Abstract
Adolescent stress exposure increases the likelihood of alcohol misuse and alcohol use disorder (AUD) in adulthood; however, it is not clear how genetic and environmental factors interact to increase risk. This study examined how adolescent social isolation affects adult binge-like ethanol drinking and levels of the stress hormone corticosterone in male and female mice with a genetic predisposition toward high alcohol preference (HAP). Twenty-eight HAP mice were separated into group-housed (GH) and socially isolated (SI) conditions \( n = 13, \) \( n = 13 \). Binge drinking was assessed using a drinking in the dark (DID) procedure. Blood samples were taken before DID and after the 4th (last) day of DID. Overall, adolescent social isolation increased adult binge drinking in a sex- and time-dependent manner. Analysis of Hour 1 intake across the 4 days indicated that SI males drank significantly more than GH males, and this was not the case in females. There was no significant effect of housing for Hour 2. On Day 4, after 2 hours of consumption, social isolation increased drinking regardless of sex. Plasma corticosterone (CORT) levels decreased following drinking, but there were no sex or housing group differences. There were correlations between CORT levels and drinking, but only for SI females. These findings demonstrate that adolescent social isolation promotes binge-like drinking in both male and female adult mice with a genetic predisposition for high alcohol preference; however, this relationship is time-dependent, and males may be more sensitive than females to social isolation stress. Additionally, corticosterone levels change with regard to binge-drinking and sex.

Keywords
alcohol use disorder (AUD), early life stress, animal models, HPA axis, drinking in the dark (DID), corticosterone, adolescence
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**Mentors**

**Julia Chester** is a professor of neuroscience and behavior at Purdue University. Dr. Chester’s research program focuses on the development and characterization of animal models as tools to identify biological and behavioral mechanisms that influence risk for alcohol use disorders and co-occurring conditions in order to improve the quality of human life. Through teaching, community and media engagement, and professional society advocacy work, Dr. Chester promotes public knowledge about neuroscience, research ethics, and the vital role that animal research plays in scientific discovery and the advancement of medicine. Dr. Chester’s research program has been funded by the National Institutes of Health, Brain & Behavior Research Foundation, Department of Defense, and Purdue University.

**Arbaaz A. Mukadam** is a third-year graduate student working toward a master’s degree and eventually a PhD in Neuroscience & Behavior from Purdue University. He is an international student brought up in Dubai, United Arab Emirates, although he was born in Mumbai, India. Arbaaz is particularly interested in studying the brain and how it controls behavior. He would like to contribute to the study of substance abuse and how that affects neurochemistry, because he has seen drug abuse and addiction in people close to him and would like to help people in similar circumstances.

**Soyol Enkh-Amgalan** is a second-year graduate student at Purdue University in the Neuroscience & Behavior PhD program. She earned her bachelor’s degree at Purdue in Brain and Behavioral Sciences with a minor in Art and Design Studio. Soyol is interested in substance use disorders, genetic and environmental factors behind addiction, and science communication, and will be continuing her work toward a PhD.

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INTRODUCTION

Alcohol use disorder (AUD) is a serious medical condition that vastly impacts the physical, mental, and emotional well-being of individuals as well as those of the people around them. The consequences of AUD and risky drinking (i.e., heavy and binge drinking) include a myriad of health problems, including multiple forms of cancer, as well as increased risk of car crashes, sexual assault, unsafe sexual behaviors, suicide, and homicide (NIAAA, n.d.). Additionally, approximately 414,000 adolescents (1.7% of 12–17-year-olds) in the United States were diagnosed with AUD in 2019 (SAMHSA, 2019). Gene-environment interactions increase the risk of developing AUD remain somewhat unclear, so it is crucial that they continue to be investigated to understand just how they increase risk.

Exposure to stress in adolescence is a crucial risk factor for the development of AUD in adulthood. The adolescent brain is unique in its experience of significant neurodevelopmental changes that present a vulnerability to the effects of stress. It is the rapid and hierarchical nature of these changes as well as the increased number of stress hormone receptors in the brain that may confer this vulnerability (Tottenham & Galván, 2016). Indeed, both epidemiological and clinical research have demonstrated a significant relationship between early stress exposure, risky alcohol use, and the development of AUD. These consequences typically follow affected individuals into adulthood (Chassin et al., 1991; Enoch, 2010; Hoffman & Su, 1998; Keyes et al., 2011; Labouvie, 1986; Wills, 1986). Various forms of childhood trauma and other adverse childhood experiences (ACEs) are correlated with heavy drinking, alcohol problems, and alcohol dependence in adulthood (Anda et al., 2005; Brady & Back, 2012; Dube et al., 2002; Kendler et al., 2000; Keyes et al., 2011; Khoury et al., 2010; Lee & Chen, 2017; Lloyd & Turner, 2008). This relationship is strengthened significantly when an individual’s parents or alcohol problems (Anda et al., 2005; Dube et al., 2002), a clear indication that there are parental influences on the development of alcohol-related problems in their offspring. A familial history of AUD is one of the strongest predictors for the development of AUD in young adults (Chassin et al., 1991), and it is clear that heritable factors (i.e., genes) contribute to risk for AUD. However, environmental factors as well as epigenetic factors are also contributory, and disentangling how these risk factors interact to predispose an individual to AUD development is a primary focus of research (Boden et al., 2021; Kendler et al., 2000; Siomek-Gorecka et al., 2021).

Social stressors are among the most potent stressors faced across many different species, including humans (Beery & Kaufer, 2015). Adolescents experience a host of social stressors (e.g., peer victimization, parental conflict, social pressures, etc.), which for some individuals result in deleterious effects on later mental health.

Animal models are often used to mimic and elucidate mechanisms of the relationship between early life stress and problematic alcohol drinking behaviors. Social isolation stress is a proposed model of early life stress that is often used to promote alcohol drinking and comorbid behaviors in rodents. Adolescent social isolation stress in rodents can produce robust, long-lasting changes in voluntary ethanol drinking and associated neurobiological adaptations (Butler et al., 2016). Social isolation stress can increase (Amancio-Belmont et al., 2020; Evans et al., 2020; Karkhanis et al., 2018; Lallai et al., 2016; León et al., 2017; Skelly et al., 2015; Wukitsch et al., 2019), have no effect on (Moench & Logrip, 2021), or decrease ethanol consumption (Deal et al., 2021).

The current study investigated the effects of adolescent social isolation on adult, binge-like ethanol consumption and plasma corticosterone in a mouse line selectively bred for high alcohol preference (HAP). These mice have a genetic predisposition toward high alcohol consumption and provide a model for heritable influences on alcohol drinking behavior. Previous work has established that HAP1 mice increase drinking behavior when they are exposed to foot shock stress, but only when they are exposed during adolescence and not adulthood (Chester et al., 2008). However, adolescent foot shock stress did not alter conditioned preference for alcohol in a conditioned place preference (CPP) procedure in HAP2 male and female mice (Breit & Chester, 2016).

Binge drinking was assessed using a drinking in the dark (DID) procedure in which mice are allowed access to ethanol for 2–4 hours over a 4-day period. Importantly,
DID has demonstrated that it produces reliably high levels of ethanol consumption in a relatively short period of time (Rhodes et al., 2005).

In the current study, we hypothesized that (1) adolescent social isolation would increase binge drinking in adulthood; (2) CORT levels would increase following social isolation; (3) CORT levels would increase following binge drinking; and (4) males and females would differ in levels of binge drinking following social isolation.

**MATERIALS AND METHODS**

**Subjects**

Subjects were 28 alcohol-naïve male and female HAP2 mice. Mice were derived from 5 different families. Housing conditions were balanced as best as possible across sexes from each family. At the start of social isolation mice were 40–42 days old as social isolation of rats has the most potent effects during a sensitive phase between weaning (P21) to early adulthood (P60) (Burke et al., 2017). Mice were housed in polycarbonate cages (29.2 × 19.0 × 12.7 cm) with aspen wood shavings in groups of 2–4 per cage. At PND 40–42 the socially isolated mice (n = 14) were moved to new cages and the group-housed mice (n = 14) remained in their original cages. Animals stayed in these housing conditions for 3 weeks with cage changes occurring once per week. Ambient room temperature was maintained at 21±2°C. Mice had ad libitum access to food and water and were kept in the same room under a 12:12 light-dark cycle (lights on at 6:00 a.m.). All experimental procedures were approved by the Purdue Animal Care and Use Committee (approved protocol #1810001803).

**Acclimation**

Mice were habituated to the experimental environment for 7 days before DID. The light-dark cycle was adjusted to lights off at 1 p.m. For the first 4 days mice were transferred from their home cages and singly housed in open-top cages for up to 2 hours during their light cycle. Mice were given sipper tubes filled with water and tubes were periodically checked as they would be during DID. After 2 hours, mice were transferred back to their home cages and left undisturbed until the next day. Mice were left undisturbed for 3 days after that.

**Drinking in the Dark (DID)**

A standard, 4-day DID procedure was carried out. Ethanol (20% v/v) was prepared from ethyl alcohol diluted with tap water. Mice were weighed 1 hour before the dark cycle began. All mice were then single housed until the end of each drinking session. Three hours into the dark cycle, water bottles were replaced with ethanol-filled sipper tubes and mice were allowed to drink for 2 hours. Sipper tubes were read immediately upon placement and at 1 h and 2 h. On day 4, mice were given access to ethanol for 4 hours and an additional recording was taken at that time.

**Blood Sampling**

Blood samples were obtained via tail during the light cycle before and after the DID procedure (PND 61–63; PND 75–77). Mice were held in restraint devices with tails exposed. A pair of sharp scissors were used to cut 0.5–1 mm of the distal tail at an angle perpendicular to the tail. Approximately 0.05–0.1 mL of whole blood was collected in 3–7 minutes per mouse in heparinized capillary tubes. Samples were kept on ice until centrifugation and plasma extraction, and plasma was frozen at -80°C until CORT analyses.

**ELISA Corticosterone Assay**

CORT levels were determined using a competitive enzyme (sheep polyclonal antibody to CORT)

**FIGURE 1.** Ethanol intake during Hour 1 across 4 days of DID.
Next, a 2 (Sex) × 2 (Housing) ANOVA for the last 2 hours of the 4-hr period on Day 4 of DID yielded a main effect of Housing (F(1,27) = 4.791, p = 0.039; SI > GH).

These analyses indicate that social isolation during adolescence increased alcohol consumption in adulthood in the DID procedure regardless of sex except for Hour 1. Males seemed to be more sensitive to the effects of social isolation than females during Hour 1.

ELISA Corticosterone Assay

To analyze differences in corticosterone (CORT) levels before and after the DID procedure, a repeated-measures ANOVA [Housing Group × Sex × Time (before vs. after DID)] was carried out. Analyses revealed a significant within-subjects difference between CORT levels before and after DID (F(1) = 6.007, p = 0.022; Before > After). A within-subjects interaction between before/after CORT levels and sex was found to be marginally significant (F(1) = 4.144, p = 0.053) in which females had a greater difference in CORT levels (Before > After) than males. Follow-up analyses indeed revealed that females had a greater difference in CORT levels than males (F(1) = 8.155, p = 0.013). Follow-up paired t-tests were performed for both sexes, and CORT levels were only significantly different before and after DID for socially isolated females (t(7) = 2.469, p = 0.043; Before > After).

Correlative analyses showed that CORT levels before and after DID were positively correlated (r = .728, p < 0.001,

RESULTS

Drinking in the Dark

Two subjects’ data, one from each housing group, had to be removed from analyses because it was suspected that their bottles leaked during the DID procedure. A 2 (Sex) × 2 (Housing) × 4 (Day) repeated-measures ANOVA on ethanol consumption (g/kg) revealed main effects of Sex (F(6.002), p = 0.022), Housing (F(5.359), p = 0.030), and a Sex × Housing interaction (F(7.787), p = 0.010).

Analysis of Hour 1 of DID yielded a main effect of Sex (F(1,23) = 10.104, p = 0.004; Female > Male) and an interaction of Sex × Housing (F(1,23) = 9.008, p = 0.006). Follow-up ANOVAs of Housing within each Sex for Hour 1 revealed no significant differences between females (F(1,14) = 1.192, p = 0.293) but a significant difference between males (F(1,9) = 9.498, p = 0.013; SI > GH).

For Hour 2, there were no main effects of Sex or Housing nor an interaction.

![FIGURE 2](image) Ethanol intake during Hour 2 across 4 days of DID.

![FIGURE 3](image) Ethanol intake at Hour 4 Day 4. At Hour 4 on Day 4 mice had had access to ethanol for 2 hours (Hour 2 – Hour 4).
Sex differences in drinking behavior were apparent, in that females drank more than males overall. Importantly, despite greater ethanol consumption in females, males were more sensitive to the effects of social isolation stress; socially isolated males drank more than group-housed males during Hour 1 across the 4 days and Hour 4 on Day 4. Group-housed and socially isolated females drank the same amount during Hour 1 across the 4 days and during Hour 4 on Day 4. These sex differences in binge drinking may depend on the concentration of ethanol offered. Our study used 20% v/v, which was sufficient to produce sex differences. Previous studies confirm that 15–30% v/v ethanol, as opposed to 10% v/v, is sufficient to produce overall sex differences (Evans et al., 2020; Lopez et al., 2011; Lopez & Laber, 2015). However, it is difficult to compare studies due to differences in species, length of social isolation, and/or the drinking procedure used. Evans et al. had the experimental design most similar to our study in that they used a variation of DID to evaluate binge drinking in male and female B6 (a high-alcohol-preferring strain) mice that had either been socially isolated or pair-housed. The use of 20–30% ethanol with a DID procedure in their study reveals those specific sex effects in which socially isolated males drank more than group-housed males, but the effect was not present in females, or it was reversed (group-housed females drink more than socially isolated females) (Evans et al., 2020). It is uncertain why females show this reversed effect or are unaffected as the concentration of ethanol changes.

Differences emerged between sexes depending on the hour during the drinking session. Males’ heightened sensitivity to social isolation was only apparent during the first hour (Hour 1) across the 4 days, and this effect disappeared by Hour 2 across the 4 days and Hours 2–4 during Day 4. Limited- and intermittent-access to ethanol following social isolation stress produce transient, sex-dependent increases in drinking behavior in rodents (Lopez et al., 2011; Lopez & Laber, 2015; Moench & Logrip, 2021; Skelly et al., 2015).

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mice on days 1, 8, and 10 of the limited-access drinking period (Lopez et al., 2011). A later study by Lopez and Laber (2015), also using a limited-access procedure, confirmed previous results in which differences in drinking between group-housed and chronically isolated mice were most significant during the first 5 days of drinking. Moench and Logrip (2021) observed different patterns of drinking in an intermittent-access 2-bottle choice procedure, based on stress history in pair-housed rats during the first hour of drinking, an effect that was not replicated in isolated rats. In this study, neither a significant effect of day nor an interaction between sex and days were observed. However, the observed sex differences only for Hour 1 suggest that transient, sex-dependent differences in drinking behavior exist across hours in addition to days.

Corticosterone is released in response to environmental stressors following activation of the hypothalamic-pituitary-adrenal (HPA) axis, which is a commonly used indicator of HPA axis activity. Early life stress in rodents produces hyperactivity of the HPA axis (Ladd et al., 1996; Vázquez et al., 2000) and increases anxiety- and depressive-like behaviors (Juruena, 2014; Juruena et al., 2020). Most of this evidence comes from studies that use maternal separation as a model of early life stress. Other stressors, like foot shock stress, may decrease HPA axis activity, exemplified by lower corticosterone levels following foot shocks, as was found in a previous study using HAP2 mice (Chester et al., 2008).

We only measured CORT before and after the DID procedure and not before social isolation. Socially isolated females were the only group to significantly differ in CORT levels before and after DID (lower levels of CORT). This result is particularly interesting considering there was no significant difference in binge drinking between housing groups for females.

Butler et al. (2014) previously reported a positive correlation between drinking behavior and baseline (in between social isolation and drinking) CORT levels for socially isolated male rats, despite not finding housing group differences in baseline CORT before drinking. We uncovered a similar finding in female mice; however, the correlations were negative, not positive. Females did not show differences in binge drinking between housing groups, but binge drinking and CORT levels were negatively correlated for socially isolated females. This relationship was also time-dependent; it was only during Hours 2–4 on Day 4 that both before and after CORT levels were correlated with binge drinking in socially isolated females. The observed negative correlations suggest that lower CORT levels before ethanol is accessed predict higher levels of binge drinking and that higher levels of binge drinking then predict lower CORT levels after ethanol is consumed. Our laboratory has previously shown that HAP mice have lower CORT levels following a stressor compared to their low-drinking counterparts, indicating possible differences in a negative feedback mechanism. Chester et al.’s study also showed that adolescent mice that experienced foot shock stress drank more than mice that weren’t given foot shocks (Breit & Chester, 2016). Though the current study did not measure CORT following social isolation stress, we found that lower CORT levels in HAP mice predict higher binge drinking, which may also suggest a negative feedback mechanism.

All things considered, social isolation does promote binge drinking in high-alcohol-prefering mice. This relationship is sex- and time-dependent with males and females responding differently to social isolation and drinking behaviors changing within each hour of the drinking period. Lower CORT levels predict higher binge-like ethanol consumption, and in turn higher binge drinking predicts lower corticosterone levels; however, this was only observed in socially isolated females. Additional research is needed to further explore sex- and housing-dependent differences in drinking and plasma corticosterone.

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