GENERATIONAL EFFECTS OF CHRONIC STRESS

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Abstract

Traumatic events that affect behavior in the current generation may also impact future generations. Although the effects of stress on pregnant mothers have been extensively explored, we know little about how trauma *prior to conception* affects offspring conceived afterwards. Here we demonstrate that chronic predator stress in adult mice alters behavior, as well as the first filial (F1) generation. Adult F0 mice were chronically exposed to a predator rat or control condition and assessed for anxiety-like (EPM, OFT, LDB) and social (SIT) behaviours. F0 mice were also assessed on circadian locomotor activity patterns prior to and following predator exposure or control conditions. F0 predator stressed mice froze more than F0 control mice overall and each day of the chronic exposure. In addition, F0 predator stressed mice exhibited increased anxiety-like behaviours (ALB) on the EPM relative to F0 controls. Following behavioral testing, F0 mice were group matched and bred to generate the F1s. Anxiety-like and social behaviors were assessed in the F1s during adolescence and again, following a mild stressor, in adulthood. Circadian locomotor activity was also monitored in the F1 adults. Adolescent offspring from predator stressed parents (PSO) exhibited increased ALB on the EPM compared to offspring of controls (CO). However, in adulthood, following the mild stressor, PSO mice showed decreased ALB compared to CO mice, suggesting increased resilience. These findings indicate that chronic pre-conception predator stress results in lasting effects on future generations, an outcome that may lead to improved understanding of the etiology of anxiety and stress-related pathologies.

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F0	Primary generation and initial group to be tested
F1	First offspring generation from F0 breeding
ALB	Anxiety-like behaviour
EPM	Elevated plus maze
OFT	Open field test
LDB	Light-dark box
SIT	Social interaction test
NSF	Novelty-suppressed feeding test
PSO	Predator stressed offspring; F1 from predator stressed F0 parents
CO	Control offspring; F1 from control F0 parents
PTSD	Post-traumatic stress disorder
APA	American Psychological Association
GAD	Generalized anxiety disorder
CCHS	Canadian Community Health Survey
NCS	National Comorbidity Study
CMS	Chronic mild stress
RET	Rat exposure test
PND	Post-natal day
DD	Constant dark (no lighting)
SCN	Suprachiasmatic nucleus
GC	Glucocorticoid
mA	Milliampere
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, 5th edition

Generational Effects of Chronic Stress

1.1 Chronic Stress in Humans

Mental health and wellness—a prevalent concern in society even prior to the COVID-19 pandemic—has come to the forefront of many discussions. Prior to the pandemic, anxiety disorders were reported to be the most common worldwide mental health disorders ranging in prevalence from 2.4-18.2% (Demyttenaere et al., 2004) and stress-related disorders such as post-traumatic stress disorder (PTSD) that manifest following a traumatic incident have been estimated to impact 9.2% of Canadians and 6.1% of Americans (Goldstein et al., 2016; Van Ameringen et al., 2008). Anxiety disorders have a variety of negative consequences on individuals' lives, which can result in impaired social function, lethargy, and suicidal ideation (American Psychological Association, 2013). Additionally, anxiety disorders can be characterised by chronic stress—both a risk factor and a symptom of the disorder—and persistent stress has been associated with disruption of other regulatory systems throughout the body (Martire et al., 2020). The American Psychological Association (APA) defines chronic stress as physiological or psychological reactivity to an extended stressful event (APA, 2022), and recent research has shown that with the ongoing COVID-19 pandemic, both chronic stress and mental health concerns are on the rise (Quadros et al., 2021).

1.1.1 Anxiety and Stress-Related Disorders

Chronic stress is a known risk factor and symptom of various psychiatric illnesses—notably stress-related and anxiety disorders. The most common anxiety disorder seen in primary care is generalized anxiety disorder (GAD; DeMartini et al.,

2019). The 2012 Canadian Community Health Survey (CCHS) reports a lifetime prevalence of GAD compatible symptoms in Canadians aged 15 years or older to be 2.5% (Pelletier et al., 2017) and the National Comorbidity Study (NCS) from the United States reports GAD to have an overall lifetime prevalence of 5.1% (Kessler et al., 1994). The same report indicates a prevalence of 3.6% for men and 6.61%, nearly twice the likelihood of incidence, for women. GAD is characterized by a minimum of 6 months of pervading and excessive anxiety, worrying about common lifetime events and practices, physical symptoms including insomnia, muscle tension, and fatigue, and impairments in personal and/or occupational daily living (APA, 2013). Furthermore, individuals living with GAD are at an increased risk of additional physical and mental health conditions. Chronic pain, asthma, chronic obstructive pulmonary disease, and inflammatory bowel disease have been found to be highly comorbid with GAD (Sareen et al., 2006), and approximately 35% of people with GAD self-medicate with alcohol and drugs to reduce anxious symptoms, further perpetuating the pre-existing symptoms and rendering them vulnerable to substance use disorders (Robinson et al., 2011).

Social anxiety disorder (also referred to as social phobia) is another common anxiety disorder notarized by its early development and high comorbidity (Martin, 2003). The disorder is highlighted by an intense fear of scrutiny in social situations and an anticipation of being negatively evaluated (Leichsenring & Leweke, 2017), symptoms that often develop in early childhood or adolescence (Martin, 2003). The CCHS reports that 8.1% of Canadians meet the criteria for lifetime social anxiety disorder, with a prevalence of 39.6% for males and 60.4% for females (MacKenzie & Fowler, 2013). The

United States reports a higher incidence at 13.3% of lifetime prevalence (Patrick, 2003). People with social anxiety disorder have been found to be highly dissatisfied with various aspects of their lives and report difficulty executing daily tasks, educational responsibilities, and occupational roles (Stein & Kean, 2000). Due in part to the early onset, social anxiety disorder is highly comorbid. Social anxiety disorder often cooccurs with additional anxiety disorders, increased risk of major depression disorder and suicidality, persistent substance-use disorder, and cardiovascular disease (Bögels et al., 2010; Kessler, 2003; Ruscio et al., 2008).

One of, if not the most discussed stress-related disorder is post-traumatic stress disorder (PTSD). PTSD can result as a pathological consequence following exposure to traumatic events (APA, 2013), such as violence, injury, and death. Not everyone who experiences a traumatic event will go on to develop PTSD, but the lifetime prevalence for those that do ranges from 1.3 to 12.2% (Karam et al., 2014) depending on geographic region. The current lifetime prevalence estimate in Canada is 9.2% and 6.1% in the United States (Goldstein et al., 2016; Van Ameringen et al., 2008). PTSD is characterized by intense, fearful reactions to triggering events associated with the traumatic event, a persistent sense of danger or threat, hypervigilance, disturbed sleep, and changes in mood and cognitive function (APA, 2013). More than 50% of people with PTSD also deal with comorbid anxiety, mood, or substance-use disorders (Piertzak et al., 2011), and it is often associated with physical disability and illness, premature death, and suicidality (Piertzak et al., 2011; Sareen et al., 2007; Schlenger et al., 2015).

1.1.2 Chronic stress and Disease

In addition to the burden on mental wellbeing, chronic stress is widely regarded as detrimental to our physical health. The interconnectedness of the bodily systems can result in impairments in regulatory function in the immune, circulatory, and circadian systems when subjected to chronic stress (Agorastos et al., 2020; Gouin, 2011; Yao et al., 2019).

The heart is susceptible to the negative consequences of chronic stress and regulation of blood pressure and blood flow is directly impacted by the stress system via cortisol and catecholamine secretion from the adrenal glands. Chronic stress can lead to damage of circulatory vessels, a build up of plaque in the arteries—a condition known as atherosclerosis—and an increased risk of cardiovascular diseases and stroke (Lagraauw et al., 2015; Tawakol et al., 2017; Yao et al., 2019). In addition to the direct connection between the stress and circulatory systems leading to detrimental cardiovascular outcomes, chronic stress can result in lifestyle changes and activities that perpetuate stress-induced cardiovascular events, such as a decrease in physical activity, smoking habits, and poor nutrition leading to obesity (Golbidi et al., 2015; Joynt et al., 2003).

Chronic stress affects immune function though activation of neuroendocrine and sympathetic systems (Gouin, 2011). Persistent activation of these systems can wear down the body over time, leading to long term overuse of bodily organs—the cumulative burden known as allosteric load (Guidi et al., 2021; Segerstrom & Miller, 2004). This systemic burden can result in either an over or under-expression of required immune molecules, leading to impaired immune responses or lack of regulation that normally

protects the body against its own immune system (Gouin, 2011). Furthermore, chronic stress promotes chronic low-grade inflammation throughout the body (Black, 2003), a delay in wound healing (Kiecolt-Glaser et al., 1995), and a susceptibility to upper-respiratory infections (Cobb & Steptoe, 1996). These, along with other immune system deficits, can lead to poorer overall quality of life and increased susceptibility to disease (Mariotti, 2015; Salleh, 2008).

Circadian rhythmicity and the stress system are bidirectionally linked thus chronic stress creates disturbances in the normally synchronized circadian rhythms (Dumbell et al., 2016; Fishbein et al., 2021; Kalsbeek et al., 2012; Koch et al., 2017; Nader et al., 2010). The circadian system is responsible for the cyclical regulation of the body across 24 hours. Outputs such as activity, rest, endocrine secretion, body temperature, and food-related processes are all influenced by the steady rhythms governed by the circadian system (Balsalobre et al., 2000; Brown et al., 2002; Damiola et al., 2000; van Oosterhout et al., 2012). These fundamental shifts and discrepancies with the normally circadian rhythms, should they persist, can have implications for physical wellbeing and can manifest as the classical sleep-wake disorders, neurological, psychiatric, and many more chronic medical disorders (Sletten et al., 2020). Patients diagnosed with psychiatric disorders such as major depressive disorder, anxiety disorders, schizophrenia, and bipolar disorder have misalignment in their circadian rhythms (Bellivier et al., 2015; Coulon et al., 2016; Emens et al., 2009: Vadnie & McClung, 2017; Zanini et al., 2013), leading to further debilitating consequences on their health and wellbeing.

The debilitating consequences of chronic stress on our emotional, mental, and physical wellbeing are detrimental to living a full and healthy life. As discussed above, psychological disorders and physical conditions alike are all impacted by chronic stress, necessitating further understanding pertaining to the mechanisms by which chronic stress impacts the brain and body. However, the research describing the effects of chronic stress on humans is limited in the degree of information gathered. Humans live complex, multifaceted lives, making it difficult to discern the causes and effects of chronic stress. Therefore, pre-clinical animal models are imperative to tease apart the nuances of the stress experience while working within a controlled environment and ethical limitations.

1.2 Animal Models of Chronic Stress

Researchers have developed a variety of experimental paradigms to study chronic stress in rodents. Animal models provide an opportunity for studying the outcomes of stress by overcoming ethical limitations associated with human research. Furthermore, animal models permit exposure to a stressor in a controlled fashion, as well as the ability to study the effects on behaviour as they develop. Behaviour tests measuring anxiety-like behaviour in rodents such as the elevated plus maze (EPM), open field test (OFT), light-dark box (LDB), and social interaction test (SIT) are widely used in chronic stress research. Animal models of chronic stress also allow for more in-depth investigation into normal daily activity patterns, such as monitoring home cage locomotor activity. Furthermore, anxiety-like behaviour and activity patterns vary between male and female rodents based on the nature of the test used (Bishnoi et al., 2021; Scholl et al., 2019). While it is not possible to measure every aspect of the human stress experience, several

rodent models have been developed which that have been shown to be robust in producing a chronic stress phenotype. The most common are described below.

1.2.1 Chronic Mild Stress (CMS)

Chronic mild stress (CMS; also referred to as chronic unpredictable stress) is a popular chronic stress paradigm where, over a period of days to weeks, rodents are exposed to a variety of mild stressors that vary in order of application (Mahar et al., 2014). The mild stressors (i.e., micro-stressors) used are non-debilitating, inescapable, and uncontrollable and are applied in unpredictable, randomized sequences spanning several weeks (Bambico et al., 2009; Grippo et al., 2006; Mahar et al., 2014). The CMS model has good predictive validity, face validity, and construct validity in reference to other animal models of stress and depression (Willner, 1997; Willner, 2005), and has been used for research exploring depressive and anxious phenotypes. Daily application of CMS stressors over a 4-week period is sufficient to produce depressive-like behaviour in mice demonstrated by a deterioration in fur state, a decreased latency to immobility in the tail suspension task and forced swim test (Mineur et al., 2006). While the same research found evidence of an anxious phenotype with mice spending more time in the dark side of the LDB, the researchers were unsuccessful in demonstrating increased anxiety-like behaviour in the EPM (Mineur et al., 2006). Similar results have been reported in rats exposed to 5-9 weeks of CMS. Stressed animals did not display anxiety-like behaviour and indeed spent more time in the open arms of the EPM relative to controls, whereas CMS resulted in increased depressive-like behaviours and decreased hedonic activities such as decreased sexual behaviours, decreased sucrose intake in the sucrose preference

test, and reduced aggression to conspecifics (D'Aquila et al., 1994). More recent research using a 3-week CMS protocol has also produced evidence of depressive-like behaviours, but ambiguous anxiety-like behaviours (Kompagne et al., 2008). Rats displayed increased anhedonia and depressive-like behaviour with lower preference of sucrose solution and increased immobility in the forced swim test, while spending more time in the open arms of the EPM—the opposite of what would be expected of an anxious phenotype (Kompagne et al., 2008). Overall, these results from the CMS model indicate that a depressive phenotype is not contingent on the presence of anxiety-like behaviour for this animal model of chronic stress. Hence, the CMS model may not be best suited for understanding anxious phenotypes.

1.2.2 Chronic Restraint/Immobilization

Restraint or immobilization stress requires that a rodent is placed in an enclosed container or chamber, thereby preventing movement and/or escape from the procedure. Chronic restraint can be done repeatedly for varying durations and procedures differ between studies on the length and number of sessions used in the chronic stress paradigm. Recently, Zhvania et al. (2022) demonstrated that 4 hours of restraint for 20 consecutive days was sufficient to produce anxiety-like behaviour in male rats, indicated by increased rearing and grooming in the OFT and reduced exploration of the EPM open arms. Previously identified high anxiety rats have also displayed enhanced anxiety-like behaviour in the OFT following 5 weeks of 3-hour daily restraint stress (Wisłowska-Stanek et al., 2016). Chronic restraint has consistently produced behavioural alterations in the EPM, as 3 hours of restraint for 14 days (Guedri et al., 2017), 6 hours for 28 days

(Chiba et al., 2012), and 2 hours for 10 days (Mitra et al., 2005; Vyas et al., 2002) have all resulted in increased anxiety-like behavior as measured in the EPM. Sex differences for anxiety-like behaviour have also been reported following chronic restraint. Female rats display decreased open arm exploration on the EPM, and lower time spent in the centre of the OFT following 1 hour of restraint for 10 days (Vieira et al., 2018); however, results are mixed as additional chronic restraint research has reported female rats to display reduced anxiety-like behaviour following restraint (Bowman et al., 2009; Noschang et al., 2009). Chronic restraint is a useful paradigm, as it allows for the use of male and female rodents, and the duration of the restraint and overall chronic procedure can be easily manipulated. However, no overall consensus has determined what "appropriate" duration is sufficient to be considered a chronic stressor for restraint. Additionally, restraint stress has low ethological validity.

1.2.3 Social Defeat

A naturally-occurring stressor in rodents is the opposition faced for social dominance. The social defeat paradigm is another frequently used chronic stress model (Pryce & Fuchs, 2017), whereby animals are exposed to a conspecific that is higher in the social hierarchy. This elevated status may be demonstrated in the form of social or physical dominance over the subdued or defeated animal (Golden et al., 2011; Hammels et al., 2015). Grooming, a behaviour known to reduce stress and arousal (Spruijt et al., 1992), is significantly impacted by 15-minutes of chronic social defeat for 15-17 days (Denmark et al., 2010). Furthermore, chronic social defeat produces increased anxiety-like behaviors as measured in the EPM, OFT and LDB (Harris et al., 2017; Hayashida et

al., 2010; Iñiguez et al., 2014; Kinsey et al., 2007). The primary disadvantage to this paradigm is the lack of female representation; this test can only be done using male animals, as females do not display aggression to dominate a social order. One possible solution to the sex-specific problem is a variation of social defeat, known as a) witnessed social defeat stress, b) trauma witness, c) vicarious social defeat, or d) emotional stress (Iñiquez et al., 2018; Verbitsky et al., 2020). In this social defeat variation, female rodents are witnesses to the social defeat and watch as a male conspecific is subdued by a more aggressive counterpart (Iñiguez et al., 2018). It is theorized that by witnessing the defeat, a similar outcome may be seen in female rodent behaviour. Indeed, female mice exposed to chronic witnessed social defeat stress exhibit increased anxiety-like behavior as measured in the OFT and EPM (Qi et al., 2022). A critique of the witnessed social defeat stress is the lack of contingency between the male and female variations. Female mice, while subjected to the defeat taking place, are not being defeated (i.e., physically subdued by an aggressor). Thus, while witnessing a defeat may produce behavioural outcomes in females, it is uncertain if the outcomes are attributable to the same mechanisms occurring in the male social defeat.

1.2.4 Chronic Predator Stress

The predator-prey model involves exposure(s) of a prey species (i.e., mouse or rat) to a predator cue or animal (e.g., urine, rat, cat, etc.). If the exposure(s) involves a live predator, it can be protected or unprotected. Predator stress has high ethological validity as prey-predator interactions are common in wild animals for a diverse number of species across many different taxa (Muñoz-Abellán et al., 2008). Adaptions of predator

stress have been modified for use in laboratory settings, from exposure to a live predator in a controlled environment to the presence of predator odours.

1.2.4.1 Chronic Predator Odour Exposure

Predator odours are salient cues for prey animals as they convey the recent and/or territorial presence of a predator. Even though laboratory rats and mice have never encountered a live predator, both display immediate defensive responses when confronted with a predator odour (Blanchard et al., 1989; Dielenberg & McGregor, 2001; Zangrossi & File, 1992). Given that this innate behavior elicited by a predator cue is both ethologically relevant and convenient for laboratory chronic stress studies, predator odour studies have been prevalent over the last few decades (Hegab et al., 2015). Exposure to high amounts of predator odour across 7 consecutive days has produced defensive behaviours that are resistant to fear extinction (Takahashi et al., 2005), and 5 days of cat odour exposure to rats results in persistent anxiety-like behaviour, as demonstrated in the EPM (Zangrossi & File, 1992). While predator odour is a highly salient cue, there is variability across studies (Hegab et al., 2015) and hence, research has examined the behavioural outcomes of live predator exposure.

1.2.4.2. Chronic Live Predator Exposure

Exposure to a live predator not only allows for olfactory cues from predator odour, but also includes visual and auditory components. Research examining the behavioral effects of chronic live predator stress in adult rodents, however, is sparse. Burgado et al. (2014) found that a 15-day protected exposure of a mouse to two predator rats was sufficient to produce anxiety-like behaviour in the OFT and decreased social interaction

in the SIT persisting up to 2 weeks following the end of the predator stress. Similarly, a 28-day chronic live predator exposure elicited increased marble-burying behavior—another metric of anxiety-like behaviour—relative to control and CMS (Barnum et al., 2012). Predator exposure is a comprehensive testing paradigm as it permits researchers to understand subsequent behaviors in both males and females (Adamec et al., 2006; Cohen & Yehuda, 2011; Diehl et al., 2007); however, little is known about the effects of chronic predator stress on females. Hence, future studies should include both males and females to fully understand the effects of chronic predator stress on behavior.

1.3 Generational Effects of Chronic Predator Stress on Offspring

The effects of chronic stress are not solely limited to those subjected to the experience. In line with human studies examining the effects of inherited stress effects (Hankerson et al., 2022; Yehuda et al., 1998, 2005), stress research has explored the consequences of stress transmission across generations (Archer & Blackman, 1971). Exposure to predator stress during gestation has been shown to impact the survival rate of mammals pre-weaning, reduce the size of the litter, and can decrease the length of the pregnancy itself (Apfelbach et al., 2005; de Catanzaro, 1988; Delhaes et al., 2014; Green et al., 2018; Korgan et al., 2014; MacLeod et al., 2018; Sheriff, Krebs et al. 2009; Voznessenskaya & Malanina, 2013). Pups that do survive are likely to exhibit abnormal development, such as variability in body weight and susceptibility to seizures (Ahmadzadeh et al., 2011; Apfeblach et al., 2005; Delhaes et al., 2014; Korgan et al., 2014; Saboory et al., 2011; Tavassoli et al., 2013; Toumi et al., 2013, 2016; Weinstock et al., 1988; Zang et al., 2019).

The effects of predator stress on pregnant mothers include abnormal offspring sociability, increased susceptibility to learning and memory deficits, elevated levels of corticosterone, heighted predator avoidance, and increased anxiety in novel environments or situations (Brachetta et al., 2018; Green et al., 2018; Sheriff et al., 2009; St-Cyr et al., 2017, 2018; St-Cyr & McGowan, 2015; Thayer et al., 2018; Toumi et al., 2016). The impact of predator stress on subsequent generations is not limited to the mammalian species. Indeed, predator cues such as visual, tactile, auditory, or olfactory stimuli can lead to alterations in non-vertebrate species, such as altered telomere length in offspring of pied flycatchers (Kärkkäinen et al., 2019) and notable anti-predation activities and behaviours in offspring of marine snails (Donelan & Trussell, 2015) and crickets (Storm & Lima, 2010).

It is increasingly clear that predator stress *prior to* pregnancy also has the potential to affect offspring (reviewed in Tariel et al., 2020). The ecological implications of such changes are profound since it extends the 'window' for stress exposure effects well beyond the short period of pregnancy. Preconception paternal exposure to an artificial predator odor can also alter antipredator behavior in F1 mice (Brass et al., 2020). Similarly, exposing both male and female rats to chronic preconception cat exposure (two hours/day for 15-50 days) increased epileptic behaviors and anxiogenic responses in offspring (Azizi et al. 2019; Mahmoodkhani et al. 2018; Saboory et al., 2020).

Overall, these studies suggest that chronic predator stress causes phenotypic changes in offspring. However, the data are limited to a small number of behavioral measures in both the parental and F1 generation, and even less is known about potential

sex differences. Hence, the goal of the current study is to provide both a comprehensive examination of the behavioral consequences of chronic stress in naïve animals, as well as the consequences of preconception chronic predator stress on offspring behaviors in both male and female mice.

1.4 Current Study

We describe research exploring whether an ecologically realistic degree of parental predation risk alters the behavior of both predator-naïve and F1 offspring. We examined defensive behaviors during the chronic exposures, subsequent anxiety-like and social behaviors and circadian activity in predator-naïve mice. Similarly, adolescent F1 offspring from preconception predator-stressed or control parents underwent a behavioral battery to assess anxiety-like and social behaviors. These behaviors were also assessed following a mild stressor in adult F1s different from the F0 to determine if parental experience altered offspring stress sensitivity. A mild foot shock was used for the F1 in lieu of predator stress to differentiate the type of stressor that was used with the F0 generation. Previous research in our lab has demonstrated results using predator stress in the F1 generation, leading the current research to determine if results can be seen when using a different stressor. For this study, F0 predator exposed mice froze more often and exhibited elevated anxiety-like behaviour on the elevated plus maze. Similarly, F1 offspring of predator exposed parents showed increased anxiety-like behaviour on the EPM relative to controls in adolescence. Interestingly, following a mild stressor in adulthood, F1 offspring demonstrated an unexpected shift, displaying increased anxiety-like behaviour relative to the offspring of predator stressed offspring.

As stated above, the relevance of this study is profound. The ongoing pandemic has escalated stress levels across the globe and has further attenuated psychological wellbeing. Furthermore, the COVID-19 pandemic is not the only source of stress in many adult lives. Chronic stress can persist in numerous areas of our day-to-day lives, disrupting our homeostatic biological rhythms and precipitating detrimental effects on our physical and mental health. Therefore, the proposed research will be a significant contribution to our current understanding of chronic stress and its implications for future generations. Furthermore, by understanding the mechanisms of generational stress, we may be able to assess how future generations will fare following parental chronic stress. In the future, this research may contribute to improved understanding of the etiology of anxiety and stress-related pathologies.

Method

2.1 Ethical Approval

All animals were treated in accordance with the guidelines of the Canadian Council on Animal Care and all procedures were approved by the Institutional Animal Care Committee of Memorial University.

2.2 Animals

All C57BL/6 mice were individually housed and given *ad libitum* access to food and water in standard laboratory conditions (i.e., temperature and humidity) on a 12-hour light-dark cycle (lights on at 7:00AM), unless described otherwise. Male Long-Evans rats (300-350g in weight) purchased from Charles River Laboratories (St. Constant, QC) were used as stimulus animals for the rat exposures. Rats were kept on a reverse light/dark

cycle (lights off at 7:00AM), handled daily, and food restricted to 85% of expected body weight for nine days (two days prior to and during the seven-day RET) to increase activity and interaction rate with mice. F0 Mice (parental generation) were purchased from Charles River Laboratories (St. Constant, QC, CA) and left undisturbed in their cages for at least one week after arrival prior to experimentation.

2.3 General Procedures

2.3.1 Predator stress*:* The exposure chamber was a standard plexiglass rat cage (47 cm x 26 cm x 20 cm) containing a clear plexiglass partition to divide the cage width into two compartments. Small holes in the partition allowed free olfactory flow. A piece of clear perforated plexiglass was placed on top of the cage to prevent animals from escaping or entering the opposite side of the cage. Mice were exposed to a rat (predator stressed) or empty chamber (control) for five minutes per day for seven consecutive days.

Rats and mice were habituated to the exposure chamber once a day for the seven days preceding exposure by placing the mouse or rat inside the cage for five minutes and allowing it to explore their side of the partitioned cage while the opposite side was unoccupied. We used two identical cages for habituation so that no mouse was habituated in a cage used to habituate a rat and vice versa. Mouse habituation always occurred before rat habituation, and the two species were never in the same or adjacent rooms until the day of exposure. On exposure days (days 8-15), the mouse was placed in the left side of the exposure chamber; the right side of the chamber contained either a live rat (Predator Stressed group) or was left empty (Control group). Control mice were run before predator exposed mice to reduce rat scent exposure. Following exposures, mice

were returned to their home cages. All exposures were video recorded and hand-scored for mouse freezing duration and frequency as an index of fear and innate defensive behavior using Behavioural Observation Research Interactive Software (BORIS, Torino, Italy). Freezing was defined as immobility except for respiration. All chambers were wiped down with 70% ethanol between habituation trials and exposures.

2.3.2 Breeders and F1 offspring*:* Ten days after the last exposure, we bred male and female control mice together and male and female PS mice together. As a general note, two different F0 cohorts were used for the study, with F0 mice in cohort one born in the late spring and F0 cohort two born in the mid-summer. Breeding pairs were housed together for seven days. All F1 offspring were left undisturbed with their mothers, except when ear notched for identification, until weaning. All F1 mice were weaned on approximately PND 21 and housed individually.

2.4 Experiments

2.4.1 The effects of chronic predator stress on behavior and circadian rhythm in F0 generation.

Male (n = 48) and female (n = 48) 6–8-week-old sexually inexperienced mice (F0) were randomly divided into one of two groups: control or predator stressed. Two days following the last RET session, 72 (36 predator stressed and 36 control) mice underwent a four-test behavioral battery (one test per day for four days). The behavioral battery started with the elevated plus maze (EPM), followed by the open field test (OFT), light-dark box (LDB) test, and the social interaction test (SIT). Detailed descriptions of each test are provided in Section 2.5. These mice were used to generate the F1

generation.

Twenty-four (12 predator stressed, 12 controls) mice were monitored for circadian rhythm activity which included 12 consecutive days of pre-RET monitoring prior to habituation and RET exposure. After day 7 of RET, mice began another 12-day post-RET monitoring period. One cohort (n = 16) began 12 days of constant dark activity monitoring, and another cohort (n = 8) continued with an extended post-RET monitoring period for 16 days. Information on all circadian monitoring periods can be found in Sections 2.5.1.-2.5.4. Timelines for behaviour and circadian mice can be seen in Figure 1 in Appendix B.

2.4.2 The effects of preconception parental chronic predator stress on behavior and circadian rhythms in F1 mice.

Offspring mice (F1) were weighed on postnatal day (PND) 5, 10, and 15 to monitor litter weight gain and development. Beginning on PND 25, F1 mice (CO male n = 22; CO female n = 22; PSO male n = 17; PSO female n = 18) underwent a four-test behavioral battery (one test per day for four days) similar to F0 mice. Detailed descriptions of each test are provided in Sections 2.5.

F1 mice were left undisturbed for 30 days following the end of the adolescent behaviour battery. On PND 55, all F1 mice were exposed to a mild foot shock (Section 2.4.6) and tested for the contextual fear memory the following day. Two days after the mild foot shock, F1 mice were tested for ALB using the behavioral battery (similar to F0 or adolescent F1), with the addition of the novelty-supressed feeding (NSF).

A subset (n = 16) of male and female control offspring (CO) and predator stressed offspring (PSO) were assigned to post-shock locomotor activity monitoring. The day following the NSF, mice began 12 days of post-shock monitoring. This monitoring period continued for another 16 days to be reflective of the extended post-RET monitoring done in the F0 generation. Information on offspring activity monitoring can be found in Section 2.5.5. Timeline can be seen in Figure 2 in Appendix B.

2.4.3 Pilot study: The effects of mild foot shock amplitude on behaviour in male mice

A preliminary study used the F0 male mice (n = 15) following breeding to determine the appropriate amplitude of a mild foot shock as a stressor in the F1 generation. Mice followed the same procedures outlined in Section 2.4.1, apart from the foot shock amplitude. Mice were assigned to a .3 mA (n = 5), .5 mA (n = 5), or .7 mA (n = 5), shock, with control and stressed mice evenly distributed among the groups. Mice were then tested on the EPM (Section 2.5.1) and assessed on anxiety-like behaviour to determine the minimal amplitude necessary to constitute a mild shock without incident of a ceiling effect.

2.5 Behavioural Tests

Prior to each behaviour test (apart from the NSF test), all mice were given 20 minutes to acclimate to the laboratory with *ad libitum* access to food and water following transport from colony rooms.

2.5.1 Elevated Plus Maze (EPM)

The EPM was constructed from white polycarbonate (.6cm thick) with four 29.0cm x 5.1cm arms connected at right angles to a central square, 10.2cm^2. Of the four

arms, there were two closed arms and two open arms, both running opposite to their equivalent. The two closed arms had 14cm high walls enclosing the arm, keeping the top and entrance to the arms open. The open arms had no walls but had a .5cm high lip around the perimeter edge of the arms. The entire maze was elevated off the floor with 45cm high reinforced legs. Mice were placed on the end of an open arm facing away from the centre. Each trial was 5 minutes, and the maze was wiped down with 70% ethanol between each trial. Two EPMs were used simultaneously with a divider blocking the mice from seeing the other maze. EthoVision XT10 (Noldus, Wageningen, Netherlands) was used to assess frequency of visits and time spent in the open and closed arms of the EPM. Ratio visits was calculated by dividing the number of visits to the open arms by the number of visits to the all the arms. Ratio time was calculated by dividing the amount of time spent in the open arms by the amount of time spent in all the arms.

2.5.2 Open Field Test (OFT)

The OFT was constructed from a 48cm x 48cm x 48cm grey acrylic box with an open top. A square area was taped off within the box 10cm away from the inner wall. This area was defined as the centre of the arena. Additionally, mouse-sized corners of the box were defined as the corners of the arena (not taped). Mice were placed in the centre of the box and each trial was 5 minutes. Boxes were wiped down with 70% ethanol between each trial. Two OFTs were used simultaneously with a divider blocking the mice from seeing the other box. Frequency of visits and time spent in the centre and corners of the box were assessed using EthoVision XT10 (Noldus, Wageningen, Netherlands).

2.5.3 Light-Dark Box (LDB)

The LDB was made from a pair of grey acyclic 20.3cm x 20.3cm x 14.9cm boxes (.5cm thick). These boxes were connected with a confined 10.2cm x 6.4cm x 14.9cm tunnel. The dark box was fitted with a grey, opaque acrylic lid and the light box had a transparent acrylic lid with 25 ventilation holes. Above the light box, a 9w 550lm light was positioned to shine into the box. Mice were placed into the dark box and each trial was 5 minutes. All boxes were wiped down with 70% ethanol between each trial. Four LDBs were used simultaneously. Frequency of visits and time spent in the light box were assessed using BORIS (Torino, Italy).

2.5.4 Social Interaction Test (SIT)

The SIT used the same arena described in Section 2.3.1. Within the arena was an empty rectangular prism cage used to hold stimulus mice for visual and olfactory contact, without physical interaction. The base was made of metal wire mesh 12.7cm x 10cm x 9.7cm and the rest was made from transparent acrylic 12.7cm x 10cm x 15.2cm (.5cm thick). Each trial was 5 minutes total, broken down into two parts. The experimental mouse was placed in the centre of the box for the first 2.5 minutes with no sex-matched stimulus mouse present in the cage. The experimental mouse was then removed briefly so that said sex-matched stimulus mouse was placed inside the cage. The experimental mouse was then placed in the centre of the box for another 2.5 minutes. Boxes were wiped down with 70% ethanol between each trial. Two SITs were used simultaneously with a divider blocking the mice from seeing the other box. Ratio visits was calculated by dividing the number of visits to the interaction area around the stimulus mouse cage when

the stimulus mouse as present by the total visits to the interaction area between both trials. Ratio time was calculated by dividing the time spent in the interaction area around the stimulus mouse cage when the stimulus mouse as present by the time spent in the interaction area between both trials. Frequency of visits and time spent in the corners close to the interaction area, far from the interaction area, and all of the corners were also assessed using EthoVision XT10 (Noldus, Wageningen, Netherlands).

2.5.5 Mild Foot Shock Fear Conditioning

The conditioning chamber was comprised of Plexiglas walls on the front and back, stainless steel side walls and top, a shockable floor with 26 stainless steel rods, a removable drop pan beneath the rods, a speaker, and house light. The conditioning chamber itself was situated within a sound attenuating isolation cubicle (Habitest, Coulbourn Instruments, Holliston, MA, US). Per the outcomes of the pilot study outlined in Section 2.4.3, all adult offspring mice were trained with a mild shock in a single 210 second fear conditioning trial. Mice were placed within the conditioning chamber and enclosed within the sound attenuating isolation cubicle. Ninety seconds after the mouse was placed within the chamber, an 80 dB tone played through the speaker. The tone played for 30 seconds and co-terminated with a 2 second .3 mA foot shock. Mice remained within the chamber for another 90 seconds following the termination of the foot shock before being removed. The following day, mice were returned to the conditioning chamber for contextual retrieval of the associated fear memory with the mild shock. No tone or shock was present for the recall trials. The recall trial durations remained the same as the training trials (210 seconds). Following both training and recall trials, floors

of the conditioning chambers were wiped down with 70% ethanol. Percent freezing, frequency of freezing, and duration of freezing were assessed from the contextual retrieval trials using FreezeFrame (Actimetrics, Wilmette, IL, US).

2.5.6 Novelty-Suppressed Feeding (NSF)

The NSF apparatus was made from a standard plastic 59.7cm x 42.9cm x 14.9cm storage box. In the middle of the arena was a round platform (9cm diameter) elevated 2cm high from the bottom of the box. Standard rodent bedding was spread around the platform so that the bottom of the box was covered but the platform was still visible. Attached to the platform using a piece of tape was a piece of standard laboratory rodent chow. Twenty-four hours prior to the test, food was removed from the home cage. Mice were placed halfway from the wall and the food pellet, and each trial was 5 minutes. The food platform was wiped down with 70% ethanol between each trial and the food pellet replaced. Two NSFs were used simultaneously with a divider blocking the mice from seeing the other box. Latency to first visit, frequency of visits, and time spent on the food platform, along with the frequency of visits and time spent in the corners of the arena were all assessed using EthoVision XT10 (Noldus, Wageningen, Netherlands).

2.6 Circadian Rhythm Activity Monitoring

A timeline of all activity monitoring periods can be seen in Figure 3, Appendix B.

2.6.1 Pre-RET

Prior to RET habituation, a subset of mice were allocated to have their home cage locomotor activity monitored using infrared wireless motion sensors (ClockLab, Actimetrics, Wilmette, IL, US) in lieu of behaviour testing. Sensors were placed in the

food hoppers of mouse cages. Activity was recorded non-stop for 12 days prior to habituation to develop a baseline assessment of locomotor circadian rhythms prior to the RET or control equivalent. Metrics of period, onset of activity, and nocturnal activity were analyzed. Nocturnal activity was calculated by dividing the amount of activity occurring during lights off by the total amount of activity. All data were assessed using ClockLab Analysis V6 (Actimetrics, Wilmette, IL, US).

2.6.2 Post-RET

Following the chronic RET exposure or control equivalent, sensors were placed in the food hoppers of mouse cages. Activity was recorded non-stop for 12 days following the RET exposure to measure changes in the metrics listed. Metrics of period, onset of activity, and nocturnal activity were analyzed. Nocturnal activity was calculated by dividing the amount of activity occurring during lights off by the total amount of activity. All data were assessed using ClockLab Analysis V6 (Actimetrics, Wilmette, IL, US).

2.6.3 Constant Dark (DD)

After the post-RET monitoring, one cohort of mice was moved to a separate colony room under constant dark conditions to assess the free-running period. Activity was recorded non-stop for 12 days to measure changes in the metrics listed. Metrics of period, onset of activity, and nocturnal activity were analyzed. Nocturnal activity was calculated by dividing the amount of activity occurring during lights off by the total amount of activity. All data were assessed using ClockLab Analysis V6 (Actimetrics, Wilmette, IL, US).

2.6.4 Extended Post-RET

After the post-RET monitoring, one cohort of mice remained in the same colony room and were recorded for another 16 days. Activity was recorded non-stop to measure changes after an extended length of time had passed since the RET. Metrics of period, onset of activity, and nocturnal activity were analyzed. Nocturnal activity was calculated by dividing the amount of activity occurring during lights off by the total amount of activity. All data were assessed using ClockLab Analysis V6 (Actimetrics, Wilmette, IL, US).

2.6.5 Offspring Extended Post-RET

Seven days following the mild shock, one cohort of mice was recorded for 28 days. Activity was recorded non-stop to measure changes after an extended length of time had passed since the mild foot shock. Metrics of period, onset of activity, and nocturnal activity were analyzed. Nocturnal activity was calculated by dividing the amount of activity occurring during lights off by the total amount of activity. All data were assessed using ClockLab Analysis V6 (Actimetrics, Wilmette, IL, US).

2.7 Statistical Analysis

All analyses were conducted using jamovi V1.6 (Sydney, Australia). A 2 (control vs stressed) x 2 (male vs female) x 7 (RET days) mixed model ANOVA was used to assess frequency and cumulative duration of freezing behaviour. Significant interactions were broken down into simple main effects using the general linear model. For all other experiments, a 2 (control vs stressed) x 2 (male vs female) between-subjects ANOVA was used to assess each metric (EPM, OFT, LDB, SIT, NSF) for F0 and F1 mice. Mean

differences between groups (control vs stressed), sex (male vs female), and interactions between the two were considered significant when p values were < .05. However, non-significant (NS, $p > .05$) statistical results trending towards significance, $p < .10$, were highlighted. This was done due to the small effect size of predator stress and an acknowledgement that a trending effect may be meaningful itself. NS statistical results are not represented in the Results Section.

Results

3.1 Chronic rat exposure produces persistent changes in freezing behaviour in mice

Two behaviour metrics were assessed during the seven-day chronic RET—freezing frequency and cumulative duration frozen. Using a 2 (Group) x 2 (Sex) x 7 (Day) mixed model ANOVA, we assessed the frequency of mouse freezing behaviour as the dependent variable (See Figure 4, Appendix B). There was a significant effect for RET Day, $F(3.57,321.47) = 50.901$, $p < .001$, $\eta^2 = .132$, a significant main effect for Group, $F(1,90) = 120.463$, $p < .001$, $\eta^2 = .187$, a significant main effect of Sex, $F(1,90) = 4.367$, $p = .039$, $\eta^2 = .007$, and a significant interaction of RET Day and Group, $F(3.57,321.47) = 5.701$, $p < .001$, $\eta^2 = .015$. There was no significant interaction between RET Day and Sex, $F(3.57,321.47) = 1.598$, $p = .181$, $\eta^2 = .004$, between Group and Sex, $F(1,23963) = .990$, $p = .322$, $\eta^2 = .002$, or between RET Day, Group, and Sex, $F(3.57,321.47) = .474$, $p = .733$, $\eta^2 = .001$. For the between-subjects effects, stressed mice froze significantly more often than control mice and females froze significantly more than males. Figures 4B and 4C in Appendix B display the main between-subjects effects of Group and Sex, respectively. Descriptive statistics and ANOVA outputs can be

found in Tables 1 and 2 in Appendix A. For the effect of RET Day, all mice froze significantly less over the course of the chronic exposure. Estimated marginal means and post hoc comparisons for freezing frequency across RET day can be found in Tables 3 and 4, respectively, in Appendix A. Estimated marginal means are plotted in Figure 4D in Appendix B.

To follow up on the interaction of RET Day and Group, simple main effects analyses were conducted using the general linear model looking at the difference between Groups for each RET Day. There were significant effects of Group at all seven RET Days; Post hoc analyses found that stressed mice froze significantly more often than control mice for all seven RET days (all *p*s < .05; See Table 5, Appendix A). Estimated marginal means can be found in Table 6 and plotted in Figure 4A, Appendix B.

Using a 2 (Group) x 2 (Sex) x 7 (Day) mixed model ANOVA, we assessed the cumulative duration of freezing as the dependent variable. There was a significant effect for Time, $F(3.80, 341.68) = 25.040$, $p < .001$, $\eta^2 = .093$, a significant main effect for Group, $F(1,90) = 115.346$, $p < .001$, $\eta^2 = .161$, and a significant interaction of Time and Group, $F(3.80, 341.68) = 6.559$, $p < .001$, $\eta^2 = .024$. There was no significant main effect of Sex, $F(1,90) = .902$, $p = .345$, $\eta^2 = .001$, and no significant interactions between Group and Sex, $F(1,90) = .954$, $p = .331$, $\eta^2 = .001$, between RET Day and Sex, $F(3.80,341.68) = 1.623$, $p = .171$, $\eta^2 = .006$, or RET Day, Group, and Sex, $F(3.80,341.68) = .497$, $p = .729$, $\eta^2 = .002$. Descriptive statistics and ANOVA outputs can be found in Tables 7 and 8 in Appendix A. For the between-subjects effect of Group, stressed mice spent significantly more time frozen than control mice, as seen in Figure 5B in Appendix B.

For the effect of RET Day, all mice spent significantly less time frozen over the course of the chronic exposure. Estimated marginal means and post hoc comparisons for freezing duration across RET day can be found in Tables 9 and 10, respectively, in Appendix A. Estimated marginal means are plotted in Figure 5C in Appendix B.

To follow up on the interaction of RET Day and Group, simple main effects analyses were conducted using the general linear model looking at the difference between Groups for each RET Day. There were significant effects of Group at all seven RET Days; Post hoc analyses found that stressed mice froze significantly longer during the five-minute trials than control mice for all seven RET days (all ps $< .05$; see Table 11, Appendix A). Estimated marginal means can be found in Table 12 in Appendix A and plotted in Figure 5A, Appendix B.

3.2 Chronic predator stress caused increased anxiety-like behaviour in the EPM, but not in the OFT or LDB in mice.

Two behaviour metrics were assessed following the EPM—ratio frequency of visits to the open arms and ratio of time spent in the open arms. Both metrics were calculated by the amount in the open arms divided by the amount in all four arms of the maze. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the ratio of visits to the EPM open arms. There was a significant main effect of Group, $F(1,67) = 5.089$, $p = .027$, $\eta^2 = .066$, and a significant main effect of Sex, $F(1,67) = 4.712$, $p = .034$, $\eta^2 = .061$. There was no significant interaction, $F(1,67) = .020$, $p = .888$, $\eta^2 < .001$. Control mice visited the open arms significantly more often than stressed mice (Figure

6A, Appendix B). Male mice visited the open arms significantly more often than female mice (Figure 6B, Appendix B).

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the ratio of time spent in the EPM open arms. There was no significant main effect of Group, $F(1,62) = .566$, $p = .455$, $\eta^2 = .006$, or a significant interaction, $F(1,62) = 3.567$, $p = .064$, $\eta^2 = .041$. However, there was a significant main effect of Sex, $F(1,62) = 21.608$, $p < .001$, $\eta^2 = .246$. Male mice spent more time in the open arms than female mice (Figure 6C, Appendix B).

We assessed the frequency of visits to and cumulative duration in the OFT centre using 2 (Group) x 2 (Sex) between-subjects ANOVAs. No significant effects or interactions were seen (all $F < 1110.741$, all $p > .143$).

The frequency of visits and cumulative duration in the light box of the LDB box were analyzed using 2 (Group) x 2 (Sex) between-subjects ANOVAs. No significant effects of interactions were found (all $F < 810.197$, all $p > 141$).

3.3 Male mice visited and spent more time socializing or close to socializing in the SIT

Two metrics were assessed following the SIT—ratio frequency of visits and duration of time spent socializing. Both metrics were calculated by the amount in the interaction area when a stimulus mouse was present divided by the total amount in the interaction area.

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the ratio of visits to the SIT social area. There was no significant main effect of Group, $F(1,67) =$

1.079, $p = .303$, $\eta^2 = .015$, or interaction between Group and Sex, $F(1,67) = 1.480$, $p = .228$, $\eta^2 = .020$. There was a trending main effect of Sex, $F(1,67) = 3.902$, $p = .051$, $\eta^2 = .053$. Male mice trending towards visiting the interaction more when the stimulus mouse was present compared to female mice.

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the ratio of time in the SIT social area. There was no significant main effect of Group, $F(1,67) = .072$, $p = .789$, $\eta^2 = .001$, or interaction between Group and Sex, $F(1,67) = 1.057$, $p = .308$, $\eta^2 = .015$. There was a significant main effect of Sex, $F(1,67) = 4.735$, $p = .033$, $\eta^2 = .065$. Male mice spent significantly more time in the interaction area when the stimulus mouse was present compared to female mice (Figure 6D, Appendix B).

3.4 Chronic predator stress did not alter locomotor activity in mice

3.4.1 No underlying differences were seen between-subjects prior to rat exposure

Mice were assessed on three metrics—length of period, onset of activity, and nocturnality. Nocturnality was calculated as a ratio proportion of activity occurring in the dark phase divided by the total daily amount of activity. Exemplar actograms demonstrating activity patterns between male and female mice can be seen in Figures 7A and 7B in Appendix B, respectively.

An independent samples *t*-test was used to assess the length of period between sexes. No significant difference was seen between males and females, $t(20) = 1.889$, $p = .074$, *Cohen's d* = .809. An independent samples *t*-test was used to assess the average onset of activity between sexes. No significant difference was seen between males and females, $t(20) = 1.284$, $p = .214$, *Cohen's d* = .550. An independent samples *t*-test was

used to assess nocturnality between sexes. No significant difference was seen between males and females, $t(20) = -.443$, $p = .663$, *Cohen's d* = -.189.

3.4.2 No differences were seen between-subjects for Group or Sex post rat exposure

Mice were assessed on three metrics—length of period, onset of activity, and nocturnality. Nocturnality was calculated as a ratio proportion of activity occurring in the dark phase divided by the total daily amount of activity. Exemplar actograms demonstrating activity patterns for control male, control female, stressed male, and stressed female can be seen in Figures 8A-8D in Appendix B, respectively. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the length of period post-RET. The Shapiro-Wilke test of normality was significant. There was no significant main effect of Group, $F(1,19) = .879$, $p = .360$, $\eta^2 = .043$, Sex, $F(1,19) = .010$, $p = .921$, $\eta^2 = .001$, or interaction between the two, $F(1,19) = .727$, $p = .404$, $\eta^2 = .035$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the onset of activity post-RET. There was no significant main effect of Group, $F(1,18) = .299$, $p = .591$, $\eta^2 = .016$, Sex, $F(1,18) = .576$, $p = .458$, $\eta^2 = .031$, or interaction between the two, $F(1,18) = .004$, $p = .949$, $\eta^2 < .001$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the nocturnality post-RET. We found no significant main effect of Group, $F(1,19) = 2.799$, $p = .111$, $\eta^2 = .126$, Sex, $F(1,19) = .361$, $p = .555$, $\eta^2 = .016$, or interaction between the two, $F(1,19) = .001$, $p = .977$, $\eta^2 < .001$.

3.4.3 Chronic predator stressed mice show a trend of decreased nocturnality two weeks post rat exposure

Mice were assessed on three metrics—length of period, onset of activity, and nocturnality. Nocturnality was calculated as a ratio proportion of activity occurring in the dark phase divided by the total daily amount of activity. Exemplar actograms demonstrating activity patterns for control male, control female, stressed male, and stressed female can be seen in Figures 9A-9D in Appendix B, respectively.

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the length of period after extended post-RET. The Shapiro-Wilke test of normality was significant. There was no significant main effect of Group, $F(1,4) <.001$, $p = 1.000$, $\eta^2 <.001$, Sex, $F(1,4) = 1.661$, $p = .267$, $\eta^2 = .158$, or interaction between the two, $F(1,4) = 4.881$, $p = .092$, $\eta^2 = .463$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the onset of activity after extended post-RET. The Shapiro-Wilke test of normality was significant. There was no significant main effect of Group, $F(1,4) = 1.194$, $p = .336$, $\eta^2 = .166$, Sex, $F(1,4) = 1.626$, $p = .271$, $\eta^2 = .225$, or interaction between the two, $F(1,4) = .394$, $p = .564$, $\eta^2 = .055$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed nocturnality after extended post-RET. There was no significant main effect of Sex, $F(1,4) = .848$, $p = .409$, $\eta^2 = .071$, or interaction between the Group and Sex, $F(1,4) = .205$, $p = .674$, $\eta^2 = .017$. There was a trending main effect of Group, $F(1,4) = 6.921$, $p = .058$, $\eta^2 = .578$. Stressed mice trended towards decreased nocturnality relative to control mice.

3.4.4 No differences were seen between-subjects for Group or Sex during constant dark

Mice were assessed on three metrics—length of period, onset of activity, and nocturnality. Nocturnality was calculated as a ratio proportion of activity that would be occurring in the dark phase divided by the total daily amount of activity during a normal 12:12 light-dark cycle. Exemplar actograms demonstrating activity patterns for control male, control female, stressed male, and stressed female can be seen in Figures 10A-10D in Appendix B, respectively.

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the length of period under DD conditions. There was no significant main effect of Group, $F(1,11) = .2481$, $p = .628$, $\eta^2 = .018$, Sex, $F(1,11) = .384$, $p = .548$, $\eta^2 = .027$, or interaction between the two, $F(1,11) = 2.455$, $p = .145$, $\eta^2 = .174$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the onset of activity under DD conditions. There was no significant main effect of Group, $F(1,11) = .012$, $p = .916$, $\eta^2 = .001$, Sex, $F(1,11) = 2.102$, $p = .175$, $\eta^2 = .152$, or interaction between the two, $F(1,11) = .754$, $p = .404$, $\eta^2 = .054$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed nocturnality under DD conditions. There was no significant main effect of Group, $F(1,11) < .001$, $p = .979$, $\eta^2 < .001$, Sex, $F(1,11) = .112$, $p = .745$, $\eta^2 = .010$, or interaction between the two, $F(1,11) = .523$, $p = .485$, $\eta^2 = .045$.

3.4.5 No differences were seen within-subjects comparing pre rat exposure to post

Mice were assessed on three metrics—length of period, onset of activity, and nocturnality. Nocturnality was calculated as a ratio proportion of activity that would be

occurring in the dark phase divided by the total daily amount of activity. These metrics were compared pre- and post-RET.

A repeated measures ANOVA was used to assess length of period across time. No significant difference was found between Time and Group, $F(1,17) = 1.991$, $p = .175$, $\eta^2 = .024$, between Time and Sex, $F(1,17) = .005$, $p = .945$, $\eta^2 < .001$, or within Time, Group and Sex, $F(1,17) = .894$, $p = .357$, $\eta^2 = .012$. A repeated measures ANOVA was used to assess onset of activity across time. No significant difference was found between Time and Group, $F(1,17) = .021$, $p = .885$, $\eta^2 < .001$, between Time and Sex, $F(1,17) = 1.811$, $p = .196$, $\eta^2 = .008$, or within Time, Group and Sex, $F(1,17) = .666$, $p = .426$, $\eta^2 = .003$. A repeated measures ANOVA was used to assess nocturnality across time. No significant difference was found between Time and Group, $F(1,17) = 1.260$, $p = .276$, $\eta^2 = .012$, between Time and Sex, $F(1,17) = .004$, $p = .949$, $\eta^2 < .001$, or within Time, Group and Sex, $F(1,17) = 1.112$, $p = .306$, $\eta^2 = .009$.

3.5 Preconception chronic predator stress had no impact on birth success or F1 development

Of the 35 breeding pairs across cohorts, 17 litters were produced. Therefore, the percentage of successful birth rate for the study was 48.57%. One (predator stressed) of the 17 litters was unavailable to be weighed and counted as the litter was found dead or destroyed (FDD) on PND1. Frequencies for litter distribution can be found in Table 13 in Appendix A.

Using an independent samples *t*-test, successful control (M = 7.22, SE = .465) and stressed (M = 8.38, SE = .324) litter size was compared. No significant difference was detected between groups, $t(15) = -1.98$, $p = .066$, *Cohen's d* = -.964.

Offspring were left undisturbed for five days following birth. Afterwards, mice were weighed at PND 5, 10, and 15. Additionally, four CO did not develop between PND10 and PND15 and were FDD. Using an independent samples *t*-tests, we assessed weight at each developmental point between groups. No significant differences were detected between groups at PND5, $t(130) = -.452$, $p = .652$, *Cohen's d* = -.079, PND10, $t(130) = -1.569$, $p = .119$, *Cohen's d* = -.273, or PND15, $t(126) = -1.051$, $p = .295$, *Cohen's d* = -.186. Descriptive statistics can be found in Table 14 in Appendix A.

3.6 Preconception chronic predator stress produced mixed anxiety-like behaviour in F1 adolescent mice

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the ratio of visits to the EPM open arms. There was no significant main effect of Group, $F(1,75) = .805$, $p = .373$, $\eta^2 = .010$, no significant main effect of Sex, $F(1,75) = 2.375$, $p = .128$, $\eta^2 = .030$, or interaction, $F(1,75) = .068$, $p = .794$, $\eta^2 = .001$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the ratio of time spent in the EPM open arms. There was no significant main effect of Group, $F(1,74) = 2.426$, $p = .124$, $\eta^2 = .029$, or a significant main effect of Sex, $F(1,74) = 2.914$, $p = .092$, $\eta^2 = .035$. However, there was a significant interaction between Group and Sex, $F(1,74) = 4.532$, $p = .037$, $\eta^2 = .054$. Tukey's post hoc comparisons found that control offspring (CO) males spent significantly more time in the open arms compared to CO females, $t(74) = 2.863$, $p =$

.027, and predator stressed offspring (PSO) males, $t(74) = 2.573$, $p = .057$. Mean differences can be seen in Figure 11A in Appendix B.

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the frequency of visits to the OFT centre. The Shapiro-Wilke test of normality was significant. There was a significant main effect of Group, $F(1,73) = 5.943$, $p = .017$, $\eta^2 = .074$. However, there was no significant main effect of Sex, $F(1,73) = 1.025$, $p = .315$, $\eta^2 = .013$, or interaction between the two, $F(1,73) = 33.826$, $p = .502$, $\eta^2 = .006$. CO mice visited the centre of the OFT arena significantly more than PSO mice (Figure 11B, Appendix B).

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the duration of time spent in the OFT centre. The Shapiro-Wilke test of normality was significant. There was no significant main effect of Group, $F(1,70) = 2.033$, $p = .158$, $\eta^2 = .028$, Sex, $F(1,70) = .603$, $p = .440$, $\eta^2 = .008$, or interaction between the two, $F(1,70) = 177.823$, $p = .412$, $\eta^2 = .009$.

We assessed the frequency of visits to and cumulative duration in the light box of the LDB using 2 (Group) x 2 (Sex) between-subjects ANOVAs. No significant effects or interactions were seen (all $F < .248$, all $p > .620$).

3.7 Chronic predator stressed adolescent offspring spent more time socializing than controls in the SIT

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the ratio of visits to the SIT social area. There was no significant main effect of Group, $F(1,74) = .845$, $p = .364$, $\eta^2 = .011$, Sex, $F(1,74) = 2.303$, $p = .133$, $\eta^2 = .030$, or interaction

between the two, $F(1,74) = .136$, $p = .713$, $\eta^2 = .002$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the ratio of time in the SIT social area. The Shapiro-Wilke test of normality was significant. There was no significant main effect of Sex, $F(1,74) = 1.050$, $p = .309$, $\eta^2 = .013$, or interaction between Group and Sex, $F(1,74) = 2.026$, $p = .159$, $\eta^2 = .024$. There was a significant main effect of Group, $F(1,74) = 6.384$, $p = .014$, $\eta^2 = .076$. PSO mice spent significantly more time in the interaction area when the stimulus mouse was present compared to CO mice (Figure 11C, Appendix B).

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the frequency of visits to the SIT corners. There was no significant main effect of Group, $F(1,74) = .904$, $p = .345$, $\eta^2 = .011$, Sex, $F(1,74) = 2.162$, $p = .146$, $\eta^2 = .027$, or interaction between the two, $F(1,74) = 3.532$, $p = .064$, $\eta^2 = .044$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the duration of time in the SIT corners. The Shapiro-Wilke test of normality was significant. There was no significant main effect of Group, $F(1,73) = .109$, $p = .742$, $\eta^2 = .001$, Sex, $F(1,73) = 2.082$, $p = .153$, $\eta^2 = .028$, or interaction between the two, $F(1,73) = .110$, $p = .741$, $\eta^2 = .001$.

3.8 Preconception predator stress did not alter freezing behaviour following a .3 mA foot shock in F1 mice

Mice were returned to the context one day after a .3mA foot shock. Percent freezing, freezing bouts, and cumulative duration of freezing bouts were measured.

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed percent freezing within the shock context. There was no significant main effect of Group, $F(1,74) = .602$, $p = .440$, $\eta^2 = .008$, Sex, $F(1,74) = .261$, $p = .611$, $\eta^2 = .003$, or interaction between

Group and Sex, $F(1,74) = .303$, $p = .584$, $\eta^2 = .004$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA we assessed the frequency of freezing bouts within the shock context. There was no significant main effect of Group, $F(1,74) = .208$, $p = .649$, $\eta^2 = .003$, Sex, $F(1,74) = 1.021$, $p = .316$, $\eta^2 = .014$, or interaction between Group and Sex, $F(1,74) = .008$, $p = .931$, $\eta^2 < .001$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the cumulative duration of freezing bouts within the shock context. There was no significant main effect of Group, $F(1,74) = .396$, $p = .531$, $\eta^2 = .005$, Sex, $F(1,74) = .854$, $p = .358$, $\eta^2 = .011$, or interaction between Group and Sex, $F(1,74) = .104$, $p = .748$, $\eta^2 = .001$.

3.7 Preconception chronic predator stress produced mixed effects on anxiety-like behaviour in F1 following a mild stressor (foot shock) in adult mice

3.7.1 PSO showed decreased anxiety-like behavior in the EPM compared to CO

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the ratio of visits to the EPM open arms. There was no significant main effect of Sex, $F(1,73) = .316$, $p = .576$, $\eta^2 = .004$, or interaction, $F(1,73) = .288$, $p = .593$, $\eta^2 = .004$. There was a significant main effect of Group, $F(1,73) = 5.010$, $p = .028$, $\eta^2 = .064$. PSO visited the open arms significantly more than CO, as seen in Figure 12B in Appendix B. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the ratio of time spent in the EPM open arms. The Shapiro-Wilke test of normality was significant. There was no significant main effect of Group, $F(1,72) = .500$, $p = .482$, $\eta^2 = .007$, no significant main effect of Sex, $F(1,72) = .001$, $p = .971$, $\eta^2 < .001$, or interaction, $F(1,72) = .683$, $p = .411$, $\eta^2 = .009$.

3.7.2 PSO females showed increased anxiety-like behavior in the OFT compared to CO females

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the frequency of visits to the OFT centre. The Levene's test of homogeneity of variance and Shapiro-Wilke test of normality were significant. There was no significant main effect of Group, $F(1,74) = 2.670$, $p = .106$, $\eta^2 = .032$, or Sex, $F(1,74) = .003$, $p = .958$, $\eta^2 < .001$. There was, however, a significant interaction, $F(1,74) = 5.680$, $p = .020$, $\eta^2 = .069$. Tukey's post hoc comparisons showed that CO females visited the centre of the OFT arena significantly more than PSO females, $t(74) = 2.879$, $p = .026$ (Figure 12C, Appendix B). Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the duration of time spent in the OFT centre. The Levene's test of homogeneity of variance and Shapiro-Wilke test of normality were significant. There was no significant main effect of Group, $F(1,72) = 2.322$, $p = .132$, $\eta^2 = .029$, or Sex, $F(1,72) = .021$, $p = .886$, $\eta^2 < .001$. There was, however, a significant interaction between the two, $F(1,72) = 4.963$, $p = .029$, $\eta^2 = .063$. Tukey's post hoc comparisons showed that CO females spent significantly more time in the centre of the OFT arena than PSO females, $t(72) = 2.739$, $p = .038$ (Figure 12E, Appendix B).

3.7.3 Females visited and spent more time in the light side of the LDB

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the frequency of visits to the LDB light box. The Shapiro-Wilke test of normality was significant. There was no significant main effect of Group, $F(1,72) = .255$, $p = .615$, $\eta^2 = .003$, or interaction between Group and Sex, $F(1,72) = .049$, $p = .826$, $\eta^2 = .001$. There

was significant main effect of Sex, $F(1,72) = 5.183$, $p = .026$, $\eta^2 = .067$. Female mice visited the light box significantly more than males, as seen in Figure 12A, Appendix B. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the duration of time spent in the LDB light box. The Shapiro-Wilke test of normality was significant. There was no significant main effect of Group, $F(1,71) = 1.913$, $p = .171$, $\eta^2 = .024$, or interaction between Group and Sex, $F(1,71) = .215$, $p = .644$, $\eta^2 = .003$. There was significant main effect of Sex, $F(1,71) = 8.226$, $p = .005$, $\eta^2 = .101$. Female mice spent significantly more time in the light box than males, as seen in Figure 12D in Appendix B.

3.7.4 Preconception chronic stress had no effect on social behaviors in offspring

Using 2 (Group) x 2 (Sex) between-subjects ANOVAs, we assessed the ratio of visits to, and ratio of time spent in the SIT social area. No significant effects or interactions were seen (all $F < 2.717$, all $p > .104$).

3.7.5 Preconception predator stress did not alter behaviors in the novelty suppressed feeding (NSF) test following a mild stressor in adult offspring

Three metrics were developed from the data: latency to, frequency of visits, and cumulative duration of time spent on the food platform. Using 2 (Group) x 2 (Sex) between-subjects ANOVAs, no significant differences were found (all $F < 1.843$, all $p > .179$).

3.8 Preconception chronic predator stress did not impact locomotor activity in F1 mice in the three weeks following a mild stressor

Mice were assessed on three metrics—length of period, onset of activity, and nocturnality. Nocturnality was calculated as a ratio proportion of activity occurring in the

dark phase divided by the total daily amount of activity. Exemplar actograms demonstrating activity patterns of CO male, CO female, PSO male, and PSO female mice can be seen in Figures 13A-13D in Appendix B, respectively.

Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed period of activity post-shock. The Shapiro-Wilk test of normality was significant. There was no significant main effect of Group, $F(1,12) = 2.04$, $p = .179$, $\eta^2 = .081$, or interaction between Group and Sex, $F(1,12) = 4.22$, $p = .062$, $\eta^2 = .168$. There was a significant main effect of Sex, $F(1,12) = 6.82$, $p = .023$, $\eta^2 = .272$, however the mean difference is not meaningfully different. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the onset of activity post-shock. We found no significant main effect of Group, $F(1,12) = 1.127$, $p = .309$, $\eta^2 = .066$, Sex, $F(1,12) = .055$, $p = .818$, $\eta^2 = .003$, or interaction between the two, $F(1,12) = 3.780$, $p = .076$, $\eta^2 = .223$. Using a 2 (Group) x 2 (Sex) between-subjects ANOVA, we assessed the nocturnality post-shock. We found no significant main effect of Group, $F(1,12) = .064$, $p = .805$, $\eta^2 = .004$, Sex, $F(1,12) = 2.905$, $p = .114$, $\eta^2 = .188$, or interaction between the two, $F(1,12) = .511$, $p = .488$, $\eta^2 = .033$.

Discussion

It is well known that chronic stress can have deleterious effects on an individual's mental health. Indeed, results from both humans and other animals suggests that the harmful effects of chronic stress during one's lifetime can propagate into future generations (Blaze et al., 2015; Blaze and Roth, 2015; Dias and Ressler, 2014; Yahyavi et al., 2015; Yehuda, 2002; Yehuda et al., 2000, 2008), perhaps leading to an increased

vulnerability to the development of psychopathology. The goal of this thesis was to 1) develop a chronic predator stress model that produces persistent changes in behavior, 2) examine the effects of chronic predator stress across generations, and 3) determine if there is a sex difference in response to chronic stress (within and across generations). As expected, repeated exposure to a rat caused increased anxiety-like behavior in both male and female mice. In addition, we demonstrated that chronic exposure to a predator prior to conception produced lasting changes in behavior in the F1 generation. Finally, there were significant sex differences, particularly in the social interaction test. Our findings suggest generational effects of stress and open a new avenue for future research investigating the mechanisms underlying these phenotypic changes. Ultimately, the goal is to better understand the risk and resilience factors associated with trauma in order to identify novel pharmacological interventions to improve mental health.

4.1 Chronic stress effects within generation

4.1.1 Chronic stress effects on defensive and anxiety-like behaviors

We examined defensive behaviors (freezing duration and frequency) during the rat exposure test (RET) in both experimental (with a rat) and control (without a rat) conditions. As expected, mice exposed to the predator froze more often and for longer than mice exposed to the control condition. This is consistent with previous research in our lab using an acute rat exposure in which rat-exposed mice freeze more than control mice (Bhattacharya, 2021, as well as Amaral et al., 2010). This is the first time, however, that we have examined the effects of multiple exposures (7 exposures). While there was a decline in freezing across days, stressed mice continued to freeze more than controls

across all seven exposure days. These data suggest that while there was some habituation to the predator, the paradigm produced sustained fear behavior. The sustained fear response across days is consistent with previous studies assessing chronic exposure to predator odors (File et al., 1993; Wallace and Rosen, 2000; Zangrossi & File, 1994). Additionally, predator-stressed female mice froze significantly more often than males in the same condition. This finding is important as female mice may be more susceptible to the effects of predator exposure and is consistent with the sex bias of anxiety disorders in humans (Blanchard et al., 1995).

Next, we examined anxiety-like and social behaviors in both predator stressed and control mice two days following the final exposure. Consistent with previous studies (Adamec et al., 2004; Bhattacharya, 2021; Zangrossi & File, 1992), predator stressed mice showed increased anxiety-like behavior in the elevated plus maze (EPM). These data, coupled with the increased freezing during rat exposures, suggests that our chronic stress model produces persistent changes in behavior. This is consistent with studies using a live predator exposure over time. Barnum et al. (2014) used a hamster ball to protectively expose mice to a predator rat for 30 minutes a day for 28 days and found that predator stressed mice showed elevated anxiety-like behaviour in the marble-burying test relative to controls and mice exposed to 28 days of chronic mild stress. Additional research using the hamster ball technique with two rats demonstrated sustained anxiety-like behaviour in the OFT and SIT (Burgado et al., 2014). After 15 days of 30-minute predator exposures, the predator stressed mice travelled less distance in the centre of the

OFT and spent significantly less time socializing in the SIT following the stressor (Burgado et al., 2014).

Despite increased anxiety-like behavior in the EPM, there were no differences across groups in the open field test (OFT) and light/dark box (LDB) in the current study. To our knowledge, the effects of chronic stress on LDB behavior have not been assessed prior to the current study. The lack of effects in the LDB is not surprising as acute predator stress have also shown no effects in this test (Adamec et al., 2005, 2006). The lack of differences in the OFT is surprising as a chronic predator stress study found differences in this test (Burgado et al., 2014). However, the metrics scored in our OFT analysis differ from previous research. In lieu of distance travelled in the centre of the arena, we measured the frequency and duration of visits to the centre. These metrics were chosen for two reasons: consistency with other metrics of additional tests used in our behavioural battery and to exclude potential outliers of highly mobile mice. Not reported in the Results Section were the observer's notes seen when auditing the Ethovision scoring. Additionally, it may be that the EPM measures aspects of anxiety-like behaviour that are not seen in the other tests, namely explorative agoraphobic behaviour. The OFT arena and the LDB arena are boxes with high or enclosed walls that may offer a semblance of protection to the experimental animal. The EPM, on the other hand, has two closed arms and two completely open arms, allowing the animals to explore freely without any semblance of protection. This open exploration without the protection of walls is not possible in the other behaviour testing arenas. In the context of predator stress, staying hidden is a key survival technique for mice. Therefore, on a test with the

possibility of exploring the open arms, it does not come as a surprise that predator stressed mice do not readily explore the open arms as much as control mice.

Chronic predator stress did not alter social behavior in the current study. This is inconsistent with Burgado et al. (2014), who reported deficits in the SIT following chronic predator stress. While this inconsistency may be a result of the difference in chronic stress exposure between the current study and that of Burgado et al. (2014), it may be due to the housing of the animals. In the current study, all animals were single-housed throughout the experiment, remaining undisturbed for 26 days following arrival at the facility. Afterwards, the animals were habituated, and predator stressed for a total of 14 days. The SIT did not take place until the end of the behaviour battery, 6 days following the last rat exposure. Therefore, mice were not social with another conspecific for a minimum of 46 days prior to the SIT. While the predator stressed mice may have displayed an avoidant phenotype in the SIT, control mice may have been demonstrating behavioural impairments due to social isolation stress. Additionally, adolescent offspring mice were not group housed, as males that are separated and re-housed together will often fight, resulting in significant injury or death. Mice are naturally social creatures and previous studies have confirmed that social isolation in housing can lead to elevated anxiety-like behaviour (Berry et al., 2012; Ieraci et al., 2016; Koike et al., 2009). Therefore, while there may be a socially avoidant phenotype in the SIT for predator stressed mice, there may be a masking effect due to isolation stress experienced by both control and predator stressed mice.

There were sex differences in both the EPM and the SIT. Males showed less anxiety-like behavior in the EPM and more social behavior in the SIT. This is consistent with previous studies that suggest that female mice are more susceptible to the anxiogenic effects of predator stress. Following exposure to a cat or cat-exposure room in C57BL/6 mice, female mice display increased anxiety-like behavior in the EPM (Adamec et al., 2006). It is not obvious why female mice are less likely to enter the open arms of the EPM, but it can be speculated that conservation and self perseverance may have something to do with it. In terms of natural selection, females are more valuable in the generational inheritance of "passing on." The gestational period and maternal care needed to rear offspring requires more time for females relative to the male's contribution to reproduction. Given that this sex effect did not interact with the group differences (stressed or control), it could be hypothesized that female mice unconditionally avoid open areas. In the SIT, male mice spent significantly more time socializing than female mice, regardless of group. There are a couple of explanations that may explain this sex difference, beginning with territorial social competition. Male mice naturally establish a hierarchy in groups, with more aggressive mice becoming dominant males and passive or defeated mice being subordinates (Pryce & Fuchs, 2017). In the SIT, the stimulus mouse is separated from the experimental mouse by a physical wire mesh, preventing any attacks or other physical interaction. Because of this protective barrier, it allows the male mice to smell each other and learn about the characteristics of the other male mouse. Another explanation for the lack of female social interaction may be the colony housing arrangement. In this study, all mice were individually housed to allow for n = 1 for each

motion sensor for the circadian monitored mice. Therefore, to allow for generalizability between the circadian and behavioural mice, the housing situations had to be the same. Due to the solitary housing, female mice may have been experiencing isolation stress and displaying an apprehension to socializing, despite the protective mesh separating the stimulus and experimental mice. Further exploration looking at the impact of isolation stress on social interaction is needed to support the current speculations.

4.1.2 Chronic predator stress does not impact locomotor activity

Across all monitoring periods, predator stress did not alter locomotor metrics. Previous research using variable exposure of mice to predator rats has demonstrated mice to have impaired home cage activity patterns (Dalm et al., 2009). While mice spent more time in the shelter, spent less time exploring and foraging in the home cage, and were more cautious in preservative behaviour overall, there was no general change in locomotor activity (Dalm et al., 2009). Home cage observations of locomotor activity are not as readily used relative to measuring locomotor activity in the OFT (Burgado et al., 2014) and researchers have argued that more research measuring home cage behaviours would measure features of animal behaviour that are more ethologically relevant compared to the standard behaviour testing approach (Grieco et al., 2021).

The lack of an effect of chronic predator stress may reflect the robustness of the SCN to circulating glucocorticoids (GCs; Kong et al., 2022; Ota et al., 2018, 2020). Because glucocorticoid receptors are absent in the SCN, circulating GCs would not impact the functions governed by the SCN (i.e., locomotor activity). Instead, recent studies looking at the effects of chronic stress using the social defeat paradigm report

circadian rhythmicity in peripheral tissues such as the lungs and kidneys, but not the liver or white adipose tissue (Kong et al., 2022). These delays in peripheral tissues may be more representative of what occurs following chronic rat exposure, though further study is required. Hence, future studies ought to explore alternative means of investigating circadian rhythmicity such as monitoring levels of plasma corticosterone or internal peripheral rhythms such as body temperature.

4.2 Preconception chronic predator stress causes both resilience and anxiety-like behaviours in adolescent offspring, depending on the behavioral test

Many studies, including the current one, report alterations in behavior following a chronic stress (Barnum et al., 2012; Burgado et al., 2014; Takahashi et al., 2005; Zangrossi & File, 1992, 1994). Less is known about the effects of stress on future generations. Hence, we examined a suite of behavioral tests measuring anxiety-like and social behaviors in the offspring of stressed and control parents. We demonstrated that a chronic predator stress prior to conception causes behavioral changes in offspring. During adolescence, offspring from predator stressed parents spent less time in the open arms of the elevated plus maze compared to offspring from control parents. In addition, adolescent offspring from stressed parents visited the center of the open field less often than offspring from control parents. Preconception predator stress, however, had no effect on behaviors in the light/dark box. While limited, previous research has also demonstrated that preconception predator stress can impact future generations in both rats and mice. Azizi et al. (2019) found that adolescent rats with one or both parents previously exposed to a predator cat explore the open arms of the elevated plus maze less

than rats of control parents. Our data suggest that the experiences in the previous generation manifest in offspring behaviours when tested under the same conditions with the exception of the stressor. No differences existed between the CO and PSO groups aside from lineage. Therefore, these results are consistent with an inherited stress phenotype.

Surprisingly, offspring from predator stressed parents show increased social behavior compared to offspring from control parents. One possible explanation for these results (i.e., lack of ALB) is a resilience effect. Resilience is defined as the resistance to pathological manifestations of stress (Cabib et al., 2012). This generational neuroadaptive process that we see in the current study would allow offspring mice to be better prepared to face potential challenges previously experienced by the preceding generations. For the SIT, increased social interaction would prove to be mutually beneficial for predated species. Cohesive group environments and higher social interactivity would allow for a "safety in numbers" approach, the probability of predation spread among the group, and increased chances of reproducibility (Brewer & Caporael, 2006). While these results were not anticipated, they demonstrate another aspect of generational stress that is not often discussed.

Given that there was no difference in treatment or housing conditions between control males and females, these significant findings may support the previous assumption given in the parental generation—a potential unconditioned response in female mice to avoid free exploration. This assumption is also supported given that the

mean time spent in the open arms for CO and PSO female mice are nearly equal, despite the difference in heritage.

4.2.2 Preconception predator stress alters behavior in response to a mild stressor in adult offspring (F1 generation)

Post-traumatic stress disorder develops in response to a traumatic event and patients often show sensitized reactions to mild stressors associated with the trauma, a response more suitable for the original traumatic event (Bremner et al., 1995; Dykman et al., 1997; Friedman et al., 1995). Furthermore, children of people with PTSD are more likely to have psychiatric conditions such as PTSD (Copeland et al., 2007; La Greca, 2007; Silva et al., 2000). In light of these factors, we assessed the behavioral response to a mild stressor in offspring from stressed or control parents. The mild stressor was a .3mA shock previously determined in a pilot study. In the fear conditioning literature, the appropriate foot shock amplitude ranges from .1-1.0 mA for adequate fear conditioning association between the context and cue (tone; Butler et al., 2018; Curzon et al., 2009; Kim & Cho, 2020). We tested amplitudes on the lower portion of the shock range (.3, .5, & .7mA) to find a foot shock that would serve as a mild stressor to invoke potential inherited susceptibility to stress from the PSO while reducing the likelihood of creating a ceiling effect by over-stressing the CO. Additionally, we chose this mild stressor because it is unlike the stressor experienced by the parental generation (predator stress). As expected, we show that female offspring from predator stressed parents spent less time in the center of the open field than female offspring from control parents. In contrast to the

adolescent results, however, offspring from predator stressed parents spent more time in the open arms of the elevated plus maze compared to offspring from control parents.

Given that the parental generation of PSO had experienced stress prior to conception, there is potential that PSO mice, as an aspect of resilience, perform better in anxiety-inducing situations following a salient stressor (i.e., foot shock) than the CO mice due to the previous experiences in the parental generation. Another possible explanation could be that PSO mice are more prone to risk taking behaviour following a stressor than CO mice. Where CO mice are reserved in judgment about potential risks to their safety following a mild shock, PSO mice may opt to explore and learn more about the environment that they are in, opting for an understanding of their location as opposed to retreating behind the walls. Research using chronic restraint stress has found that 60 days of restraint to rat sires and dams produced decreased anxiety-like behaviour in the F1 offspring (He et al., 2016). Adult offspring demonstrated decreased anxiety-like behaviour on the EPM and OFT, with increased exploration relative to CO, leading researchers to speculate that the parental stress experience may lead to alleviated stress responses in offspring via reprogramming of the parental germline due to environmental influence (He et al., 2016). This resilience phenotype has not been extensively shown in chronic stress research, and further investigation is warranted.

Unexpectedly, given the results from the previous batteries, female offspring mice spent more time in the light box of the LDB than male offspring mice. Given that no differences were seen in the previous batteries and the only separation between the adolescent and adult batteries was a mild stressor, it may be that females are not as

readily impacted by a physical stressor as male mice. There was no interaction of group and sex, thus the parental generation's experience did not impact the LDB results. It is not entirely clear why this sex effect is present in the offspring generation and not the parental generation.

4.2.3 Preconception predator stress does not impact SCN driven locomotor activity circadian rhythms

As was previously seen in the parental generation, the parental stress experience did not impact locomotor activity. While there was a significant sex effect for the length of period, the mean difference was not meaningful between means of 24.0 and 24.1 hours (a difference of 6 minutes). Overall, locomotor activity metrics may not be the best means of measuring circadian rhythm disturbances in the offspring generation. As discussed in the parental locomotor activity section, very little research has explored the home cage behaviours and locomotor activity of laboratory rodents following chronic stress paradigms (Dalm et al., 2009; Grieco et al., 2021), with findings indicating changes in behaviours, but not in locomotor activity. However, research exploring the circadian rhythm of plasma corticosterone is a potential alternative for exploring the effects of predator stress on the circadian system. Amaral et al. (2010) have shown that an acute exposure of a mouse to a predator rat is sufficient to produce a peak in corticosterone secretion 5 minutes following the exposure. The rat exposure model requires further exploration to determine further resulting physiological changes (Amaral et al., 2010); however; the investigation of plasma corticosterone is a promising avenue for better understanding the influence of chronic stress on circadian function.

4.3 Implications and future directions

Chronic stress itself is not considered a mental disorder. However, stress and the persistent feeling of being overwhelmed are highly characteristic with many different psychological disorders. Anxiety disorders, in particular, are majorly represented by fear and abnormalities or inappropriate reactivity from the stress system, as described by the Diagnostic and Statistical Manual of Mental Disorders (5th ed.; DSM-V; APA, 2013). Indeed, public mental health for the general population has been significantly impacted by stress and anxiety related disorders due to the COVID-19 pandemic. The 2020 Stress in America ™ survey from the American Psychological Association (APA) found that 78% of American adults deem the COVID-19 pandemic a significant source of stress in their lives and 67% have reported increased stress over the course of the ongoing pandemic. In Canada, nearly 38% of adults have reported feelings of isolation and loneliness as a result of the pandemic (Statistics Canada, 2021). Meta-analysis research published in mid-2020 has demonstrated that anxiety and depression in the general population have prevalence of 29.6% and 33.7%, respectively (Salari et al., 2020). Further meta-analysis from the end of 2020 has reported an increased in anxiety disorder incidence, growing from 6.3% prior to the COVID-19 global outbreak to 50.9% following the onset of the pandemic (Xiong et al., 2020). In 2010, the prevalence of anxiety disorders was approximately 4.5% (Vos et al., 2012). Furthermore, the impact of stress is not confined to the impacted generation. Yehuda et al. (1998) demonstrated that offspring of Holocaust survivors who did not have significantly higher instances of traumatic events relative to demographically similar counterparts, remarkedly, had a

significantly greater prevalence of lifetime PTSD. Indeed, structural racism and cumulative trauma have also been postulated to be sources of generational affective disorders for non-Hispanic White Americans (Hankerson et al., 2022). These findings then lend to the conclusion that the impact of chronic stress is dire.

Healthcare systems are already overtaxed; therefore, it is crucial for researchers to understand proactive and preventative approaches relating to the consequences of chronic stress and the propagational effects in generations to come. Resources and successful clinical interventions do exist for different mental health concerns, but not without impaired accessibility. It is therefore a challenge and a source of stress itself to get the help needed for those who are impacted. Further research in this area of study should investigate different models of chronic stress in relation to the others to better understand the complexity of the outcomes of chronic stress. While there may not be a ”one size fits all” paradigm that is best used for investigating chronic stress, understanding what aspect of a multifaceted phenomenon such as stress would allow for a more holistic picture. Furthermore, investigating risk assessment behaviour may provide further insight about ALB following chronic stress. While outside the scope of this research project, risk assessment on behavioural tests would allow researchers to understand additional aspects of stress responses not seen in normal behavioural metrics. For generational studies, researchers should look further into the outcomes of resilience. While generational stress may result in the propagation of stress-induced behaviours, the role of resilience during development and adulthood may allow us to better understand inherited protective characteristics when faced with adverse situations.

For the present study, future direction for the current research method may benefit from incorporating measures of depressive-like behaviour. Previous research in our lab has found that predator stress may elicit depressive-like behaviours in mice and their offspring, as seen in the forced swim test and sucrose preference tests (Bhattacharya, 2021). Therefore, in addition to measures of ALB, depressive-like behaviour testing may elucidate additional behavioural changes that result as a consequence of generational predator stress.

4.4 Limitations

In the parental generation, we found significant freezing behaviour, elevated ALB, but no changes to central clock activity. In the subsequent offspring generation, we found a mix of resilience and ALB during adolescence and later in adulthood following a mild stressor, but no changes to locomotor activity. While these behavioural changes in both generations are intriguing, it is only appropriate to address limitations encountered during this research process.

A primary concern throughout the study was the impact of isolation stress. All mice were single-housed to ensure continuity between animals in behavioural and circadian subgroups. Mice are social animals, and it is recommended to avoid single housing as it can greatly influence behaviour (Van Loo et al., 2003). This is an important limitation to highlight, as one of the purposes of the study was to explore the behavioural outcomes of chronic stress on multiple generations of mice. These results might therefore be skewed given the single housing home cage environment. Future research ought to

explore how group housed mice behave in the same paradigm to determine the impact of isolation stress in a chronic predator stress experiment.

While every effort was made to ensure inter-rater reliability and non-biased responses of the hand-scored RET videos, it was not possible to score the RET videos blindly. The scorers were able to see when the rat was present in the box and when it was not, therefore scorers were aware of which condition the experimental mouse belonged to. To ensure consistent scoring practices were adhered to, scorers were coached by the lead researcher for what constituted freezing behaviour. Scorers responses were double-checked by the lead researcher to point out any discrepancies or missed behaviours. Additionally, when additional scorers were not available, most of the videos were scored by the same person. While this allowed for consistency in scoring, there may also be consistent discrepancies that would not be obvious during analysis (i.e., deviation from the mean).

Interruptions with equipment and space were a large limitation to the locomotor activity data collection and analysis in this study. The wireless sensors within the food hoppers of the mouse cages encountered some interference during a number of the monitoring periods, including disconnection from the data collection wireless gateway, and mice chewing on the casing and turning the sensors around in the hopper so that the sensor no longer faced into the living area of the cage. Additionally, only half the normal number of mice could be monitored in the constant dark room as the space was needed by other ongoing research in the facility. Furthermore, locomotor activity was measured for only one offspring cohort as the sensors were in use for the other. These limitations mean

that the entire scope of the locomotor activity data results may not be complete due to a lack of numbers and, therefore, statistical power.

Given the nature of the current study was chronic stress, it was difficult to measure every potential contravening variable should the measurement induce further stress on the mice. One such measure would be the estrous cycle of the female mice. In the results, we found a few sex effects within the freezing and ALB. Unfortunately, we cannot report on the stage of estrous for these female mice as it was determined early on in the project that it would implement another stressor that would be unique to the female mice. Vaginal cytology is an uncomfortable procedure for female mice, where a sterile swap is inserted into the vaginal canal and a cell cluster is collected. The cells can later be visually inspected to determine what stage of estrous the mouse is in at the time of collection. This limitation prevents the association of ALB and estrous phase. However, future research exploring chronic predator stress and female mouse reproduction may find interesting outcomes.

Breeding pairs in the F0 were group-matched, meaning control males were paired with control females and stressed males were paired with stressed females. This breeding strategy is useful for determining the propagation of generational stress when both parents are exposed. However, it is limited in that tracing what the impact of each parent is remains unknown. Parental sex differences would be better elucidated should a combination of four breeding pairs be used, with a mixed of control and stressed males and females used to determine if a single parental chronic stress exposure is sufficient to produce generational effects in offspring.

4.5 Conclusion

The findings from the present study provide meaningful insight about the generational effects of chronic stress. There may be more than simply negative consequences of chronic stress. Indeed, our findings suggest that preconception stressful experiences can have lasting impacts that result in behaviours likely to support offspring in stressful or novel situations that may be dangerous. It is with this in mind, that we hope future research will build on the present findings so that we may better understand the consequences of stress across generations. Furthermore, by understanding the mechanisms of generational stress, we may be able to assess how future generations will fare following parental chronic stress. In the future, this research may contribute to improved understanding of the etiology of anxiety and stress-related pathologies, which could lead to more effective treatment strategies.

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Appendix A

Table 1

Descriptive Statistics and Repeated Measures ANOVA Results for F0 Freezing Frequency with Greenhouse-Geisser Sphericity Corrections

Independent Variable	SS	df	MS	F	p	η^2
RET Day	22555	3.57	6314.7	50.901	<.001	.132
RET Day*Group	2526	3.57	707.2	5.701	<.001	.015
RET Day*Sex	708	3.57	198.3	1.598	.181	.004
RET Day*Group*Sex	210	3.57	58.8	.474	.733	.001
Residuals	39881	321.47	124.1			

Table 2

Descriptive Statistics and Repeated Measures ANOVA Between-Subjects Results for F0 Freezing Frequency

Independent Variable	n	M	SE	SS	df	MS	F	p	η^2
Group				32074	1	32074	120.463	<.001	.187
Control	336	8.80	.452						
Stressed	328	22.72	.802						
Sex				1163	1	1163	4.367	.039	.007
Male	335	14.4	.710						
Female	329	17.0	.710						
Group*Sex				264	1	264	.990	.322	.002
Control Male	168	8.11	.615						
Control Female	168	9.50	.661						
Stressed Male	167	20.78	1.079						
Stressed Female	161	24.73	1.172						
Residuals				23963	90	266			

Table 3

Estimated Marginal Means and Descriptive Statistics for F0 Freezing Frequency Across RET Day

RET Day	n	M	SE
Day 1	95	28.94	1.253
Day 2	95	15.01	1.166
Day 3	94	13.34	.885
Day 4	95	18.03	1.231
Day 5	95	13.18	.933
Day 6	95	9.82	.740
Day 7	95	12.18	.951

Table 4

Post Hoc Comparisons Using Tukey's HSD for Mean Differences of F0 Freezing Frequency Across RET Day

	Day 1	***Day 2***	***Day 3***	***Day 4***	***Day 5***	***Day 6***
Day 1						
Day 2	13.935***					
Day 3	15.602***	1.667				
Day 4	10.914***	-3.020	-4.687*			
Day 5	15.761***	1.827	.159	4.847*		
Day 6	19.120***	5.185***	3.518***	8.205***	3.358**	
Day 7	16.764***	2.829	1.162	5.849***	1.002	-2.356

*Note: *, **, & *** denote mean difference is significant at the .05, .005, & <.001 levels, respectively.*

Table 5

Post hoc Comparisons Using Tukey's HSD for Estimated Marginal Mean Differences of F0 Freezing Frequency Across RET Day Between Group

RET Day	Contrast	Mean Dif	SE	df	t	p	Cohen's d
Day 1	Stressed-Control	20.61	2.08	650	9.92	<.001	1.660
Day 2	Stressed-Control	13.49	2.08	650	6.50	<.001	1.089
Day 3	Stressed-Control	13.10	2.08	650	6.27	<.001	1.058
Day 4	Stressed-Control	17.96	2.08	650	8.65	<.001	1.448
Day 5	Stressed-Control	13.06	2.08	650	6.29	<.001	1.053
Day 6	Stressed-Control	7.24	2.08	650	3.48	<.001	.538
Day 7	Stressed-Control	11.92	2.08	650	5.74	<.001	.960

Table 6

Estimated Marginal Means and Descriptive Statistics for Simple Main Effect Analysis of F0 Freezing Frequency Between Groups Moderated by RET Day

RET Day	Group	n	M	SE
Day 1	Control	48	18.60	1.46
	Stressed	47	39.21	1.48
Day 2	Control	48	8.10	1.46
	Stressed	47	21.60	1.48
Day 3	Control	48	6.79	1.46
	Stressed	46	19.89	1.49
Day 4	Control	48	9.17	1.46
	Stressed	47	27.13	1.48
Day 5	Control	48	6.62	1.46
	Stressed	47	19.68	1.48
Day 6	Control	48	6.10	1.46
	Stressed	47	13.34	1.48
Day 7	Control	48	6.23	1.46
	Stressed	47	18.15	1.48

Table 7

Descriptive Statistics and Repeated Measures ANOVA Within-Subjects Results for F0 Freezing Cumulative Duration with Greenhouse-Geisser Sphericity Corrections

Independent Variable	SS	df	MS	F	p	η^2
RET Day	46867	3.80	12345	25.040	<.001	.093
RET Day*Group	12277	3.80	3234	6.559	<.001	.024
RET Day*Sex	3039	3.80	800	1.623	.171	.006
RET Day*Group*Sex	930	3.80	245	.497	.729	.002
Residuals	168455	341.68	493			

Table 8

Descriptive Statistics and Repeated Measures ANOVA Between-Subjects Results for F0 Freezing Cumulative Duration

Independent Variable	n	M	SE	SS	df	MS	F	p	η^2
Group				80806	1	80806	115.346	<.001	.161
Control	326	8.78	.648						
Stressed	328	30.78	1.525						
Sex				632	1	632	.902	.345	.001
Male	335	18.7	1.25						
Female	329	20.6	1.37						
Group*Sex				668	1	668	.954	.331	.001
Control Male	168	8.81	1.128						
Control Female	168	8.76	.642						
Stressed Male	167	28.70	1.947						
Stressed Female	161	32.94	2.353						
Residuals				63049	90	701			

Table 9

Estimated Marginal Means and Descriptive Statistics for F0 Freezing Cumulative Duration Across RET Day

RET Day	n	M	SE
Day 1	95	38.0	2.87
Day 2	95	16.0	1.52
Day 3	94	15.4	1.27
Day 4	95	26.3	2.30
Day 5	95	15.8	1.26
Day 6	95	13.3	2.54
Day 7	95	14.2	1.36

Table 10

Post Hoc Comparisons Using Tukey's HSD for Mean Differences of F0 Freezing Cumulative Duration Across RET Day

	Day 1	***Day 2***	***Day 3***	***Day 4***	***Day 5***	***Day 6***
Day 1						
Day 2	22.023***					
Day 3	22.667***	.644				
Day 4	11.717*	-10.305*	-10.950***			
Day 5	22.287***	.264	-.380	10.570***		
Day 6	24.751***	2.728	2.084	13.034**	2.464	
Day 7	23.800***	1.777	1.133	12.082***	1.513	-.952

*Note: *, **, & *** denote mean difference is significant at the .05, .005, & <.001 levels, respectively.*

Table 11

Post Hoc Comparisons Using Tukey's HSD for Estimated Marginal Mean Differences of F0 Freezing Cumulative Duration Across RET Day Between Group

RET Day	Contrast	Mean Dif	SE	df	t	p	Cohen's d
Day 1	Stressed-Control	39.3	3.93	650	10.02	<.001	1.679
Day 2	Stressed-Control	18.9	3.93	650	4.82	<.001	.808
Day 3	Stressed-Control	16.9	3.95	650	4.27	<.001	.720
Day 4	Stressed-Control	30.4	3.93	650	7.74	<.001	1.298
Day 5	Stressed-Control	17.7	3.93	650	4.35	<.001	.729
Day 6	Stressed-Control	15.1	3.93	650	3.85	<.001	.646
Day 7	Stressed-Control	16.1	3.93	650	4.09	<.001	.686

Table 12

Estimated Marginal Means and Descriptive Statistics for Simple Main Effect Analysis of F0 Freezing Cumulative Duration Between Groups Moderated by RET Day

RET Day	Group	n	M	SE
Day 1	Control	48	18.13	2.76
	Stressed	47	57.47	2.79
Day 2	Control	48	6.33	2.76
	Stressed	47	25.26	2.79
Day 3	Control	48	6.95	2.76
	Stressed	46	23.81	2.82
Day 4	Control	48	11.14	2.76
	Stressed	47	41.55	2.79
Day 5	Control	48	7.14	2.76
	Stressed	47	24.22	2.79
Day 6	Control	48	5.56	2.76
	Stressed	47	20.70	2.79
Day 7	Control	48	6.24	2.76
	Stressed	47	22.31	2.79

Table 13

Frequency Distribution for Breeding Birth Success Rate, Litter Size, and Sex of F0 Offspring

Cohort	Breeding Pairs		Litters	Mean Litter Size	Males	Females	Birth Success Rate
2	Control	7	4	4	17	12	57.14%
	Stressed	8	3	2	8	8	37.5%
3	Control	10	5	5	27	9	50%
	Stressed	10	6	6	23	28	60%

Table 14

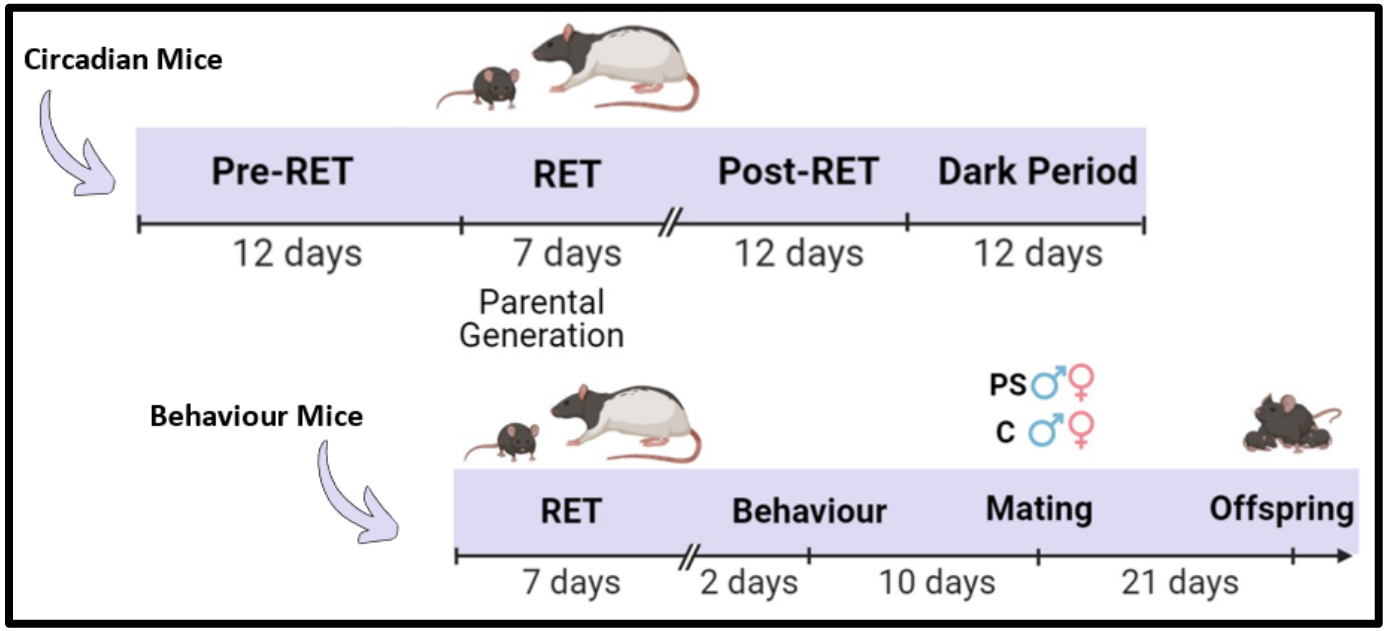
Frequency Distribution for Breeding Birth Success Rate, Litter Size, and Sex of F0 Offspring

PND	Group	N	M	SE
5	CO	65	2.89	.105
	PSO	67	2.96	.091
10	CO	65	5.82	.183
	PSO	67	6.15	.111
15	CO	61	7.31	.163
	PSO	67	7.51	.098

Appendix B

Figure 1

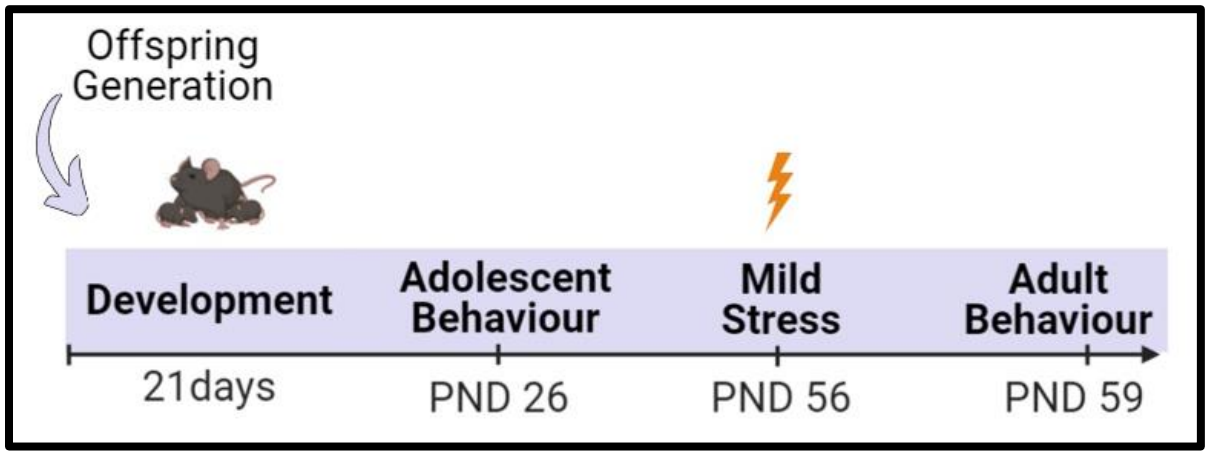
Timeline of Parental Generation For Circadian and Behavioural Groups



Note: Both groups in the F0 generation experienced the rat exposure test (RET) at the same time. Circadian mice began pre-RET monitoring before the onset of habituation (not shown) and RET. Circadian mice began post-RET monitoring the day after the last RET day and behaviour mice began their testing battery after two idle days post-RET. Abv: predator stress (PS); control (C).

Figure 2

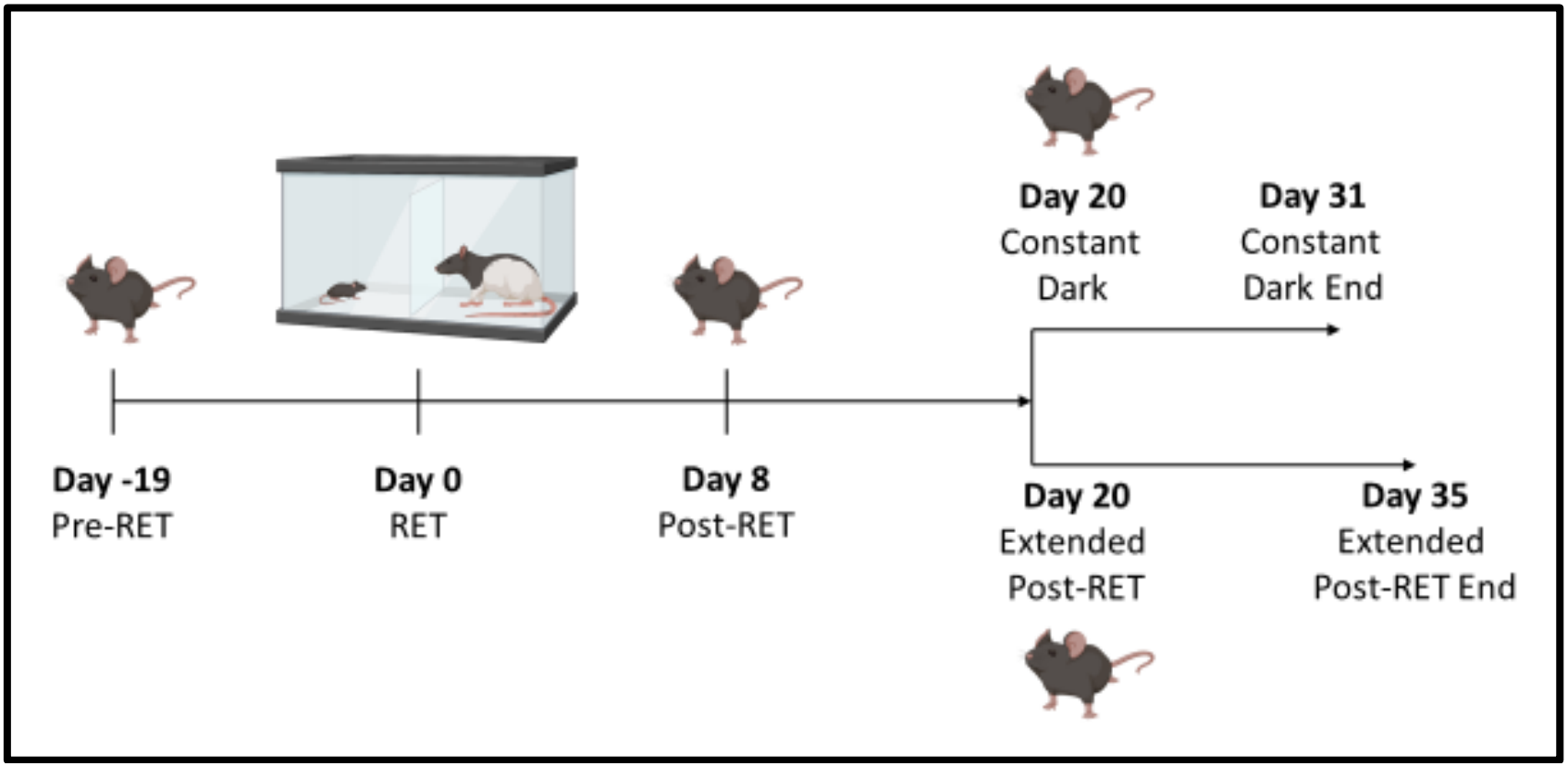
Timeline of Offspring Generation



Note: Both predator-stressed offspring (PSO) and control offspring (CO) experienced the same procedures throughout the experiment. The mild stress experienced was a .3mA foot shock administered once.

Figure 3

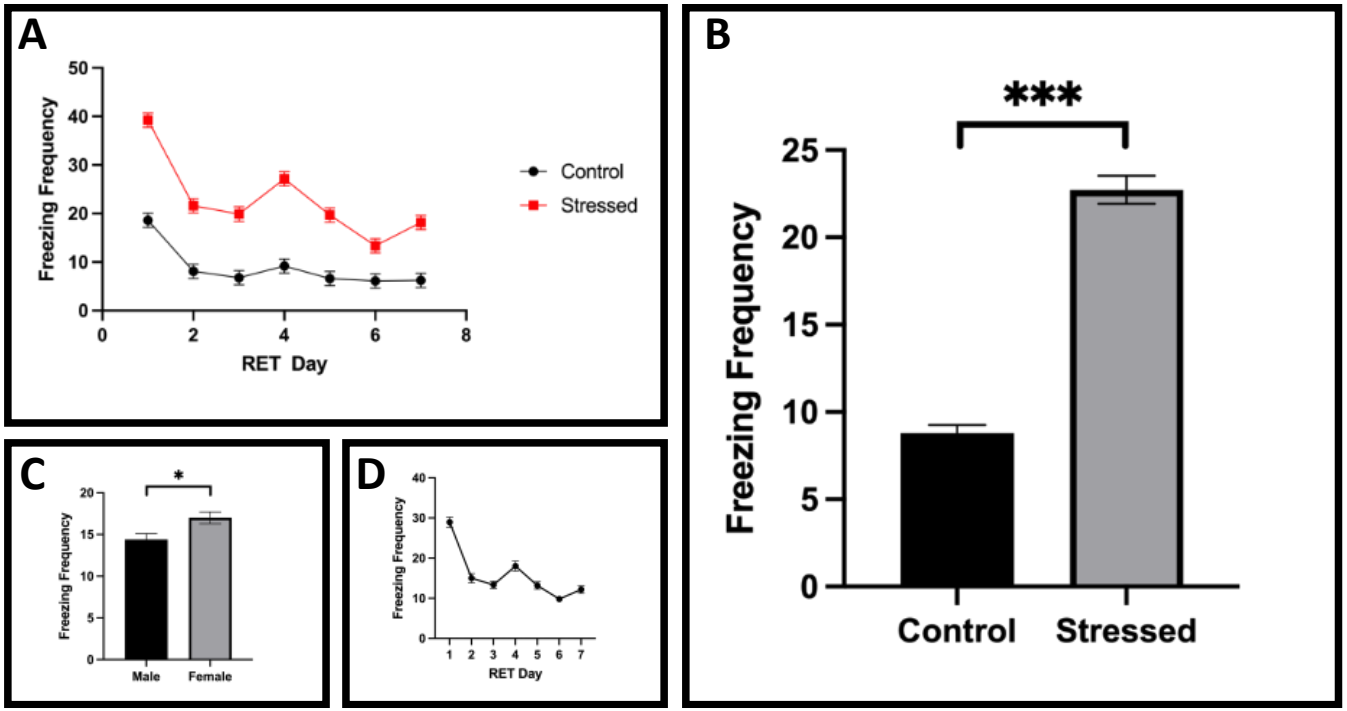
Timeline of Circadian Mice



Note: Abv: rat exposure test (RET).

Figure 4

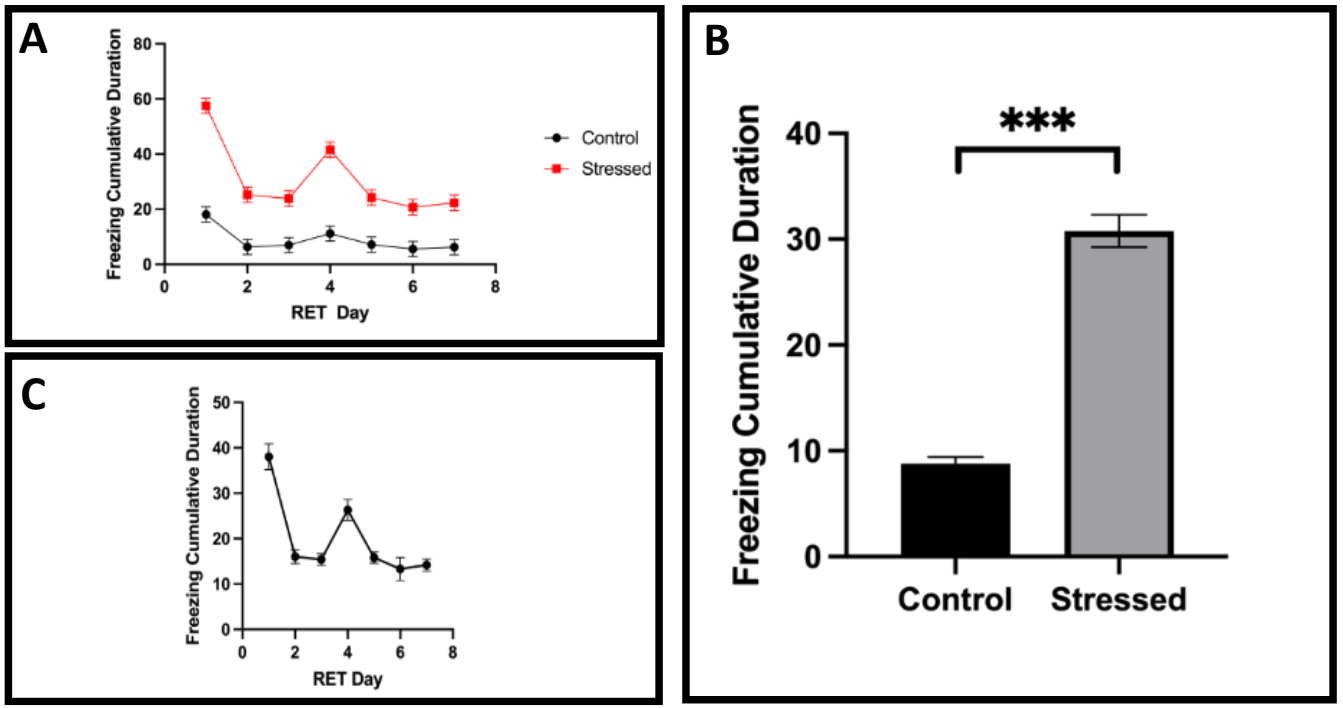
F0 Freezing Frequency for 7-Day Rat Exposure Test (RET)



Note: Changes in freezing frequency behaviour for the rat exposure test (RET) between control and predator stressed groups, male and female mice, and over the chronic RET time. *A*. Number of freezes between predator stressed RET or control condition across RET day. *B*. Number of freezes between groups. *C*. Number of freezes between sexes. *D*. Number of freezes across RET Day (*p<.05; ***p<.001).

Figure 5

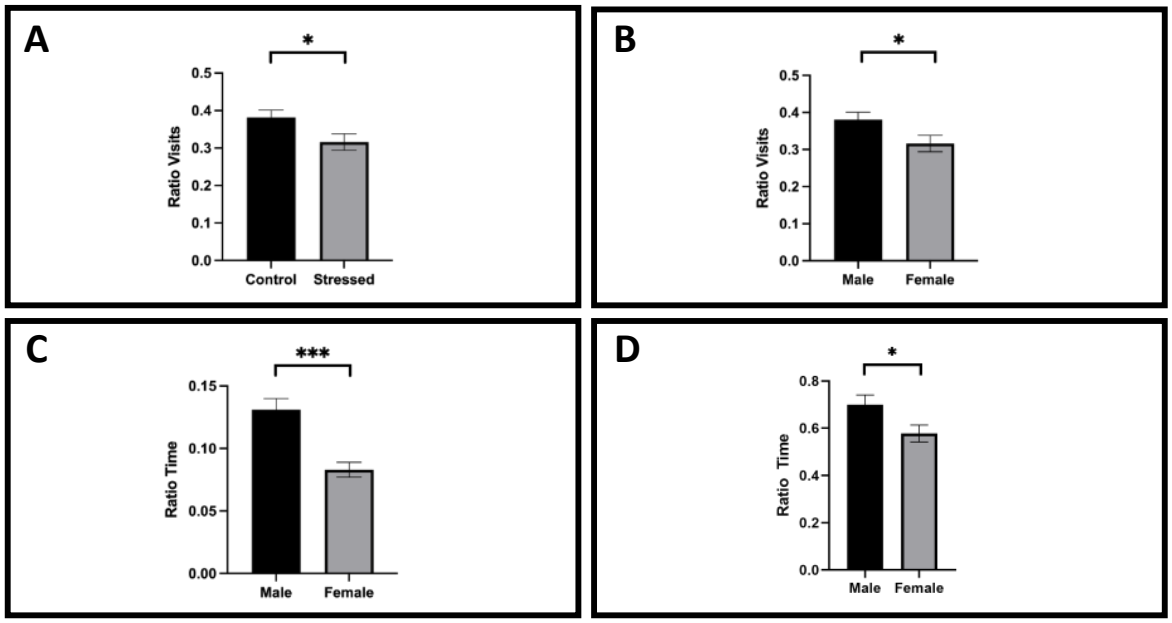
F0 Freezing Cumulative Duration for 7-Day Rat Exposure Test (RET)



Note: Changes in cumulative duration of freezing behaviour for the rat exposure test (RET) between control and predator stressed groups, male and female mice, and over the chronic RET time. *A*. Duration of freezes between predator stressed RET or control condition across RET day. *B*. Number of freezes between groups. *C*. Duration of freezes across RET Day. (***p<.001).

Figure 6

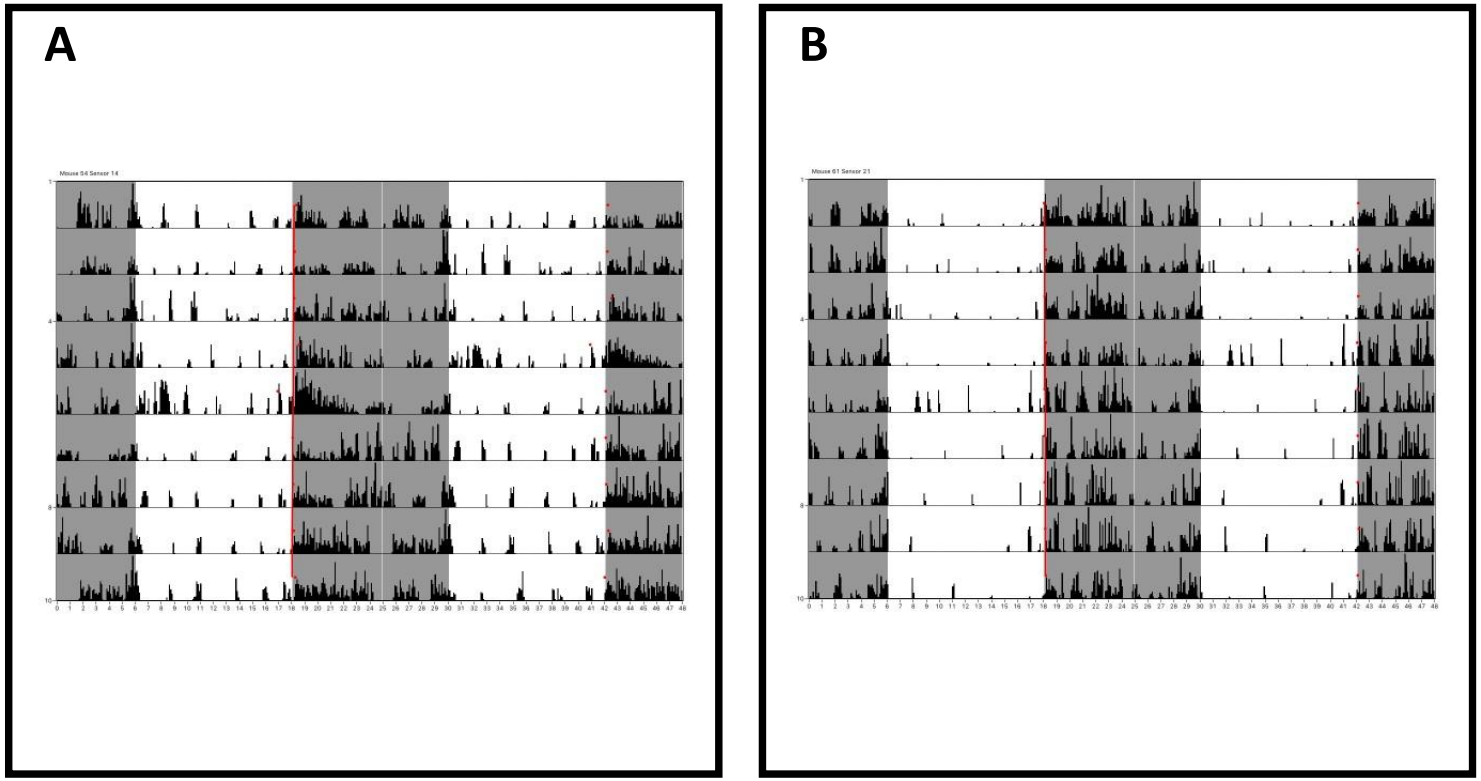
F0 Behaviour



Note: Changes in parameters of the elevated plus maze (EPM) and social interaction test (SIT) between control-predator stressed groups and male-female mice. *A*. Ratio visits of entries to the EPM open arms between groups. *B*. Ratio visits of entries to the EPM open arms between sexes. *C*. Ratio time spent in the EPM open arms between sexes (sec). *D*. Ratio visits of entries to the SIT interaction area between sexes. (*p<.05; ***p<.001).

Figure 7

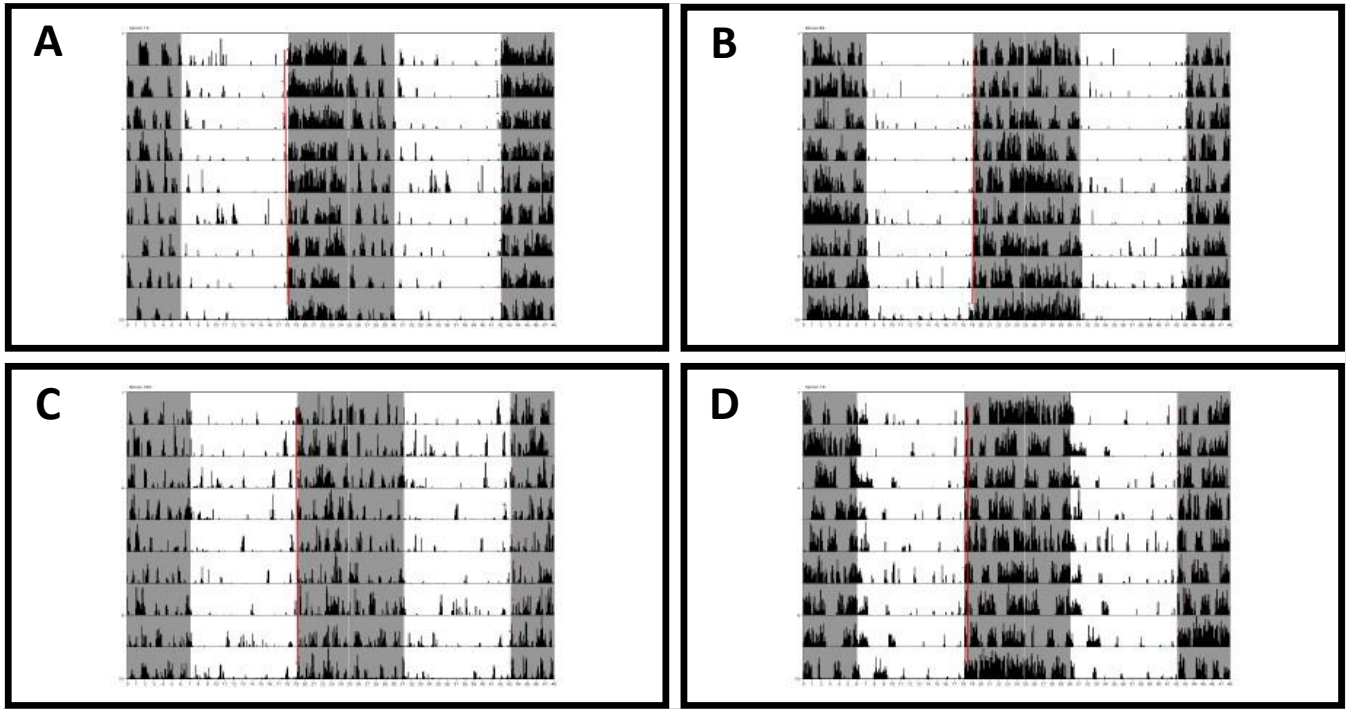
F0 Actograms of Pre-Rat Exposure Test (RET) Activity



Note: Activity patterns between (A) male and (B) female mice 12 days prior to habituation and rat exposure test (RET). Actograms are double-plotted with red regression lines denoting onset of activity at the hour (x-axis) across days (y-axis). Light schedules are indicated, with lights-off represented by shaded ranges.

Figure 8

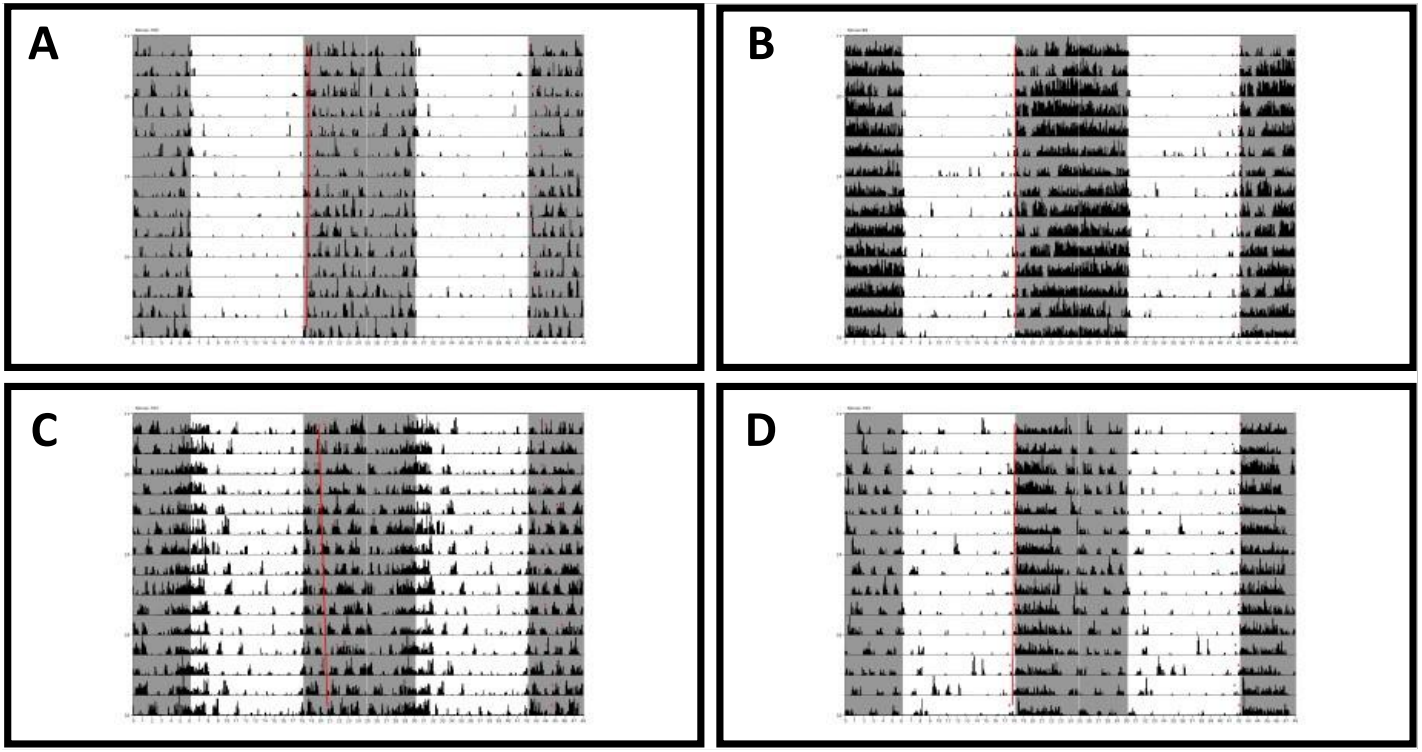
F0 Actograms of Post-Rat Exposure Test (RET) Activity



Note: Activity patterns of (A) control male, (B) control female, (C) stressed male, and (D) stressed female mice for 12 days following the rat exposure test (RET). Actograms are double-plotted with red regression lines denoting onset of activity at the hour (x-axis) across days (y-axis). Light schedules are indicated, with lights-off represented by shaded ranges.

Figure 9

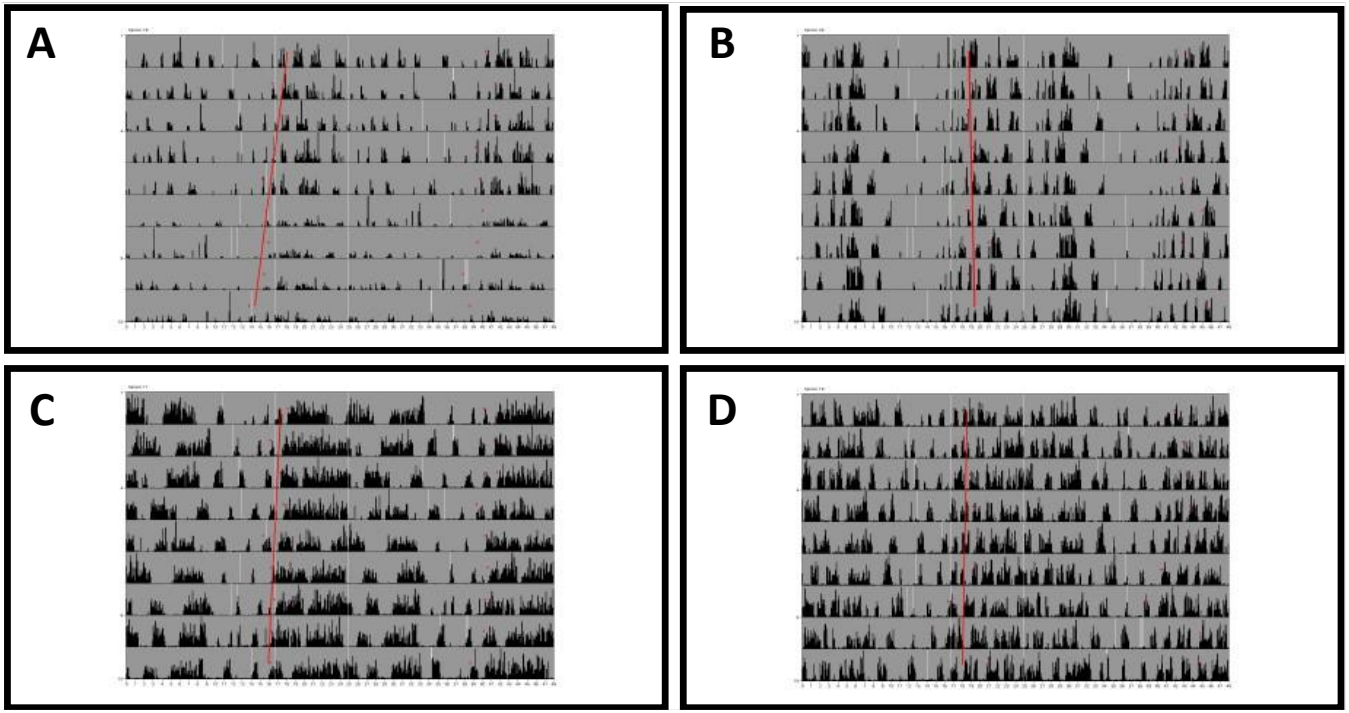
F0 Actograms of Extended Post-Rat Exposure Test (RET) Activity



Note: Activity patterns of (A) control male, (B) control female, (C) stressed male, and (D) stressed female mice for 16 days following the post rat exposure test (RET) activity monitoring. Actograms are double-plotted with red regression lines denoting onset of activity at the hour (x-axis) across days (y-axis). Light schedules are indicated, with lights-off represented by shaded ranges.

Figure 10

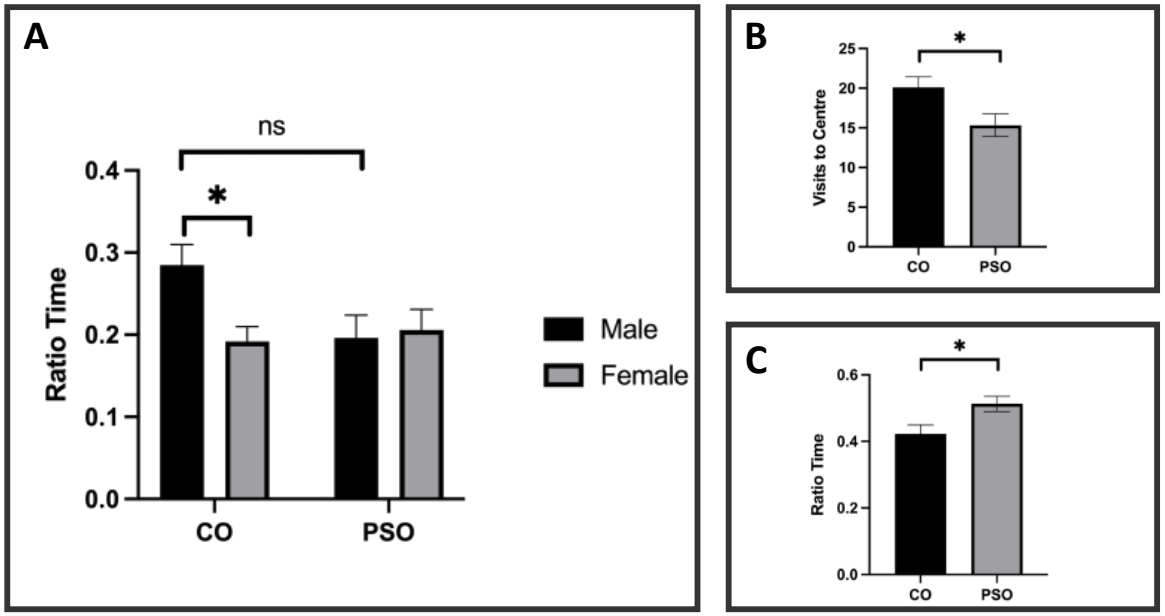
F0 Actograms of Constant Dark Post-Rat exposure Test (RET) Activity



Note: Activity patterns of (A) control male, (B) control female, (C) stressed male, and (D) stressed female mice for 12 days following the post rat exposure test (RET) activity monitoring. Actograms are double-plotted with red regression lines denoting onset of activity at the hour (x-axis) across days (y-axis). Light schedules are indicated, with lights-off represented by shaded ranges.

Figure 11

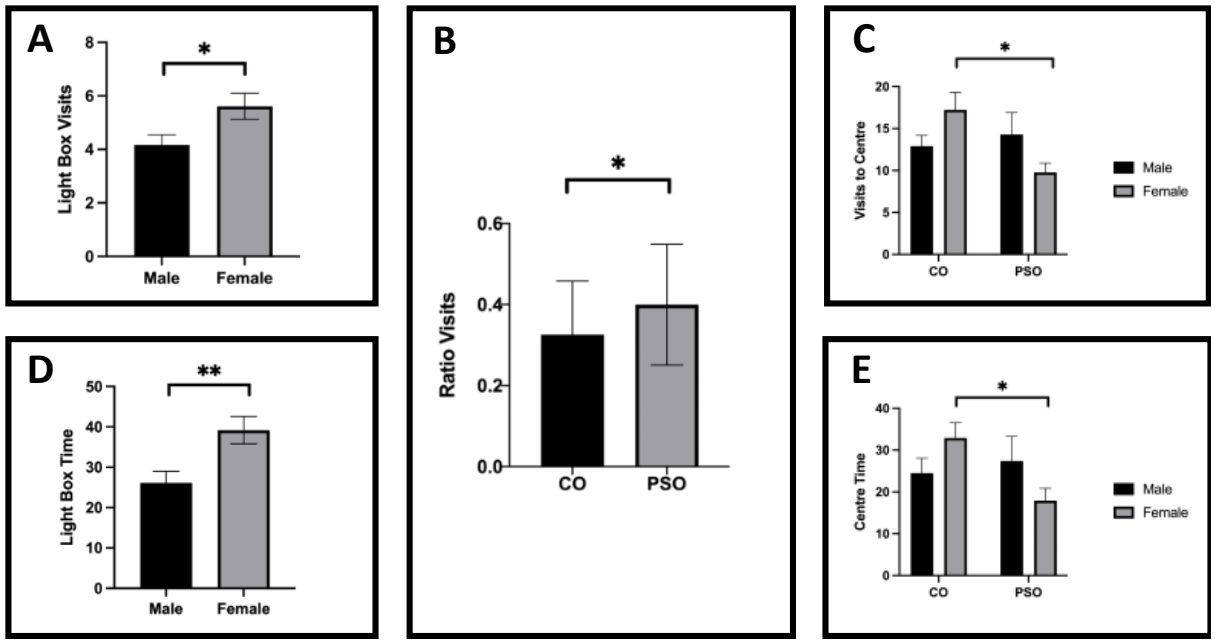
F1 Adolescent Behaviour



Note: Changes in parameters of the elevated plus maze (EPM), open field test (OFT) and social interaction test (SIT) between CO-PSO groups and male-female mice. *A*. Ratio time spent in the EPM open arms between groups and sexes (sec). *B*. Visits to OFT centre between groups. *C*. Ratio time spent in the SIT interaction area (sec). (*p<.05).

Figure 12

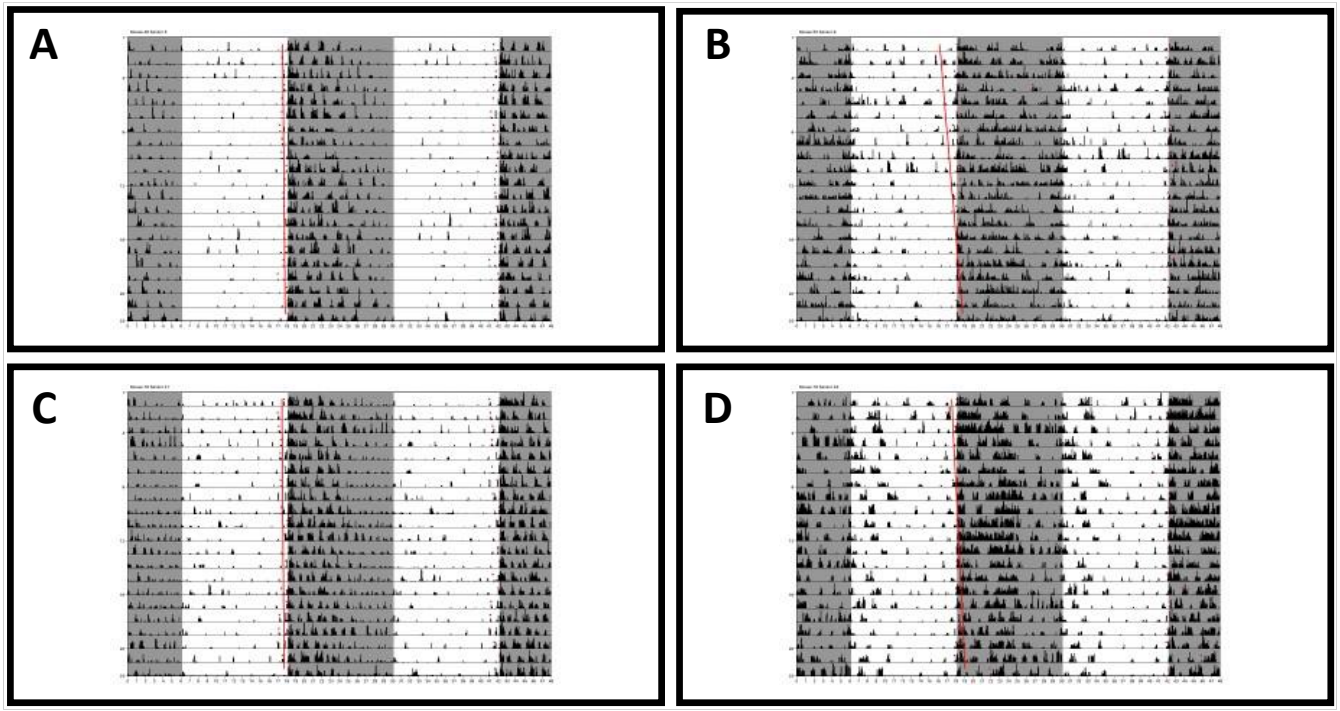
F1 Adult Behaviour



Note: Changes in parameters of the elevated plus maze (EPM), open field test (OFT) and light-dark box (LDB) between CO-PSO groups and male-female mice. *A*. Frequency of visits to LDB light box between sexes. *B*. Ratio visits to EPM open arms between offspring groups. *C*. Frequency of visits to OFT centre between groups and sexes. *D*. Duration spent in LDB light box between sexes. *E*. Time spent in OFT centre between groups and sexes. (*p<.05; **p<.01).

Figure 13

F1 Actograms of Post-Rat Exposure Test (RET) Activity



Note: Activity patterns of (A) CO male, (B) CO female, (C) PSO male, and (D) PSO female mice for 22 days following the adult behaviour battery. Actograms are double-plotted with red regression lines denoting onset of activity at the hour (x-axis) across days (y-axis). Light schedules are indicated, with lights-off represented by shaded ranges. Abv: control offspring (CO); predator stressed offspring (PSO).