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Title: A History of Chronic Repeated Predation Stress Reduces Aversion to Ethanol Adulteration in Male, but not Female, C57Bl/6J Mice

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Abstract

Background: Trauma related psychiatric disorders, such as posttraumatic stress disorder (PTSD), and alcohol use disorder (AUD) are highly comorbid illnesses that separately present an opposing, sex-specific pattern, with increased prevalence of PTSD in females and increased prevalence of AUD diagnoses in males. Likewise, PTSD is a risk factor in the development of AUD, with conflicting data on the impact of sex in the comorbid development of both disorders. Because the likelihood of experiencing more than one traumatic event is high, we aim to utilize chronic exposure to adolescent and early adult predator stress to query the extent to which sex interacts with chronic stress to influence alcohol consumption, or cessation of consumption.

Methods: Male (n=16) and female (n=15) C57BL/6J mice underwent chronic repeated predatory stress (CRPS) or daily handling for two weeks during adolescence (P35-P49) and two weeks during adulthood (P65-P79). All mice were subject to open field testing and marble burying analysis as metrics of anxiety-like behavior. Mice subsequently underwent a two-bottle choice intermittent ethanol access (IEA) phase (P90-131) with the options of 20% ethanol or water. After establishing drinking behavior, increasing concentrations of quinine were added to the ethanol to assess ethanol seeking behavior in the presence of an aversive stimuli, as a metric of compulsive-like drinking.

Results: CRPS increased baseline corticosterone and anxiety-like behaviors in the open field in both male and female mice as compared to control mice that had not been exposed to CRPS. Consistent with previous reports, we observed a sex difference in alcohol consumption such that females consumed more ethanol per gram of body mass

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than males. In addition, CRPS increased both alcohol intake by weight and preference, suggesting compulsive-like drinking behavior in male mice during quinine adulteration.

Conclusion: Collectively, we demonstrate that CRPS during late adolescence and early adulthood can induce anxiety-like behavior in both sexes but selectively influences ethanol intake in males. Male mice with a history of CRPS continue to engage in ethanol seeking behaviors despite being paired with an aversive gustatory stimulus, suggesting dependence-like drinking behavior. Our results suggest that stress may play a role in the development of anxiety-like behaviors and also drive a sex-specific alteration in drinking behavior.

Keywords: alcohol, adolescence, stress, anxiety, sex difference, predatory stress

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1. Introduction

Traumatic events are highly prevalent, with upwards of 70% of adults worldwide reporting a history of exposure to at least one type of trauma (Benjet et al., 2016). Exposure to trauma increases risk for a number of psychiatric disorders across the internalizing spectrum, including posttraumatic stress disorder (PTSD), the signature trauma-related disorder, but also other disorders such as major depressive disorder and anxiety disorders (Brown et al., 2014). A wealth of data underscore the cumulative effect of trauma, such that higher trauma load across the lifespan is associated with increased risk of psychopathology and severity of symptoms/impairment (Karam et al., 2013). Traumatic life events are also associated with increased risk for aberrant drinking and resulting alcohol use disorder (AUD) (Erbes et al., 2007; Hodge et al., 2004, 2006; Jacobson et al., 2008; Kilpatrick et al, 2003; Smith et al., 2008; Wright et al., 2002), particularly among those with PTSD (Head et al., 2016; Kessler et al., 1995; Petrakis et al., 2011; Sampson et al., 2015). Given the public health burden of trauma and resulting psychopathology, as well as the clinical importance of comorbid PTSD-AUD (e.g., worse treatment prognosis, increased risk for suicide) (Blanco et al., 2013; Isper et al., 2015; Read et al., 2004; Rojas et al., 2014; Shorter et al., 2015) there exists a need for preclinical models of these phenotypes as a critical piece to better understand disease etiology.

The link between mood disorders and ethanol consumption is well studied, with the understanding that prior stress may exacerbate excessive ethanol consumption (Edwards et al., 2013; Gilpin and Weiner, 2017; Koob and Moal, 2001). This connection may be strengthened by the antidepressant effects of ethanol, with its consumption

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acting as a mechanism of self-medication to mitigate the unwanted psychological effects of stress (Putman et al., 2016, Wolfe et al., 2018, 2016). Extensive research has been done to uncover the impact of early life and adult stress on later development of alcohol use disorder (Evren et al., 2011; Keyes et al., 2011; Khoury et al., 2010; Lopez et al., 2016; Pietrzak et al., 2012), but few studies look at the link between cumulative stress during adolescence and early adulthood and the development of alcohol use disorder in adulthood. Additionally, previous studies that consider development mainly focus on social stressors in weanlings through adulthood (Caruso et al., 2018; Roeckner et al., 2017; Skelly et al., 2015; Varlinskaya et al., 2017). Because extensive neuromodulatory events that shape the adult brain occur throughout adolescence, and the prevalence of traumatic stress in the adolescent age range (Berton and Stabb, 1996; Pelcovitz et al., 1994), examination of how this critical period of development shapes adult behaviors long after the stressor is removed is a crucial void to fill.

Another understudied area in the literature is in the realm of sex differences. Separately, in regard to mood disorders and alcohol use disorder, males and females exhibit differences in prevalence, with males exhibiting a nearly two fold increased rate of alcohol disorders compared to females (Grant et al. 2015; Kilpatrick et al., 2003) and females with higher incidences of mood disorders, namely PTSD, such that the prevalence is twice that of males (Grant et al., 2015; Naninck et al., 2011; Nolen-Hoeksema and Girgus, 1994; Piccinelli and Wilkinson, 2000; Steiner et al., 2003; Wade et al., 2002). However, studies of comorbidity between PTSD and AUD are inconclusive with regard to sex differences (for review see Gilpin and Weiner, 2017). Historically, neurobiological research and definitions have taken place in males, yet

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emerging data has shown fundamental differences in the sexes in regards to social behavior (Kopec et al., 2018), synaptic plasticity (Hyer et al., 2018), and gene expression (Bekhbat et al., 2018; Rowson et al., 2019). The evidence showing differential neural activity between the sexes makes the research of sex differences even more important to obtain a complete view of the alterations various environmental events may have on the adult onset of disease.

The prevalence of alcohol use disorder and anxiety disorders are highly comorbid, yet the exact mechanism remains unclear on how a history of stress may influence the risk of developing alcohol use disorder later in life and whether biological sex influences rates of development of subsequent disorders (Gilpin and Weiner, 2017). Moreover, the sequence of events is unclear, such that it is not definitively known if stress precipitates alcohol use disorder, or alcohol misuse generates an increase in stress that differs between the sexes, a potential unexplored confound that may play an important role in the assessment of those at risk of the development of these disorders (Boschloo et al., 2012; Buckner and Schmidt, 2008; Wolitzky-Taylor et al., 2012). As a first step, we aim to determine if alcohol consumption or the cessation of alcohol consumption is influenced by a history of stress in a sex dependent manner.

2. Methods

2.1 Animals

Juvenile C57BL/6J mice (n = 7-8/group) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and arrived at our facilities on postnatal day 22 (**Figure 1A**). Temperature and humidity was maintained at 21°C (± 1°C) and 47%-66%

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respectively. Mice were pair housed in ventilated rack cages with a sex-matched cage mate in an AAALAC-approved facility. All mice were kept on a 12:12 light:dark cycle (lights on at 0600) with water and Teklad LM-485 7012 standard rodent chow (Envigo, Madison, WI, USA) provided *ad libitum*. On postnatal day 35, mice were randomly assigned into one of two groups (chronic repeated predator stress [CRPS] vs. control [non-stress], **Figure 1B**). Eight male and eight female mice remained pair housed and assigned to the control group. The remaining eight males and seven females were individually housed and assigned to the predator stress group. Enrichment in the form of a single nestlet square was withheld in the CRPS group for the duration of stress and behavior and was reintegrated into their cages at PND 87. CRPS mice remained individually housed for the remainder of the study. All animal protocols were approved by Virginia Commonwealth University's Animal Care and Use Committee. All studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2 Chronic Repeated Predatory Stress (CRPS)

As previously described (Barnum et al., 2012; Burgado et al., 2014), predatory stress was completed daily for a total of fifteen days in adolescence (post-natal day [PND] 35-49) and fifteen days in adulthood (PND 65-79) (**Figure 1A**). Predatory stress was comprised of a thirty-minute exposure of the mice to a Long Evans rat. All Long Evans rats were retired male breeders, restricted to 15g rodent chow per day, and had reduced cage changes from twice to once a week throughout the duration of the stress to increase aggression. During their light cycle, mice were placed in a dwarf hamster ball measuring five inches in diameter (Lee's Aquarium & Pet Products, San Marcos,

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CA, USA, Cat. #20198 and #20193) with a strip of crushed rodent chow on Fisher brand tape securing the lid and effectively luring the Long Evans rat to the ball which contained the mouse (Burgado et al., 2014).

2.3 Fecal corticosterone assessment

Fecal boli were collected at six separate time points throughout the span of adolescent and adult predator stress (**Figure 1A**) to assess alterations in baseline corticosterone between control mice and mice undergoing CRPS. Collections were obtained from the following time points: pre stress (PND 35, before the beginning of adolescent predatory stress), acute adolescent (PND 37, after the three exposures to predatory stress), chronic adolescent (PND 49, after the final day of adolescent predatory stress), pre adult (PND 65, before the first exposure to adult predatory stress, at the end of a 15 day abstinence from predatory stress experienced during adolescence), acute adult (PND 67, after the third exposure to adult predatory stress) and chronic adult (PND 79, after the final day of adult predatory stress). Mice were briefly placed in an empty mouse cage that had been cleaned with 70% ethanol. Mice remained in the cage for approximately two minutes per collection. Two fecal boli per mouse were collected using a clean, irradiated wooden toothpick, placed in a clean 2.0mL micro centrifuge tube. Freshly collected fecal boli were stored on dry ice for transport and moved to a -80°C freezer for long term storage. Fecal samples were processed according to methods described in (Bardi et al., 2011). Samples were thawed for one hour at room temperature preceding corticosterone assessment. Thawed fecal samples were weighed and collected in glass test tubes with 500µL of 100% methanol per 45mg fecal coli. Methanol volume was scaled proportionally to the

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weight of fecal matter collected. Glass tubes were covered in Parafilm, vortexed for approximately 30s to homogenize the sample, and centrifuged at 2500xg for 10 minutes at 23°C. The supernatant was removed and diluted 1:20 in assay buffer included in the Enzo Corticosterone ELISA kit (Cat. No. ADI-901-097). Assay was completed in duplicate according to manufacturer's instructions. Plate was read at both 405nm and 535nm using an automated plate reader to determine concentration of metabolized corticosterone at each time point.

2.4 Estrus Assessment

On postnatal day 79, estrus was measured in all female mice using a visual assessment based on the characterization found in Byers et al., 2012. Estrus tracking continued daily throughout behavioral assessments (PND 82-87). Males were handled in a similar fashion to mitigate the confound of differential handling between the sexes.

2.5 Open Field Test

The open field test was used to evaluate both locomotor activity and anxiety-like behavior (Carola et al., 2002; Choleris et al., 2001; Prut and Belzung, 2003). Testing was conducted between PND 82-85 using methods similar to those previously described (Burgado et al., 2014). Three to four hours into their light cycle, mice were placed in a 13.5" x 13.5" white bottom polyethylene square box with 16" high white walls and permitted to explore for ten minutes. Activity was recorded using an overhead camera, and metrics were assessed using EthoVision XT13 Software (Noldus Technologies, Leesburg, VA, USA). The resulting variable was the percentage of time spent in the center of the arena, with decreased time in the center suggestive of

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increased anxiety-like behaviors. Additionally, velocity, percent of total time spent moving, and total distance traveled was measured to assess locomotor activity.

2.6 Marble Burying

Marble burying has been demonstrated to be a signifier of an anxiety-like phenotype (Deacon, 2006). On PND 84-87, mice were placed in a standard, clean mouse cage containing five inches of fresh, lightly pressed Teklad 7097 ¼" corncob bedding (Envigo, Madison, Wisconsin, USA) and twenty marbles, arranged into four rows of five. Each mouse was placed into the cage for 30 minutes and tracked using EthoVision XT13 Software (Noldus Technologies, Leesburg, VA, USA). Screenshots were taken at the end of thirty minutes and marbles were counted by two observers blinded to stress group, with the key variable being the number of marbles that were buried at the end of the task. Marbles were considered buried if at least two-thirds of the marble was under bedding at the end of the task.

2.7 Intermittent Ethanol Access (IEA)

Eight days after the last stress exposure, mice were transferred from ventilated rack cages to static home cages in preparation for ethanol assessments. Mice were given three days (PND 87-89) to habituate to their new cage. Non-stressed control mice were individually housed, with enrichment, to ensure accuracy in assessment of drinking volumes. The mice without a history of stress were reintroduced to enrichment via nestlet squares. Two bottle choice intermittent ethanol exposure was executed as described (Hwa et al., 2011). Over the course of four weeks, mice were given a choice between 20% (v/v) ethanol made from 100% ethanol and tap water, or unaltered tap

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water in sipper tubes, constructed from 10mL serological pipettes and fitted with metal ball-bearing sipper tubes. All mice were given access to water or 20% ethanol three days a week, every Mon-Wed-Fri, *ad libitum*, on a twenty-four hour on/off cycle, one to two hours before their dark cycle (Hopf et al., 2010). Starting and ending volumes were noted and used to determine consumption during the twenty-four-hour time period. Sipper tube volumes, not weights, were used to assess fluid consumption and to remove the confound of fluid loss due to the removal of the tubes from the cage. Two empty control cages were used to assess fluid loss due to leaking and evaporation of both water and 20% ethanol. Starting and ending volumes of the tubes in the control cages were noted during each twenty-four hour time period, averaged, and subtracted from the consumption values in each mouse cage.

2.8 Quinine Exposure (QuiA)

To determine dependence-like drinking behavior, quinine, a bitter tastant, was incorporated into the 20% ethanol as previously described (Hopf et al., 2010). Quinine exposure is commonly used to determine dependence-like drinking behavior, as it produces a negative effect linked to ethanol consumption, a defining factor of the human definition of AUD (Hopf et al., 2010). Increasing concentrations of quinine were added into 20% ethanol, at the concentrations of 5mg/L, 10mg/L, 50mg/L, 100mg/L, 150mg/L, and 200mg/L over the course of twelve days. Mice were given twenty-four hours to consume either water or 20% ethanol with the specified concentration of quinine in the same two-bottle choice model described above. Methods to assess ethanol-quinine consumption and preference were measured identically to the initial IEA measurements.

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2.9 Statistical analysis

GraphPad Prism 8.0.2 for Windows (GraphPad Software, La Jolla, CA) was used for assessment of all subsequent analyses. An alpha value of 0.05 was used in all cases. Fecal corticosterone data were analyzed using a repeated measures three-way ANOVA with the factors of time, sex, and group (CRPS vs. control) across the six fecal collections. Behavioral data were analyzed using a two-way ANOVA with the factors of sex and group (CRPS vs. control). Significant interactions were further assessed using Tukey's post hoc analysis. Due to the significant sex difference in ethanol consumption reported throughout the literature for C57BL/6J mice (Cozzoli et al., 2014; Middaugh et al., 1999) IEA data were analyzed first using a three-way repeated measures ANOVA with the factors time, sex, and group (CRPS vs. control) to verify that females consumed more ethanol than males. Following analyses were separated between male and female and run using a two-way repeated measures ANOVA with the factors time and group (CRPS vs. control) to determine 20% ethanol intake by volume, ethanol preference, or total fluid volume consumed. In the incidence of interactions, post hoc analysis was completed using Tukey's post hoc analysis. A post hoc power analysis (F test, ANOVA: Repeated Measures within Factors) was conducted using G*Power 3.1 (Faul et al., 2007) to determine the achieved power in IEA consumption, IEA preference, QuiA consumption, and QuiA preference. Subsequently, an a priori power analysis was conducted using this data to determine the sample size required to obtain the recommended statistical power of 0.8.

3. Results

3.1 Fecal corticosterone

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Three-way ANOVA with the factors of time, sex, and group was used to determine differences between cumulative corticosterone concentrations in fecal boli between the sexes by stress condition (**Figure 2**). Corticosterone concentrations were significantly different between male and female mice ($F_{(1, 27)} = 17.97$, $p = 0.0002$) and exposure to CRPS marginally increased corticosterone concentrations ($F_{(1, 27)} = 4.022$, $p = 0.055$). In addition, time in the study influenced corticosterone concentrations with a general decrease over time ($F_{(2.569,69.36)} = 134.1$, $p < 0.0001$). Data show both factors of time and stress interacted ($F_{(5, 134)} = 7.707$, $p < 0.0001$) and time and sex interacted ($F_{(5,135)} = 2.626$, $p = 0.0267$). To tease these interactions apart, post hoc assessment using a repeated measures 2-way ANOVA was done with the factors of time and stress between each sex separately. Data demonstrated a significant difference between males with and without CRPS history in the chronic adolescent and acute adult time points ($p = 0.0421$ and $p = 0.0102$ respectively) such that those in the CRPS group had higher corticosterone concentrations at those times compared to the males in the control condition. Female corticosterone concentrations were also impacted by CRPS, which were significantly higher than the female non-stress mice at the chronic adult time point ($p = 0.0101$).

3.2 Open Field

Two-way ANOVA with the factors of sex and stress group of the open-field test confirmed that mice in the CRPS group spent less time in the center of the arena ($F_{(1,27)} = 8.839$; $p = 0.0061$, **Figure 3A**) and more time in the periphery ($F_{(1,27)} = 8.478$, $p = 0.0071$), suggesting increased anxiety-like behavior when compared to the non-stressed controls. There was no effect of sex on time spent in the center of the arena (p

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> 0.05). Mice with a history of stress displayed signs of hyperactivity, as these mice spent more time moving ($F_{(1,27)}$ = 18.44, p = 0.0002), covered a greater distance ($F_{(1,27)}$ = 15.22, p = 0.0006), and moved at a significantly higher speed ($F_{(1,27)}$ = 14.54, p = 0.0007) than non-stressed control mice (**Figure 3B-D**). Three of the seven female mice with a history of stress were in estrus during the time of the open field assessment. Pearson's correlation analysis showed no significant effect of estrus stage in open-field performance within this group (p > 0.05). No non-stressed control females were in estrus at the time of testing.

3.3 Marble Burying

Two-way ANOVA analysis with the factors of sex and stress group demonstrated a main effect of sex ($F_{(1,27)}$ = 5.68, p = 0.0245; **Supplementary Figure 1A**), but not stress (p > 0.05) in number of marbles buried such that females buried more marbles than males (mean ± SEM: Females = 13.33 ± .6449; Males = 11.00 ± 0.0701). Furthermore, we found no difference between duration of time spent in the center (p > 0.05; **Supplementary Figure 1B**) or periphery (p > 0.05) yet saw a significant main effect of stress in number of center visits ($F_{(1,27)}$ = 4.385, p = 0.0458; **Supplementary Figure 1C**), with non-stress control mice visiting the center more than mice with a history of stress. Similarly, there was a main effect of stress in time spent moving, as non-stress control mice spent more time moving than mice with a history of stress ($F_{(1,27)}$ = 6.969, p = 0.0136; **Supplementary Figure 1D**). No females were in estrus at the time of testing.

3.4 Intermittent Ethanol Access

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Consistent with the literature (Middaugh et al., 1999), female mice, regardless of stress background, consumed an increased volume of ethanol when controlled for weight; therefore, after initial analysis to verify females consume more ethanol than males, we present the data separately for male and female subjects in order to better illustrate the effects of stress within each sex (**Figure 4A-D**).

Three-way repeated measures ANOVAs with the factors of sex, stress, and time was used to measure ethanol intake and preference by weight and total fluid intake for all mice. As expected with the IEA model and consistent with the literature, the mice significantly increased their ethanol consumption ($F_{(5.711, 154.2)} = 21.51$, $p < 0.001$; **Figure 4A**) and preference ($F_{(4.691, 126.7)} = 13.77$, $p < 0.0001$; **Figure 4B**) over time (Hwa et al., 2011). Female mice consumed more alcohol (adjusted for body mass) than male mice ($F_{(1,27)} = 43.87$, $p < 0.0001$). A history of CRPS did not significantly increase either alcohol consumption ($p > 0.05$) or preference for the 20% alcohol solution ($p > 0.05$). In males, CRPS demonstrated a numeric but not statistically significant impact on 20% intake ($F_{(1,14)} = 4.232$, $p = 0.0588$) and 20% preference ($F_{(1,14)} = 3.672$, $p = 0.0760$).

The post hoc power analysis to compute achieved power showed a low to moderate effect size within the 4 groups analyzed (IEA consumption: N=31, groups = 4, $\lambda = 2.859$, $F=2.188$, $\alpha = 0.05$, power = 0.194; IEA preference: N=31, groups = 4, $\lambda = 5.333$, $F=2.329$, $\alpha = 0.05$, power = 0.381). In order to achieve the recommended power of 0.8, a total sample size of 132 and 72 mice would be required for IEA consumption and IEA preference respectively.

Although total fluid intake differed by sex ($F_{(1,27)} = 37.71$, $p < 0.0001$) and time ($F_{(7.497, 202.4)} = 19.41$, $p < 0.0001$), consistent with the sex difference in consumption

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reported above, exposure to CRPS did not alter total fluid intake ($p > 0.05$), suggesting differences in ethanol intake and preference are not due to differences in total fluid intake following stress exposure (data not shown).

3.5 Quinine

Exposure to CRPS delayed the impact of quinine adulteration (QuiA) on alcohol intake such that higher concentrations of quinine were necessary to reduce drinking behaviors (main effect of time: $F_{(4.106,110.9)} = 91.22$, $p < 0.0001$). Data showed a main effect of sex ($F_{(1,27)} = 11.62$, $p = 0.0021$) but not stress ($p > 0.05$) when analyzed using a three-way ANOVA with the factors time, sex, and stress (**Figure 4C**). When separated by sex and analyzed using a 2-way repeated measures ANOVA, as done in the IEA data, we saw a main effect of stress ($F_{(1,14)} = 6.448$, $p = 0.0236$) in the males. Males with a history of CRPS consumed more quinine adulterated ethanol than non-stressed males. Data showed no significant effect of stress in the females ($p > 0.05$). Quinine-adulterated ethanol preference data (**Figure 4D**) showed a main effect of sex ($F_{(1,27)} = 5.792$, $p = 0.0232$) and a sex by stress interaction ($F_{(1,27)} = 6.889$, $p = 0.0141$) and a time by sex by stress interaction ($F_{(6,162)} = 2.752$, $p = 0.0142$). Analysis using a 2-way repeated measures ANOVA separating the sexes showed that, in males, there was a main effect of stress ($F_{(1,14)} = 8.406$, $p = 0.0117$) and a significant interaction ($F_{(6,84)} = 2.438$, $p = 0.0320$). Post hoc analysis using a Tukey's test showed non-stressed males consume significantly less quinine laced ethanol when quinine levels reached 100mg/L ($p = 0.0133$) compared to baseline, whereas the males with a history of CRPS did not significantly decrease consumption until quinine levels reached 200mg/L ($p = 0.0049$). There was no significant difference in ethanol consumption or preference between

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females regardless of stress background ($p > 0.05$) or non-stressed control male and female mice ($p > 0.05$).

Assessment of achieved power was completed using a post hoc power analysis. Like the IEA data, output show a modest effect between the 4 groups (QuiA consumption: N=31, groups = 4, λ= 2.186, F=2.384, α = 0.05, power = 0.172; QuiA preference: N=31, groups = 4, λ= 6.651, F=2.434, α = 0.05, power = 0.492). A total sample size of 160 and 56 for QuiA consumption and QuiA preference would be needed respectively in order to achieve the recommended statistical power of 0.8.

4. Discussion

The data presented in this manuscript extend previous findings (Burgado et al., 2014) to demonstrate that predation stress beginning in adolescence alters anxiety-like behaviors in both male and female mice. Fecal corticosterone analysis shows a sustained significant increase in basal corticosterone with a history of CRPS when compared to non-stressed controls. Although we observed sex differences in the impact of CRPS on ethanol behaviors, CRPS altered anxiety-like behavior in both male and female mice. Importantly, the behavioral assessments reported here were conducted prior to ethanol consumption and do not reflect an impact of ethanol intoxication or withdrawal (Lee et al., 2015). The open field and marble burying tests are well established behavioral assessments for the measurement of anxiety-like phenotypes in rodent models (Diniz et al., 2018; Kedia and Chattarji, 2014; Njung'e and Handley, 1991). In the open field, our data extend previous findings regarding the impact of CRPS on anxiety-like behavior in male mice. Similar to the previous reports from both adolescent and adult mice, we demonstrate an increase in anxiety-like behavior in male

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mice following CRPS (Barnum et al., 2012; Burgado et al., 2014) and we extend this previous report to now demonstrate that repeated exposure to CRPS also increases anxiety-like behavior in female mice. Although not assessed in the current study, predation stressors have been reported to give rise to depressive-like behaviors after the cessation of stress (Burgado et al., 2014; Zoladz and Diamond, 2016), and these types of behaviors may be of interest in future studies.

One of the hallmarks of AUD is the continuance of drinking despite pervasive negative consequences (Goltseker et al., 2018; Hopf et al., 2010). One laboratory mechanism by which to model the presence of conflict-resistant alcohol consumption is to adulterate the ethanol solution with quinine or other bitter agents (Darevsky et al., 2019). Despite similar impacts on anxiety-like behavior, the effects of CRPS on ethanol consumption were disproportionately represented in male mice such that a history of CRPS increased ethanol-related consumption behaviors to near significant levels. Although control males, and females regardless of stress history, reduced QuiA consumption, similar to a previous report of no impact of sex on QuiA consumption (Sneddon et al., 2018), male mice that had a history of CRPS demonstrated conflict-resistant alcohol drinking. Most remarkably, a history of CRPS caused male mice to be resistant to QuiA such that they continued preferentially choosing to consume the 20% ethanol solution over water until 2x the quinine was added to the solution that was necessary to dissuade drinking in male controls. A similar effect has been demonstrated previously through the use of repeated cycles of alcohol exposure (Olney et al., 2018) but has not been reported following a chronic stress paradigm in mice. However, similar effects of exposure to a predator stimulus have been reported to increase compulsive-

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like drinking of QuiA in a subset of male rats, suggesting that the impact of predatory stress may generalize across species (Edwards et al., 2013). This disassociation in anxiety-like behavior and ethanol consumption behaviors may provide a model system in which to assess the neural underpinnings of chronic stress exposure on alcohol behaviors with high translational potential given the role of drinking despite consequences and impairment as a cardinal symptom of AUD. Although neural underpinnings are beyond the scope of the current manuscript, work in rats suggests that both the medial prefrontal cortex and amygdala (Edwards et al., 2013) may be brain regions for focus of future studies aimed at understanding mechanism of compulsive-like drinking. Furthermore, previous reports show that ethanol exposure alters neural composition in the hippocampus (Enman et al., 2014; Ewin et al., 2018; Wolfe et al., 2018).

The utilization of this predation model to induce stress not only produces robust, long lasting effects in mice, but also, due to the nature of the stressor, is less likely to give rise to sex-specific differences in affective-like behaviors, unlike other models of predation stress, such as predator odor (Adamec et al., 2008; Caruso et al., 2018). This particular model of predatory stress is a close simulation of the stressor that mice would experience in the wild; a genuine model of face to face interactions between predator and prey, and thus, has high validity. Because of the combination of sensory ques that signal the potential for danger, this stressor is more likely to produce equal results from males and females alike, more so than odor exposure alone. Furthermore, the CRPS model mimics the criterion for life threat or threat of personal integrity needed for a stressor to be deemed traumatic in humans under DSM-5 criteria for PTSD (American

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Psychiatric Association, 2013). Despite the results of the marble burying task, we observed a main effect of CRPS in the open field; showing both males and females with a history of CRPS exhibit an anxiety-like phenotype that is comparable between the sexes.

Although our sample sizes were not robust enough to allow for assessment of individual variation in anxiety-like behavior as a predictor of QuiA drinking behavior and achieved power may restrict some statistical conclusions in this study, a previous assessment in rats demonstrated that the behavior of avoidance could be used to predict manifestation of compulsive drinking (Edwards et al., 2013) and future studies could consider leveraging individual variation to elucidate underlying mechanisms of compulsive drinking behaviors. Assessment of transcript expression in the hippocampus and nucleus accumbens could be ideal areas of interest where these changes may occur (Khisti et al., 2006; Wolstenholme et al., 2011; Åberg et al., 2005). It has been shown in the literature that ethanol consumption alters gene expression in brain regions such as the nucleus accumbens and prefrontal cortex (Wolstenholme et al., 2011). Moreover, the interaction between stress and sex could prime these areas to increase the incidence of dependent-like drinking behaviors leading to the results we see in our study. This potential for an epigenetic alteration triggered by CRPS could be the defining factor of resilience in the development of the comorbidity of PTSD- and AUD-like phenotype in males but not female rodents.

In summary, these data report CRPS increases basal corticosterone levels in male and female mice. Moreover, these mice with a history of stress exhibit behaviors consistent to those with an anxiety-like phenotype. Male mice with a history of stress

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exhibited dependent-like drinking behaviors, which was not observed in females. Collectively, this stress- and sex- specific alteration in dependent- like drinking behavior may hint toward a sex- and stress- specific epigenetic alteration that are not apparent in behavioral assessments and reflects the importance of the assessment of both sexes and their inherent differences.

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6. Figures:

Figure 1: Experimental Design and Timeline.

A) Experimental timeline. Adolescent stress also was the first day of isolation for mice in the predator stress group. Non-stress mice remained pair housed until the end of behavior (PND 87), where they were isolate housed for the duration of the ethanol consumption phase. Fecal collections occurred at six separate time points, before the first adolescent stress exposure (PND 35), after the third adolescent stress exposure (PND 37), after the last adolescent stress exposure (PND 49), before the first adult stress exposure (PND 65), after the third adult stress exposure (PND 67), and after the final adult stress exposure (PND 79). **B)** 31 mice were used in this experiment, 16 males and 15 females. Each sex was separated equally into non stress and CRPS groups. Mice subject to stress were chosen at random at PND 35 before the beginning of stress. 7 females were chosen to undergo stress to allow pair housing of the non-stress female mice.

Figure 2: Chronic Stress Increases Basal Corticosterone Expression.

Corticosterone levels collected from six different time points spanning the duration of both adolescent and adult predator stress show that chronic repeated predatory stress produced a sustained increase in basal corticosterone extracted from fecal boli. Reported values depict mean ± SEM. #p = 0.06, *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 3: Predator stress increases anxiety like behaviors in the open field test.

A) Chronic repeated predator stress decreases time spent in the center regardless of sex when compared to controls. **B-D)** Likewise, a history of chronic repeated predator stress increases locomotor activity in the open field. Together, this data suggests

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increased anxiety-like behavior in mice who underwent chronic repeated predator stress. Data points collected between three and six days post the last day of predator stress (PND 82-85). Reported values depict mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4: Chronic repeated predator stress increases alcohol seeking behavior in male mice.

A) Baseline ethanol intake by weight shows a sex difference between ethanol consumption (p < 0.0001), with females consuming more ethanol by weight than males regardless of stress history. Within the sexes, there is a numeric increase in 20% ethanol intake by males with a history of CRPS when compared to male controls (p = 0.0588) There is no significant difference within the females in regards to 20% ethanol intake between stress histories. **B)** There was no baseline difference in ethanol preference in terms of sex or stress history. Within the sexes, males with a history of CRPS show a numeric but not significant increase in 20% ethanol preference when compared to male controls (p = 0.0760). There is no significant difference within females in regards to 20% ethanol preference between stress histories. **C)** Addition of increasing levels of quinine in 20% ethanol show a main effect of sex in ethanol consumption, with females as a whole consuming more quinine adulterated ethanol that males (p = 0.0021), with a time by sex (p = 0.0001) and sex by stress (p = 0.0304) interaction. Within the sexes, data show a main effect of stress (p = 0.0236) with males with a history of CRPS consuming more quinine adulterated ethanol than male controls. Within females, there was no significant difference in quinine adulterated ethanol consumption between the stress backgrounds. **D)** Quinine adulterated 20% ethanol

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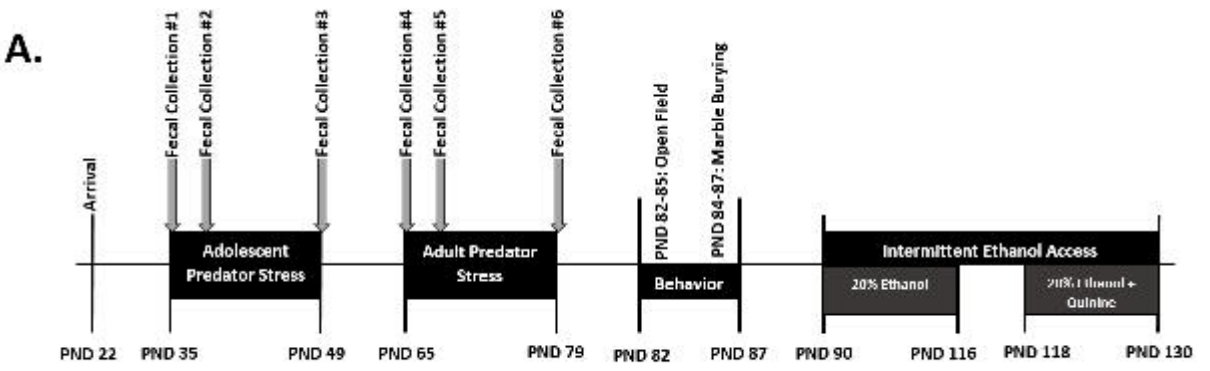
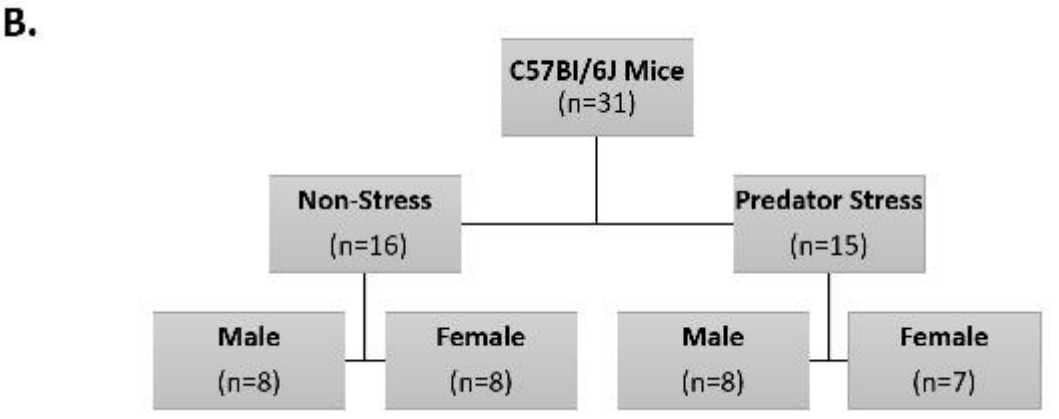
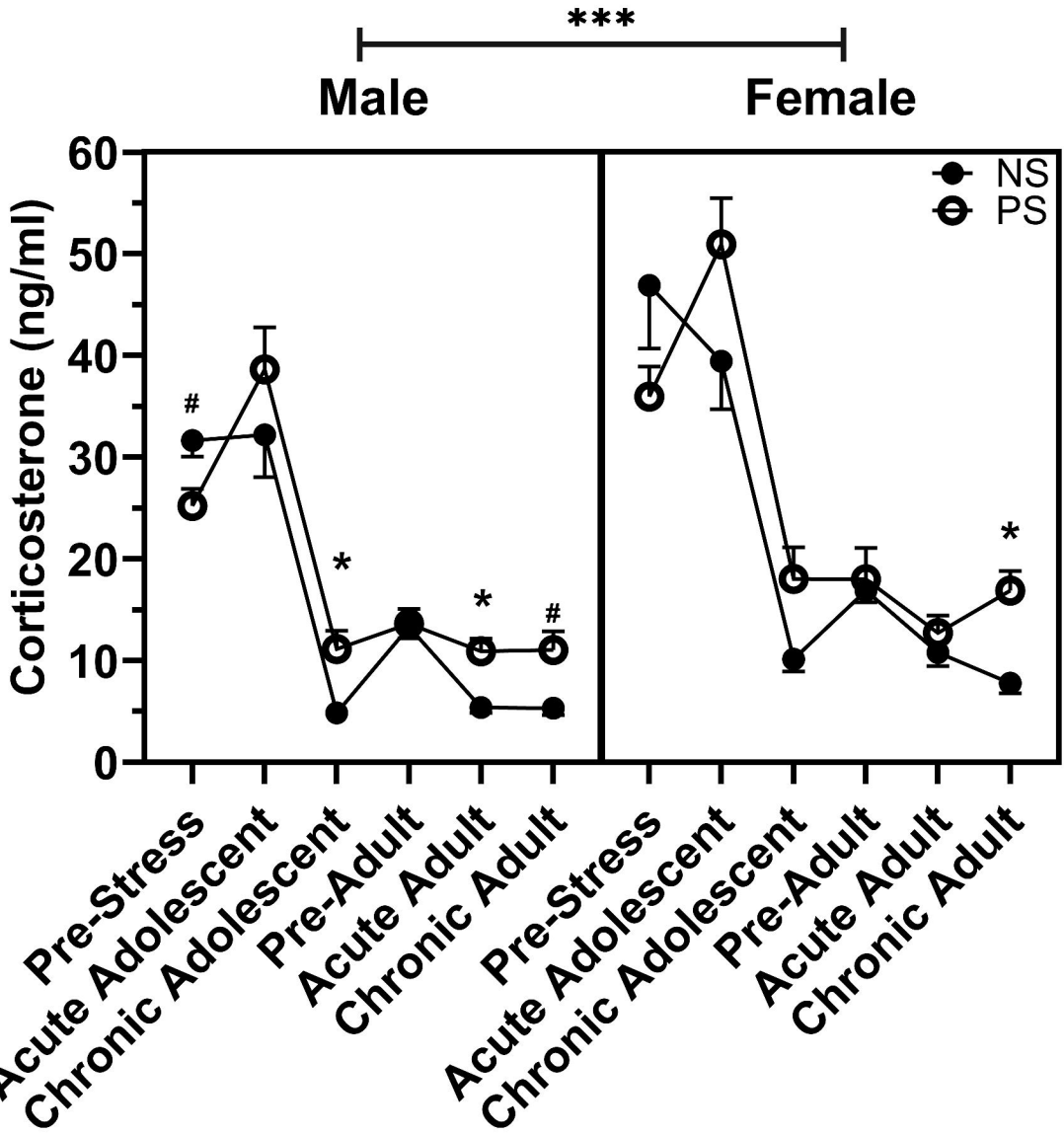
preference shows a main effect of sex ($p = 0.0232$) with both sex by stress ($p = 0.0141$) and time by sex by stress ($p = 0.0142$) interactions, with males with a history of CRPS exhibiting significantly increased preference towards quinine adulterated ethanol across both females and male controls. Within the males, data show a main effect of stress ($p = 0.0117$) driven by the males with a history of CRPS. When compared to baseline, control males significantly decrease quinine adulterated ethanol consumption at 100mg/L quinine whereas males with a history of CRPS significantly decrease at 200mg/L quinine adulterated ethanol. Within females, there is no significant difference in quinine adulterated ethanol preference between the stress histories. Reported values depict mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

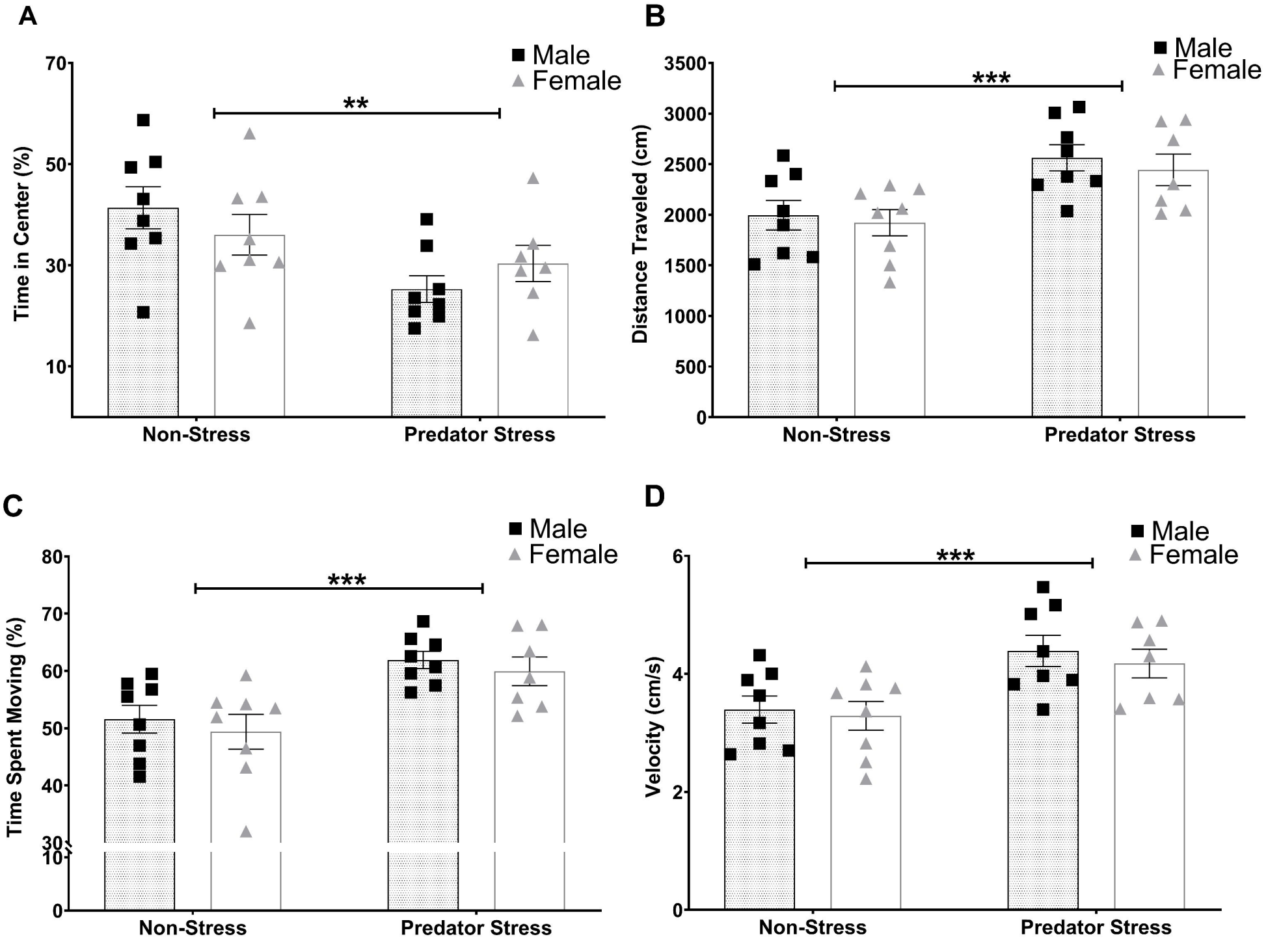
Supplementary Figure 1: Chronic Repeated Predation Stress induces no change in marbles buried.

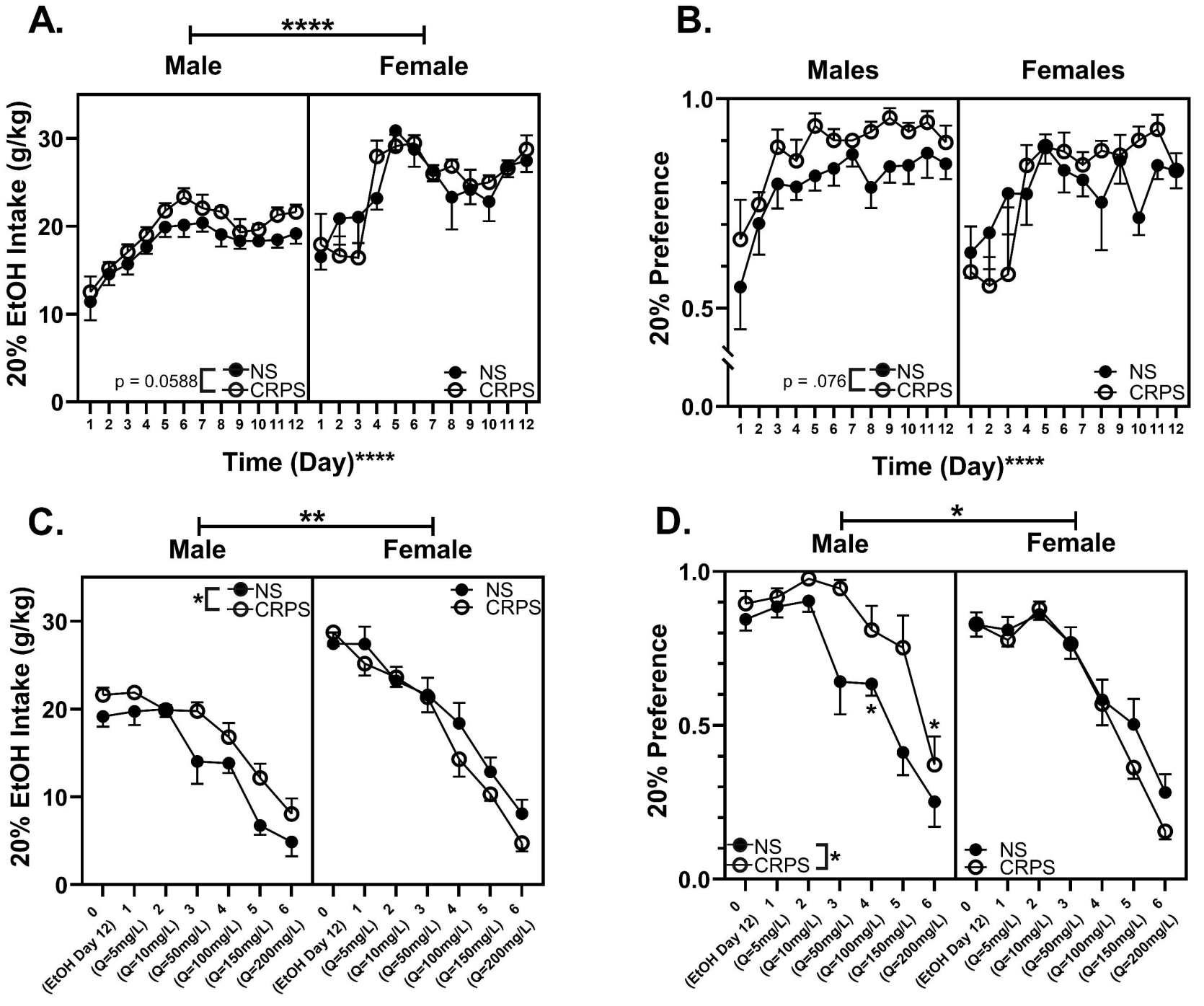
A) A two-way ANOVA shows a main effect of sex in total number of marbles buried, with females, regardless of stress background, burying more marbles than males. **B)** Percentage of total time spent in the center of the arena shows no significant difference between either sex or stress. **C)** Data show a main effect of stress in the number of center visits, with animals with a history of stress visiting the center less than their non-stress counterparts regardless of sex. **D)** Analysis of movement in the field shows a main effect of stress in time spent moving, with mice with a history of stress showing hypoactivity when compared to their non-stressed counterparts. Reported values depict mean ± SEM. *$p < 0.05$.


A.
Arrival
Fecal Collection #1
Fecal Collection #2
Fecal Collection #3
Fecal Collection #4
Fecal Collection #5
Fecal Collection #6
PND 82-85: Open Field
PND 84-87: Marble Burying
Adolescent Predator Stress
Adult Predator Stress
Behavior
Intermittent Ethanol Access
20% Ethanol
PND 22
PND 35
PND 49
PND 65
PND 79
PND 82
PND 87
PND 90
PND 116
PND 118
PND 130


B.
C57Bl/6J Mice
(n=31)
Non-Stress
(n=16)
Predator Stress
(n=15)
Male
(n=8)
Female
(n=8)
Male
(n=8)
Female
(n=7)


***
Male
Female
NS
PS
Corticosterone (ng/ml)
60
50
40
30
20
10
0
#
*
*
#
*
Pre-Stress
Acute Adolescent
Chronic Adolescent
Pre-Adult
Acute Adult
Chronic Adult
Pre-Stress
Acute Adolescent
Chronic Adolescent
Pre-Adult
Acute Adult
Chronic Adult


A
Male
Female
Time in Center (%)
70
50
30
10
**
Non-Stress
Predator Stress
B
Male
Female
Distance Traveled (cm)
3500
3000
2500
2000
1500
1000
500
0
***
Non-Stress
Predator Stress
C
Male
Female
Time Spent Moving (%)
80
70
60
50
40
30
10
0
***
Non-Stress
Predator Stress
D
Male
Female
Velocity (cm/s)
6
4
2
0
***
Non-Stress
Predator Stress


A.
****
Male
Female
20% EtOH Intake (g/kg)
p = 0.0588
NS
CRPS
Time (Day)****
B.
Males
Females
20% Preference
p = .076
NS
CRPS
Time (Day)****
C.
**
Male
Female
20% EtOH Intake (g/kg)
*
(EtOH Day 12)
(Q=5mg/L)
(Q=10mg/L)
(Q=50mg/L)
(Q=100mg/L)
(Q=150mg/L)
(Q=200mg/L)
D.
*
Male
Female
20% Preference