phenotype, in females only (Alonso et al., 1991). In contrast, when exposed to 45 min restraint 3X/day during the third trimester, only male offspring showed less mobility but increased sugar consumption in the forced swim and sucrose preference tests, respectively (van den Hove et al., 2014). Regarding anxiety-like behaviors, third trimester restraint stressors have been shown to increase these phenotypes in adult female offspring, with no effect (Zagron and Weinstock, 2006; Pallarés et al., 2007; van den Hove et al., 2014) or reduced anxiety (Ordyan and Pivina, 2004) in males. However, these studies utilized different restraint stressor paradigms, rodent species and strains, and behavioral tests, thus precluding any conclusions from being made. Unfortunately, as it is often the case in animal research, many studies have not included female offspring in their analyses, and

these studies reported no difference (Rimondini et al., 2003; Pascual et al., 2010) or increased anxiety-like behaviors (Szymańska et al., 2009; Szczesny et al., 2014; Lussier and Stevens, 2016) in adult male offspring. Importantly, most investigations that have tested prenatally stressed adult female offspring for anxiety- or depressive-like behaviors did not mention if the estrus cycle stage was considered. This is a crucial variable in the context of prenatal stress because depressive- and anxiety-like behaviors vary with cycle stage (Frye et al., 2000; Jaric et al., 2019), particularly in gestationally stressed offspring (Brunton and Russell, 2010; Grundwald and Brunton, 2015), and *in utero* stressors may cause irregular cycling in females (van Camp et al., 2018). Thus, failure to consider the female hormonal cycle at the time of testing could have contributed to the discrepancies between studies.

Curiously, very few studies in rodents have investigated the anxiety- or depressive-like effects of prenatal stressors when administered during the first or second trimester of pregnancy. Variable stressor exposure during the first or second trimester of pregnancy did not affect the anxiety-like behaviors in adult C57/BL6:129 hybrid mice of either sex, however depressive-like behaviors were increased in first trimester stressed males, with their female counterparts being unaltered (Mueller and Bale, 2008). A later study from this research group found hyperactivity and reduced anxiety-like behaviors in male, but not female, offspring exposed to first trimester variable stress (Bronson and Bale, 2014), suggesting that males may be more susceptible to the behavioral effects of early gestational stressors. Immune challenges known to promote mental health and metabolic outcomes in adult rodents (Reisinger et al., 2015) similarly promoted depressive-like outcomes when administered prenatally. Lipopolysaccharide (LPS; a

bacterial cell wall component) administered to rodents during the second trimester of pregnancy increased male and female offspring immobility in the forced swim test, but had no effect on sucrose consumption, suggesting that prenatal bacterial-like challenges may lead to behavioral despair but not anhedonia (Lin and Wang, 2014). LPS exposure during this trimester similarly resulted in increased depressive- and anxiety-like phenotypes, but only male C57BL/6 offspring were studied (Depino, 2015). In contrast, only male mouse offspring exposed to Polyinosinic:Polycytidylic acid (Poly I:C; a synthetic double stranded RNA analog which mimics viral infection) during the second trimester showed anxiety- and depressive-like behaviors in the light-dark, tail suspension, and forced swim tests (Majidi-Zolbanin et al., 2015), although no effect of second trimester Poly I:C on these behaviors have also been reported (Li et al., 2014). Overall, very few studies have investigated the anxiogenic or depressogenic impacts of early prenatal challenges in rodents and thus the long-term behavioral effects of stressors administered at these time points remain unclear.

1.4.3 The anxiety- and depressive-like behaviors of prenatally stressed offspring may manifest in juvenility

In humans, not only do adults born to mothers stressed during their pregnancy display higher rates of anxiety and depression, but there is also evidence that symptoms may start to manifest early in life, including in children (ages 4-11) (Davis and Sandman, 2010; Rice et al., 2010; Kingsbury et al., 2016) and in youth (age 15-17) (Maxwell et al., 2018). Notably, characteristics indicative of later-life mental health outcomes may be detected very early in postnatal development, which is important for designing early-life interventions for individuals born to mothers who were distressed during pregnancy. For

instance, infants born to stressed mothers displayed a higher incidence of such predictive features, such as difficult or negative temperament, negative affect, and externalizing/internalizing symptoms (Huizink et al., 2002; de Weerth et al., 2003; Hentges et al., 2019). Likewise, research from Project Ice Storm suggests that young adolescents born to mothers pregnant during this natural disaster may also exhibit these externalizing symptoms, particularly when stressed occurred during late gestation (Jones et al., 2019; Yong Ping et al., 2020). One study reported higher levels of these negative traits in prenatally stressed females as early as weeks of age (Braithwaite et al., 2017), suggesting that there may be sex differences in this regard prior to the onset of puberty.

Rodent studies have confirmed the possibility of early onset of anxiety- and depressive-like behaviors in the context of prenatal stress. Male and female juvenile rodents exposed to third trimester restraint stress showed increased anxiety-like behaviors (Schroeder et al., 2013; Jia et al., 2015; Qulu et al., 2015) and depressive-like behaviors (Guan et al., 2013; Sun et al., 2013a; Zhang et al., 2013; Jia et al., 2015) as well as altered locomotor activity (Borsonelo et al., 2011; Sun et al., 2013b). In line with the possibility that these outcomes may be sex-specific, a few studies have reported that only juvenile males showed increased anxiety- and depressive-like behaviors following exposure to third trimester restraint stress (Laloux et al., 2012; Lee et al., 2016; Dang et al., 2018; Iturra-Mena et al., 2018). In contrast, one study found no effect third trimester stress on juvenile male anxiety-like behaviors (Pascual et al., 2010), and curiously two studies reported reduced depressive-like behaviors in prenatally stressed males and females (Rayen et al., 2011; Schroeder et al., 2013). The use of different rodent species and

strains, restraint stressor timings and lengths, age of rodent testing, and behavioral tests used varied greatly between these studies, which may account for result discrepancies.

Notably, while very few studies have examined mental health outcomes stemming from early prenatal stress in adult rodents, no previous studies have measured anxiety- and depressive-like behaviors in juvenile rodents exposed to first trimester stress, and a limited number have investigated these phenotypes following second trimester exposure. Increased immobility in the tail suspension test as well as more anxiety-like behaviors in the open field test have been reported in male and female juveniles stressed during the second or third trimester (Zhang et al., 2013; Jia et al., 2015). However, offspring of both sexes stressed during the third, but not second, trimester of pregnancy displayed depressive-like behavior in the forced swim test (Jia et al., 2015), indicating that the later gestational period may be more vulnerable to stressful encounters in terms of depressive-like behavioral outcomes. Other changes to open field behaviors have been reported in second trimester stressed juvenile rodents, with a shock stressor reducing the incidence of crouching (Hutchings and Gibbon, 1970), hydrocortisone injection increasing activity, and an overcrowding stressor increasing fecal production but reducing jumping (Lieberman, 1963), although it is unclear how these behavioral changes relate to anxiety. Overall, most studies have observed increased depressive- and anxiety-like behaviors in juvenile offspring exposed to third trimester prenatal stressors, few have investigated these outcomes following second trimester exposures, and none have tested juvenile first trimester stressed offspring. Furthermore, the age of onset of these behaviors and the role of sex hormones in this regard remain unclear, particularly considering that none of the aforementioned studies reported whether juvenile offspring were assessed for pubertal

onset at the time of behavioral assessment. This is an important variable to consider as sex hormones can alter anxiety- and depressive-like behaviors (Boivin et al., 2017; Delevich et al., 2020) and prenatal stressors can shift the age of pubertal onset in offspring of both sexes (Smith and Waddell, 2000; Pallarés et al., 2013). Standardization of stressor paradigms and behavioral test batteries, as well as study replication, is also needed before definite conclusions can be made regarding the influence of stressor timing, as well as offspring sex and age, on the behavioral outcomes of prenatal stressors.

1.5 Potential mechanisms by which prenatal stressors may influence offspring health

The HPA and microbiota-gut-brain axes are two systems that have been proposed to underlie some of the physical and mental health outcomes observed in prenatally stressed offspring. Stress-induced increases in maternal glucocorticoid production has been shown to increase the permeability of the placental barrier and downregulate the 11β-HSD2 enzyme, which may allow higher levels of maternal stress hormones to enter fetal circulation and/or activate fetoplacental glucocorticoid secretion, ultimately altering the trajectory of fetal development (Seckl and Holmes, 2007; Harris and Seckl, 2011). These stress hormone elevations in the fetus have been correlated with depressive- and anxiety-like behaviors, as well as with changes to the physical growth and expression of genes involved in metabolism, seen in prenatally stressed offspring (Mairesse et al., 2007b; Mueller and Bale, 2008; Carpenter et al., 2017), possibly through glucocorticoid-mediated GR and MR methylation altering the epigenetic control of the offspring HPA axis (Radtke et al., 2011; Yao et al., 2014; Kertes et al., 2016; Luft et al., 2020). In addition to glucocorticoid elevations *in utero*, offspring exposed to gestational stressors

exhibited a dysregulated HPA axis in adolescence and adulthood (Maccari and Morley-Fletcher, 2007; Huizink et al., 2008; Enayati et al., 2020). In a context where HPA axis dysfunction may have significant consequences for offspring responses to later life stressful encounters, these prenatally stressed individuals may show altered susceptibilities to future stressors (Chung et al., 2005; Ishiwata et al., 2005; Entringer et al., 2009; Oosterhof et al., 2016).

It has been suggested that abnormalities in the colonization patterns of microorganisms residing within the gastrointestinal tract (the gut microbiota) may be another mechanism by which prenatal stress promotes vulnerability to mental illnesses (Kelly et al., 2015). In humans, the gut microbiota is altered in individuals with depression and anxiety (Jiang et al., 2018; Valles-Colomer et al., 2019). As well, depressive- and anxiety-like behaviors in adult rodents stressed *in utero* (Sun et al., 2013b; Gur et al., 2017; Soares-Cunha et al., 2018; Enayati et al., 2020) were accompanied by changes in the composition and diversity of gut microbial communities (Golubeva et al., 2015; Gur et al., 2017, 2019). Stressful experiences during pregnancy changed the composition of the maternal vaginal microbiota (Jašarević et al., 2015, 2017), resulting in the newborn receiving altered microbes (Jašarević et al., 2015, 2017, 2018; Zijlmans et al., 2015), suggesting that prenatal stress may alter the offspring microbiota from birth to have metabolic and behavioral consequences later in life. The immune system is one communication pathway which allows crosstalk between gut bacteria and the host brain (Carabotti et al., 2015), and inflammatory changes have been shown to produce cognitive and behavioral impairments (Cryan and Dinan, 2012). Increased pro-inflammatory expression has been detected in the placental (Bronson and

Bale, 2014; Gur et al., 2017), fetal (Gur et al., 2017; Jašarević et al., 2018), and adult (Diz-Chaves et al., 2012, 2013; Gur et al., 2019), gut-brain tissues of prenatally stressed mice. It may be through disruption of the maturation of the offspring inflammatory immune system and microbiota that prenatal stressors lead to increased depressive- and anxiety-like behaviors. Furthermore, microbiota-gut-brain crosstalk can be further influenced by glucocorticoids (Carabotti et al., 2015), thus by altering HPA axis function prenatal stressors may have additional influences on the microbiota and its communication pathways with the brain (Jašarević and Bale, 2019).

It may also be through the HPA axis actions on the gut microbiota that prenatal stress leads to physical development and metabolic changes in the offspring. Newborns which experience an abnormal colonization event, such as delivery by caesarean section instead of vaginally, show persistent microbiota signature alterations which correlate with childhood obesity (Salminen et al., 2004; Blustein et al., 2013). The gut microbiota is involved in regulating fat storage (Bäckhed et al., 2004) and dysbiosis may increase the expression of pro-inflammatory cytokines by adipose tissue (Virtue et al., 2019). Maternal food intake patterns and weight gains during pregnancy can be altered by prenatal stressors (D'mello and Liu, 2006; Borsonelo et al., 2011; Amugongo and Hlusko, 2013), and these changes may accelerate the postnatal growth of the offspring (Berghänel et al., 2017). As there is also evidence that maternal obesity and diet can influence the offspring inflammatory immune system (Pimentel et al., 2012; Edlow et al., 2016) as well as the microbiota (Paul et al., 2016), it is thus possible that if an offspring receives an abnormal microbiota from its prenatally stressed mother, this microbial

signature may increase risk of later-life obesity, potentially through immune and metabolic signals.

1.6 Objectives and hypothesis

This thesis compares the effects of a prenatal stressor administered during two distinct trimesters of pregnancy on physical growth and fat distribution, as well as on anxiety- and depressive-like behaviors in prepubertal male and female mouse offspring. This study aimed to answer three major questions: 1) Can prenatal stress alter physical growth, fat distribution, and anxiety- and depressive-like behaviors in mouse offspring prior to the onset of puberty? 2) Do male and female prepubertal offspring stressed *in utero* show differential physical and behavioral changes? and 3) Will distinct trimester gestational stressors have unique effects on offspring outcomes? There is strong evidence from the literature and our preliminary observations that adult male and female offspring respond differently to prenatal stressors in terms of physical growth, fat distribution, and stress-related behaviors, therefore all experimental manipulations were conducted in both sexes. Furthermore, as it has been alluded to before, previous studies suggest that the timing of prenatal stress is important for mediating physical and behavioral effects but limited research has investigated early pregnancy exposures, therefore two trimesters of stressor exposure were compared, namely the first (E1.5-6.5) and second (E7.5-12.5) trimesters.

This study fills several gaps in the available literature on rodent prenatal stress. Firstly, no previous studies have investigated how stressors applied during early fetal development alter the physical growth of neonatal offspring. The present thesis tracked offspring body weights daily across the first three weeks of postnatal development

(PND1-21). Secondly, sex differences in physical growth following first or second trimester stressors have not been previously elucidated, therefore in this thesis separate analyzes were conducted on offspring mice of both sexes. Thirdly, it has not been studied how first or second trimester gestational stressors can alter juvenile offspring body fat composition. This thesis investigated the percentage of fat relative to body weight across five locations in offspring at the prepubertal (PND21) stage. Finally, there have been no studies investigating whether prenatal stress (of any paradigm) during the initial phases of pregnancy promotes anxiety- or depressive-like behaviors in rodents confirmed to be prepubertal. The few rodent studies that have reported behavioral effects of first or second trimester stressors tested adult offspring (Mueller and Bale, 2008; Bronson and Bale, 2014) or did not confirm the puberty status of juvenile offspring (Lieberman, 1963; Zhang et al., 2013; Jia et al., 2015). Thus, the present study is instrumental in elucidating whether anxiety and depressive phenotypes may be detected prior to the onset of sex hormones when stress is experienced in the very early stages of fetal development. C57BL/6 mice were used in the present experiment such that future analyses of tissues could be done to investigate the gut microbiota-immune-brain axis as a potential underlying mechanism of the behavioral and metabolic effects of prenatal stress, as this mouse strain is the model used by researchers investigating the effects of stress on this axis in the context of mental health.

Based on the literature, it is hypothesized that prenatally stressed offspring will have a lower weight at birth but will gain more weight than non-stressed controls across the first three weeks of postnatal development and grow more fat at specific body sites. Anxiety- and depressive-like behaviors are also expected to be increased in prenatally

stressed offspring compared to controls. All outcomes are expected to be sex- and trimester specific, however the limited number of previous studies prevents the directionality of these differences from being predicted with exactitude. This research will advance knowledge on the physical and behavioral outcomes of offspring exposed to stressors early in pregnancy and assess whether these changes manifest in a sex-specific manner prior to the onset of puberty. This study will also provide insight into potential developmental windows where interventions or treatments could be administered to potentially prevent and/or alleviate the negative effects of gestational stressors.

2. Methods

2.1 Animals

Naïve female and male C57BL/6 mice (6-8 and 7-9 weeks old upon arrival, respectively) were used as parent stocks (Charles River Laboratories, St-Constant, Québec, Canada). Females were pair-housed and males were single-housed in 19 centimeter (cm) by (x) 29 cm x 13 cm polycarbonate N10 Mouse Cages (Ancare) with a cotton nestlet, a cardboard house and standard woodchip bedding, and had free access to water and food (Teklad Global 18 % Protein Rodent Diets, catalog number 2018, Envigo). Mice were maintained on a 12 h light-dark cycle (lights on 0700 to 1900 h) with temperature and humidity kept within the ranges of 21.0-23.0 degrees Celsius (ºC) and 30-50 %. Housing cages were changed once a week. All experimental procedures were approved by the University of Ottawa's Animal Care Committee and met the CCAC guidelines.

2.2 Summary of experimental procedures

Mice acclimated for one week following their arrival to the animal facility and then underwent several rounds of breeding. Following detection of a copulation plug (E0.5), pregnant females were randomly assigned to a control condition (no stressor) or to one of the two prenatal stressor conditions. Dams in the prenatal stressor conditions were physically restrained for 30 min, 3X daily, throughout the first (E1.5-6.5) or the second (E7.5-12.5) trimester of pregnancy. Dams in the control condition were left undisturbed. Once born (PND1), pups remained with their mothers and were weighed daily until they were euthanized on PND21. On PND2, litters were standardized to 8 pups, with a focus on maintaining an even sex ratio (4 males and 4 females when

possible). One pup of each sex from each litter was euthanized on PNDs 7 and 14 for analyses not included in this thesis. Starting on PND19, external examinations were conducted daily on the remaining male and female offspring to confirm prepuberty status. These mice were tested for anxiety- (open field, elevated plus maze) and depressive-like (splash test, tail suspension test) behaviors on PNDs 19 and 20. On PND21, all pups were euthanized by rapid decapitation, and various tissues and organs were collected for analyses not included in this thesis. Mouse bodies were frozen for later fat dissection. Figure 1 depicts a visual timeline of the experimental procedures.

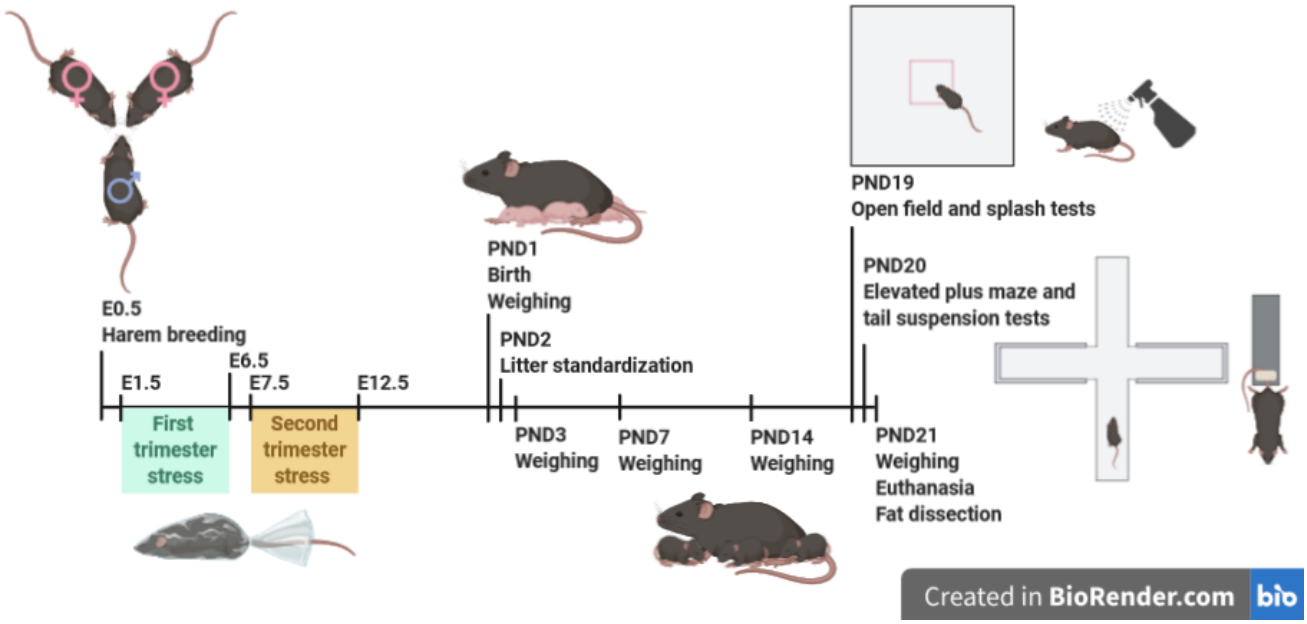

Figure 1. Timeline of experimental procedures.
E = embryonic day, PND = postnatal day. Image created using BioRender.com free licence.

2.3 Breeding and prenatal stressor procedures

Harem mating was used as the breeding scheme (Braden et al., 2017). Estrus was induced between 1600-1700 h by sprinkling woodchip bedding and nestlet from the cage of a male into a cage containing two females (Braden et al., 2017). The next day between

1600-1700 h the pair of females was introduced into the male's housing cage and left undisturbed overnight. At 0700 h the following morning, females were examined for copulation plugs using a cotton swab to gently assess the vaginal opening (Behringer et al., 2016). After detection of a copulation plug, pregnant females were singly housed in new cages and randomly assigned to their experimental condition. A physical restraint stressor (reviewed in Buynitsky and Mostofsky, 2009) was administered to the pregnant females in a prenatal stressor condition during the first (E1.5-6.5) or the second (E7.5-12.5) trimester of their pregnancy. This stressor procedure was selected based on previous reports demonstrating that physical restraint during pregnancy elicited anxiety- and depressive-like behaviors in male and female juvenile (Laloux et al., 2012; Guan et al., 2013; Schroeder et al., 2013; Jia et al., 2015) and adult (Alonso et al., 1991; Morley-Fletcher et al., 2004; Akatsu et al., 2015; Gur et al., 2017) rodent offspring. Each mouse was gently picked up by the tail and quickly placed into a triangular transparent plastic bag. A small hole at the nose end of the bag allowed the mouse to breathe while tape closing the tail end of the bag prevented physical movement or escape. The mouse remained in the bag for 30 min during which breathing and limb positioning were continuously monitored. The stressor procedure was carried out 3X daily at 0900, 1200 and 1500 h, at a location outside the mouse housing room to prevent stress pheromones from affecting mice in the other cages. Mice in the no stressor control condition were left undisturbed.

2.4 Offspring developmental measures

2.4.1 Birth and litter characteristics

Pregnant mice were checked daily at 0800 h for birth, which was denoted PND1. All mice gave birth between days 18.5-20.5 of gestation. The total number of pups at birth (including live and dead) were recorded along with the number of males and females.

2.4.2 Body weights

Pups were weighed daily from PND1 (birth) to PND21. The percentage of weight change from PND1 was calculated for PNDs 3, 7, 14 and 21 by subtracting the body weight at PND1 from the body weight at each of these timepoints, and these values were divided by the body weight at PND1 and multiplied by 100 ([(PND X – PND 1)/PND1] *100). All weight measures were in grams (g). These timepoints were selected to provide a measurement of physical growth shortly after birth (PND3) and then once every postnatal week until euthanasia (PNDs 7, 14, 21).

2.4.3 Puberty onset assessment

Prior to behavioral testing or euthanasia on PNDs 19-21, all offspring were assessed for pubertal onset. This was done by external examination of the males for balanopreputial separation and of the females for vaginal opening (Caligioni, 2009; Hoffmann, 2018). Confirmation of prepubertal status was conducted based on previous studies showing that prenatal manipulations, including physical stressors, shifted pubertal onset in male and female rodent offspring (Smith and Waddell, 2000; Pallarés et al., 2013).

2.4.4 Behavioral testing

Tests for anxiety- and depressive-like behaviors took place over two days (PND19-20). The order of testing was the same for every mouse: the open field (morning) and splash (afternoon) tests were administered on PND19 and the elevated plus maze (morning) and tail suspension (afternoon) tests were conducted on PND20. Morning tests always took place between 1000 and 1300 h and afternoon tests between 1200 and 1700 h. Litters with their mother acclimated to the testing environments for 1 h, in their home cage, prior to each behavioral test. For the open field and elevated plus maze tests, the acclimation occurred at 100 level of illuminance (luminous flux per unit area; lux) in a soundproof room next to the testing room. For the splash and tail suspension tests, acclimation occurred in the same rooms in which testing took place at 530 and 100 lux, respectively. All behavioral testing rooms had soundproofing material around the doorframes to reduce outside noise. Testing arenas were cleaned with Quato 78 Plus Disinfectant (Swish Maintenance Limited) between each mouse to limit odor carryover.

2.4.4.1 Tests for anxiety-like behaviors

Open field and elevated plus maze testing rooms included a video camera mounted from the ceiling over the test arena and connected to a computer located outside of the room. Mouse movements in the two apparatus were tracked in real time and subsequently analyzed using EthoVision XT (version 11.5, Noldus Information Technology). The center-point detection setting (which places a detection point in the middle of the mouse's back for movement tracking) was used for both tests.

2.4.4.1.1 Open field test

On PND19, prepubertal mouse offspring were tested in the open field, a well-validated test of anxiety-like behaviors in rodents that measures their natural conflict between exploring a new environment and fear of open and brightly lit areas (Seibenhener and Wooten, 2015). Mice were individually placed into the bottom left corner of an arena (45 cm x 45 cm x 45 cm; custom-made by Canus Plastics Incorporated [Inc.]) and allowed to freely explore the environment for 10 min under light at 300 lux (verified by a luxmeter). For each mouse, the cumulative duration spent (seconds [s]) and frequency of entries made in the center (15 cm x 15 cm) and the four corners (each 10 cm x 10 cm) of the arena were measured, as well as the latency of first entry into the center (s) and the total distance travelled (cm). Supplementary measurements, including cumulative time spent (s) and frequency of entries made in a larger center of the arena (25 cm x 25 cm) and the number of feces were also determined. Jumping behaviors including the total time spent jumping (s), frequency of jumping bouts, total number of jumps, and latency to first jump (s) were also manually scored from the videos using ODLog (Macropod Software) and included as supplementary data.

2.4.4.1.2 Elevated plus maze test

The elevated plus maze test is another well-validated test for anxiety-like behaviors and assesses an animal's aversion for brightly lit, elevated open areas and preference for safer dark and enclosed areas (Komada et al., 2008). This test was performed on PND20. The acrylic plastic maze (Noldus Information Technology) consisted of two white perpendicularly crossed arms (6 cm wide x 75 cm long). One arm had 20 cm tall black walls and the other was open (no walls or edges). The maze was

located 74 cm off the ground. Each mouse was individually placed into the bottom left corner of the open center of the maze, facing the intersection of an open and closed arm, and allowed to freely explore the maze for 10 min under 100 lux lights (verified by luxmeter). For each mouse, the cumulative time (s) spent and frequency of entries made in the open and closed arms were measured, as well as the latency to first entry into the open arms (s) and the total distance travelled (cm). Supplementary measurements included cumulative duration (s) spent and frequency of entries made in the center and the number of feces produced.

2.4.4.2 Tests for depressive-like behaviors

The splash and tail suspension tests were filmed using a video camera mounted on a tripod. Behaviors were scored manually using The Observer XT software (version 15, Noldus Information Technology).

2.4.4.2.1 Splash test

The splash test can be used to assess the grooming behavior of rodents. Reduced grooming in the splash test correlates with increased depressive-like behaviors in the forced swim and tail suspension tests (Shiota et al., 2016), and thus the splash test has been used as a less stressful alternative for assessing depressive-like phenotypes (Isingrini et al., 2010; Reis-Silva et al., 2019). Alternatively, increased grooming can be indicative of elevated stress and anxiety, as rodents exposed to acute or mild stressors groom more frequently (Kalueff et al., 2016). The splash test was conducted on PND19 and involved applying two gentle mist sprays of water to the lower back of the mouse, and immediately placing the mouse into an individual empty cage of same dimensions to

the litter's home cage, but with only woodchip bedding on the bottom (i.e., no house, nestlet, food or water). Mice were left in the empty cage for 10 min and the room lighting was consistently set at 530 lux using a luxmeter, to ensure well-lit video recordings. The total time spent grooming during the 10 min recording period (s), total number of grooming sessions, average length of a grooming session (s), and latency to first groom (s) were determined for each mouse. These parameters were also scored for rearing, a non-grooming behavior indicative of anxiety (Kalueff and Tuohimaa, 2005), and digging, a natural behavior in rodents which may be disrupted in animals with negative affect (Deacon, 2009) and included as supplementary data. Supplementary measurements also included total number of jumps and the latency to first jump (s).

2.4.4.2.2 Tail suspension test

The tail suspension test exposes a mouse to the inescapable and uncomfortable stress of being hung upside down by the tail. The amount of time the mouse spends without moving (referred to as immobility) is measured, which reflects the mouse halting escape attempts. A mouse with increased behavioral despair, a depressive-like behavior in rodents, will spend more time immobile in this test (Cryan et al., 2005). On PND20, mice were suspended by the tail by being attached to a metal paddle with surgical tape for 6 min (Tail Suspension Test Cubicle [SOF-821], Med Associates Inc.). To help prevent tail climbing, short plastic tubing was placed onto the tail, such that the part of the tail closest to the body was covered. The lighting of the room was set to 100 lux using a luxmeter. For each mouse, the total time spent immobile (s), average length of immobility session (s), total number of immobility sessions, and the latency to immobility (s) were measured. As previously described, immobility was scored when the

mouse exhibited no limb or body movements for longer than 3 s consecutively and included respiration and passive swaying of the body (Mitchell et al., 2013; Murata et al., 2018).

2.4.5 Fat dissections

On PND21, prepubertal offspring were euthanized by rapid decapitation and the bodies were frozen (-80°C) for later dissection of fat pads. Euthanasia and tissue collection procedures occurred between 0900 and 1200 h to minimize effects related to diurnal factors. Bodies were later thawed and the mesenteric, perigonadal, perirenal/retroperitoneal, posterior subcutaneous, and brown fat pads were collected and weighed based on Mann et al., 2014, de Jong et al., 2015, and Bagchi and MacDougald, 2019 (Figure 2). Mesenteric fat and perigonadal fat surrounded the digestive tract and the reproductive organs, respectively. The two fat pads located at the site of the kidney (perirenal, surrounding the kidney, and retroperitoneal, against the abdominal wall and dorsal to the kidney) were dissected together. The posterior subcutaneous fat deposit was comprised of the dorsolumbar, inguinal, and gluteal pads, found beneath the skin of the lower half of the body from the ribcage downward. Brown fat was the combination of the interscapular and subscapular deposits which are found between or underneath the shoulder blades, respectively. A fat percentage was calculated for each fat pad type for each mouse by dividing the fat pad weight by the mouse's body weight on PND21 (both in g) and multiplying by 100 ([fat pad weight / PND21 body weight] *100). The weights of the fat obtained from the five dissected adipose sites were summed together to obtain the total body fat measurement, which was similarly converted into a fat percentage.

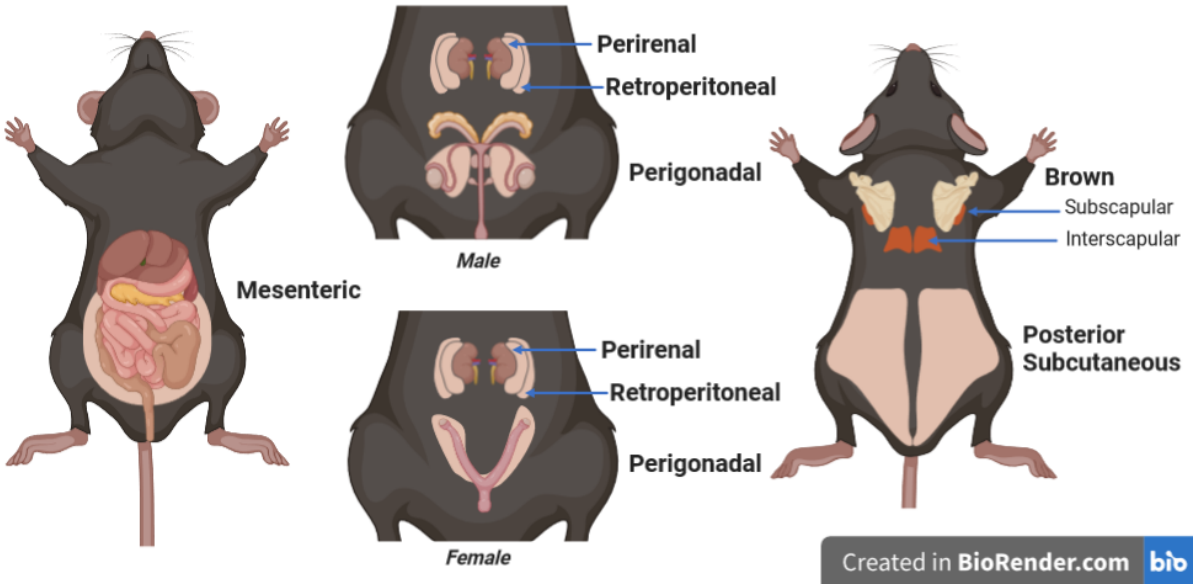

Figure 2. Locations of fat pads dissected and weighed from male and female offspring aged 21 days.
Mesenteric fat surrounds the gastrointestinal tract and perigonadal fat is associated with the epididymis and testes (males) or uterus and ovaries (females). Perirenal fat encompasses the kidney and retroperitoneal fat is posterior to the kidney against the abdominal wall. Posterior subcutaneous fat is located just beneath the skin in the lower half of the body, surrounding the hindlimbs and hips, and includes the dorsolumbar, inguinal, and gluteal pads combined. Brown fat pads were dissected from the interscapular and subscapular regions, respectively located between or underneath the shoulder blades. Locations were distinguished based on Mann et al., 2014, de Jong et al., 2015, and Bagchi and MacDougald, 2019. Image created using BioRender.com free licence.

2.5 Statistical Analyses

Statistical analyses were conducted using IBM SPSS Statistics version 26.0. Litter size at birth (which included live and dead pups) was analysed using the nonparametric Kruskal-Wallis test due to the dependent variable not being continuous. The sex ratio at birth (which only included live pups) was analyzed using the nonparametric Chi-Squared Test of Independence applied to a 3 x 2 contingency table (Categorical predictor variable: Prenatal Stressor (No Stressor [CON], First Trimester Stressor [1TRI], Second Trimester Stressor [2TRI]; Categorical outcome variable: Sex [Males, Females]). The remaining data was first analyzed for normality and equality of variances using the Shapiro-Wilk

and Levene's tests, respectively. All data met the assumption of homogeneity of variance (Levene's Test $p > 0.05$). Normally distributed data was analyzed using a series of one-way analyses of variance (ANOVAs) with Prenatal Stressor (CON, 1TRI, 2TRI) serving as the between-group factor. Data not meeting the assumption of normality (Shapiro-Wilk Test $p < 0.05$) was analyzed using the nonparametric Kruskal-Wallis test. If the overall ANOVA or Kruskal-Wallis test was significant ($p < 0.05$), follow-up comparisons were conducted, comprised of *t* tests with a Bonferroni correction to maintain the family-wise error rate at 0.05. Males and females were analyzed separately in all datasets. For the offspring body weight changes, the degrees of freedom decreased across PNDs due to litter standardization on PND2 and pup euthanasia on PNDs 7 and 14 (for analyses not included in this thesis).

3. Results

3.1 Birth and litter characteristics

During the breeding process a skewed sex ratio and variations in litter sizes were observed, with second trimester stressed litters appearing to be larger and to exhibit a different sex ratio compared to the other groups. The potential influence of the prenatal stressor on pregnancy outcomes was thus investigated. The length of gestation was not affected by prenatal stressor exposure, $F(2,16) = 1.000$, $p = 0.390$ (Figure 3A) but there was a non-significant trend for the prenatal stressor to influence the litter size at birth (which included live and dead pups), $H(2) = 5.156$, $p = 0.075$ (Figure 3B). When offspring sex was considered, it was found that there was a non-significant trend for the number of live males or females at birth to be influenced by the prenatal stressor, $\chi^2(2, N = 146) = 5.280$, $p = 0.071$ (Figure 3C).

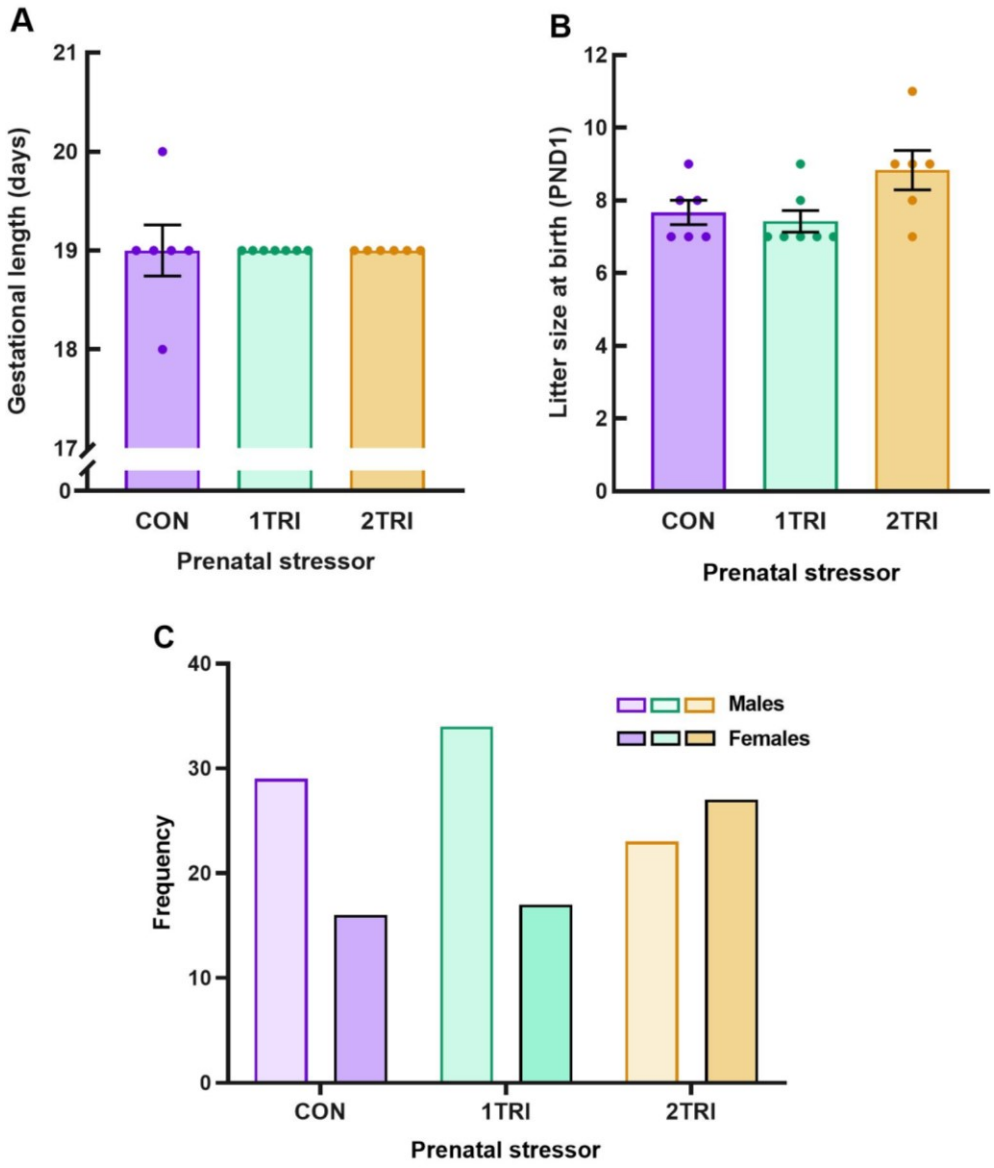

Figure 3. Characteristics of litters born to prenatally stressed and non-stressed mothers. Gestational length (A), litter size (includes number of live and dead pups) (B), number of males or females (live pups only) (C) at birth (postnatal day [PND] 1) in mothers exposed to stress during pregnancy (first or second trimester; 1TRI or 2TRI) or not stressed (controls; CON). Data represents means ± S.E.M. Number of litters per group: CON = 6, 1TRI = 7, 2TRI = 6.

3.2 Body weight changes across development (relative to birth weight)

To investigate whether first and second trimester prenatal stressors had differential effects on the early postnatal development of male and female mouse offspring, body weight changes (relative to birth weight) were determined shortly after birth as well as weekly until PND21. Although birth weights were unaffected by gestational stressor exposure, weight gains across the first three weeks of life varied depending on the trimester of stressor exposure and offspring sex. The factor Prenatal Stressor did not alter offspring weights at birth (PND1) in males, $H(2) = 1.613$, $p = 0.446$, but tended to increase female birth weights, $H(2) = 5.406$, $p = 0.067$, and follow-up tests revealed that the tendency for second trimester stressed females to be born larger than first trimester stressed did not reach significance, $p = 0.061$ (Figure 4A). In male offspring, there was a main effect of Prenatal Stressor on the percentage of weight change on PNDs 3, 7 and 21 (PND3: $H(2) = 7.874$, $p = 0.020$; PND7: $F(2,77) = 3.509$, $p = 0.035$; PND21: $F(2,31) = 3.938$, $p = 0.030$), but not on PND14, $F(2,54) = 2.198$, $p = 0.121$ (Figures 4B-E). Post hoc analyses confirmed that male pups born to dams stressed during the second trimester of pregnancy had gained more weight than non-stressed controls on PNDs 3 ($p = 0.015$), 7 ($p = 0.032$) and 21 ($p = 0.039$) (Figures 4B-C, 4E). In female offspring, the percentage of weight change on PND3 was influenced by whether offspring had been stressed during gestation, $F(2,54) = 4.807$, $p = 0.012$, and there was a trend on PND7 that did not reach significance, $F(2,54) = 2.916$, $p = 0.063$ (Figures 4B-C). Follow-up tests confirmed that first trimester stressed females had gained more weight than controls on PND3 ($p = 0.009$) and showed a trend for having gained more weight on PND7 as well ($p = 0.066$) (Figures 4B-C). On PND14, percentage of weight

change was comparable in prenatally stressed and non-stressed females, $F(2,40) = 0.185$, $p = 0.832$, and unlike males, the percentage of weight change on PND21 was comparable among prenatally stressed and non-stressed females, $F(2,25) = 0.102$, $p = 0.903$ (Figures 4D-E). Figures showing male and female offspring daily body weights from PNDs 1-21 can be found in the Supplementary Data section (Appendix 1).

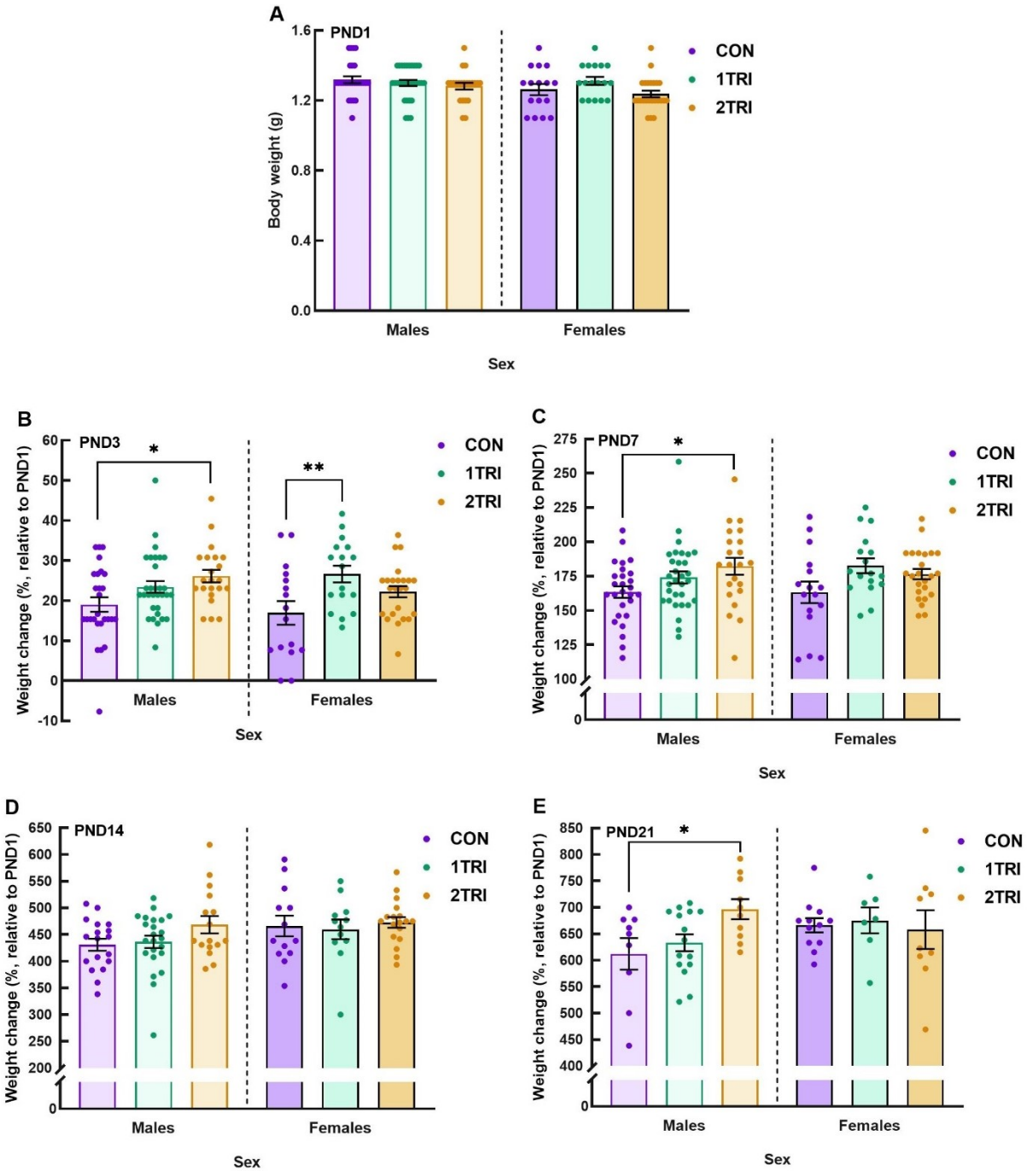

Figure 4. Physical growth of prenatally stressed and non-stressed male and female offspring across development

Body weight (in grams [g]) at birth (postnatal day [PND] 1; A) and percentage (%) of body weight change relative to weight at birth on PNDs 3 (B), 7 (C), 14 (D) and 21 (E) in male and female pups born to prenatally stressed mothers (first or second trimester; 1TRI or 2TRI) or non-stressed controls (CON). Data represents means ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$ relative to CON offspring. Sample sizes follow left to right for each figure: (A-C) N's = 27, 31, 22, 16, 17, 24; (D) N's = 18, 23, 16, 13, 12, 18; (E) N's = 9, 15, 10, 9, 7, 12.

3.3 Offspring body fat composition (relative to body weight)

The effect of first or second trimester prenatal stressors on rodent offspring body fat composition has only been previously studied at the adulthood stage (Dahlgren et al., 2001; Pankevich et al., 2009), therefore fat pads were dissected and weighed from PND21 prepubertal male and female mouse offspring. According to Figure 5 and confirmed by statistical analyses, body fat was increased in prenatally stressed male offspring, but not females. In males, there was a main effect of Prenatal Stressor on the percentage of fat at the perirenal/retroperitoneal location only, $F(2,34) = 3.790$, $p = 0.033$. As seen on Figure 5A, follow-up tests confirmed that second trimester stressed males had a higher fat percentage than the first trimester stressed males at the perirenal/retroperitoneal site ($p = 0.045$). In contrast, prenatal stress in males did not alter the mesenteric ($F(2,33) = 0.705$, $p = 0.501$), perigonadal ($H(2) = 4.367$, $p = 0.113$), subcutaneous ($F(2,34) = 1.749$, $p = 0.189$) or brown $F(2,34) = 0.650$, $p = 0.528$) fat pad percentages, nor did it alter the percentage of total body fat, $F(2,33) = 1.240$, $p = 0.302$ (Figure 5A). Unlike males, Prenatal Stressor did not alter the percentage of fat at any location in female offspring (Mesenteric: $F(2,25) = 0.944$, $p = 0.402$; Perigonadal: $F(2,25) = 1.537$, $p = 0.235$; Perirenal/Retroperitoneal: $F(2,25) = 0.320$, $p = 0.729$; Subcutaneous: $F(2,25) = 1.824$, $p = 0.182$; Brown: $F(2,25) = 2.374$, $p = 0.114$; Total: $F(2,25) = 1.988$, $p = 0.158$) (Figure 5B).

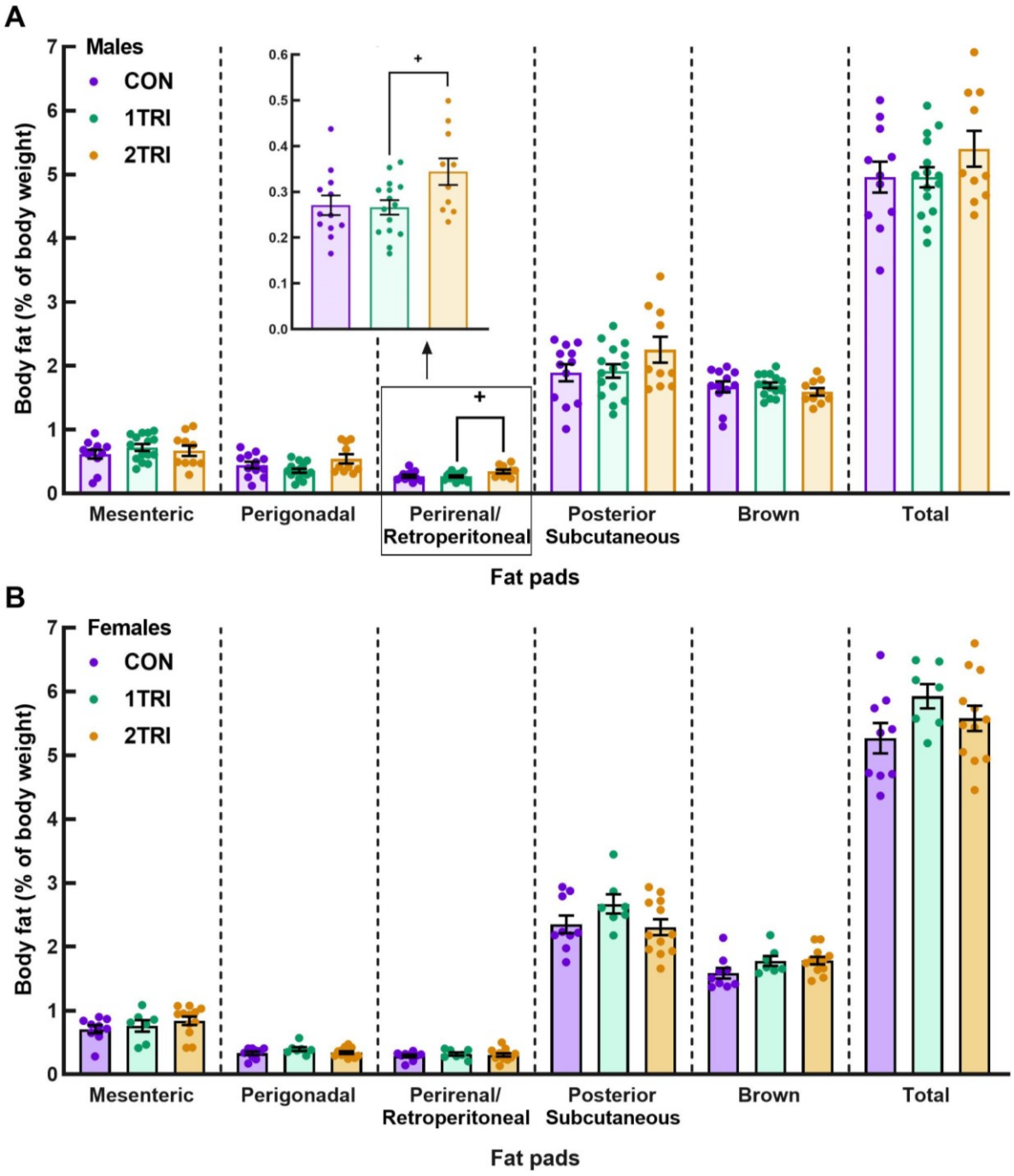

Figure 5. Fat pad distribution (percentage [%], normalized to body weight) of prenatally stressed and non-stressed males and females at 21 days old.
Mesenteric, perigonadal, perirenal/retroperitoneal, subcutaneous (includes dorsolumar, inguinal, and gluteal depots), brown (includes interscapular and subscapular depots) and total (mesenteric, perigonadal, perirenal/retroperitoneal, subcutaneous, and brown combined) body fat (%, relative to body weight) of postnatal day [PND] 21 male (A) and female (B) mouse offspring pups born to mothers stressed during pregnancy (first or second trimester; 1TRI or 2TRI) or not stressed (control; CON). Data represents means ± S.E.M. $^{+}p < 0.05$ relative to 1TRI males. Sample sizes for CON, 1TRI and 2TRI are N's = 12, 15, 10 for males and N's = 9, 7, 12 for females, except for CON males mesenteric and total fat where n = 11 (due to one mouse missing mesenteric fat).

3.4 Offspring anxiety-like behavior: Open field test

Whether first or second trimester gestational stressors modulated the anxiety-like behaviors of offspring confirmed to be prepubertal was examined, of relevance since previous investigations into these outcomes did not confirm puberty status (Lieberman, 1963; Jia et al., 2015). Sex-specific behaviors were displayed by the prenatally stressed prepubertal offspring, with only females exhibiting phenotypic differences in the open field test. Prenatally stressed male offspring showed no differences in open field behaviors compared to non-stressed controls. The time spent, $H(2) = 0.106$, $p = 0.948$, number of entries in, $H(2) = 0.143$, $p = 0.931$, and latency to first enter, $H(2) = 0.782$, $p = 0.676$, the center of the apparatus, as well as the time spent, $F(2,34) = 0.099$, $p = 0.906$, and number of entries in, $F(2,34) = 1.170$, $p = 0.323$, its four corners and the total distance traveled in the field, $F(2,34) = 0.306$, $p = 0.738$, did not differ between prenatally stressed and non-stressed males (Figure 6). In contrast, although time spent in the center of the open field was unchanged in prenatally stressed females, $H(2) = 4.632$, $p = 0.099$, they made more entries into this part of the field, $H(2) = 7.269$, $p = 0.026$, and took less time to make their first entry into it, $H(2) = 5.847$, $p = 0.054$, compared to their non-stressed counterparts (Figures 6A-C). Likewise, time spent in the four corners of the open field was similar in all female groups, $F(2,25) = 1.262$, $p = 0.300$, but the number of entries into the four corners was increased by the Prenatal Stressor factor, $F(2,25) = 3.628$, $p = 0.041$, as was the total distance travelled in the field, $F(2,25) = 4.457$, $p = 0.022$ (Figures 6D-F). Post hoc tests confirmed that first trimester stressed female offspring entered the center more quickly ($p = 0.051$) and more frequently ($p = 0.022$), entered the four corners more often ($p = 0.048$), and travelled more ($p = 0.026$) compared

to non-stressed female controls (Figures 6B-C, 6E-F) Additional open field measures can be found in the Supplementary Data section of Appendix 1.

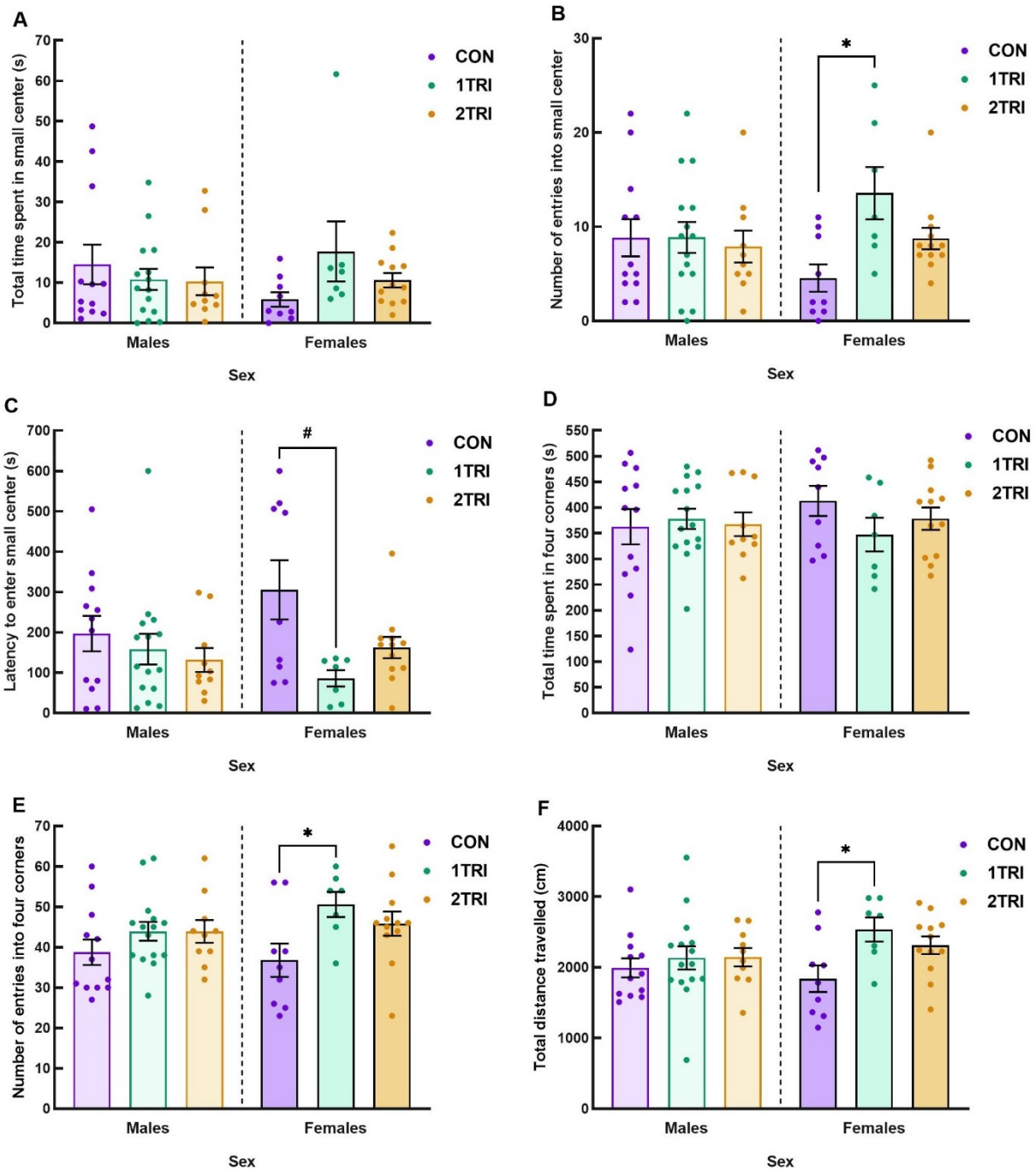

Figure 6. Open field test behaviors of male and female mouse offspring aged 19 days. Offspring were born to mothers stressed during pregnancy (first or second trimester; 1TRI or 2TRI) or not stressed (control; CON). Data shown is the cumulative time spent (in seconds [s]) in the center (A), frequency of entries into the center (B), latency (s) to first entry the center (C), cumulative time (s) spent in the four corners (D), frequency of entries into the four corners (E), and the total distance travelled in the apparatus in centimeters (cm) (F). Data represents means ± S.E.M. $^{*}p < 0.05$ and $^{\#}p = 0.051$ relative to CON females. Sample sizes for CON, 1TRI and 2TRI are N's = 12, 15, 10 for males and N's = 9, 7, 12 for females.

3.5 Offspring anxiety-like behavior: Elevated plus maze test

Similar to open field behaviors, prenatal stress in male offspring had no effect on behavioral parameters measured in the elevated plus maze. The time spent in open arms ($H(2) = 0.177$, $p = 0.915$), number of entries into open arms ($H(2) = 2.108$, $p = 0.348$), latency to first entry into open arms ($H(2) = 1.852$, $p = 0.396$), time spent in closed arms ($H(2) = 0.361$, $p = 0.835$), number of entries into closed arms ($H(2) = 2.053$, $p = 0.358$), or total distance travelled in the maze ($F(2,33) = 0.117$, $p = 0.890$) were comparable between prenatally stressed and non-stressed males (Figure 7). In contrast, in females prenatal stress increased the frequency of entries into the closed arms, $F(2,25) = 3.881$, $p = 0.034$, and tended to increase the frequency of entries into the open arms as well, $H(2) = 4.882$, $p = 0.087$ (although the time spent in either of these arms was unchanged, $H(2) = 2.505$, $p = 0.286$ and $H(2) = 2.473$, $p = 0.290$, respectively) (Figures 7A-B, D-E). There was also a trend for the Prenatal Stressor to influence the total distance moved in the apparatus, $F(2,25) = 3.215$, $p = 0.057$ (Figure 7F). Follow-up tests showed non-significant trends for second trimester stressed females entering the closed arms more often and travelling greater distances than first trimester stressed females, $p = 0.079$ and $p = 0.067$, respectively (Figures 7E-F). Finally, prenatal stressor exposure in females did not influence latency to enter the open arms, $H(2) = 0.061$, $p = 0.970$ (Figure 7C). Additional elevated plus maze measures can be found in the Supplementary Data section of Appendix 1.

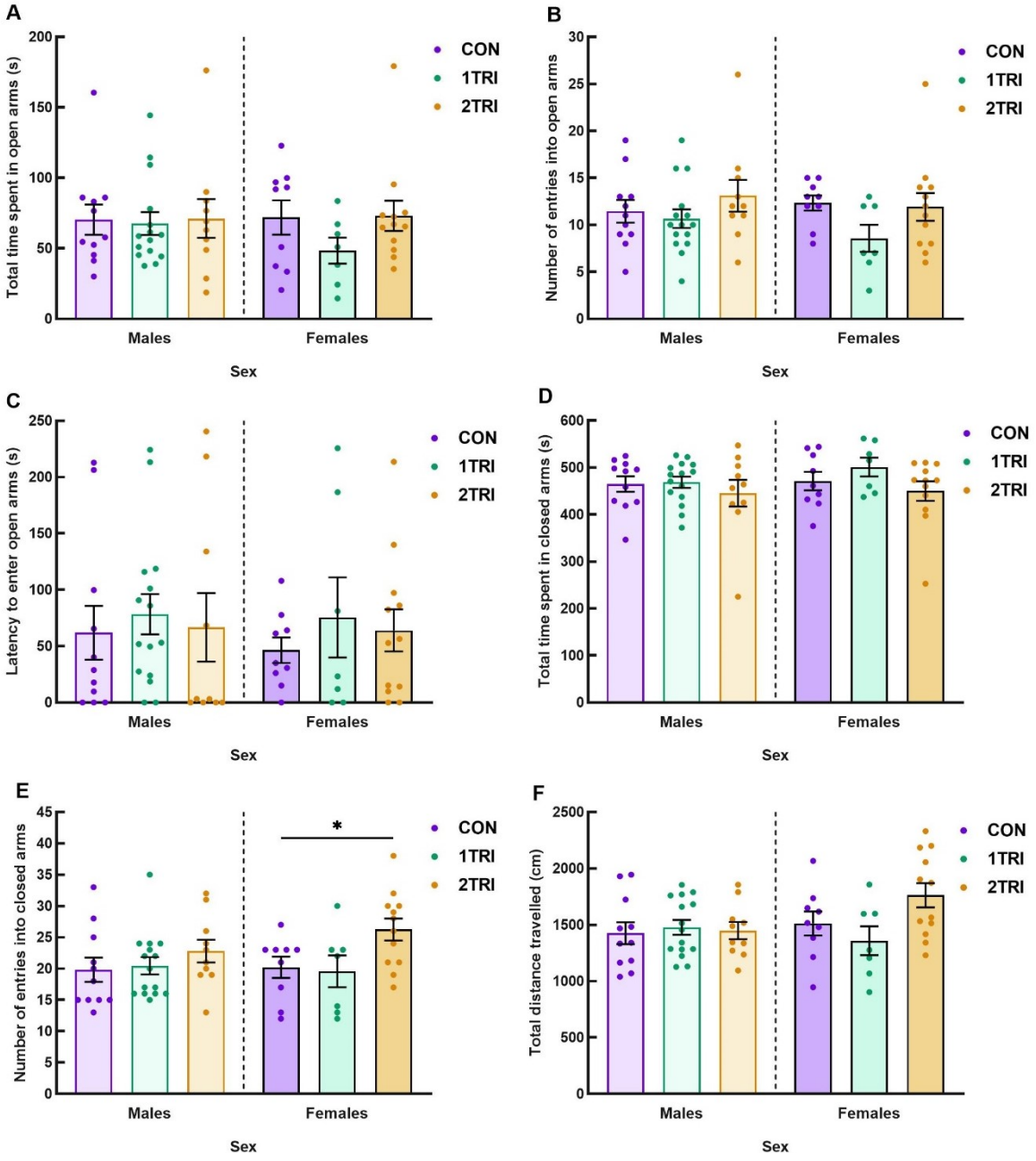

Figure 7. Behaviors of male and female offspring in the elevated plus maze test at 20 days of age.

Offspring were born to mothers stressed during pregnancy (first or second trimester; 1TRI or 2TRI) or not stressed (control; CON). Data shown is the cumulative time (in seconds [s]) spent in the open arms (A), frequency of entries into open arms (B), latency (s) to first entry into open arms (C), cumulative time (s) spent in the closed arms (D), frequency of entries into closed arms (E), and the total distance travelled in centimeters (cm) (F). Data represents means ± S.E.M. $^{*}p <$ 0.05 main effect of prenatal stress. Sample sizes for CON, 1TRI and 2TRI are N's = 11, 15, 10 for males and N's = 9, 7, 12 for females. One CON male was excluded from the analyses due to falling off the maze during testing.

3.6 Offspring depressive-like behavior: Splash test

Previous studies have reported increased depressive-like behaviors in juvenile rodent offspring exposed to stressors early in gestation, however whether these offspring had reached puberty at the time of testing is unknown (Zhang et al., 2013; Jia et al., 2015). Unlike in the tests for anxiety-like behaviors, only prenatally stressed male offspring displayed abnormal behaviors in the splash test. In males, the Prenatal Stressor tended to affect the total time spent grooming, $H(2) = 5.030$, $p = 0.081$, whereas the average duration of grooming session and the total number of grooming sessions, $H(2) = 10.216$, $p = 0.006$ and $F(2,32) = 4.449$, $p = 0.020$, significantly differed between prenatally stressed and non-stressed male offspring (Figures 8A-C). Follow-up tests confirmed that second trimester stressed males had fewer grooming bouts ($p = 0.018$) compared to first trimester stressed males, but when they did groom their grooming sessions lasted longer than first trimester stressed ($p = 0.007$) and non-stressed control males ($p = 0.023$) (Figures 8B-C). There was also a trend for prenatal stress to influence the latency to start rearing in male offspring, $H(2) = 5.846$, $p = 0.054$, with post hoc tests confirming first trimester stressed males had a longer latency to rear than non-stressed controls, $p = 0.048$ (Figure 8H). No differences in the latency to start grooming ($H(2) = 1.066$, $p = 0.587$) or other rearing behaviors in the splash test (Total time: $F(2,32) = 2.136$, $p = 0.135$; Average length: $F(2,32) = 0.516$, $p = 0.602$; Total number: $F(2,32) = 1.980$, $p = 0.155$; Latency: $F(2,32) = 0.683$, $p = 0.512$) was apparent in male offspring (Figures 8D-G).

In females, there was a non-significant trend for the Prenatal Stressor to affect total time spent grooming, $F(2,25) = 2.830$, $p = 0.078$ (Figure 8A). No effects of the

prenatal stressor were observed for the average grooming session length, $H(2) = 4.170$, $p = 0.124$, total number of grooming sessions, $F(2,25) = 0.242$, $p = 0.787$, or the latency to start grooming, $H(2) = 2.272$, $p = 0.321$ (Figures 8B-D). Rearing behaviors were also unaltered in female offspring exposed to prenatal stressors (Total time: $F(2,25) = 0.478$, $p = 0.625$; Average length: $F(2,25) = 1.231$, $p = 0.309$; Total number: $F(2,25) = 0.945$, $p = 0.402$; Latency: $H(2) = 2.256$, $p = 0.324$) (Figures 8E-H). Additional digging and jumping behaviors can be found in the Supplementary Data section of Appendix 1.

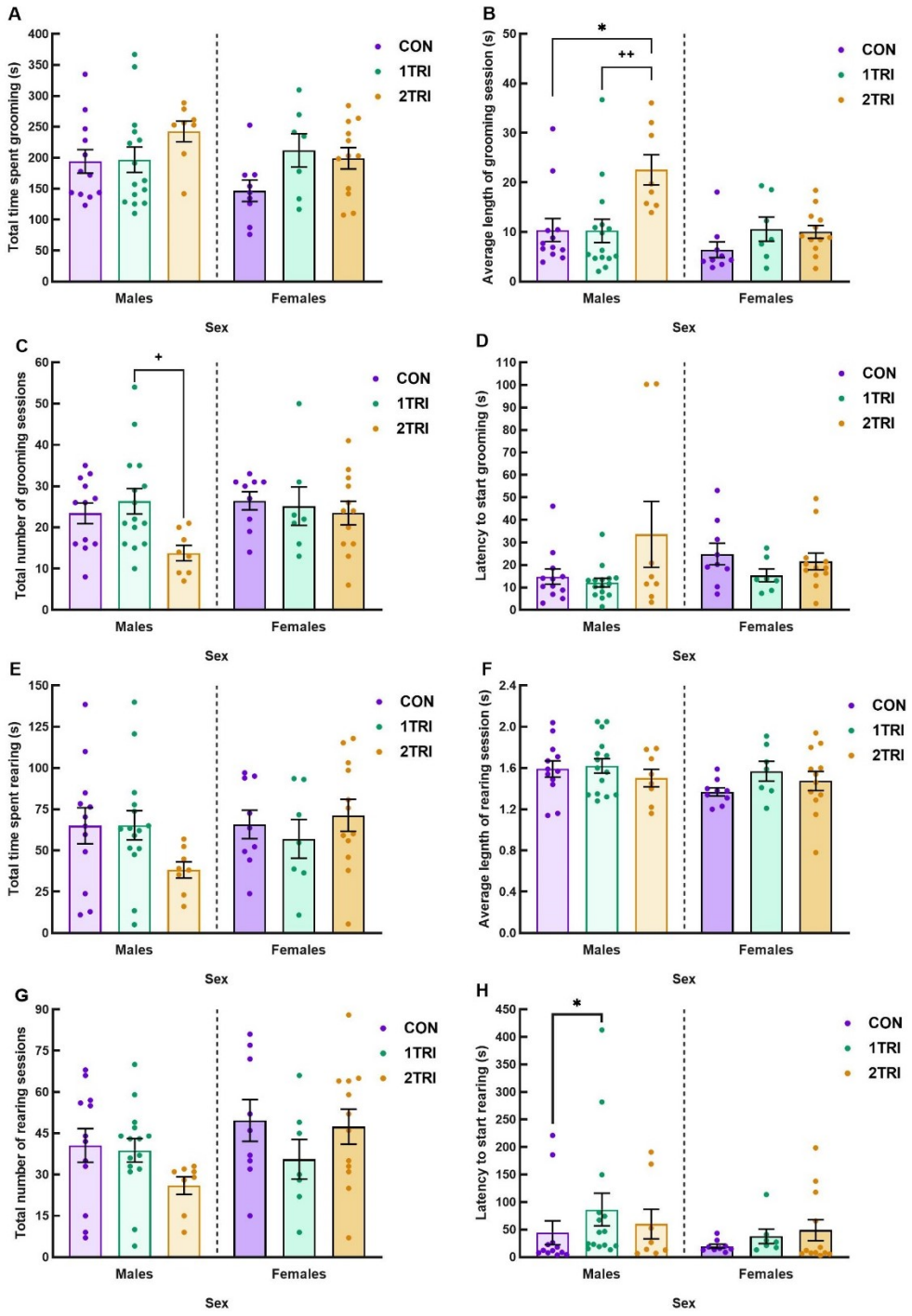

Figure 8. Splash test behaviors of male and female mouse offspring aged 19 days.
Dams were stressed during pregnancy (first or second trimester; 1TRI or 2TRI) or not stressed (control; CON). Data shown is the cumulative time (in seconds [s]) spent grooming (A), average length (s) of grooming sessions (B), frequency of grooming sessions (C), latency (s) to start grooming (D), total time (s) spent rearing (E), average length (s) of rearing sessions (F), frequency of rearing sessions (G), and latency (s) to start rearing (H). Data represents means ± S.E.M. $^{*}p < 0.05$ relative to CON males, $^{+}p < 0.05$ and $^{++}p < 0.01$ relative to 1TRI males. Sample sizes for CON, 1TRI and 2TRI are N's = 12, 15, 8 for males and N's = 9, 7, 12 for females. Two 2TRI males were excluded due to problems with the video recordings.

3.7 Offspring depressive-like behavior: Tail suspension test

Prenatal stressor exposure had no effect on depressive-like behaviors in the tail suspension test in either male or female offspring. The total time spent immobile (males: $F(2,31) = 0.849$, $p = 0.438$; females: $F(2,21) = 2.55$, $p = 0.102$) and the latency to immobility (males: $F(2,31) = 0.970$ $p = 0.390$; females: $F(2,21) = 0.278$, $p = 0.760$) were comparable between prenatally stressed and non-stressed offspring (Figures 9A-B). The average length and total number of immobility sessions can be found in the Supplementary Data section of Appendix 1.

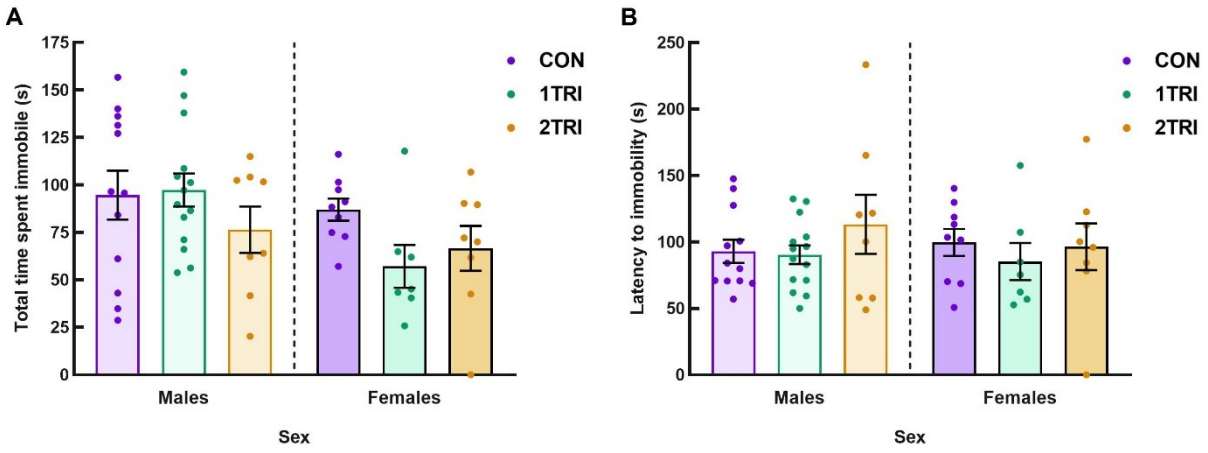

Figure 9. 20-day old male and female mouse offspring behaviors in the tail suspension test. Pregnant mice were stressed during the first (1TRI) or second (2TRI) trimester or were not disturbed (control; CON). Data shown is the total time spent immobile (A) and the latency to immobility (B), both in seconds (s). Data represents means ± S.E.M. Sample sizes for CON, 1TRI and 2TRI are N's = 12, 14, 8 for males and N's = 9, 7 and 8 for females. Seven mice were excluded from the final dataset due to the following testing issues: one 1TRI male and two 2TRI females continuously grabbed onto the tape/bar which their tails were attached to; a 2TRI male and a 2TRI female had their back paws stuck to the tape for entire test; one 2TRI male and one 2TRI female video files were lost.

4. Discussion

The main objective of this study was to investigate the effects of prenatal maternal stress experienced in early pregnancy on physical growth and fat pad distribution, as well as on anxiety- and depressive-like behaviors in prepubertal male and female mouse offspring. The current findings show that the impacts of early prenatal stress on these outcomes were dependent on the trimester of pregnancy during which the stressor was experienced and varied as a function of the sex of the offspring. Male offspring were most susceptible to the metabolic effects of prenatal stress when applied during the second trimester of pregnancy, showing alterations in the form of exaggerated weight gains across the first three weeks of postnatal development and greater body fat percentages on PND21. In contrast, female offspring stressed *in utero* mainly demonstrated behavioral alterations, exhibiting a hyperactive phenotype in tests designed to evaluate anxiety-like phenotypes. These findings demonstrate that the sex- and trimester-specific physical and behavioral health outcomes of offspring stressed during early fetal development manifest prior to the onset of puberty. This research contributes to the very few studies that have investigated the physical and behavioral effects of prenatal stressors at a prepubertal stage. Importantly, these findings highlight gestational periods and offspring sex that are the most vulnerable to the effects of prenatal stressors to direct research efforts into the development of possible preventive interventions/strategies for the adverse physical and mental outcomes.

4.1 Sex ratio was changed in litters exposed to second trimester stress

As there is previous evidence for reduced uterine receptivity following early pregnancy stressors (Wiebold et al., 1986; Pratt and Lisk, 1989; Clark et al., 1993;

deCatanzaro et al., 1994; Arck et al., 1995; Lee et al., 2008; Jafari et al., 2017), whether pregnancy outcomes and litter characteristics would be modulated by the first and second trimester prenatal stressors was first examined. Although pregnancy duration was the same in prenatally stressed and non-stressed dams, dams that were stressed during the second trimester tended to birth larger litters with an equal sex ratio, in contrast to non-stressed and first trimester stressed litters that tended to have a male bias at birth. The apparent sex bias observed in the non-stressed litters agrees with the C57BL/6 strain reportedly yielding a higher number of males relative to females (Weir, 1960). Rodents exposed to first or second trimester prenatal stressors have been previously found to have smaller litters with fewer males (Wiebold et al., 1986; Pratt and Lisk, 1989; deCatanzaro et al., 1994), but intact gestational lengths, pup numbers, and sex ratios at birth have also been reported (Hutchings and Gibbon, 1970; D'mello and Liu, 2006; Mueller and Bale, 2006; Amugongo and Hlusko, 2013; Zhang et al., 2013; Bronson and Bale, 2014; Jeje and Raji, 2017). The impact of early pregnancy stressors on birth outcomes of C57BL/6 mice has not been previously investigated and thus the current findings are the first to demonstrate that prenatal stressors during the second, but not first, trimester can equalize the sex ratio in this mouse strain. Importantly, human populations exposed to severe stressors such as natural disasters or terrorist attacks, especially during early pregnancy, showed a subsequent decline in male births compared to males born under non-stressed conditions (Hansen et al., 1999; Catalano et al., 2006; Torche and Kleinhaus, 2012; Doğer et al., 2013). As previously alluded to, the different sex ratio in second trimester stressed C57BL/6 litters is a novel finding which supports previous observations that

stressful encounters during gestation can have significant implications for birth outcomes, but whether these repercussions can be maladaptive remains unclear.

4.2 Metabolic parameters were altered in prepubertal male offspring exposed to stress during the second trimester of pregnancy

Prenatal stressor exposure affected offspring physical growth in a sex- and trimester-specific manner, with only males born to dams stressed during the second trimester of pregnancy being significantly impacted. These mice gained more weight than their non-stressed counterparts across the first three weeks of postnatal development and had larger fat deposits surrounding the kidneys (perirenal/retroperitoneal locations). In contrast, females born to dams stressed during the first trimester were slightly heavier at birth and had gained more weight on PND3, but these changes normalized across development so that by the end of the second and third postnatal weeks, weight changes were comparable in all groups, as was fat composition on PND21. Few studies have investigated the effects of early pregnancy stressors on postnatal development in rodents. Unlike the current findings, three studies comparing first and second trimester stressors did not see alterations in offspring body weights across the first three weeks of postnatal development (D'mello and Liu, 2006; Mueller and Bale, 2006; Jeje and Raji, 2017). However, these experiments utilized different rodent strains (Sprague-Dawley or Wistar rats, C57BL/6:129 mice) or stressor paradigms (variable mild stress, dexamethasone injection), making comparisons with the current findings difficult, as physical growth of prenatally stressed rodents may vary depending on the strain (Stöhr et al., 1998) and the type of stressor used (Fride and Weinstock, 1984). Additionally, in the present study the body weights of male and female offspring were tracked separately, while female

offspring were not included in the analyses of Jeje and Raji (2017) or D'mello and Liu (2006), and Mueller and Bale (2006) pooled male and female offspring weights, which may have obscured any sex differences.

Regarding the influence of gestational stressors on fat pad distribution, no previous study has investigated how first or second trimester exposures may cause alterations at the prepubertal stage. When adults were considered, however, first trimester stress reduced body fat percentages in males only (Pankevich et al., 2009), while in the present study males stressed during this time were unaltered at the prepubertal stage. In contrast, adult male and female rats exposed to dexamethasone during the second trimester of pregnancy had heavier retroperitoneal fat pads in adulthood (as observed in the present study), while their female counterparts displayed larger perigonadal adipose weights (Dahlgren et al., 2001). Perirenal and retroperitoneal fat pads normally grow during postnatal development, but abnormally large increases in visceral fat levels (including perirenal and retroperitoneal deposits) have been linked to an elevated risk of obesity and other metabolic disorders (Berry et al., 2013). Thus, in the present study, the exaggerated fat weight increase seen at this location in second trimester stressed male offspring, along with the more pronounced weight gains displayed by these mice, may be indicative of an increased risk for later-life obesity and/or metabolic disorders. Detection of these early signs of metabolic dysfunction, which have also been reported in human infants and children exposed to gestational stressors (Gillman et al., 2006; Watt et al., 2013; Dancause et al., 2015), is critical for determining when interventions may be administered to reduce the heightened risk which prenatally stressed offspring have of developing later-life obesity and diabetes (Virk et al., 2010, 2012; Hohwü et al., 2014).

4.3 Prepubertal mouse offspring exposed to prenatal stressors showed sex- and trimester-specific behavioral changes

In the present thesis, the tests for anxiety- and depressive-like behaviors revealed sex- and trimester-dependent outcomes in gestationally stressed prepubertal mice, with second trimester stressed males exhibiting altered grooming behaviors, and females that had been stressed *in utero* displaying a hyperactive phenotype. Although the prenatally stressed female offspring did not exhibit a strong preference for darkened or well-lit areas in anxiogenic environments, they transitioned between these zones more often, entering more frequently into the corners and small/large centers of the open field (markedly by the first trimester stressed offspring) and into the closed arms of the elevated plus maze. As these offspring also travelled a greater distance in both tests, taken together these results suggest that they may have developed a hyperactive phenotype across development. In contrast, prepubertal male offspring showed no behavioral changes in either the open field or elevated plus maze tests, but displayed abnormalities in their grooming response to water spray stimulation, with specifically second trimester stressed males being affected, further pointing towards a higher male susceptibility to the effects of mid-gestational stressors, as was shown in the growth and metabolic measures.

The present thesis is the first study that investigated anxiety- or depressive-like behaviors in prepubertal offspring that had been stressed during the very first days of fetal development. In previous experiments which considered adult offspring, C57/BL6:129 hybrid males exposed to variable stress during the first trimester of pregnancy were hyperactive, had reduced anxiety-like behaviors in the light-dark test (Bronson and Bale, 2014), and increased depressive-like behaviors in the tail suspension,

forced swim, and sucrose preference tests, whereas females were unaffected (Mueller and Bale, 2008). Conversely, in the present study, prior to puberty a hyperactive phenotype was displayed by first trimester stressed females, while their male counterparts had unaltered anxiety- or depressive-like behaviors, which suggests that prior to sex hormone development there are baseline phenotypic differences between male and female offspring that have been exposed to early gestational stressors. This may suggest that the surge in sex hormones that accompanies pubertal onset may modify stress-sensitive biological systems in a way which normalizes the female hyperactivity phenotype, while possibly stimulating the emergence of anxiety- and/or depressive-like behaviors in male offspring. Alternatively, the variable stressors which were administered during the first trimester by Mueller and Bale (2008) and Bronson and Bale (2014) may elicit differential behavioral phenotypes than the predictable restraint stressor paradigm which was used in the present experiments (Fride and Weinstock, 1984).

In accordance with the current findings, previous studies have suggested that male offspring may be more sensitive to the effects of stress experienced during the second trimester of pregnancy. Adult males exposed to a mid-gestational immune challenge displayed increased anxiety-like behaviors in the open field, elevated plus maze, and light-dark tests (Depino, 2015; Majidi-Zolbanin et al., 2015; Golub et al., 2016), reduced locomotor activity (Li et al., 2014; Depino, 2015), and more pronounced depressive-like phenotypes (Depino, 2015; Majidi-Zolbanin et al., 2015), although changes in both sexes (Lin and Wang, 2014) or neither sex (Mueller and Bale, 2008; Li et al., 2014) have also been reported. In humans, the strongest association between prenatal stressors and adulthood anxiety and depressive symptoms are found in men that have been exposed to

early pregnancy stressors (Watson et al., 1999; Kleinhaus et al., 2013; Herbison et al., 2017), further suggesting increased male stress susceptibility during this time of gestation. In the present research, although second trimester stressed male offspring were unaltered in terms of their anxiety- and depressive-like behaviors prior to puberty, they did show abnormal grooming patterns. Sex hormones may exacerbate the behavioral alterations displayed by these offspring to promote stronger perturbations after puberty.

Few studies have tested second trimester stressed rodents for anxiety- or depressive-like behaviors in juvenility (PND25-35), and for those that did, mixed results have been reported, possibly due to these offspring not being assessed for puberty status. Juvenile male and female offspring exposed to a second trimester restraint stressor showed increased anxiety-like behaviors and reduced locomotion in the open field test (Jia et al., 2015), but in contrast a shock stressor during this trimester reduced crouching, suggestive of reduced emotionality (Hutchings and Gibbon, 1970). Conversely, the second trimester stressors of overcrowding or hydrocortisone injection produced differential open field behaviors in mouse offspring, the former increasing activity but the latter reducing jumping and increasing defecation (Lieberman, 1963). The modulation of depressive-like behaviors in juvenile offspring by second trimester restraint stressors also remains unclear, with neither males or females showing altered forced swim behaviors (Jia et al., 2015), or rats of both sexes displaying increased immobility in the tail suspension test (Zhang et al., 2013). Importantly, these studies did not report whether the puberty status of the juvenile rodents was assessed, an important consideration as stressful encounters *in utero* can shift pubertal onset in both male and female rodent offspring (Smith and Waddell, 2000; Pallarés et al., 2013), and sex hormones have

previously been demonstrated to be capable of altering anxiety- and depressive-like behaviors (Boivin et al., 2017; Delevich et al., 2020). As male and female prepubertal offspring in the present experiment did not exhibit anxiety- or depressive-like behaviors in response to second trimester stress, but previous studies have detected these behavioral changes in juvenility, this may suggest that sex hormone surges are required for the development of these phenotypes. Indeed, most mood and anxiety disorders emerge during puberty or in the following years (Cohen et al., 1993; Kessler et al., 1994; de Lijster et al., 2017).

In the present study, no differences were observed in the tail suspension test in prepubertal male and female offspring exposed to prenatal stressors. This contrasts with previous studies that have consistently reported increased depressive-like behaviors in rodent offspring aged 24-34 days exposed to second or third trimester physical restraint stressors (Guan et al., 2013; Sun et al., 2013a; Zhang et al., 2013; Jia et al., 2015; Lu et al., 2017; Dang et al., 2018; Iturra-Mena et al., 2018). As previously discussed, however, confirmation of pubertal status in these studies was not mentioned, thus it is possible that these offspring were post-pubertal and depressive-like behaviors may have only emerged after sexual development (Boivin et al., 2017). Overall, it remains unclear whether the diverse neurological and hormonal changes that occur during puberty onset are required for the emergence of depressive-like behaviors in rodent offspring that are exposed to prenatal stressors. The results of the present study do indicate that prior to puberty, differences in activity and grooming patterning exist between female and male offspring that have been stressed during early gestation.

A complex microstructure underlies grooming behavior which when analyzed can yield significant insight into an animals affective state (Kalueff and Tuohimaa, 2005), which was not assessed in the present study but could be explored at a future time. Furthermore, the stereotypical grooming patterns of adult rodents are not established until several weeks after birth (Golani and Fentress, 1985), suggesting that the rodent's postnatal age may be an important consideration when interpreting stress-induced grooming alterations, and making it difficult to make conclusions about what the altered grooming in the second trimester stressed prepubertal males means in terms of affective state. A grooming pattern which has been characterized to be abnormal in an adult rodent may not be diagnostic of an issue at the juvenile stage, thus more research is needed to compare how stressors influence grooming behaviors across the lifespan. In the current experiment, individual placement of the rodent offspring into a novel environment for splash testing may have been a particularly stressful experience and contributed to the grooming changes that were observed.

Procedural differences in the behavioral testing done in the present work and that of previous research may account for the differences seen in results. Firstly, previous studies did not specify the illumination level of the open field and elevated plus maze tests (Lieberman, 1963; Hutchings and Gibbon, 1970; Li et al., 2014; Jia et al., 2015; Golub et al., 2016) or had lower lighting than what was utilized in the present study (Mueller and Bale, 2008; Depino, 2015; Majidi-Zolbanin et al., 2015), which is of importance since higher light levels have been correlated with decreased ambulation and increased anxiety-like phenotypes (Bouwknecht et al., 2007; Albani et al., 2015). Furthermore, these behaviors in prenatally stressed offspring have been shown to vary

across the light-dark cycle (Ehrlich et al., 2015), but previous studies tested during the dark phase (Mueller and Bale, 2008) or did not specify the time of testing (Lieberman, 1963; Hutchings and Gibbon, 1970; Jia et al., 2015). Finally, different rodent species or strains show baseline differences in tail suspension activity which adds to the complexity of making comparisons between studies (Cryan et al., 2005). Overall, procedural differences between the present experiments and prior studies, in addition to the possibility of previously tested juvenile rodents being post-pubertal, may account for the behavioral patterns which were observed in this thesis differing from those of previous studies.

4.4 Glucocorticoid modulation of fetoplacental gene expression may contribute to the sex- and trimester-specific effects of prenatal stressors

There is evidence that the rise in maternal glucocorticoid levels that occurs in response to stressors applied during the first, second, or third trimester of pregnancy (D'mello and Liu, 2006; Amugongo and Hlusko, 2013) can induce epigenetic changes in the fetoplacental unit that may contribute to accelerated maturation in postnatal offspring. Under healthy conditions, placental expression of 11β-HSD2 protects the fetus from excess levels of maternal corticosterone (rodents) or cortisol (humans) by chemically inactivating these hormones (Holmes et al., 2006). However, prolonged exposure to stressors can downregulate the expression of placental 11β-HSD2 (Peña et al., 2012; Mina et al., 2015; Seth et al., 2015; Briffa et al., 2017) and allow greater passage of maternal glucocorticoids to the fetus (Montano et al., 1991; Staud et al., 2006; Vackova et al., 2009). Since optimal levels of glucocorticoids are necessary for proper growth and development of fetal organs and systems, abnormal elevations of these hormones can

have significant consequences for the developing fetus, including modifying metabolic-related systems and the brain (Harris and Seckl, 2011). Importantly, stress-induced 11β-HSD2 reductions have been shown to differ between male and female placentas, with vulnerabilities changing over the course of pregnancy. Chronic variable stressor exposure in the first trimester of pregnancy reduced 11β-HSD2 expression in E12 female but not male placentas (Pankevich et al., 2009), suggesting a heightened female vulnerability to first trimester corticosterone elevations, as seen in the present study with restraint stress during this time only affecting female offspring in terms of the PND3 body weights and open field behaviors. Conversely, physical restraint stress from mid pregnancy onward resulted in 11β-HSD2 downregulation in E21 male placentas (but females were not assessed) (Mairesse et al., 2007a), and continuous corticosterone exposure during mid pregnancy reduced 11β-HSD2 expression only in male placentas on E17.5 (Cuffe et al., 2012), suggesting that the male sex may be more susceptible to mid-pregnancy stressors, as seen in the current study with increased growth and fat and altered grooming patterns observed.

Corticosterone elevations in the fetus resulting from stress-induced placental 11β-HSD2 downregulation can lead to widespread gene expression changes through GR and MR binding (Harris and Seckl, 2011), including changes to genes which control neurodevelopment, growth and metabolism. For example, third trimester restraint stress disturbed the neurogenesis and morphological development of hippocampal neurons from the neonatal stage to adulthood through a GR-dependent mechanism (Fujioka et al., 2006), suggesting that changes in the regulation of the offspring HPA axis contribute to the neurodevelopmental effects of *in utero* stressors. Adult mouse offspring that had been

exposed to third trimester dexamethasone treatment displayed diverse metabolic perturbations, including hyperinsulinemia, increased adiposity, and mitochondrial dysfunction (Chen et al., 2020). These changes were associated with elevated corticosterone and GRs in the neonatal stage, and were attributed to the reduced expression of the *Ppargc1a* gene, an essential regulator of energy metabolism, through *in utero* GR-mediated methylation of the *Ppargc1a* promotor (Chen et al., 2020). Prenatal HPA activation has also been shown to disturb amino acid, vitamin and leptin transport across the placenta (Sugden et al., 2001; Smith and Waddell, 2002; Vaughan et al., 2012; Schroeder et al., 2018), suggesting that gestational stressors interfere with fetal nutritive supply. Furthermore, altered methylation patterns in the placental and fetal hypothalamus in prenatally stressed mice correlated to altered metabolic profiles, eating habits and adiposity in adulthood (Schroeder et al., 2017, 2018), and in humans stress *in utero* caused epigenetic changes to inflammatory and stress response genes which were associated with reductions in birth weights and early childhood body fat (Kertes et al., 2016; Wu et al., 2018). In summary, a potential mechanism underlying the physical growth and behavioral effects of prenatal stressors could be through sex-and trimester-specific 11β-HSD2 downregulation, leading to fetoplacental corticosterone elevations and global gene expression changes.

4.5 The influence of prenatal stressors on offspring outcomes may be modulated by inadequate food consumption during pregnancy

Stress-induced changes to maternal food intake patterns during pregnancy may have contributed to the litter and metabolic alterations observed in the gestationally stressed mouse offspring. Previous studies have reported that on the days of prenatal

stressor exposure, maternal weight gains and food/water consumption were decreased (Kinsley and Svare, 1986; Ward and Wainwright, 1988; D'mello and Liu, 2006; Borsonelo et al., 2011; Amugongo and Hlusko, 2013), and a reduced access to food during pregnancy has itself been shown to be stressful (Lesage et al., 2001; Belkacemi et al., 2011). Nutrient restriction in early pregnancy (independent of stress) induced placental 11β-HSD2 downregulation similarly to gestational stressors (Bertram et al., 2001; Lesage et al., 2001; Belkacemi et al., 2011) and accelerated growth of postnatal offspring (Berghänel et al., 2017), in agreement with the present study where larger weight gains were displayed by the prenatally stressed pups. It is thus possible that gestational stressors and malnutrition could act synergistically on offspring metabolic outcomes, possibly through shared biological pathways (Langley-Evans et al., 1996; Bertram et al., 2001; Lesage et al., 2001; Belkacemi et al., 2011). As previously discussed, placental 11β-HSD2 downregulation can occur in a trimester- and sex-specific manner in response to prenatal stressors (Mairesse et al., 2007a; Pankevich et al., 2009; Cuffe et al., 2012) and this enzyme may also be decreased following stress-induced reductions in maternal food intake (Bertram et al., 2001; Lesage et al., 2001; Belkacemi et al., 2011). An important finding in the current study was the exaggerated growth and increased body fat of the male offspring that had been exposed to second trimester stressors, which could have been modulated by placental 11β-HSD2 changes.

In the present work, the second trimester stressed litters showed an equal sex ratio at birth, while first trimester stressed and non-stressed litters exhibited a male birth bias. In mice, embryos implant into the uterine horns between E4 and E5 (Namiki et al., 2018). A stressor applied during the second trimester of pregnancy, which began on E7.5,

therefore would not have interfered with the initial embryonic implantation, but rather could have influenced the uterine environment in a way that made it less favorable for the survival of male embryos. Indeed, there is evidence for preferential loss of male embryos following early gestational insults, with increased rates of embryonic resorption (Wiebold et al., 1986; Clark et al., 1993; deCatanzaro et al., 1994; Arck et al., 1995; Lee et al., 2008; Jafari et al., 2017) and heightened male embryo susceptibility (Pratt and Lisk, 1989) reported in rodents exposed to early pregnancy stressors. In line with heightened male vulnerability to second trimester exposures, previous studies have demonstrated a link between inadequate nutrition during gestation and a reduced sex ratio (e.g., fewer males than females) at birth (Rivers and Crawford, 1974; Mitra and Chowdhury, 1989; Meikle and Thornton, 1995; Rosenfeld et al., 2003). There is also evidence that male fetuses may have a faster growth rate and require a higher caloric intake *in utero* than females (Scott and Holson, 1977; Tsunoda et al., 1985; Douhard, 2017), thus under conditions of nutrient restriction and/or stress, weaker male fetuses are preferentially aborted to increase the chances of the few stronger male fetuses surviving (Torche and Kleinhaus, 2012). From an evolutionary perspective, in polygynous species such as rodents weaker males get outcompeted by the stronger members of their sex, thus poorly nourished female offspring are more likely than males to have reproductive success, and are preferentially born (Trivers and Willard, 1973; Douhard, 2017). Mice are multiparous animals which under normal conditions experience a basal level of embryonic resorption over the course of pregnancy (Holinka et al., 1979; Clark et al., 1993; Prell et al., 2016). Stress-induced absorptions may therefore occur without significantly altering litter size at birth, particularly if this occurs in a male-directed fashion and simply increases the

proportion of viable female offspring. Thus, in the present study the second trimester stressor altered the uterine environment in such a way to result in an equal sex ratio at birth. Furthermore, there is a critical period in early gestation where stressor exposure results in reduced maternal investment, with fewer resources directed towards the developing fetus (Berghänel et al., 2017). From an evolutionary perspective, this is adaptive for the mother as it conserves resources for later reproductive attempts. For the fetus, however, the resource restriction limits development, therefore to buffer against the stress-induced reductions to maternal investment, the offspring adapt by accelerating physical growth (Berghänel et al., 2017). This eventually leads to exaggerated growth during the postnatal period and increased offspring survival (Berghänel et al., 2017). In summary, the second trimester stressor may have increased the survival of female embryos to produce the observed effect of an altered sex ratio at birth. Furthermore, the prenatal stressor may have reduced *in utero* maternal investment towards the surviving offspring and thus caused these pups to accelerate their postnatal growth as a compensatory mechanism. The exaggerated weight gains displayed by the male offspring reflected adaptive metabolic changes that hastened maturation in order to increase the chances of these offspring having later-life reproductive success.

The stress-related behaviors seen in the prepubertal male and female mouse offspring could have also potentially been modulated through the gestational stressors reducing maternal food intake. In human populations, mothers exposed to famine while pregnant birthed offspring with increased later-life anxiety and mood disorders (Brown et al., 2000; Huang et al., 2013; van den Broek and Fleischmann, 2019), and rodents fed a nutrient restricted diet during pregnancy produced adult offspring which showed

increased depressive- and anxiety-like behaviors (Belluscio et al., 2014) and altered responses to later-life stressors (Nätt et al., 2017; Ye et al., 2018). Furthermore, although *in utero* malnutrition has been associated with altered neurodevelopment (Debassio et al., 1994; Gressens et al., 1997; González-Maciel et al., 2015) and reduced adult brain volume (de Rooij et al., 2016), it is unknown whether stress-induced modulation of maternal food consumption during gestation may change the brain and contribute to these phenotypes, particularly at the prepubertal age. Thus, it is possible that the prenatally stressed female hyperactivity and male grooming alterations that were observed in the present thesis could have been at least partially influenced by *in utero* nutrient deficits.

4.6 Stress-induced disruptions to the establishment of the nervous system during early pregnancy may alter later-life behaviors

Prenatal stressor exposure may have altered the trajectory of offspring neurodevelopment to contribute to the altered behaviors displayed by prenatally stressed prepubertal offspring. In mice, E7.5 marks the formation of the neural plate, a cellular structure which is the primordial nervous system (Chen et al., 2017; DeSesso and Williams, 2018). The following four days consist of highly coordinated and complex processes which allow for the establishment of the neural tube (E8.5), cerebral hemispheres (E10.0), and primary and secondary brain vesicles (E's 9.0-11.0) which become the forebrain, midbrain and hindbrain. By E12.5 rapid growth of the brain has established well-defined structures which continue to mature during early postnatal life (reviewed in Chen et al., 2017). Thus, prenatal stressor exposure during the first and second trimesters of pregnancy correspond to a developmental window of rapid cellular division and growth, and a critical time period where the earliest determinants of nervous

system structure and function are being defined. Structures are most sensitive to teratogens when they first begin to differentiate (DeSesso and Williams, 2018), therefore the establishment of nervous system progenitors during the first two weeks of gestation correspond to a time of high susceptibility. Perturbations during this developmental window, as in the current study, may have altered form and function of the developing fetal brain to result in the behavioral changes observed in the prepubertal offspring.

In the present study, prepubertal female offspring exposed to first trimester prenatal stress displayed increased activity in the open field and elevated plus maze tests. The neurobiology of rodent exploratory behavior is not fully understood, however. Hippocampal lesions have been shown to induce hyperactivity in the open field (Kimble, 1963; Jarrard, 1968; Thompson et al., 2018), and prenatal stressors have been shown to cause disrupted hippocampal development (Fujioka et al., 2006). A number of cell types in this brain region are also thought to be involved in modulating locomotor activity in novel environments (Thompson et al., 2018). Thus, possible disturbed brain development caused by early gestational stress could have altered hippocampal neurocircuitry in the brains of first trimester stressed females. However, the underlying biological mechanisms by which sex and stressor timing may have interacted to cause locomotor changes remains to be investigated.

In the current experiments, the splash test was selected for as a less stressful way of assessing the depressive-like behaviors of prenatally stressed offspring, as reduced grooming in this test correlates with increased immobility in the forced swim and tail suspension tests (Shiota et al., 2016). However, none of the prenatally stressed offspring displayed reduced grooming after having received water sprays. In fact, second trimester

stressed males exhibited fewer but more lengthy grooming sessions. Increased levels of self-grooming and disordered grooming patterns are often displayed by rodents exposed to stressors, including water sprays, or with high levels of anxiety (Kalueff and Tuohimaa, 2005; Kalueff et al., 2016), thus the grooming changes exhibited by the second trimester stressed male offspring may reflect these affective states. Grooming behaviors are modulated by multiple brain areas including the neocortex, striatum, cerebellum, hypothalamus, amygdala and brainstem (Kalueff et al., 2016), all regions established in their primitive forms during the second trimester of mouse pregnancy (Chen et al., 2017). As previous studies have indicated heightened male susceptibility to the behavioral effects of mid-gestational challenges (Li et al., 2014; Depino, 2015; Majidi-Zolbanin et al., 2015; Golub et al., 2016), possibly the altered grooming displayed by the second trimester stressed prepubertal males indicates disrupted neurodevelopment initiated during this developmental window, which may progress into the more clearly defined anxiety- and/or depressive-like phenotypes that have been previously displayed by adult males that have been stressed during this time.

Prepubertal mouse offspring behaviors in the tail suspension test were unaltered by the prenatal stressor conditions. The amount of movement displayed by an animal in the tail suspension test is controlled by the activation of cortical and limbic circuitry (Carlson et al., 2017) involving multiple neurotransmitter systems (O'Leary and Cryan, 2009). Juvenile rodents may behave differently than adults in the tail suspension test due to their relevant brain regions still maturing. For example, structurally the serotonergic system of PND21 mice is comparable to that of adults (Loizou and Salt, 1970; Miranda-Contreras et al., 1998), but the expression and binding affinity of the serotonin reuptake

transporter differs in juvenility compared to adulthood which may result in altered functioning of this monoamine system in younger mice (Mitchell et al., 2013, 2016). As prenatal stressors can influence the trajectory of serotonergic development in juvenility and into adulthood (van den Hove et al., 2006; Akatsu et al., 2015), this may interact with puberty onset to promote the manifestation of depressive-like behavioral changes in later life, particularly as there is evidence that post-pubertal male and female rodents display more behavioral despair than their pre-puberty counterparts (Boivin et al., 2017). Thus, the prepubertal offspring that were tested in the current study may have experienced the tail suspension test differently than they would have in adulthood due to immature neurocircuitry.

4.7 Postnatal handling can attenuate the physical and behavioral consequences of gestational stressors

A major difference between the procedures performed by previous rodent studies investigating the effects of early pregnancy stressors and those conducted in the present experiments is the amount of experimenter contact with the litters. Prior investigations into the growth and behavioral outcomes of first or second trimester stressors left litters undisturbed during the first three weeks of life, or only briefly handled the offspring during weekly cage changes (Lieberman, 1963; Hutchings and Gibbon, 1970; D'mello and Liu, 2006; Mueller and Bale, 2006; Jia et al., 2015; Jeje and Raji, 2017). In contrast, in the current study all mice were handled daily for weighing procedures, with the dam moved manually into an empty new cage during these times. Daily short handling of rodent pups (where a dam is separated from her young offspring) has previously been shown to be sufficient to influence offspring physiology and brain function (Meaney et

al., 1991; Papaioannou et al., 2002), particularly when combined with prenatal stressors. For instance, the reduction of maternal care behaviors observed in prenatally stressed litters (Brummelte and Galea, 2010; Del Cerro et al., 2010; Gatta et al., 2018) was limited when the corticosterone-injected mothers also experienced brief daily separation from their young pups (as in the current study), showing increased levels of pup care post-reunion (Castelli et al., 2020). Nursing is one of the maternal care behaviors reported to be increased following pup handling (Pryce et al., 2001; Macrí et al., 2004; Wei et al., 2010), which may lead to offspring receiving more milk from their mother. Indeed, third trimester stressed offspring that experienced neonatal handling gained more weights during the first three weeks of postnatal development (Castelli et al., 2020) and into adulthood (Vallée et al., 1996) compared to their non-handled prenatally stressed counterparts, possibly due to increased nursing. Our previous work which used identical prenatal stressor procedures to the present study (Osborne et al., 2020, in preparation) suggested an effect of gestational stress on maternal care behaviors in a novel cage environment, with increased passive contact with pups displayed by first trimester stressed dams and increased sniffing of pups shown by second trimester stressed mothers. Notably, there is evidence that under normal conditions mothers may direct their care behaviors in a sex-specific fashion, showing male offspring increased licking and nursing in both single-sex and mixed-sex litters (Cirulli et al., 1997; Hao et al., 2011). In the present study, it is possible the second trimester stressed dams showed increased maternal care behaviors towards their male offspring, contributing to their larger weight gains across postnatal development, but unfortunately nursing was not assessed and thus conclusions cannot be made at present. How prenatal stressor timing and postnatal

handling may interact to direct maternal care behaviors in a sex-specific fashion to influence offspring growth, however, remains to be investigated.

In addition to possibly contributing to the increased weight gains displayed by prenatally stressed offspring, postnatal handling could have interfered with the emergence of an anxiety-like phenotype. In non-stressed rodent pups, daily handling during the first 21 days of life has been shown to reduce anxiety-like behaviors in juvenility (Ferré et al., 1995; Núñez et al., 1995) and adulthood (Meerlo et al., 1999; Severino et al., 2004; Skripuletz et al., 2010; Siviy, 2018). Likewise, neonatal handling attenuated anxiety-like behaviors normally displayed by offspring exposed to prenatal stressors and increased locomotor activity (Wakshlak and Weinstock, 1990; Bogoch et al., 2007; Akatsu et al., 2015; Castelli et al., 2020), similar to the hyperactivity exhibited by prenatally stressed handled females in the current study. Importantly, increased exploration may be indicative of reduced anxiety (Kelley et al., 1989; Choleris et al., 2001) and hyperactivity can reflect an animal engaging in an active coping strategy in response to experiencing a novel environment or mildly stressful situation (Yen et al., 2013). Thus, the hyperactive phenotype displayed by the prenatally stressed female offspring in the present study may suggest an improved ability of these females to deal with later life stressors. In support of this, previous studies have suggested that prenatally stressed female offspring may be more resilient to a secondary stressor in adolescence (Iturra-Mena et al., 2018) or adulthood (Bowman et al., 2004; Mueller and Bale, 2006), possibly due to increased MR expression in the hippocampus and amygdala beginning at the fetal stage (Lan et al., 2017). Furthermore, the increased mother-infant interaction which occurs following brief daily separations (Pryce et al., 2001; Garoflos et al., 2007,

2008; Akatsu et al., 2015) has been shown to upregulate GR expression in the hippocampi of handled offspring (Liu et al., 1997; Champagne et al., 2008; van Hasselt et al., 2012). Hippocampal GR upregulation may promote the development of a more efficient negative feedback pathway of the HPA axis (Meaney et al., 1985, 1989; Fenoglio et al., 2004), reduce corticosterone responses to stressors and increase resilience to later-life stressful situations encountered by the offspring (Liu et al., 1997; Meerlo et al., 1999; Garoflos et al., 2005; Claessens et al., 2012). Thus, in the present study the daily handling procedures may have prevented the emergence of anxiety-like behaviors in prenatally stressed offspring, and additionally caused adaptive changes to HPA axis receptors which promoted active coping behaviors (in the form of increased exploration) in female offspring exposed to the mildly stressful situation of behavioral testing. It is also possible the daily offspring weighing procedure contributed to the absence of depressive-like behaviors in the prenatally stressed offspring, as postnatal handling was previously reported to reduce the time which *in utero* corticosterone-treated juvenile rats spent immobile in the forced swim test (Castelli et al., 2020), however no other studies have investigated how handling may mitigate depressive-like phenotypes at baseline or following gestational stressors.

4.8 Summary of outcomes in prenatally stressed offspring in the context of prepuberty

The results of this thesis demonstrate that male and female prepubertal mouse offspring are differentially affected by stressful experiences during gestation, and that the resulting metabolic and behavioral outcomes are dependent on the trimester of pregnancy which stressor was applied. Maternal prenatal challenges are disruptive to both the

mother and fetus, reducing maternal food consumption during pregnancy (Kinsley and Svare, 1986; D'mello and Liu, 2006; Borsonelo et al., 2011; Amugongo and Hlusko, 2013) and affecting how the dam will care for her neonatal offspring (Brummelte and Galea, 2010; Del Cerro et al., 2010; Gatta et al., 2018; Osborne et al., 2020, in preparation), while simultaneously increasing fetal glucocorticoid exposure *in utero* (Montano et al., 1991; Staud et al., 2006; Vackova et al., 2009), and altering offspring brain development (Fujioka et al., 2006). Female offspring appeared to be more susceptible to the metabolic and behavioral effects of stressors when applied during the first trimester of pregnancy, exhibiting exaggerated growth in early neonatal life and hyperactivity at the preweaning stage. In contrast, male offspring were specifically affected by second trimester stress, which largely impacted their weight gains and fat pad distribution into the third gestational week and altered their grooming patterns in the splash test on PND19. Interestingly, neither male nor female offspring exhibited anxiety- and depressive-like behaviors following prenatal stressor exposure. Importantly, all these outcomes were detected prior to the onset of puberty, which indicates that prenatal stressors can modulate some aspects of offspring health independent of sex hormones and the widespread biological changes which these molecules induce. Based on these findings, it can be surmised that periods of susceptibility to gestational stressors may change over the course of fetal development in a sex-specific manner, resulting in diverse consequences for offspring growth and behavior prior to sexual maturation. Further investigations into the proposed mechanisms that may underlie these changes are required to fully elucidate when changes are taking place during development, and how they may be prevented by targeted interventions.

Previous studies have reported increased anxiety- and depressive-like behaviors in juvenile (Zhang et al., 2013; Jia et al., 2015) and adult (Mueller and Bale, 2008; Lin and Wang, 2014; Depino, 2015; Majidi-Zolbanin et al., 2015) rodent offspring exposed to first or second trimester gestational challenges. However, these behavioral phenotypes were absent in the prepubertal offspring examined in the present study, suggesting that pubertal sex hormone surges are important for the manifestation of these outcomes. This is supported by studies showing rates of anxiety and mood disorders increase in the years following puberty (Cohen et al., 1993; Kessler et al., 1994; de Lijster et al., 2017), and post-pubertal mouse offspring of both sexes were found to spend more time immobile in the forced swim test compared to their prepubertal counterparts (Boivin et al., 2017). Testicular hormones have been shown to be important for modulating the behaviors of adult male rodents that have been stressed *in utero,* with decreased plasma testosterone levels detected in these animals (Dahlgren et al., 2001; Gerardin et al., 2005; Fedotova et al., 2017a), and these hormone reductions were associated with increased anxiety-like behaviors (He et al., 2015). Furthermore, gestationally stressed adult males which were gonadectomized had stronger anxiety- and depressive-like phenotypes compared to their intact counterparts (Frye and Wawrzycki, 2003; Fedotova et al., 2017a, 2017b). Thus, it may be that prenatal stressors modulate the behaviors of male offspring by altering post-pubertal testosterone levels, which may explain why in the present work prepubertal males showed unaltered anxiety- or depressive-like phenotypes, but previous research in both humans and rodents reported male-specific behavioral perturbations following early gestational stressors (Watson et al., 1999; Mueller and Bale, 2008; Kleinhaus et al., 2013; Depino, 2015; Majidi-Zolbanin et al., 2015; Herbison et al., 2017). Furthermore, there is

some evidence that prenatal stressors may reduce the already low levels of testosterone which are produced by male fetuses at the end of the third trimester (Ward and Weisz, 1980; Ward et al., 2003), a hormone which is critical for sexual differentiation of the brain during this time and in early neonatal life (Simerly, 2002; Lenz and McCarthy, 2010), thus the altered grooming behaviors displayed by the second trimester stressed male offspring in the present study may be an early sign that the stressor disrupted brain development, and that stronger behavioral perturbations may emerge after puberty once sex hormones start being produced in much larger quantities.

When considering adult female offspring, prenatal stressors have been shown to decrease their serum levels of follicle-stimulating hormone and estradiol (Ordyan et al., 2013; Del Cerro et al., 2015), and ovariectomy of gestationally stressed females increased their depressive-like behaviors compared to their intact counterparts (Frye and Wawrzycki, 2003). However, in contrast to males, gonadectomy did not increase the anxiety-like behaviors of females when performed on non-prenatally stressed animals prior to puberty (Boivin et al., 2017; Delevich et al., 2020) or in adult females that had been stressed *in utero* (Walf and Frye, 2007). This suggests that mechanisms other than sex hormones modulate female anxiety-like phenotypes, which agrees with observations in humans that anxiety disorders are more prevalent in females than in males prior to puberty onset (Epkins, 2002; Canino et al., 2004; Beesdo et al., 2009; Orgilés et al., 2012) (unlike depression where rates are equal between the sexes or higher in males prior to puberty; Kashani et al., 1983 and Anderson et al., 1987), and findings of the present study where prepubertal prenatally stressed females showed a hyperactive phenotype in the tests for anxiety-related behaviors. The sex hormone rises which accompany puberty

have been shown to modulate both structural and functional brain development by influencing neurogenesis (Ahmed et al., 2008), apoptosis (Nuñez et al., 2002), and neural circuit organization (Cunningham et al., 2002; Piekarski et al., 2017) through complex biological mechanisms. Thus, by altering sex steroid synthesis and activity, prenatal stressors can contribute to increased neuropsychiatric symptoms in offspring after the pubertal stage.

4.9 Strengths, limitations, and future directions

The most important strength of the present thesis was the inclusion of both male and female offspring in all analyses, as females have regrettably often been excluded from previous studies looking at the metabolic and behavioral outcomes of prenatal stressors (D'mello and Liu, 2006; Li et al., 2014; Depino, 2015; Jeje and Raji, 2017). In line with this, considering that mouse offspring were prepubescent at the time of testing, another crucial strength of this thesis was the confirmation of prepuberty status performed on all mouse offspring. Puberty has not been considered by any of the previous studies investigating the behavioral effects of prenatal stressors in juvenile rodents (Lieberman, 1963; Hutchings and Gibbon, 1970; Zhang et al., 2013; Jia et al., 2015). This can be problematic as pubertal onset may be shifted by gestational stressors (Smith and Waddell, 2000; Pallarés et al., 2013) and sex hormones can influence behavior (Brunton and Russell, 2010; Grundwald and Brunton, 2015). An additional strength of the current study was that the stressor procedures were administered during the very early stages of pregnancy, of relevance because the effects of first or second trimester prenatal stressors remain understudied compared to a large body of previous work investigating third trimester exposures. This thesis thus provides evidence that the

early gestational periods are just as crucial for defining the trajectory of offspring development as the later stages, and indicates that stressful experiences during this early developmental window can affect the growth and behavior of male and female offspring into the third postnatal week. Moreover, body weight measurements of the male and female mouse offspring were conducted daily from birth to PND21, which provided a complete picture of whether and how physical growth may be influenced by the prenatal stressors. Most research done previously have only weighed offspring on a few developmental time points (D'mello and Liu, 2006; Mueller and Bale, 2008; Jeje and Raji, 2017), which may result in crucial periods of growth being missed. Finally, two behavioral tests were conducted for each behavioral phenotype being investigated (anxiety and depression), which provides a more comprehensive and reliable assessment of these phenotypes compared to studies which have only used one test for each behavioral assessment (Mueller and Bale, 2008; Zhang et al., 2013; Bronson and Bale, 2014; Jia et al., 2015).

A limitation of the present work is the small sample size used for some of the experimental groups in the tail suspension test, partially due to several mice having to be excluded due to tail grabbing during the test. This is a common issue which occurs at a low, random baseline rate in this test, particularly when using C57BL/6 mice (Cryan et al., 2005), and may be prevented by using larger group sizes. Furthermore, since the tail suspension test is optimized for adult mice, a number of unforeseen issues arose when adapting the procedure for the smaller PND20 offspring, which may have contributed to the high degree of variability seen in the data. Slight technical modifications in this test would improve the consistency of future experiments done in mice at this age. Other

potential limitations of this thesis are related to methodology. Offspring were tested in the open field and elevated plus maze for 10 min in the present study, however 5 min is a more common testing length and anxiety-like behaviors may only be detected during early stages of the test (Ueno et al., 2020). The video footage from these tests will be reanalyzed at a future time to determine whether more distinct behavioral changes could be detected during the first 5 min versus the last 5 min of the test. In the splash test, diving into more detailed analysis of how prenatal stressors may have influenced grooming microstructure could also provide more insight into the observed changes (Kalueff and Tuohimaa, 2005), especially in light of the "arousing-like" phenotype apparent in prenatally stressed males.

Future directions for this research would be to assess anxiety- and depressive-like behaviors at a post-pubertal stage and in adulthood to determine how prenatal stress may interact with sex hormone surges to modulate the developmental trajectories of behavior. It would also possibly be of benefit to incorporate additional tests for anxiety- and depressive-like behaviors (e.g. light-dark test for anxiety, novelty suppressed feeding and forced swim tests for depressive-like behaviors) for more detailed phenotype assessment of the prenatally stressed offspring. A future experiment could also measure neonatal pup milk intake (possibly by weighing stomach contents of euthanized neonates) and record undisturbed home cage maternal behaviors to assess the influence of these factors on offspring growth. Placental gene expression (including 11β-HSD2), maternal stress hormones during pregnancy, and fetal corticosterone levels would also provide valuable insight into how the prenatal HPA axis of mother and offspring are modulated by stressful encounters. The bodies and food intake of the dams in the present study were

weighed daily throughout gestation and postpartum, therefore analysis of this data would provide insight into whether the prenatal stressors altered maternal growth and food consumption, as well as 11β-HSD2 expression, to potentially influence offspring body weights. Finally, measuring body fat composition at earlier postnatal stages, as these deposits are developing in early neonatal life (Han et al., 2011; Berry et al., 2013), would be valuable for investigating the age of onset of the increased adiposity seen in the second trimester stressed males. This could be done through euthanizing offspring at various time points, or by imaging live animals (Marzola et al., 2016) which would allow for repeated measures over time.

Additional future directions include exploring the proposed mechanisms underlying the metabolic and behavioral outcomes of first and second trimester stressors. As previously discussed, significant evidence in the literature points towards the HPA and microbiota-gut-brain axes as pathways underlying the effects of prenatal stressors on later-life metabolic and mental illnesses. Tissues were collected from the PND21 dams and pups in the present study, as well as from male and female offspring on PNDs 7 and 14. Although their analysis was not part of the current thesis, these tissues will be processed at a later date to investigate in more detail how changes to the microbiota, digestive tract, and brain may be changed in prenatally stressed developing offspring. Signaling molecules of the inflammatory immune system and HPA axis will also be studied across these tissues, as well as in the blood, to better understand how microbe-gut-brain crosstalk may be modulated by gestational challenges.

4.10 Conclusions

The data from this thesis indicates that stressors experienced during early pregnancy influence offspring physical growth and behaviors in a sex- and trimester-specific manner. Prenatally stressed offspring gained more weight than non-stressed controls during early postnatal development, however first and second trimester stressors had differential effects on the male and female offspring, with first trimester stressed females showing increased growth during the first week of life, but second trimester stressed males exhibiting exaggerated development until PND21. At the end of the third postnatal week, males from the second trimester stressed group also had increased body fat at the perirenal and retroperitoneal locations. Although prenatal stressors did not result in anxiety-like behaviors in prepubertal offspring of either sex, females stressed *in utero* did exhibit a hyperactive phenotype in the open field and elevated plus maze. Depressive-like behaviors in the tail suspension and splash tests were unaltered by prenatal stressor exposure, but second trimester stressed males showed disrupted grooming patterns in the splash test.

In summary, this data indicates that the anxiety- and depressive-like behaviors previously reported in first or second trimester stressed adult rodents do not manifest prior to puberty. Possibly, the widespread somatic changes that occur with the onset of sex hormone secretion may act to trigger the emergence of anxiety- and depressive-like behaviors in animals that have been exposed to prenatal stressors. Administrating interventions and/or treatments prior to or during the critical window of puberty onset and at the time of sexual maturity may be beneficial towards preventing the emergence of anxiety- and depressive-like phenotypes in adulthood.

Appendix 1: Supplementary Data

1. Daily changes in offspring body weights across development

Individual male and female mouse offspring were weighed daily from PND1-21, and sex- and trimester-specific outcomes were observed. Body weights over time were analyzed separately in males and females using a mixed design ANOVA with Prenatal Stressor (CON, 1TRI, 2TRI) as the between-group factor and Postnatal Day (PND1-21) as the within-group factor, and corrected for sphericity using Huynh-Felt correction. As depicted on Figure 10A, male weights varied as a function of Postnatal Day, $F(20,600) = 1764.222$, $p < 0.001$. Although the interaction between Prenatal Stressor, $F(2,30) = 2.611$, $p = 0.090$, and Postnatal Day, $F(3.31,49.59) = 2.556$, $p = 0.060$ (Huynh-Feldt sphericity correction), did not reach significance, based on the a priori prediction that prenatally stressed and non-stressed offspring would gain weight differently during early development, analyses of the simple effects comprising the Prenatal Stressor x Postnatal Day interaction were conducted. Simple effects analysis showed that on PNDs 4, 5 and 6, second trimester stressed males had significantly larger body weights than non-stressed controls, *p's* = 0.002, 0.027 and 0.006, respectively (Figure 10A).

In females, both Prenatal Stressor and Postnatal Day significantly affected body weights, $F(2,25) = 7.867$, $p = 0.002$ and $F(20,500) = 2388.417$, $p = 0.001$, respectively (Figure 10B). The interaction between these factors was not significant, $F(40,54.54) = 1.898$, $p = 0.118$ (Huynh-Feldt sphericity correction), however simple effects analyses were conducted based on the a priori prediction that the weight gains of non-stressed and prenatally stressed offspring would vary across early development. As seen in Figure 10B, across postnatal development first trimester stressed female offspring weighed more

than non-stressed controls (PND2: $p = 0.001$; PNDs 3-10: $p < 0.001$; PND11: $p = 0.030$; PND13: $p = 0.008$; PND14: $p = 0.004$; PND19: $p = 0.036$) and second trimester stressed offspring (PND2: $p = 0.021$; PND3: $p < 0.001$; PND4: $p = 0.002$; PND5: $p = 0.001$; PNDs 6-10 $p < 0.001$; PND12: $p = 0.037$; PND13: $p = 0.041$; PND14: $p = 0.007$; PND19: $p = 0.035$). On PND7, second trimester stressed offspring were significantly larger than controls as well, $p < 0.001$ (Figure 10B).

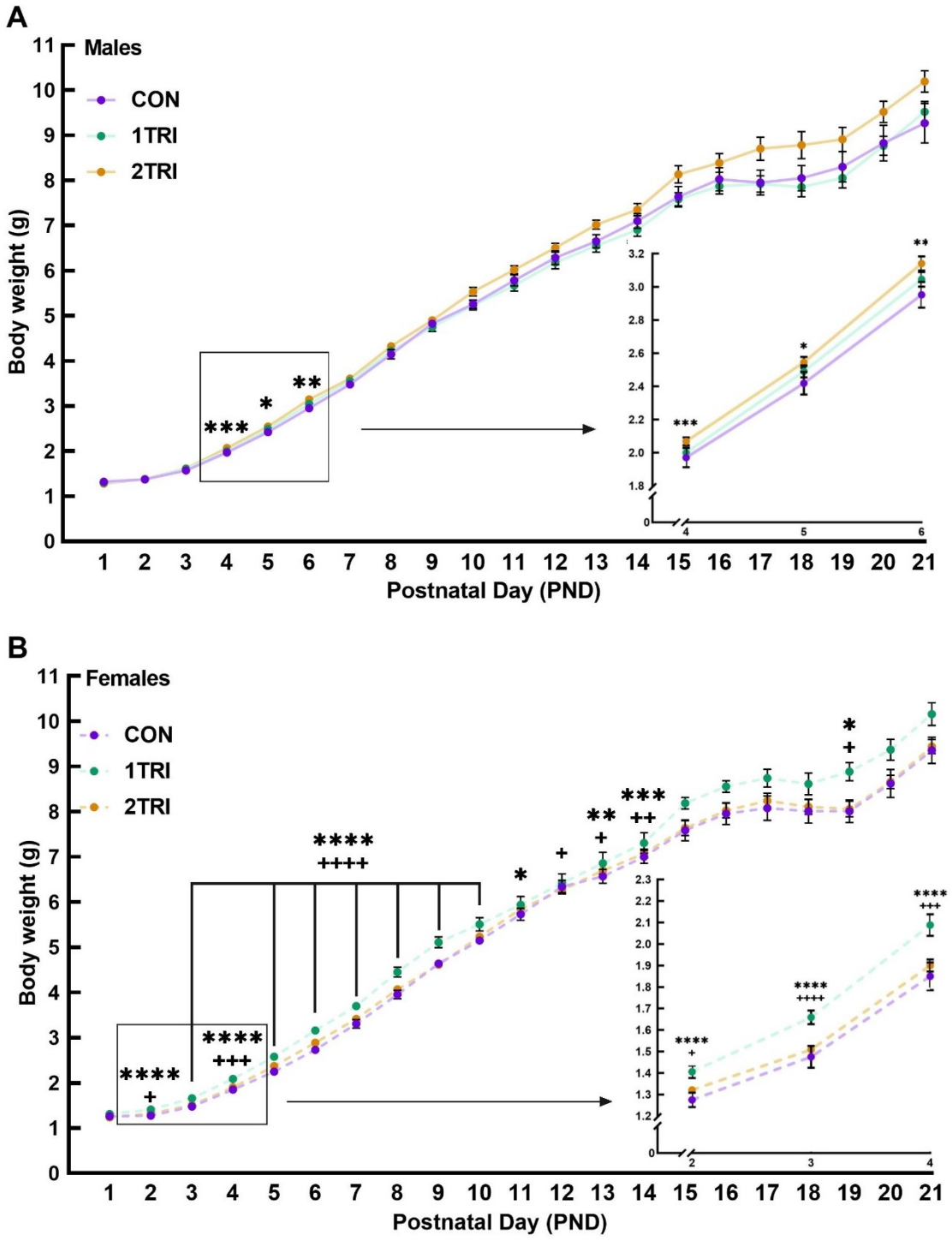

Figure 10. Daily body weights (in grams [g]) of prenatally stressed or non-stressed offspring of both sexes across development.
Male (A) and female (B) offspring were born to prenatally stressed mothers (first or second trimester; 1TRI or 2TRI) or non-stressed controls (CON). Data represents means ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$ relative to non-stressed controls. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.005$, $^{++++}p < 0.001$ relative to first trimester stressed offspring. Sample size for each timepoint is CON = 9, 1TRI = 14, and 2TRI = 10 for males (A), and CON = 9, 1TRI = 7, and 2TRI = 12 for females (B).

2. Offspring anxiety-like behavior: Open field test

Supplementary, non-traditional behavioral parameters in the open field were measured, and only mild phenotypic differences were displayed by male and female prenatally stressed offspring. In male offspring, prenatal stressor exposure did not alter the time spent ($H(2) = 0.005$, p = 0.998), frequency of entries ($F(2,34) = 0.127$, $p = 0.881$), or latency to first entry ($H(2) = 0.248$, p = 0.883) into the large center of the open field, nor did it change the number of fecal boli produced ($H(2) = 4.157$, $p = 0.125$) (Figures 11A-D). Likewise, total time spent jumping ($F(2,34) = 2.265$, $p = 0.119$), number of jumping sessions ($F(2,34) = 0.9293$, $p = 0.405$) or total number of jumps ($F(2,34) = 1.737$, $p = 0.191$) in male offspring were unaffected by Prenatal Stressor (Figures 11E-G). The only supplementary parameter affected by prenatal stressor exposure in the open field was the latency to first jump, $F(2,34) = 5.448$, $p = 0.009$, with follow-up tests confirming that first and second trimester stressed males were quicker to jump in the open field test compared to non-stressed controls, $p = 0.035$ and $p = 0.014$, respectively (Figure 11H).

In females, Prenatal Stressor did not alter the time spent or latency to first entry into the large center of the open field, $H(2) = 4.568$, p = 0.102 and $H(2) = 4.055$, p = 0.132. There was a trend for increased number of entries into this section, $F(2,25) = 3.166$, $p = 0.059$, but post hoc tests showed that the tendency for first trimester stressed females to enter the large center more frequently than controls did not reach significance, $p = 0.060$ (Figures 11A-C). The number of fecal pellets produced by females was unchanged between groups, $H(2) = 1.574$, $p = 0.455$, as was the time spent jumping, $F(2,25) = 0.287$, $p = 0.753$, total number of jumps, $F(2,25) = 1.761$, $p = 0.193$, and the

latency to first jump, $F(2,25) = 2.096$, $p = 0.144$ (Figures 11D-E, 11G-H). There was a non-significant trend for prenatal stressor exposure to increase the number of jumping sessions in female offspring, $F(2,25) = 3.077$, $p = 0.064$, but follow-up tests showed that the trend for first trimester stressed females to engage in more jumping bouts during open field testing did not reach significance, $p = 0.063$ (Figure 11F).

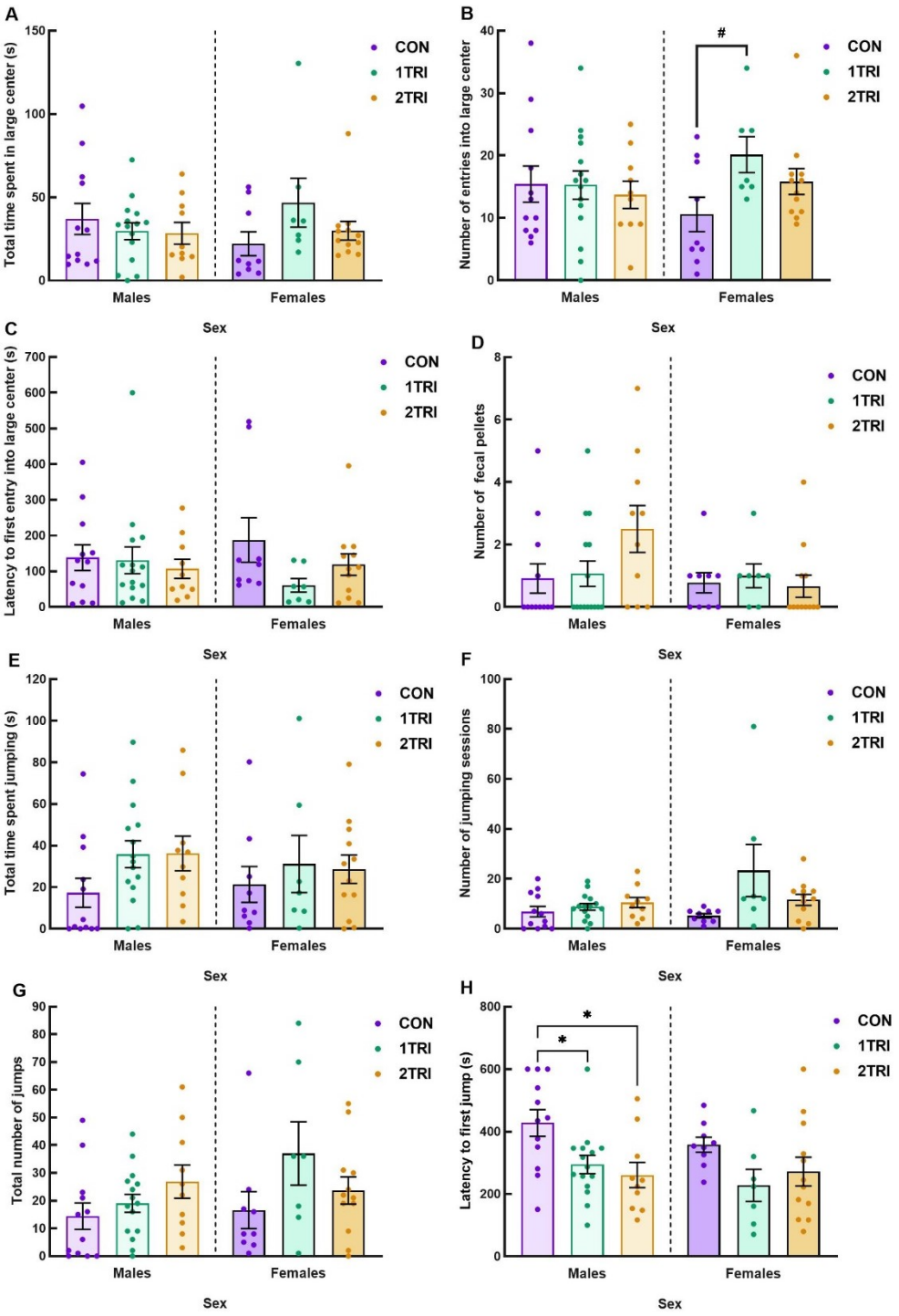

Figure 11. Additional open field behaviors in male and female offspring at 19 days of age. Dams were stressed during pregnancy (first or second trimester; 1TRI or 2TRI) or not stressed (control; CON). Data shown is the cumulative time (in seconds [s]) spent in the large center (A), frequency of entries into large center (B), latency (s) to first entry into large center (C), number of feces (D), cumulative time (s) spent jumping (E), frequency of jumping sessions (F), total number of individual jumps (G), and the latency (s) to first jump (H). Individuals with a latency of 600 s did not jump. Data represents means ± S.E.M. $^{*}p < 0.05$ and $^{\#}p = 0.060$ relative to CON females. Sample sizes for CON, 1TRI and 2TRI are N's = 12, 15, 10 (males) and N's = 9, 7, 12 (females).

3. Offspring anxiety-like behavior: Elevated plus maze test

Prenatal stressor exposure did not alter additional elevated plus maze parameters in either male or female prepubertal offspring. In males, Prenatal Stressor did not alter the time spent or the number of entries into the center of the elevated plus maze, $H(2) = 0.604$, $p = 0.739$ and $H(2) = 2.112$, $p = 0.348$, respectively, nor did it change the number of fecal pellets produced, $H(2) = 4.594$, $p = 0.101$ (Figures 12A-C). Likewise, in females center duration and frequency were also unchanged, $H(2) = 0.604$, $p = 0.739$ and $F(2,25) = 2.656$, $p = 0.090$, respectively, and prenatal stressor exposure had no effect on feces produced in the elevated plus maze test, $H(2) = 2.594$, $p = 0.273$ (Figures 12A-C).

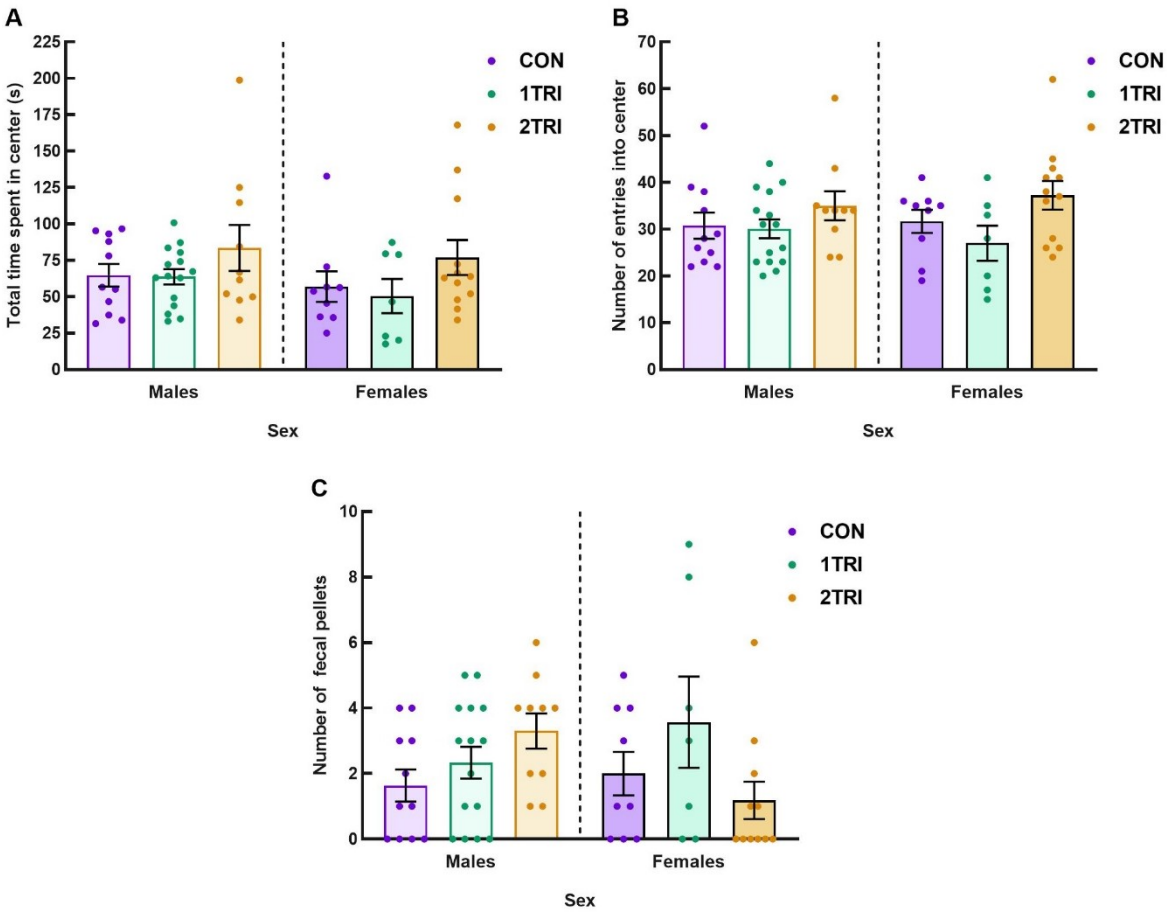

Figure 12. Additional elevated plus maze behaviors in 20-day old male and female offspring. Offspring were born to mothers stressed during pregnancy (first or second trimester; 1TRI or 2TRI) or not stressed (control; CON). Data shown is the cumulative duration (in seconds [s]) spent in the center (A), frequency of entries into center (B), and number of fecal pellets produced (C). Data represents means ± S.E.M. Sample sizes for CON, 1TRI and 2TRI are N's = 11, 15, 10 for males and N's = 9, 7, 12 for females. One CON male was excluded from the analyses due to falling off the maze during testing.

4. Offspring depressive-like behavior: Splash test

As digging and jumping behaviors were exhibited by offspring following water stimulation in the splash test, these phenotypes were also assessed. Prenatally stressed male offspring showed no differences from their non-stressed counterparts in digging behaviors during the splash test (Total duration: $H(2) = 0.414$, $p = 0.813$; Average duration: $H(2) = 0.257$, $p = 0.880$; Total number of sessions: $H(2) = 0.225$, $p = 0.893$; Latency: $H(2) = 0.172$, $p = 0.917$), and their jumping behaviors were also unchanged (Total number: $H(2) = 3.225$, $p = 0.199$; Latency: $H(2) = 3.225$, $p = 0.199$ (Figures 13A-F). Female offspring exposed to prenatal stressors showed no differences in the splash test behaviors of digging (Total duration: $H(2) = 1.037$, p = 0.596; Average duration: $H(2) = 1.521$, $p = 0.468$; Total number: $H(2) = 0.991$ $p = 0.609$; Latency: $H(2) = 0.722$, $p = 0.697$) or jumping (Total number: $H(2) = 1.745$, $p = 0.418$; Latency: $H(2) = 2.008$, $p = 0.366$) (Figures 13A-F).

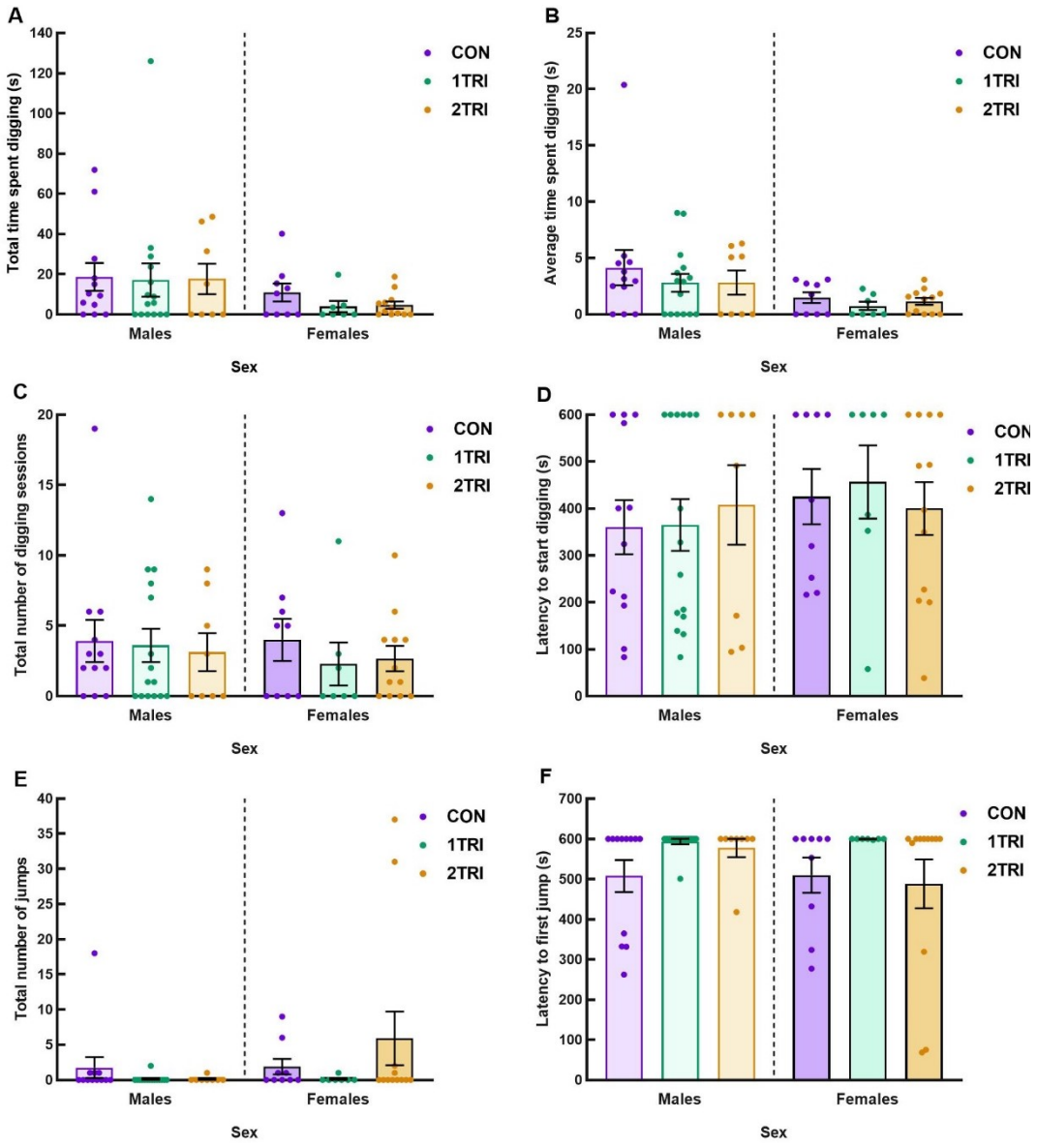

Figure 13. Splash test digging and jumping behaviors of male and female mouse offspring aged 19 days.

Dams experienced stress during pregnancy (first or second trimester; 1TRI or 2TRI) or were not stressed (control; CON). Data shown is the cumulative duration (in seconds [s]) spent digging (A), average length (s) of digging session (B), frequency of digging sessions (C), latency (s) to start digging (D), total number of jumps (E), and latency (s) to start jumping (F). Individuals with a latency of 600 s did not display the specified behaviors. Data represents means ± S.E.M. $^{*}p <$ 0.05 main effect of prenatal stressor. Sample sizes for CON, 1TRI and 2TRI are N's = 12, 15, 8 for males and N's = 9, 7, 12 for females. Two 2TRI males had to be excluded due to problems with the video files.

5. Offspring depressive-like behavior: Tail suspension test

There was no effect of prenatal stressor exposure on the average length or total number of immobility sessions in male or female prepubertal offspring (Average length males: $F(2,31) = 1.604$, $p = 0.217$; Average length females: $F(2,21) = 0.681$, $p = 0.517$; Total number males: $F(2,31) = 1.197$, $p = 0.316$; Total number females: $F(2,21) = 1.863$, $p = 0.180$) (Figures 14A-B).

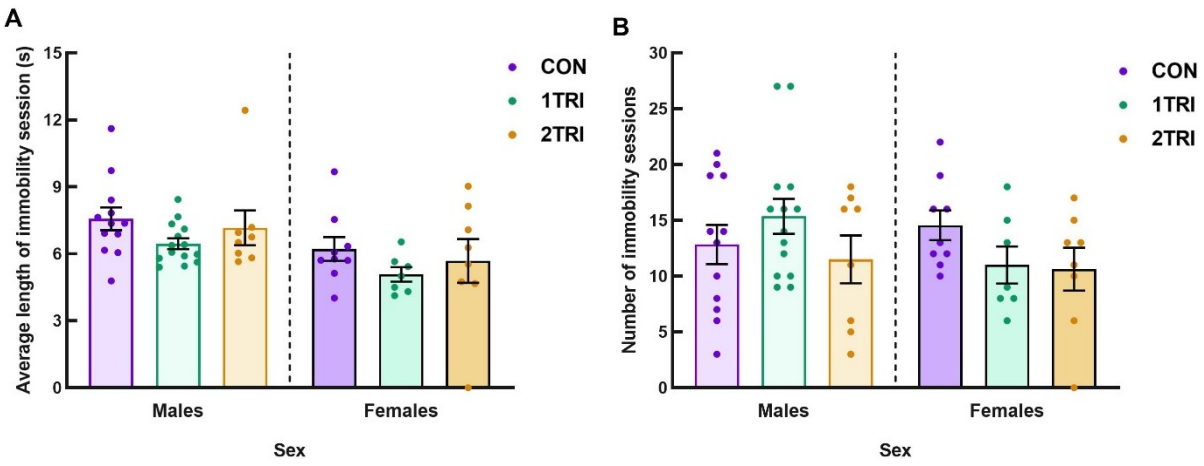

Figure 14. Supplementary tail suspension test measures in male and female mouse offspring at 20 days of age

Dams were stressed during pregnancy (first or second trimester; 1TRI or 2TRI) or not stressed (control; CON). Data shown is the average length (in seconds [s]) of immobility session (A) and the total number of immobility sessions (B). Data represents means ± S.E.M. Sample sizes for CON, 1TRI and 2TRI are N's = 12, 14, 8 for males and N's = 9, 7 and 8 for females. Seven mice in total had to be excluded from the final dataset due to the following testing issues: one 1TRI male and two 2TRI females continuously grabbed onto the tape/bar which their tails were attached to; a 2TRI male and a 2TRI female had their back paws stuck to the tape for entire test; one 2TRI male and one 2TRI female video files were lost.

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