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“Wide-Awake Drunk”: Observing the Combined Effects of Alcohol and Caffeine on Somatic Withdrawal Signs in C57BL/6J Mice

Montana Jenkins
Butler University

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Applicant Montana Dawn Jenkins
(Name as it is to appear on diploma)

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Read, approved, and signed by:

Thesis adviser(s) Jennifer Berry 5/7/19
Date

Date

Reader(s) Robert Dale 5/6/19
Date

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"Wide-Awake Drunk": Observing the Combined Effects of Alcohol and Caffeine on Somatic Withdrawal Signs in C57BL/6J Mice

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Montana Dawn Jenkins

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Abstract

Caffeine is one of the most widely used psychoactive stimulants in the world and is often used in combination with other substances. The combination of caffeine and alcohol has been shown to induce a stimulated, rather than sedated state, which may result in increased alcohol-attributable accidents (e.g., drunk driving, unprotected sex, and over intoxication). Preclinical research has found mixed results regarding the co-consumption of caffeine and alcohol – some found that caffeine increases alcohol intake while others the opposite. The current study expanded on previous research by testing the effects of combined caffeine and alcohol exposure in a binge-like, mouse paradigm. It was anticipated that the mice would consume larger amounts of alcohol in combination with caffeine compared to consuming either alcohol or caffeine alone. The present study explored drinking behaviors in 24 adult C57BL/6J mice using an intermittent access 2-bottle choice paradigm. Singly-housed mice were presented with one bottle of tap water and one bottle of tap water with incrementally increasing concentrations of alcohol (3-20% v/v), caffeine (0.01-0.05% w/v), or a mixture of alcohol and caffeine every other day. The amount of liquid consumed from each bottle was recorded at the same time every day and, approximately 24 hours after the last drinking day, mice were videotaped to assess somatic signs of alcohol or caffeine withdrawal. We hypothesized that mice in the combined alcohol and caffeine condition would drink more than the other conditions and that their physical dependence, as evidenced by increased somatic signs, would be greater than after consuming either drug alone.

"Wide-Awake Drunk": Observing the Effects of Combined Alcohol and Caffeine on Somatic Withdrawal Signs in C57BL/6J Mice

Alcohol and caffeine are two of the most commonly used substances throughout the world. The reasoning behind this is that both are known to be physically and psychologically addictive due to their changing of brain chemistry (Cauli & Morelli, 2005; Gilpin & Koob, 2008). More specifically, alcohol is classified as a depressant on the brain whereas caffeine is a stimulant. This makes them appear to be total opposites and seemingly unrelated; nevertheless, there are numerous beverages containing mixtures of the two. Whether it is in the form of soda and rum, coffee and whiskey, an energy drink and vodka, or some other concoction, these mixed drinks have become increasingly popular in the United States. In fact, a recent poll found that 13% of students in grades 8 through 12 and 33.5% of young adults aged 19 to 28 reported consuming alcoholic beverages mixed with caffeinated energy drinks within the past year (Johnston, O'Malley, Miech, Bachman, & Schulenberg, 2016). And although the combination is increasingly common, the Dietary Guidelines for Americans (2015) have cautioned against mixing alcohol with caffeine due to an observed increase in hospitalizations from these mixtures. Even so, the alcohol and caffeine combination has since been going strong. Given this popular marriage, researchers have begun to raise the question – what undesirable effects arise with the mixture of alcohol and caffeine?

Caffeine itself is the most widely consumed psychoactive drug in the world and, unlike most other substances, it is legal and unregulated in almost all countries. In both humans and animals, caffeine shows pharmalogical effects that are similar to those of other psychostimulants: increases in arousal, motor activity, and reinforcing effects; yet, it is important to point out that caffeine has a weaker reinforcing effect than other psychostimulants (Ribeiro & Sebastião, 2010). Independently, it is known to stimulate the central nervous system (CNS) by antagonizing (i.e. blocking) receptors for the inhibitory neuromodulator adenosine (Ribeiro & Sebastião, 2010). Adenosine is thought to be responsible for the onset of drowsiness, which occurs as a result of

buildup of adenosine levels throughout the duration of the wakeful day. Caffeine has also been shown to have several effects on the cardiovascular system – primarily by blocking cardiac adenosine receptors. This blockade inhibits adenosine's effects and can cause tachycardia and arrhythmias through intense β_1-receptor activity (Cappelletti, Daria, Sani, & Aromatario, 2015). By antagonizing the receptors for adenosine, caffeine ultimately promotes wakefulness, delays drowsiness, and increases heart rate. Typically, caffeine is relatively harmless and carries few to no consequences for people; however, it may present significant problems when over-consumed or combined with other substances.

Behaviorally, caffeine can have a number of positive effects, including increases in alertness, reduction of fatigue, and improved performance on tasks (Johnson, Spinweber, & Gomez, 1990). These effects are seen when individuals consume little to moderate amounts of caffeine, which is considered less than or equal to about 400 milligrams per day for healthy adults (Mayo Clinic Staff, 2017). Most people who consume caffeine stay safely within this range and do not reach levels of overdose; however, caffeine tolerance and addiction is relatively commonplace. Someone with a need for caffeine can experience caffeine withdrawal after stopping caffeine consumption. Caffeine withdrawal can lead to various aversive effects, such as headaches, decreased levels of alertness, fatigue, and increased anxiety (Ratcliff-Crain, O'Keeffe, & Baum, 1989; Smith, 2002). Withdrawal signs have been measured in both human and animal models, particularly in regard to stress and anxiety (Ferré & O'Brien, 2011). Interestingly, withdrawal signs tend to be more severe if caffeine is consumed in combination with other substances, such as alcohol or nicotine (O'Rourke, Touchette, Hartell, Bade, & Lee, 2016; Fritz, Quoilin, Kasten, Smoker, & Boehm, 2016), and hence warrants further investigation of these combinations.

Similar to caffeine, alcohol is also legal and widely used; however, its use poses a much larger risk for the drinker if used irresponsibly. Alcohol is unlike any other drug in that it can alter the levels of a wide variety of neurotransmitters – or chemicals that allow for synaptic communication – within the brain. Alcohol impacts several excitatory and inhibitory

neurotransmitter systems, including those of adenosine, dopamine, glutamate, and GABA (McIntosh & Chick, 2004). Alcohol is classified as a CNS depressant and when an individual continues to drink, they will begin to show signs of alcohol intoxication such as lowered inhibitions, trouble concentrating, loss of coordination, mood swings, slower breathing, etc. (McIntosh & Chick, 2004). Although these symptoms may seem undesirable, alcohol also results in an increased release of the neurotransmitter dopamine in the reward areas of the brain (Di Chiara & Imperato, 1988). Dopamine plays a major role in the motivational component of reward-motivated behavior and, subsequently, is associated with feelings of bliss, motivation, and euphoria - hence the nickname "feel-good hormone." These feelings motivate an individual to drink more in a single setting, which can pose a significant problem for not only the user, but also those individuals around them.

Binge-drinking is considered consuming more than four drinks for women or five drinks for men in a two-hour time span, which can quickly raise one's blood alcohol content (BAC) to a dangerous level of 0.08% or more (White, Tapert, & Shukla, 2018). Consuming excessive amounts of alcohol within a short period of time is associated with a number of health and social problems (Esser et al., 2014; Sacks, Gonzales, Bouchery, Tomedi, & Brewer, 2015). These problems include but are not limited to: drunk driving, risky sexual activity, interpersonal violence, unintended pregnancy, and, most importantly, death (Thombs et al, 2010; Marczinski & Fillmore, 2014; McKetin, Coen, & Kaye, 2015). According to the Centers for Disease Control and Prevention (2016), alcohol is responsible for around 88,000 deaths in the United States every year – with more than half of them related to binge-drinking. Given that binge-drinking can have a host of unintended consequences, many of them aversive, it is clearly important to better understand the motivation to consume substances like alcohol in excess.

Previous studies have shown that alcohol in combination with other substances can increase the chances of binge-drinking occurring. Such substances include nicotine (O'Rourke et al., 2016), caffeine (Fritz et al., 2016), and others, making it important to study these substances.

Although legal, the combination of alcohol and caffeine may pose a substantial risk for the abuser and those around them. When alcohol and caffeine are mixed, the stimulant effects of caffeine may mask the depressant effects of alcohol, causing users to feel more awake but nevertheless intoxicated (Ferré & O'Brien, 2011). Subsequently, this "wide-awake drunk" feeling can increase the chances of the individual drinking more than they should, thereby increasing the risk of binge-drinking. One study found that a sample of drinkers aged 15 to 23 who mixed alcohol with energy drinks were four times more likely to binge drink compared to those who did not mix alcohol with energy drinks (Emond, Gilbert-Diamond, Tanski, & Sargent, 2014). In 2010, colleges and universities across the United States began to see an increase in injuries and blackouts related to caffeinated alcoholic beverages (Siegel, 2011). Soon after, the Food and Drug Administration (2010) issued a warning that caffeine is an unsafe food additive to alcoholic beverages and that further action, such as seizure of these products, is possible under federal law. Consequently, the sale of drinks that were premixed with caffeine and alcohol was banned in stores throughout the United States; however, such drinks remain prevalent in bars that buy the substances separately and then mix them together. Nevertheless, the concerning reason for increased injuries due to caffeinated alcoholic mixtures is unclear and, thus, require further investigation of their mechanistic underpinnings to determine the safety of their co-consumption.

One potential reason for the increasingly popular combination of alcohol and caffeine is the mechanistic overlap between the two substances. Acute alcohol use also results in increased extracellular levels of the neuromodulator adenosine (Sharma, Engermann, Sahota, & Thakkar, 2010). As discussed previously, adenosine promotes sleep and suppresses arousal, which are common side effects of alcohol consumption; however, caffeine antagonizes these receptors, causing them to be blocked and, ultimately, promotes wakefulness rather than sleep (Butler & Prendergast, 2012). Thus, caffeine may block the adenosinergic receptors so that the increased adenosine from alcohol cannot attach, leading to a more alert feeling than would be felt with alcohol alone. Accordingly, humans report feeling less intoxicated/impaired when caffeine and alcohol are

co-administered rather than when either are taken alone (Marczinski & Fillmore, 2006). Thus, further understanding of the potential interaction between caffeine and alcohol in drinking behaviors is necessary to understand more of the mechanistic underpinnings between these substances. Preclinical models have already been a valuable tool in gaining a better understanding and, thus, may be a vital step towards the future development of the dangers associated with their co-consumption.

Given the overlap between caffeine and alcohol use as well as the mechanisms involved with both drugs, relatively few clinical and preclinical studies have examined the effects of both substances simultaneously, and those that did have found conflicting results. Fritz and colleagues (2016) found that co-consumption of alcohol with caffeine significantly increased alcohol intake and resultant blood alcohol concentration (BAC) in both adolescent and adult mice following a voluntary binge-like drinking paradigm. Others, however, have found that caffeine and alcohol co-consumption decreased alcohol intake or had no effect (Rezvani et al., 2013; Fritz, Companion, & Boehm, 2014). The current study is designed to help address the gaps that currently exist in the literature regarding caffeine and alcohol co-consumption and potential co-dependence. Due to the previously reported masked effects that caffeine has on alcohol, we predicted that the mixture of caffeine and alcohol would increase co-consumption, inducing the “wide-awake drunk” feeling. As a result of this combination and increased consumption, the mice will later exhibit more somatic withdrawal signs (i.e., a measure of anxiety-like behavior) compared to those seen in alcohol or caffeine alone. Ultimately, this study aims to examine the effects of voluntary co-administration of alcohol and caffeine in a 2-bottle choice intermittent access paradigm in a mouse model.

Method

Subjects

Male and female C57BL/6J mice (n=24) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All mice were 8 weeks of age prior to the start of the experiment. All mice were singly housed upon arrival under a 12-hour reverse light/dark cycle until the start of the experiment. Food and water were made available *ad lib* throughout the entirety of the experiment. All mice were weighed once per week throughout the duration of the experiment. Care of all animals was carried out in accordance with the guidelines of the National Institutes of Health Office of Laboratory Animal Welfare. The Institutional Animal Care and Use Committee at Butler University also approved the protocol.

Drugs and Chemicals

Caffeine, 190 proof alcohol (ethanol), and all other chemicals without a specified supplier were obtained from Sigma (St. Louis, MO, USA). Alcohol and/or caffeine were mixed with tap water to obtain the desired concentrations listed. These desired concentrations were as follows: 3%, 6%, 10% v/v, or 20% v/v alcohol and/or 0.01. 0.03, 0.05% w/v caffeine.

Drinking Model for Alcohol Consumption

Previous studies have shown that mice will consume caffeine at physiologically-relevant doses in a short-access binge-like paradigm (Fritz et al., 2016), but no studies to date have detailed the effects of caffeine alone or in combination with alcohol in an intermittent access (IA) paradigm. Thus, we utilized a 2-bottle choice, intermittent access paradigm for the current study. Every Monday, Wednesday, and Friday, singly housed mice were presented with the two bottles – one containing tap water and one containing tap water alone or with a mix of an increasing concentration of alcohol and/or caffeine (alcohol 3-20% v/v; caffeine 0.01-0.05% w/v) that were previously mentioned. The mice were exposed to this drug bottle (water, alcohol, and/or caffeine) for exactly 24 hours. For week 1, mice were exposed to 3% alcohol and/or 0.01% caffeine on

Monday, 6% alcohol/0.03% caffeine on Wednesday, and 10% alcohol/0.05% caffeine on Friday. On all other days (Tuesday, Thursday, Saturday and Sunday), mice were given only one bottle of tap water. Starting with the Monday of week 2, all mice received the highest concentration(s) of 20% alcohol/0.05% caffeine and the concentrations remained at this level for the duration of the study. The bottles were weighed at the same time daily to measure consumption. The positions of the bottles were switched every drinking day and the fluids were refreshed every 4-5 days.

Withdrawal Behavior

The anxiety-like behavior associated with caffeine and/or alcohol withdrawal was investigated using a somatic signs model. Somatic signs were chosen based off of previous research involving alcohol and nicotine and have been shown to accurately measure anxiety-like behavior in mice undergoing withdrawal (Damaj, Kao, & Martin, 2003; Locklear, McDonald, Smith, & Fryxell, 2012; O'Rourke et al., 2016).

Approximately 24 hours after the last drug drinking day, mice were placed into a clean home cage and videotaped for 20 minutes in order to assess somatic signs of alcohol and/or caffeine withdrawal. Videotaped signs were scored by reviewers blind to the conditions and included: grooming, tremors, wet dog shakes, head shakes, chattering, tail rattling, body twitching, jumping forwards, cage scratching, digging, rearing, retropulsion, mastification, hind foot scratching/shaking, hunched position, and escape attempts.

Statistical Analysis

The mice were separated into four groups with six C57BL/6J mice per group, containing three males and three females to help reduce gender bias. Within each group, all mice (males and females) were treated exactly the same and received the same drug treatment (alcohol, caffeine or alcohol and caffeine) throughout the entire experiment. Each group was given one of the following 2-bottle drinking paradigms: water/water, caffeine/water, alcohol/water, and alcohol and caffeine/water. After four weeks of consumption, the caffeine and ethanol-containing bottles were

removed, leaving the mice with only one bottle of water. Approximately 24 hours after the removal of caffeine and/or alcohol, all mice were observed for somatic withdrawal signs as described above. Amounts of consumption, preference, and withdrawal signs were then compared across groups.

The raw consumption values were first transformed by subtracting the average value of a "leak" control bottle (treated exactly the same as other cages, except that no mouse is present in that cage). This was done to control for leakage of the bottle or evaporation that may occur over time and was recorded every day at the same time that the other bottle weights are recorded. Consumption values were then transformed based on the percentage of alcohol and/or caffeine present in the bottle for that particular day, density of the substance if applicable (i.e. for alcohol), and the weight of each individual animal. These values were then averaged over the number of days for each concentration, yielding an average daily alcohol consumption (g/kg/day) and an average daily caffeine consumption (mg/kg/day) for each mouse. The percent preference for the alcohol and/or caffeine bottle was calculated as the number of mls of alcohol and/or caffeine water consumed divided by the total mls of fluid consumed * 100. Data were analyzed using GraphPad Prism version 8.0 statistical software. Consumption and preference data were evaluated using a series of 2 (sex: male vs. female) X 4 (concentration) mixed factor analysis of variance (ANOVA), with sex being a between subjects factor and concentration being a within subjects factor. Somatic signs evidenced during the acute withdrawal stage were videotaped and scored by reviewers blinded to the conditions of each mouse. Individual signs were tallied. Somatic withdrawal signs were analyzed using a 2 (sex) X 4 (group: water, alcohol only, caffeine only, alcohol+caffeine) between subjects ANOVA. Post-hoc tests were completed using a Student's t-test when applicable.

Results

Alcohol

Consumption

Mice were presented with bottles containing alcohol (3, 6, 10 and 20% v/v) for 24 hours every Monday, Wednesday, and Friday and the bottle weights were recorded in order to examine alcohol consumption. A two-way ANOVA revealed a significant main effect of concentration ($F(5,20) = 56.28$, $p < 0.001$), sex ($F(1,4) = 11.35$, $p < 0.05$), and interaction of concentration and sex ($F(5,20) = 8.126$, $p < 0.0005$). Both male and female mice consumed substantially more alcohol during exposure to the 20% v/v bottle compared to the other concentrations (Figure 1A). Males and females consumed relatively similar amounts of alcohol until the highest concentration, 20% v/v, was reached. From that point forward, females consumed significantly more alcohol than males, with the largest difference at the highest concentration (~40 g/kg/day for females vs. ~30 g/kg/day for males). Both females and males consumed the least amount of alcohol when presented with 3% v/v and the most when presented with 20% v/v.

Preference

Mice were presented with bottles containing alcohol (3, 6, 10 and 20% v/v) for 24 hours every Monday, Wednesday, and Friday and the bottle weights were recorded in order to examine alcohol preference in relation to a second bottle containing water. A two-way mixed factor ANOVA revealed no significant main effects of either sex or concentration as well as no significant interaction. Generally, males appeared to have a relatively stable level of preference throughout all concentrations, with a preference ratio of around 0.8 (Figure 1B). Females, however, appeared to have an increasing preference for alcohol as the concentrations increased, up to 0.8 for the highest concentration. Males exhibited the least preference for alcohol at 6% v/v and the most preference at 3% v/v. Females exhibited the least preference for alcohol at 3% v/v and the most preference at 20% v/v.

Caffeine

Consumption

Mice were presented with bottles containing caffeine (0.01, 0.03, 0.05% w/v) for 24 hours every Monday, Wednesday, and Friday and caffeine consumption was calculated. A two-way ANOVA revealed no interaction of sex and caffeine concentration. However, there was a significant effect of concentration, $F(5, 20) = 15.29$, $p < 0.0001$, where both male and female mice initially consumed more caffeine as the concentrations also increased (Figure 2A). Yet, this trend decreased throughout the last three weeks of the experiment. There was also a significant effect of sex, $F(1, 4) = 9.069$, $p < 0.05$, where females tended to consume more caffeine than males consistently throughout all concentrations. Females consumed the least amount of caffeine when presented with 0.01% w/v (2 mg/kg/day) and the most amount when presented with 0.05% (3-10 mg/kg/day). Males consumed the least amount of caffeine when presented with 0.01% w/v (1 mg/kg/day) and the most when presented with 0.05% w/v (2-5 mg/kg/day).

Preference

Mice were presented with bottles containing caffeine (0.01, 0.03, 0.05% w/v) for 24 hours every Monday, Wednesday, and Friday and the bottle weights were recorded in order to examine caffeine preference in relation to a second bottle containing water. A two-way mixed factor ANOVA revealed no significant main effect of sex nor an interaction of sex and concentration. There was a significant effect of concentration, $F(5, 20) = 14.16$, $p < 0.0001$, where mice initially had a higher preference for the lower concentrations as each was introduced, however, as the experiment continued, the mice drastically decreased their preference of the 0.05% w/v concentration (Figure 2B). Males exhibited the least preference for caffeine during week 4 with the 0.05% w/v (ratio of 0.15) and the most at week 1 with the 0.03% w/v (ratio of 0.8). Females

exhibited the least preference for caffeine at week four with the 0.05% w/v (ratio of 0.16) and the most when presented with 0.03% w/v (ratio of 0.83).

Alcohol + Caffeine

Consumption

Mice were presented with bottles containing alcohol+caffeine (3% v/v alcohol + 0.01% w/v caffeine; 6% v/v alcohol + 0.03% w/v caffeine; 10% v/v alcohol + 0.05% w/v caffeine; and 20% v/v alcohol + 0.05% w/v caffeine) for 24 hours every Monday, Wednesday, and Friday and the bottle weights were recorded in order to examine alcohol+caffeine consumption. Because we expected sex differences due to previous reports and data collected in our lab, separate two-way ANOVAs were conducted for male and female mice. A two-way repeated measures ANOVA on combined alcohol and caffeine consumption in male C57 mice revealed a significant main effect of substance (alcohol or caffeine; $F(1, 2) = 122.4$, $p < 0.05$), concentration ($F(5, 10) = 23.73$, $p < 0.001$), and interaction of substance and concentration ($F(5, 10) = 34.78$, $p < 0.0001$). Male mice consumed significantly more alcohol when presented with 20% v/v alcohol + 0.05% w/v caffeine compared to when presented with 3% v/v alcohol + 0.01% caffeine (Figure 3A). A two-way repeated-measures ANOVA on combined alcohol and caffeine consumption in female C57 mice revealed a significant main effect of substance ($F(1, 2) = 465.7$, $p < 0.05$), concentration ($F(5, 10) = 43.66$, $p < 0.001$), and interaction of substance and concentration ($F(5, 10) = 74.00$, $p < 0.0001$). Female mice consumed significantly more when presented with 20% v/v alcohol + 0.05% w/v caffeine than when presented with 3% alcohol + 0.01% w/v caffeine (Figure 3B). Males consumed the least alcohol+caffeine at 3% v/v alcohol + 0.01% w/v caffeine (4 g/kg/day + 6 mg/kg/day) and the most at 20% v/v alcohol + 0.05% w/v caffeine (30 g/kg/day + 29 mg/kg/day). Females consumed the least alcohol+caffeine at 3% v/v alcohol + 0.01% w/v caffeine (4 g/kg/day + 6 mg/kg/day) and the most at 20% v/v alcohol + 0.05% w/v caffeine (31 g/kg/day + 33 mg/kg/day).

Preference

Mice were presented with bottles containing alcohol+caffeine (3% v/v alcohol + 0.01% w/v caffeine; 6% v/v alcohol + 0.03% w/v caffeine; 10% v/v alcohol + 0.05% w/v caffeine; and 20% v/v alcohol + 0.05% w/v caffeine) for 24 hours every Monday, Wednesday, and Friday and the bottle weights were recorded in order to examine preference in relation to a second bottle containing water. A two-way mixed factor ANOVA on preference for alcohol+caffeine revealed a significant main effect of concentration ($F(5, 10) = 15.41$, $p < 0.0002$) and sex ($F(1, 2) = 26.96$, $p < 0.05$), but no significant interaction of concentration and sex. Collapsed across concentration, males had a relatively higher preference for alcohol+caffeine compared to the females (Figure 3C). Males exhibited the least preference for alcohol+caffeine at 20% v/v alcohol + 0.05% w/v caffeine during week 4 and the most preference at 6% v/v alcohol + 0.03% w/v caffeine (preference ratio of 0.6 vs. 0.9). Females exhibited the least preference at alcohol+caffeine at 20% v/v alcohol + 0.05% w/v caffeine during week 2 and the most preference at 6% v/v alcohol + 0.03% w/v caffeine (preference ratio of 0.55 vs. 0.9).

Alcohol vs Alcohol + Caffeine Comparison

Consumption

The average alcohol consumption in both alcohol alone mice and alcohol+caffeine mice was compared at the four alcohol concentrations: 3% v/v, 6% v/v, 10% v/v, and 20% v/v in male and female mice. A two-way mixed factor ANOVA comparing alcohol consumption in C57 female mice when presented with either alcohol alone or alcohol+caffeine revealed a significant main effect of concentration ($F(5, 24) = 58.78$, $p < 0.0001$), treatment group ($F(1, 24) = 28.26$, $p < 0.0001$), and interaction of concentration and group ($F(5, 24) = 7.042$, $p < 0.001$). Collapsed across treatment group, female mice consumed more alcohol at the 20% v/v concentration than the 3% v/v concentration. The consumption of alcohol in the alcohol alone treatment group was significantly higher compared to the alcohol+caffeine treatment group (42 g/kg/day vs. 22

g/kg/day) once the highest concentration of 20% v/v was reached (Figure 4A). A two-way mixed factor ANOVA comparing alcohol consumption in C57 male mice when presented with either alcohol alone or alcohol+caffeine revealed no significant interactions of concentration and treatment group, but did reveal a significant main effect of concentration ($F(5, 20) = 60.77$, $p < 0.0001$). Collapsed across treatment group, male mice consumed more alcohol at the 20% v/v concentrations than the 3% v/v concentration. The consumption of alcohol in both treatment groups appears to be similar until the 20% v/v was reached (~22 g/kg/day), in which the alcohol+caffeine males consumed slightly (though not significantly) more than the alcohol only males (Figure 4A).

Preference

The average alcohol preference for both alcohol alone mice and alcohol+caffeine mice was compared at the four alcohol concentrations: 3% v/v, 6% v/v, 10% v/v, and 20% v/v. A two-way mixed factor ANOVA comparing alcohol preference when presented with either alcohol alone or alcohol+caffeine revealed no significant main effects of concentration, sex, or interaction of concentration and sex. It appeared that, particularly at the highest concentrations, both males and females preferred the alcohol alone treatment over the alcohol+caffeine treatment, albeit non-significantly (Figure 4B).

Caffeine vs Alcohol + Caffeine

Consumption

The average caffeine consumption in both caffeine alone mice and alcohol+caffeine mice was compared at the three caffeine concentrations: 0.01% w/v, 0.03% w/v, 0.05% w/v in male and female mice. A two-way mixed factor ANOVA comparing caffeine consumption in C57 female mice when presented with either caffeine alone or alcohol+caffeine revealed a significant main effect of concentration ($F(5, 24) = 21.06$, $p < 0.0001$), treatment group ($F(1, 24) = 38.83$, $p < 0.0001$), and interaction of concentration and group ($F(5, 24) = 18.05$, $p < 0.0001$). Collapsed across treatment group, female mice consumed more caffeine at the 0.05% w/v concentration compared

the 0.01% w/v concentration (Figure 5A). However, the caffeine-only group's consumption drastically decreased after the initial 0.05% w/v concentration whereas the consumption of the alcohol+caffeine treatment group remained high (3 mg/kg/day vs. 8 mg/kg/day). Generally, female mice consumed more caffeine in the alcohol+caffeine group compared to the caffeine only group. A two-way mixed factor ANOVA comparing caffeine consumption in C57 male mice when presented with either caffeine alone or alcohol+caffeine revealed a significant main effect of concentration ($F(5, 24) = 6.550$, $p < 0.005$), treatment group ($F(1, 24) = 57.19$, $p < 0.0001$), and interaction of concentration and group ($F(5, 24) = 4.415$, $p < 0.01$). Collapsed across treatment group, male mice consumed more caffeine at the 0.05% w/v concentration compared to the 0.01% w/v concentration (Figure 5A). Similar to what was observed in female mice, the male caffeine-only group's consumption started to decrease after the initial sessions with the 0.05% w/v concentration whereas the male alcohol+caffeine group remained high (3 mg/kg/day vs. 10 mg/kg/day). However, this decrease was not as drastic as was observed in the female mice. Generally, male mice consumed more caffeine in the alcohol+caffeine group compared to the caffeine only group.

Preference

The average caffeine preference for both caffeine-alone mice and alcohol+caffeine was compared at the three caffeine concentrations: 0.01% w/v, 0.03% w/v, and 0.05% w/v. A two-way mixed factor ANOVA comparing caffeine performance when presented with either caffeine alone or alcohol+caffeine revealed a significant main effect of concentration ($F(5, 40) = 21.81$, $p < 0.0001$), sex ($F(3, 8) = 15.68$, $p < 0.01$), and interaction of concentration and sex ($F(15, 40) = 2.581$, $p < 0.01$). Mice exhibited a small preference of the 0.03% w/v concentration over the 0.01% w/v and 0.05% w/v concentrations during week 1 (Figure 5B). Male and female mice differed in their preference for the caffeine and alcohol+caffeine at the initial 0.05% w/v concentration until week 3; past this point, the preference for both sexes began to nearly stabilize with one another. Both sexes preferred the alcohol+caffeine over the caffeine alone group.

Somatic Signs

Twenty-four hours following withdrawal from either alcohol, caffeine, or alcohol+caffeine on day 27, 20-minute videos were taken of each mouse in which somatic signs were recorded and later measured by a reviewer who was blinded to the condition of the mouse being observed. Measured signs included: grooming, tremors, wet dog shakes, head shakes, chattering, tail rattling, body twitching, jumping forwards, cage scratching, digging, rearing, retropulsion, mastification, hind foot scratching/shaking, hunched position, and escape attempts. A two-way between subjects ANOVA revealed no significant main effect of treatment group, sex, or interaction between treatment group and sex. Generally, females tended to have more somatic signs compared to males regardless of treatment group, albeit non-significantly (Figure 6).

Discussion

The present study was designed to develop an animal model of caffeine and alcohol co-consumption that mimics that seen in the human population and investigated the effects of both alcohol and caffeine withdrawal on anxiety-like behavior in C57BL/6J mice in order to better understand human behavior associated with caffeine and alcohol consumption and withdrawal. Previous studies have largely focused on systemically injected caffeine or forced continuous access for long durations (Rezvani et al., 2013; Hughes, 2011). Additionally, while a few studies have examined voluntary binge-like caffeine and alcohol consumption in mice under a 2-bottle choice paradigm (Fritz et al., 2014), this study is the first to evaluate caffeine and alcohol consumption in mice via IA. IA has been shown to be advantageous in C57BL/6J mice because it induces an escalated and preferential intake, which may mimic a feature of human alcohol dependence (Hwa et al., 2011). For this reason, IA was chosen for use in the present study along with the two-bottle-choice paradigm (alcohol alone, caffeine alone, or alcohol+caffeine) to observe the comorbid effects on anxiety-like withdrawal behaviors between female and male mice.

Consumption levels were examined to determine potential sex differences in the consumption and preference for alcohol or caffeine (Figure 1-5). Consistent with previous studies, female rodents were found to consume significantly more alcohol than male rodents (Witt, 2007) when animals were presented with alcohol alone (Figure 1A). However, males and females did not present significant differences in their preferences for alcohol alone (Figure 1B). Caffeine consumption levels in the current study were also consistent with the results of previous research, (Figure 2A), which has found that higher concentrations result in higher consumption levels (Fritz et al., 2014). The literature has also shown that female rodents consume more caffeine compared to males (Vener and Krupka, 1982; Short, Drago, & Lawrence, 2006). This was also consistent, as females consumed more caffeine than males, and had a higher preference for caffeine alone compared to males (Figure 2B). However, both females and males significantly reduced caffeine consumption and preference starting at week 2. The reason for this could have been that the mice no longer felt the rewarding effects of the caffeine, i.e., becoming tolerant to the drug concentration and no longer wanting to consume it. It could also have been due to boredom or little feelings of reward.

Both males and females consumed more of the alcohol+caffeine solution at the highest concentration compared to the lowest concentration (20% v/v alcohol + 0.05% w/v caffeine, 3% v/v alcohol 0.01% w/v caffeine, respectively). Their preferences, however, were both highest at the second concentration, 6% v/v alcohol + 0.03% w/v caffeine, that was utilized during the first week (Figure 3C). In contrast to the alcohol and caffeine alone conditions, females in the combined alcohol+caffeine group did not generally consume or prefer the solution more than the males at any concentration (Figure 3). The exact reasons for this are unclear. Some research suggests there may be an increase of adenosine receptor density in male rats compared to females (Butler, Smith, Berry, Sharrett-Field, & Prendergast, 2009). This could indicate that females need to consume less to develop a tolerance compared to males. However, further testing and research is required to corroborate the current findings. When comparing the alcohol alone group to the alcohol+caffeine

group, there was a significant difference found between consumption levels, particularly between the sexes. Alcohol alone females consumed significantly more compared to the alcohol+caffeine females; however, this significance was not observed between the males of either condition (Figure 4A). Both alcohol alone females and males preferred their conditions at the highest concentration compared to the alcohol+caffeine males and females, albeit non-significantly (Figure 4B). There were also significant differences found between consumption levels of caffeine alone and alcohol+caffeine, with the alcohol+caffeine group consuming and preferring significantly more than the caffeine alone groups at the highest condition of 20% v/v alcohol + 0.05% w/v caffeine (Figure 5A, 5B).

In humans, anxiety has been found to be positively correlated with alcohol withdrawal (Sullivan, Sykora, Schneiderman, Naranjo, & Sellers, 1989). Caffeine administration has been shown to increase alcohol intake, and, along with it, to increase the anxiety-like behaviors associated with alcohol withdrawal (Marczinski & Fillmore, 2006). In order to measure post-withdrawal anxiety levels, the current study examined the behaviors of mice following alcohol, caffeine, or combined treatment via somatic sign models. The withdrawal signs were chosen based on studies that have examined alcohol and/or nicotine withdrawal in mice (Damaj et al., 2003; Locklear et al., 2012); thus, this was a novel measure of anxiety-like behaviors in mice experiencing alcohol and caffeine withdrawal. Unfortunately, there was no significant difference in either male or female mice in the number of somatic signs observed in the alcohol+caffeine group compared to the other experimental conditions, including the control (Figure 6). A potential reason for this could be due to the time frame in which the videos were taken, as mice have been known to have optimal windows in which to measure withdrawal signs following the final dosage. Consequently, the present study could have recorded the signs before or after the mice experienced withdrawal symptoms, resulting in non-significant results. Previous studies have also commonly utilized other measures, such as locomotor activity monitors, balance beams, and elevated plus mazes, which may offer a more sensitive measure of withdrawal-induced anxiety in mice following

alcohol+caffeine co-consumption (Fritz et al., 2014; Rezvani et al., 2013). Given this information and lack of significant findings in the current experiment, somatic sign models may not be the best indicator of anxiety-like symptoms in mice undergoing alcohol+caffeine withdrawal.

This is the first study that has surveyed the consequences of combined alcohol and caffeine consumption under this particular drinking paradigm. Thus, the present data indicate that the concentrations of caffeine and alcohol affected consumption levels. Furthermore, the data indicates that the presence of alcohol does affect the amount of caffeine consumed; however, it does not indicate that the presence of caffeine had an effect on the amount of alcohol consumed. When compared to human behavior, this data suggests that consumption and dependence on alcohol may increase caffeine consumption, but not the reverse. Prior studies examining whether caffeine influences alcohol intake in mice, however, have yielded mixed results, with some showing an increase in alcohol intake (Franklin, Hauser, Bell, & Engleman, 2013; Fritz et al., 2016), a decrease in intake (Rezvani et al., 2013), or no effect (Hughes, 2011; Fritz et al., 2014). These contrasting findings may be due to the dosages of alcohol and caffeine chosen for the experiment. A multitude of alcohol and caffeine co-consumption doses have been tested in previous literature; however, it may be that a narrow dose range of caffeine facilitates an increase in alcohol drinking behavior in laboratory rats (Kunin, Gaskrin, Rogan, Smith, & Amit, 2000). In the present study, female and male mice both seemed to prefer the caffeine concentration at 0.03% w/v in the caffeine alone and alcohol+caffeine conditions (Figures 2B, 3C). This is roughly equivalent to an energy drink (e.g., Red Bull) and has been shown to be successful in previous studies (Fritz et al., 2016). Therefore, the 0.05% w/v caffeine concentration may have been too high, and, consequently, did not inflict the predicted increase in alcohol+caffeine consumption.

There were limitations of this study that may explain the results gathered during the length of the experiment. The sample size of the study served as a significant limitation that may have impacted the results and limited the external validity of the findings. Future research should include a larger sample in order to increase confidence in extrapolating the results. Further, mice were

exposed to the first three alcohol concentrations (3% v/v, 6% v/v, and 10% v/v) and the first two caffeine concentrations (0.01% w/v and 0.03% w/v) for only a single day during the first week, whereas mice were exposed to the highest concentrations of alcohol (20% v/v) and caffeine (0.05% w/v) for the final three weeks. A more balanced distribution of these concentrations, such as presenting each concentration for one week, may have been more beneficial to observe distinct consumption levels and preferences at the different concentrations. Another option could also be to expose different groups to different concentrations altogether. Due to time constraints, the videos for the somatic signs were each scored solely by a single blinded individual. This is one likely reason for the non-significance observed in the anxiety-like behavior. Future research should utilize multiple blinded scorers per video and it would also be beneficial to include other, more objective measures of anxiety-withdrawal signs (i.e. elevated plus maze, acoustic startle response) as well. Additionally, rather than utilizing IA, future studies may employ other consumption paradigms, such as drinking-in-the-dark (DID) or continuous access (CA), to find out whether a different model would show a greater level of alcohol+caffeine co-consumption in mice. As previous studies have shown that the age of an animal can play a role in the amount of substance consumed as well as withdrawal from these substances (Squeglia, Boissoneault, Van Skike, Nixon, & Matthews, 2014), additional studies using the same two-bottle choice paradigm across various age groups for extended periods of time would provide better insight into the long-term effects of alcohol and caffeine co-dependence on measures of anxiety.

In sum, the current study is the first to make use of the IA model when investigating the effects of the combination of alcohol and caffeine. Although the current study did not find increased alcohol+caffeine consumption compared to either substance alone as expected, the IA model appears to be an effective way to mimic human binge-like consumption of both substances in mice. Animal studies investigating the co-morbidity of these substances, like the current study, aim to provide valuable insights into the mechanistic overlap between alcohol and caffeine as well as the potential dangers associated with their co-consumption in humans. Given the large and growing

number of individuals consuming alcohol and caffeine in tandem with one another, more pre-clinical and clinical research is needed in order to determine why these substances are so widely co-abused and to further lines of treatment for dependence.

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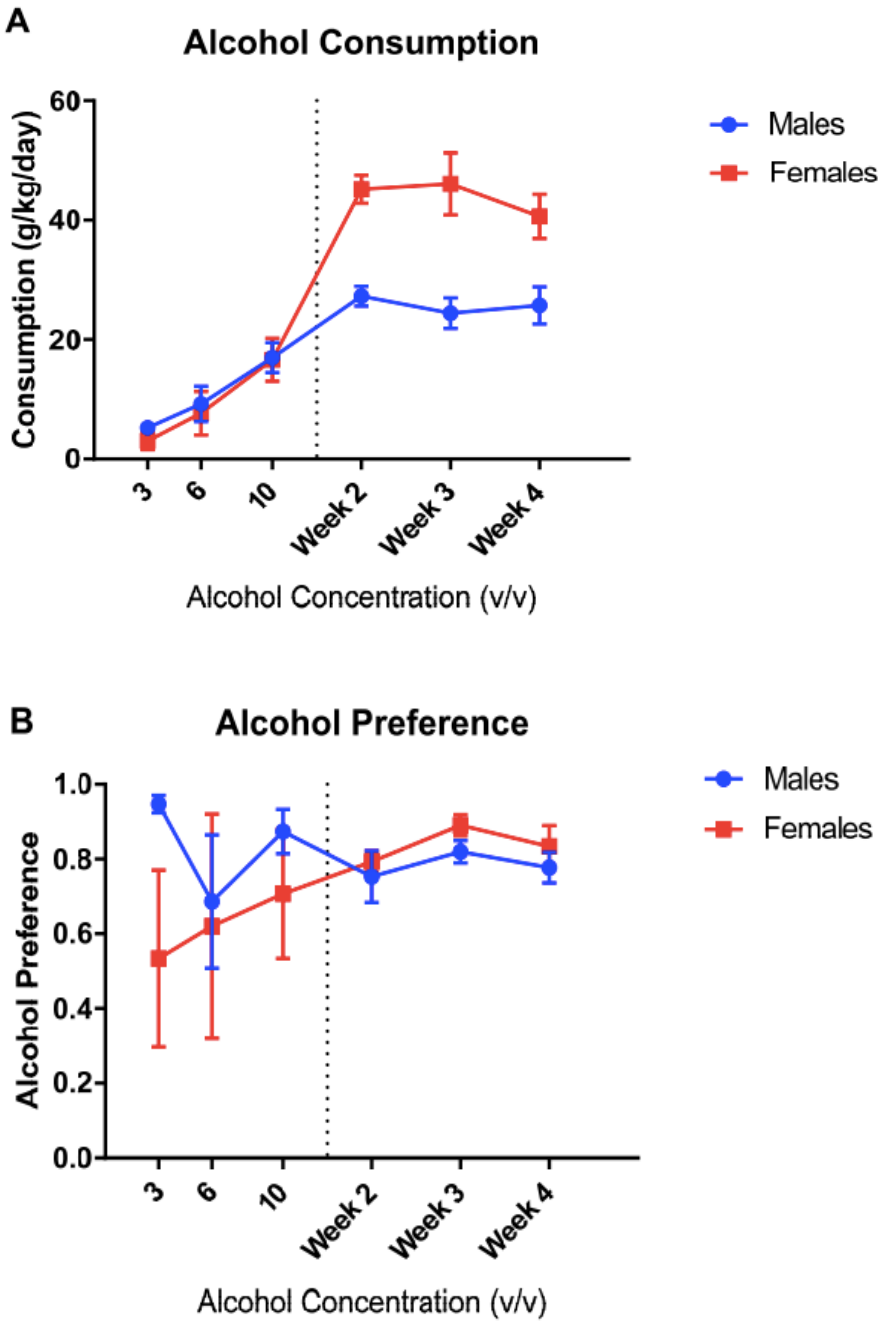


Figure 1A: Alcohol consumption in an intermittent access two-bottle choice paradigm in male (n = 3) and female (n = 3) C57BL/6J mice. The average daily consumption levels in male and female mice. Two-way ANOVA found a main effect of concentration at $p < 0.001$, sex at $p < 0.05$, and interaction of concentration and sex at $p < 0.0005$. 1B: Alcohol preference over water measured in male and female mice. No significance found.

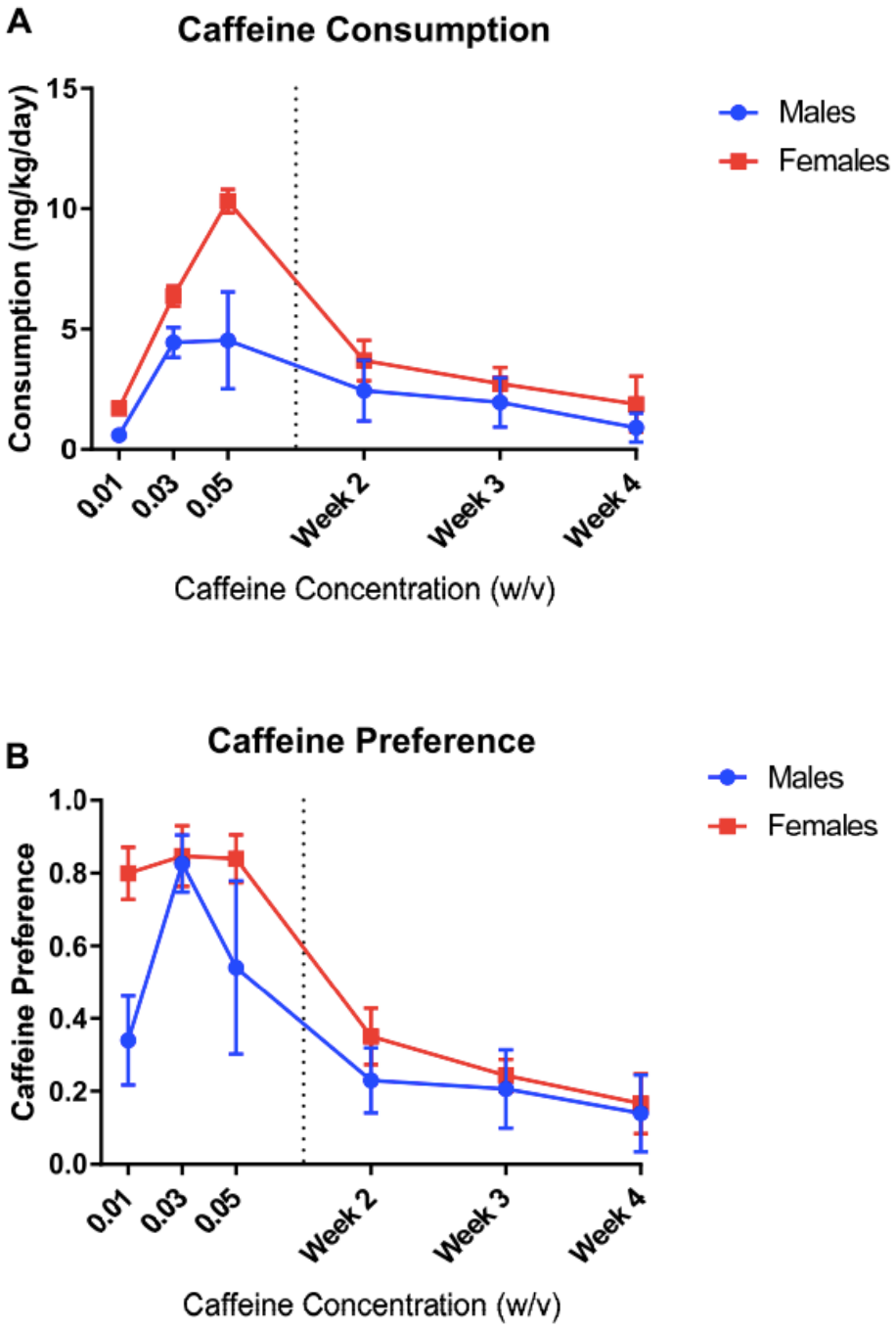
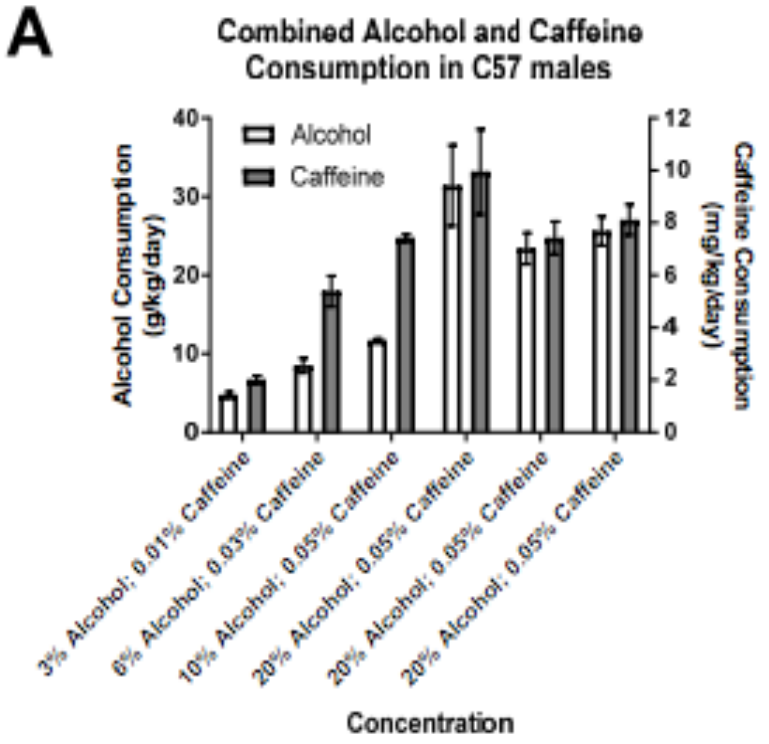
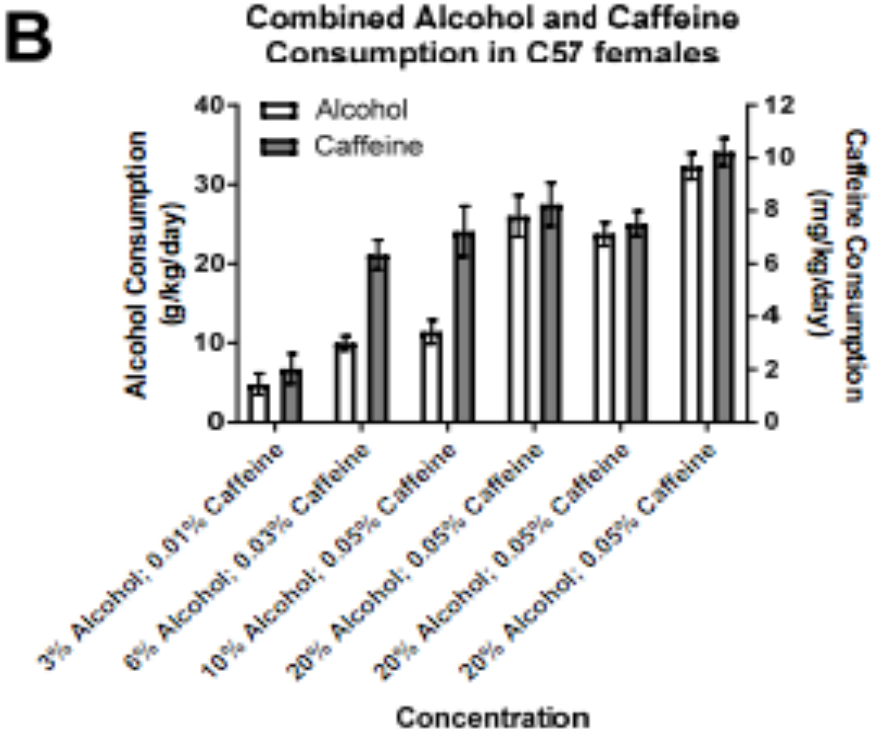


Figure 2A: Caffeine consumption in an intermittent access two-bottle choice paradigm in male (n = 3) and female (n = 3) C57BL/6J mice. The average daily consumption levels in males and female mice. Two-way ANOVA found a main effect of concentration at $p < 0.0001$ and sex at $p < 0.05$. 2B: Caffeine preference over water measure in male and female mice. Two-way ANOVA found a main effect of concentration at $p < 0.0001$.


A
Combined Alcohol and Caffeine Consumption in C57 males
Alcohol
Caffeine
Alcohol Consumption (g/kg/day)
Caffeine Consumption (mg/kg/day)
0
10
20
30
40
0
2
4
6
8
10
12
3% Alcohol; 0.01% Caffeine
6% Alcohol; 0.03% Caffeine
10% Alcohol; 0.05% Caffeine
20% Alcohol; 0.05% Caffeine
20% Alcohol; 0.05% Caffeine
20% Alcohol; 0.05% Caffeine
Concentration


B
Combined Alcohol and Caffeine Consumption in C57 females
Alcohol
Caffeine
Alcohol Consumption (g/kg/day)
Caffeine Consumption (mg/kg/day)
0
10
20
30
40
0
2
4
6
8
10
12
3% Alcohol; 0.01% Caffeine
6% Alcohol; 0.03% Caffeine
10% Alcohol; 0.05% Caffeine
20% Alcohol; 0.05% Caffeine
20% Alcohol; 0.05% Caffeine
20% Alcohol; 0.05% Caffeine
Concentration

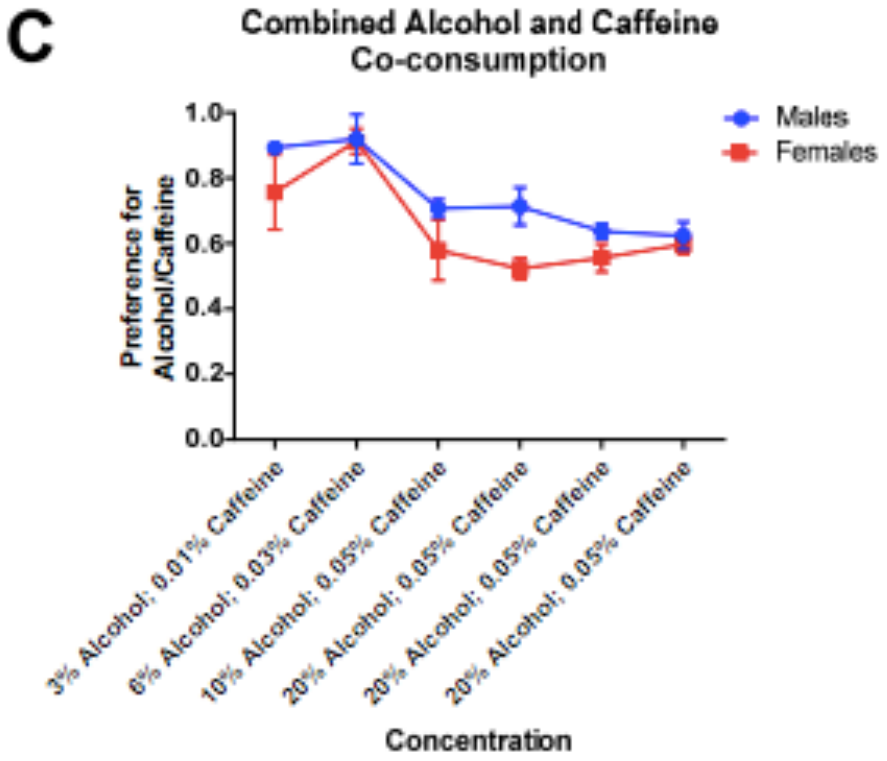


Figure 3A: Alcohol + caffeine consumption in an intermittent access two-bottle choice paradigm in male C57BL/6J mice ($n = 3$). Two-way repeated measures ANOVA found substance at $p < 0.05$, concentration at $p < 0.0001$, and interaction of concentration and substance at $p < 0.0001$. 3B: Alcohol + caffeine consumption in an intermittent access two-bottle choice paradigm in female C57BL/6J mice ($n = 3$). The average daily consumption levels in males and female mice. Two-way repeated measures ANOVA found substance at $p < 0.05$, concentration at $p < 0.0001$, and interaction of concentration and substance at $p < 0.0001$. 3C: Alcohol + caffeine preference over water in both male and female mice. Alcohol + caffeine preference over water measured in male and female mice. Two-way repeated measures ANOVA found substance at $p < 0.05$ and concentration at $p < 0.05$. Males had higher preference for alcohol + caffeine compared to females.

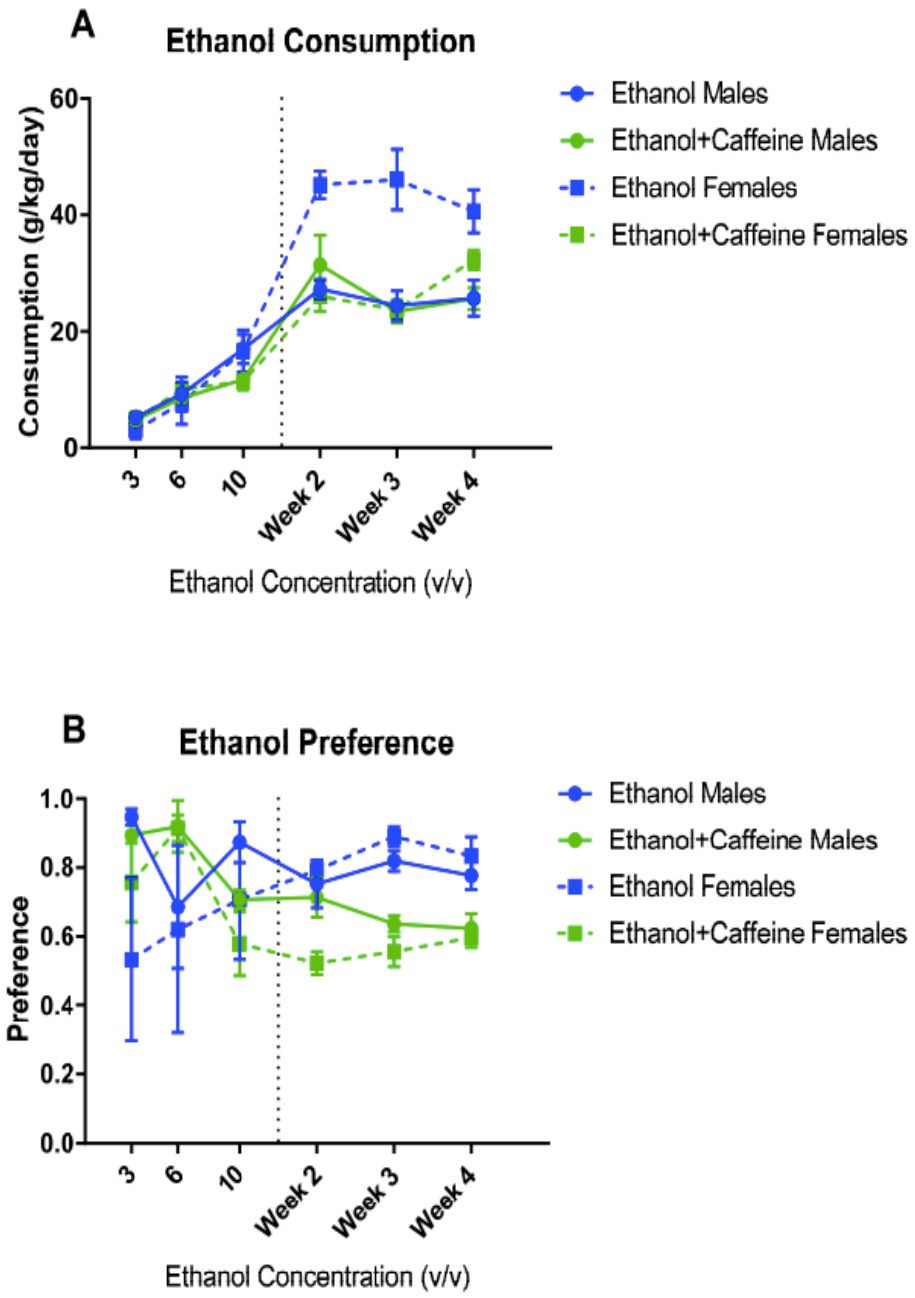


Figure 4A: Comparison of alcohol and alcohol + caffeine consumption in male (n = 6) and female (n = 6) C57BL/6J mice. Two-way mixed factor ANOVA found a main effect of concentration at $p < 0.0001$ for males. Two-way mixed factor ANOVA found a main effect of concentration at $p < 0.0001$, group at $p < 0.0001$, and interaction of concentration and group at $p < 0.05$ for females. 4B: Comparison of alcohol and alcohol + caffeine preference in male (n = 6) and female (n = 6) C57BL/6J mice. No significance found.

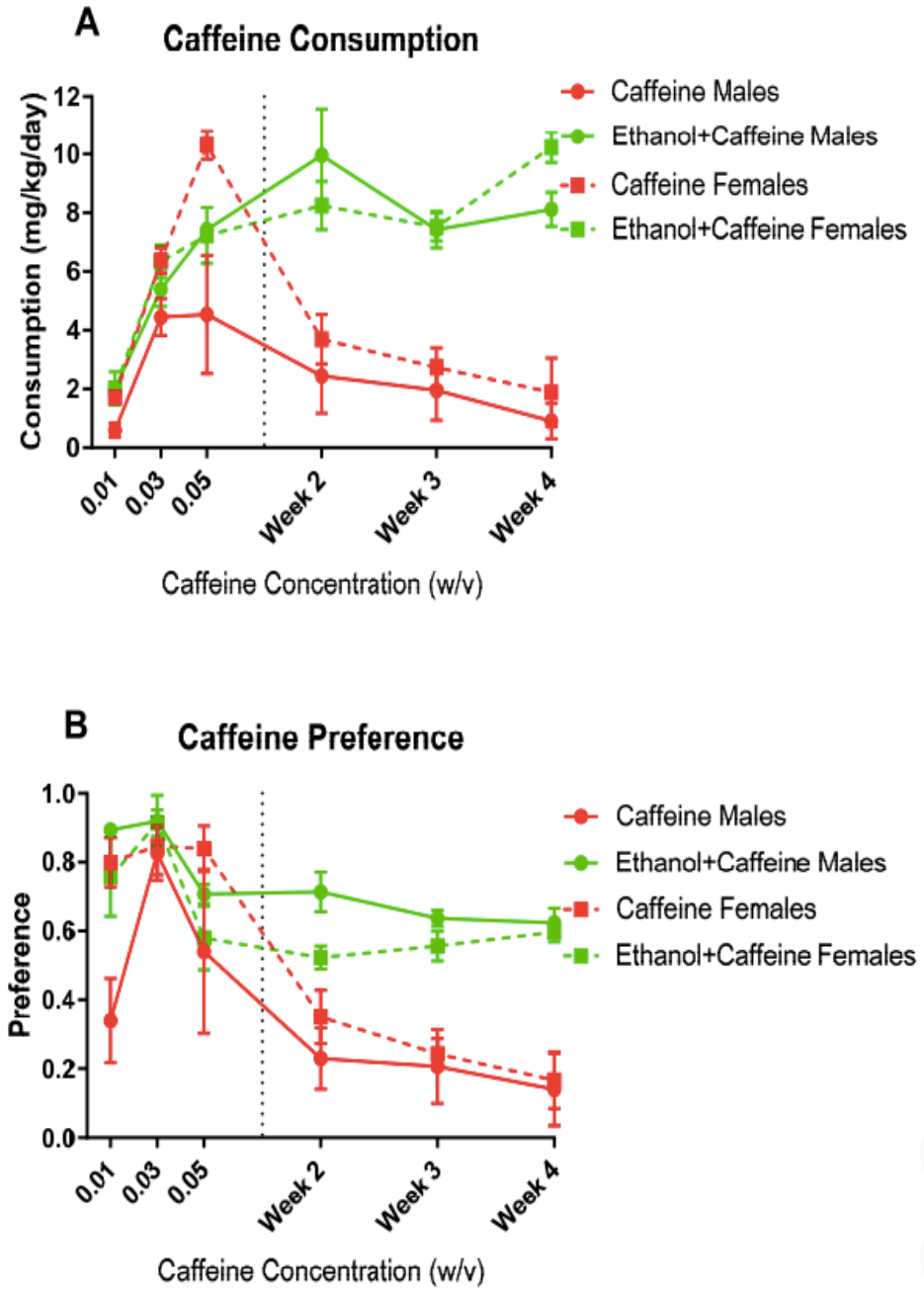


Figure 5A: Comparison of caffeine and alcohol + caffeine consumption in male (n = 6) and female (n = 6) C57BL/6J mice. Two-way mixed factor ANOVA found a main effect of concentration at $p < 0.05$, group at $p < 0.0001$, and interaction of concentration and group at $p < 0.05$ for males. Two-way mixed factor ANOVA found a main effect of concentration at $p < 0.0001$, group at $p < 0.0001$, and interaction of concentration and group at $p < 0.0001$ for females. 5B: Comparison of caffeine and alcohol + caffeine preference in male (n = 6) and female (n = 6) C57BL/6J mice. Two-way mixed factor ANOVA found a main effect of concentration at $p < 0.0001$, sex at $p < 0.05$, and interaction of concentration and sex at $p < 0.05$.

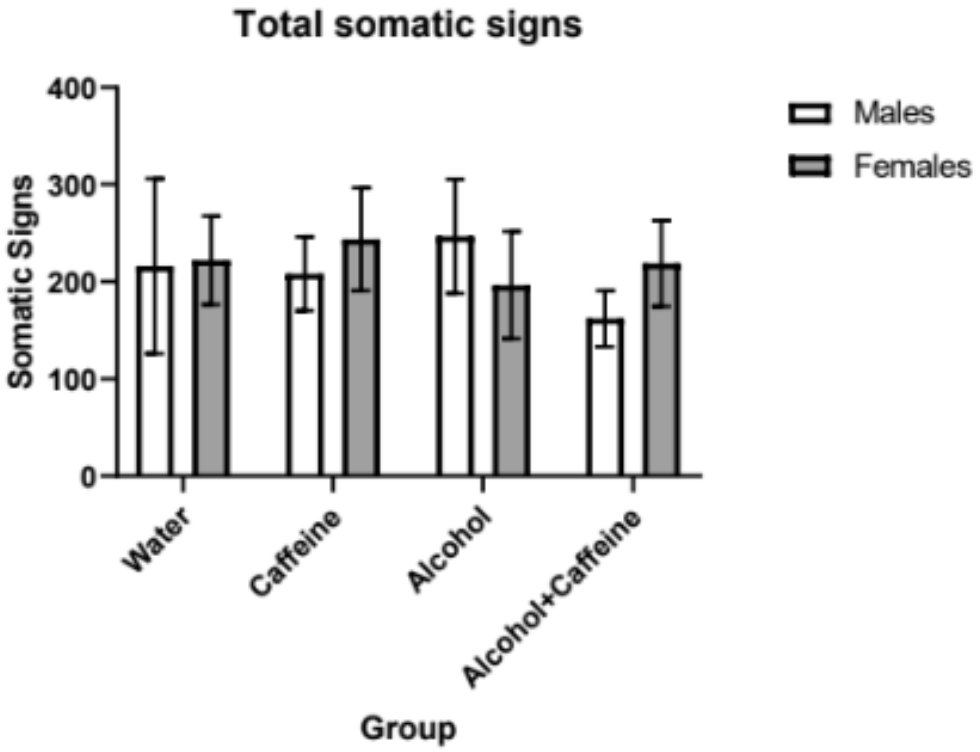


Figure 6: Somatic signs for anxiety-like behavior on Day 29 (withdrawal) in C57BL/6J male (*n* = 12) and female (*n* = 12) mice. Somatic signs in male and female mice 24 hours after withdrawal. No significance found.