Anxiety-Inducing Effects of Alcohol and Caffeine in C57BL/6J Mice

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Anxiety-Inducing Effects of Alcohol and Caffeine in C57BL/6J Mice

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of the Requirements for Graduation Honors

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Abstract

Caffeine and alcohol are two of the most popular and legal drugs around the world. In America, both drugs are easily accessible, which has contributed to frequent use of the drugs in tandem. Previous studies have revealed much about the mechanisms of each drug, but the effects of co-consumption are not yet fully understood. One concern is that caffeine could reduce how intoxicated a person feels, leading to an increase in alcohol consumption or other risky behaviors. Withdrawal is also a concern as it helps maintain the cycle of substance abuse. This study was designed to examine consumption patterns of and anxiety-like withdrawal from alcohol and caffeine in an animal model. C57BL/6J mice ($n=48$) were given caffeine (0.015% or 0.03%) and/or alcohol (3-20%) in a two-bottle choice intermittent access voluntary paradigm. Fluid consumption was recorded daily for five weeks and mice were then tested in an elevated plus maze (EPM) to assess anxiety-like behaviors during withdrawal. The amount of caffeine consumed remained steady while the amount of alcohol consumed gradually increased. Both sexes consumed more alcohol when it was paired with the lower dose of caffeine and consumed more caffeine when it was paired with alcohol. Overall, less consistent consumption patterns were found in female mice. The EPM revealed no significant differences between experimental conditions, even when sex was considered. These results suggest that a specific amount of caffeine may be necessary to impact alcohol consumption in mice, and that psychological withdrawal from these drugs may not manifest as anxiety.
Anxiety-Inducing Effects of Alcohol and Caffeine in C57BL/6J Mice

Caffeine and alcohol have become a popular combination, particularly among youth. These two drugs are largely considered commonplace and this often leads to them being trivialized or seen as harmless. While there are laws regarding the purchasing and consumption of alcohol, underage drinking is incredibly common and alcohol poisoning is not foreign to most medical professionals (NIAAA, 2017). Excessive use of alcohol (particularly binge drinking - or heavy alcohol consumption often defined as 4-5 drinks within 2 hours) can create dependence or result in deadly consequences; yet in a recent survey, nearly 40% of college students in America reported binge drinking in the previous month (NIAAA, 2017). In contrast, caffeine is viewed as less dangerous than alcohol and has fewer limitations placed upon it in America. Unlike alcohol abuse, caffeine is not frequently viewed as an addictive substance or one that can be abused (Ferre & O’Brien, 2011). When combined, the two substances can have synergistic negative health effects. The Federal Drug Administration prevents the sale of the two substances packaged together in the US, but both substances are easy to get a hold of and then ingest simultaneously. Although the availability of alcohol has decreased in the 2000s, 80% of 12th graders still reported that it would be “fairly easy” or “very easy” for them to gain access to alcohol (Johnston et al., 2016). Not only is it easy to acquire and combine caffeine with alcohol, it is increasingly popular. Over 50% of college students reported having consumed energy drinks with alcohol at parties (Malinauskas et al., 2007). Considering the increasing prevalence of this combination, research on the effects of the two together is currently lacking.
Alcohol acts as a central nervous system depressant. Alcohol affects multiple neurotransmitters, or chemicals that allow for synaptic communication (McIntosh & Chick, 2004). Alcohol can increase the release of neurotransmitters such as dopamine, norepinephrine, GABA and serotonin while decreasing the release of the primary mammalian excitatory neurotransmitter glutamate (McIntosh & Chick, 2004). Long term abuse of alcohol is associated with cerebral and subcortical atrophy (McIntosh & Chick, 2004) and liver disease (NIAAA, 2017). If an individual who is dependent on alcohol suddenly stops using the drug, it can lead to several compensatory changes that include an increase in glutamate, an influx of calcium within cells, hyperexcitability, and ultimately cell death (McIntosh & Chick, 2004). These consequences all exemplify the dangers associated with excessive alcohol use and withdrawal.

In contrast to alcohol, caffeine acts as a central nervous system stimulant. In humans, the majority of caffeine is absorbed within 45 minutes (Ferre & O’Brien, 2011). Like alcohol, caffeine operates through multiple mechanisms. Primarily, the drug acts as an antagonist for the neurotransmitter and neuromodulator adenosine, which leads to increases in the release of dopamine, noradrenaline, and glutamate (Ribeiro & Sebastiã, 2014). In larger doses, caffeine can also mobilize intracellular calcium and inhibit phosphodiesterases (Cappelletti et al., 2015). Caffeine is a competitive inhibitor that will induce the breakdown of cAMP and other molecules that “store energy” (Cappelletti et al., 2015). These mechanisms all contribute to how caffeine creates the sensation of increased energy in most humans.

While the individual mechanisms of alcohol and caffeine have been extensively studied, the mechanism behind the combination of the two drugs is still not fully
understood. The common consensus is that caffeine antagonizes the intoxicating effects of alcohol, but the molecular mechanisms beneath this opposition remains unclear (Ferre & O’Brien, 2011). Yet other studies have claimed that combining the two drugs alters the effects of caffeine alone more than it alters the effects of alcohol alone (Liguori & Robinson, 2001). While caffeine consumption does not alter blood alcohol content, it can mask the behavioral intoxication induced by alcohol (Ferreira et al., 2006). Previous studies have found a reduction in subjective measures of intoxication, but not objective measures of intoxication, when caffeine is combined with alcohol (Thombs et al., 2010). While several studies have been done in humans regarding the combined consumption, the results remain unclear as each study has used a variety of drug doses and behavioral measures (Siegel, 2011). Humans, however, have not been the only subject used to study the mechanisms behind these two drugs.

Animal models are essential to scientific research and have often been used to study substance abuse. There are several hypotheses that cannot be tested in humans due to ethical and/or realistic limitations. Animals have varying degrees of genetic differences from humans but share similarities that allow insight into the human brain and behaviors. Utilizing animal models increases internal validity as it is easier to control for confounding variables; however, it decreases external validity in terms of how a study’s findings translate to humans. The current study chose to employ a rodent model, as it allowed for voluntary consumption choices of alcohol and/or caffeine and it would be impractical and unethical to study this combination of drugs in humans in the same manner. To measure exact levels of drug consumption over five weeks, an animal model was necessary and sufficient.
A rodent model was also the optimal choice as rodents have often been utilized to examine both alcohol and caffeine consumption behaviors. In particular, C57BL/6J mice were used because they have been utilized in drug abuse research previously and found to consume more alcohol (Hwa et al., 2011). Self-administration of caffeine combined with alcohol has previously been examined and has shown that mice do have an interest in this combination (Fritz et al., 2014). However, the two drugs do not necessarily have clear additive effects when combined. For example, neither drug alone may significantly increase plasma levels of the stress hormone corticosterone. However, a combination of the two can lead to an increase in corticosterone plasma levels in rats within a half hour (Kunin et al., 2000b). Additionally, the drugs each have complex effects on anxiety that are not simplified when the two are combined (Gulick & Gould, 2009, Fritz et al., 2009). This complicated interaction effect could stem from the ability of both drugs to affect adenosine receptors (Arolfo et al., 2004; Butler & Pendergast, 2012, Cappelletti et al., 2015). Due to the complicated nature of both drugs and the interaction between the two, the current study intended to further clarify the effects of alcohol and caffeine co-consumption in mice.

While acute administration of alcohol and/or caffeine can impact anxiety and corticosterone levels, similarly complicated results have been found in studies examining withdrawal from these drugs. Alcohol and caffeine have both independently been shown to affect corticosterone levels associated with withdrawal-induced anxiety in humans and rodents. However, the effects of these drugs on anxiety remains unclear, as not only can each drug individually show either an increase or decrease in anxiety, but withdrawal from both drugs at once can also have variable outcomes (Ferre & O’Brien, 2011, Gulick &
CO-CONSUMPTION OF ALCOHOL + CAFFEINE

Gould, 2009). Although several animal models for anxiety-like behavior exist, the elevated plus maze (EPM) has been used extensively to examine anxiety in both mice and rats. Briefly, the EPM consists of two open arms and two closed arms on an elevated surface, with the notion that mice and rats prefer to spend more time in the closed arms where they are better protected against predation. It has been cited as a measure of unconditioned anxiety in over 2,000 papers in the last 20 years (Walf & Fyre, 2007). However, the EPM was initially designed to test anxiolytic drugs rather than withdrawal, and it is still debated as a reliable and valid measurement tool (Kliethermes, 2004). Some experiments have found it to be a reasonable measure of anxiety during withdrawal (Begg et al. 2005), while other studies argue that the EPM has been taken as valid and left untested (Carobrez & Bertoglio 2005).

Considering the high rates of alcohol abuse and the popularity of combining it with caffeine, there is insufficient research on the effects of the two substances together. Multiple studies have been done using both humans and animal models, but the overall findings remain inconclusive (Ferre & O’Brient, 2011; Ferreira, 2006; Fritz et al., 2014; Fulick & Gould, 2009; Kunin et al., 2000a). This study aimed to help clarify the effects that alcohol combined with caffeine can have in a mouse model. An intermittent voluntary access consumption paradigm was chosen because it is more likely to induce dependence, is better for external validity, and is typically conducive to a quick escalation of alcohol drinking (Hwa et al. 2011). Additionally, the EPM was selected to measure anxiety associated with withdrawal, as it has been useful to many previous studies (Walf & Fyre, 2007). This experiment sought to compare the consumption rates of caffeine and alcohol when presented either individually or in combination, and to observe the withdrawal effects.
of the combination of these two drugs. Specifically, varying levels of caffeine and alcohol were examined to determine how dose could mediate these effects.

Method

Subjects

The subjects of this experiment were C57BL/6J mice (n=48, 24 female and 24 male) purchased from Jackson Laboratory (Bar Harbor, ME, USA). They were 8 weeks old before the experiment began. The mice were housed in individual cages in a room set to a 12-hour reverse light/dark cycle (lights off at 7 AM) prior to and during the experiment. The mice had continuous access to food and water. Their care and conditions were maintained in accordance with the National Institutes of Health Office of Laboratory Animal Welfare as well as a protocol approved by the Institutional Animal Care and Use Committee at Butler University.

Drugs and Chemicals

Caffeine and 190 proof ethanol were purchased through Sigma (St. Louis, MO, USA). Tap water was mixed with both the caffeine and alcohol to create the desired concentrations.

Drinking Model

Each mouse was assigned to one of six condition groups (control, 0.015% caffeine, 0.03% caffeine, ethanol, 0.015% caffeine + ethanol, or 0.03% caffeine + ethanol). The mice in the ethanol condition began with 3% ethanol that was increased each week (6%, 10%) until the maximum concentration of 20% ethanol was reached. The mice always had continuous access to a bottle of tap water. The second bottle containing their condition group’s
substance was presented for 24 hours and then removed for the following 24 hours. Each mouse had the same option presented to it repeatedly throughout the entire study. The secondary (experimental) bottles were given to them and removed approximately 3 hours into their dark phase at 10 AM.

**Behavior**

An elevated plus maze (EPM) was used to observe the anxiety-like behavior that results from withdrawal following caffeine and/or alcohol. Approximately 48 hours after the drug bottles were removed, the mice were individually placed into the EPM. ANY-maze video tracking system (San Diego Instruments, San Diego, CA) tracked the movement of each mouse. The white EPM (San Diego Instruments) contained four arms, each measuring approximately 30 cm long and 5 cm wide and elevated approximately 50 cm above the floor. A lip, approximately 1 cm high, surrounded the open arms. The maze has a center platform and a wall encloses two of the four (directly opposite each other) arms. Each mouse was filmed using the ANY-maze software system for 5 min and then how long each mouse spent in each arm during the task was calculated. The EPM has been utilized in previous studies as a way to measure animal anxiety (Carobrez & Bertoglio, 2005). Mice typically prefer to spend time in the enclosed arms (Walf & Fyre 2007). The EPM was used to see if the mice were experiencing increased anxiety during withdrawal after weeks of drinking caffeine and/or alcohol. The number of entries into each arm were counted (when all four of the mouse's paws are in the arm) and the total duration of time spent in each arm was assessed. More entries into and greater time spent in the closed arms was treated as higher anxiety.
Statistical Analyses

Consumption and Preference: 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 from the bottles or evaporation that may have occurred during the experiment and was recorded every day at the same time that the other bottle weights were recorded.

Consumption values were then transformed based on the percentage of alcohol or caffeine present in the bottle for that particular day, density of the substance if applicable (i.e. 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 average daily caffeine consumption (mg/kg/day) for each mouse. The percent preference for the alcohol or caffeine was calculated as the number of mls of alcohol or caffeine water consumed divided by the total mls of fluid consumed * 100. Data was 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 (where sex is a between-subjects factor and the concentration is a within-subjects factor). In some consumption analyses (i.e. caffeine combined with alcohol), data were analyzed separately for male and female mice, as they have previously shown different consumption patterns (Butler et al., 2009).

Elevated Plus Maze: the amount of time each mouse spent in the closed arms and the open arms were separately calculated for each mouse and then averaged across condition. The number of entries into open and closed arms were also individually calculated for each mouse and then averaged across condition. The condition groups were contrasted with one
another utilizing a between-subjects ANOVA. A secondary comparison used a 2 (sex) X 6 (condition group) between-subjects ANOVA. Post-hoc tests were completed using a Student’s t-test when applicable.

Results

Caffeine Only

Consumption and preference

Mice (n=8, half male) were presented with a bottle of 0.015% caffeine every other 24 hours (every Monday, Wednesday, Friday) for 5 consecutive weeks. Another eight mice were similarly presented with 0.03% caffeine every other 24 hours. There was no statistically significant consumption or preference patterns for the mice in either of these two conditions (Figure 1). The mice consumed nearly as much water as caffeine (average preference for caffeine was 0.54 and 0.56 in the 0.015% caffeine condition and the 0.03% caffeine condition respectively), indicating that they did not prefer to consume caffeine alone compared to water, and consumption values varied largely between individual mice.

Alcohol Only

Consumption

Eight of the mice (half male) were presented with a bottle of alcohol every other 24 hours. The alcohol increased in concentration each Monday (3%, 6%, 10%, 20%) until the last week (remained at 20%). The bottles were weighed every day to observe consumption. A two-way ANOVA revealed a significant main effect of concentration ($F(2.816, 16.90) = 42.44, p<0.0001$), a significant main effect of gender ($F(1, 6) = 19.23, p<0.005$), and a
significant interaction effect \( F(14, 84) = 6.737, p<0.0001 \). As the concentration of the alcohol increased, most mice also consumed more alcohol. At the highest concentration of alcohol (20%, weeks 4-5) female mice drank significantly more alcohol than the male mice \( F(1, 6) = 19.23, p<0.005 \) (Figure 2A).

**Preference**

There was no significant main effect for sex or time, nor was there a significant interaction effect for alcohol preference in the alcohol alone condition. Across the entire experiment, both male and female mice preferred the alcohol bottles to the water bottles (male preference for alcohol was 0.62 and female preference for alcohol was 0.84).

**Alcohol + Caffeine 0.015%**

**Consumption**

The mice (n=8; 4 males and 4 females) in the alcohol + caffeine 0.015% condition (AC15) showed significant consumption patterns. Two-way ANOVAs revealed a significant main effect for time [Males: \( F(14, 90) = 36.99, p<0.0001 \); Females: \( F(14, 90) = 46.55, p<0.0001 \)] a significant main effect for substance (alcohol/caffeine) concentration [Males: \( F(1, 90) = 1062, p<0.0001 \); Females: \( F(1, 90) = 995.9, p<0.0001 \)], and a significant interaction effect [Males: \( F(14, 90) = 36.91, p<0.0001 \); Females: \( F(14, 90) = 41.12, p<0.0001 \)]. On average, the mice consumed more of the combined drugs over time, as alcohol concentration increased (Figure 3A-B).

**Preference**
When comparing the preference of water to AC15, there was no significant main effect for time, but there was a main effect for sex \((F(1, 6) = 8.232, p<0.05)\). There was no significant interaction effect (Figure 4A). On average, the mice showed a strong preference for the combined drugs over water. The male mice had a slightly stronger preference for the drug combination with an average preference of 0.92 while the female mice had an average preference of 0.85.

**Alcohol + Caffeine 0.03%**

**Consumption**

The mice \((n=8; 4\) males and 4 females) in the alcohol + caffeine 0.03% condition (AC30) also showed significant consumption patterns. There was a significant main effect for time [Males: \((F(1, 90) = 24.03, p<0.0001)\); Females: \((F(1, 90) = 25.22, p<0.0001)\)], a significant main effect for substance (alcohol/caffeine) concentration [Males: \((F(1, 90) = 371.6, p<0.0001)\); Females: \((F(1, 90) = 275.9, p<0.0001)\)], and a significant interaction effect [Males: \((F(14, 90) = 20.48, p<0.0001)\); Females: \((F(14, 90) = 17.97, p<0.0001)\)]. AC30 consumption rose relatively consistently over time as alcohol concentration increased (Figure 3C-D).

**Preference**

The same preference patterns were seen in the AC30 group as in the AC15 group. There was no significant main effect for time, but there was a main effect for sex \((F(1, 6) = 6.656, p<0.05)\). There was no significant interaction effect (Figure 4B). On average, the mice showed a strong preference for the combined drugs over water. The male mice had a
stronger preference for the drug combination with an average preference of 0.83 while the female mice had an average preference of 0.67.

**Comparison of Alcohol Conditions**

*Males*

A two-way ANOVA compared the consumption behaviors of the male mice in the three experimental conditions that involved alcohol (alcohol alone, AC15, and AC30). There was a significant main effect of treatment group ($F(2, 9) = 12.71, p<0.01$), a significant main effect of concentration ($F(4, 36) = 131.7, p<0.0001$), and a significant interaction effect ($F(8, 36) = 4.116, p<0.01$). Male mice in all three conditions drank more alcohol each time the amount of alcohol increased (however they all drank less in the 5th week). The AC15 condition drank significantly more alcohol than the alcohol alone condition in weeks 4-5 and significantly more than the AC30 condition during week 5 (figure 5A).

*Females*

A two-way ANOVA compared the consumption behaviors of the female mice in the three experimental conditions that involved alcohol (alcohol alone, AC15, and AC30). There was a significant main effect of treatment group ($F(2, 9) = 5.885, p<0.05$), a significant main effect of concentration ($F(4, 36) = 238, p<0.0001$), and a significant interaction effect ($F(8, 36) = 2.409, p<0.05$). Female mice in all three conditions drank more alcohol each time the amount of alcohol increased (however they all drank less in the final week). The mice in the AC15 condition drank significantly more alcohol than mice in the AC30 condition during weeks 3-5. Mice in the alcohol alone condition consumed significant less
than their counterparts in the AC15 condition during week 5 and consumed significantly more than their counterparts in the AC30 condition during week 4 (figure 5B).

**Comparison of Caffeine Conditions**

*Males*

A two-way ANOVA compared the consumption behaviors of the male mice in the four experimental conditions that involved alcohol (0.015% caffeine alone, 0.03% caffeine alone, AC15, and AC30). There was a significant main effect for treatment group ($F(3, 12) = 31.90, p<0.0001$), but there was no significant main effect of concentration (week) nor was there a significant interaction effect.Collapsed across weeks, the AC30 male group drank significantly more caffeine than every other male group, whereas the 0.015% caffeine alone group consumed the least amount of caffeine overall (figure 6A).

*Females*

A two-way ANOVA compared the consumption behaviors of the female mice in the four experimental conditions that involved alcohol (0.015% caffeine alone, 0.03% caffeine alone, AC15, and AC30). There was a significant main effect for treatment group ($F(3, 12) = 8.716, p<0.01$), however there was no significant main effect of concentration (week). There was also a significant interaction effect ($F(12, 48) = 2.339, p<0.05$). There were a few significant differences between treatment groups, but none that lasted for more than two weeks (figure 6B).

**Elevated Plus Maze**
After the 5-week drinking paradigm, all 48 mice were individually placed in the EPM for 5 minutes. Their entries into each arm were counted and the amount of time they spend in each arm was recorded to look for patterns indicative of anxiety. The number of entries each mouse made into the open arms and into the closed arms of the EPM were averaged within each experimental condition. There was no statistically significant difference between conditions for either type of arm (Figure 7A). The time spent in the open arms and in the closed arms of the EPM were also averaged within each experimental condition. There was no statistically significant difference between conditions for either type of arm (Figure 7B). When this data was analyzed within each sex, there was still no statistical significance.

Discussion

The current study sought to investigate the dose-dependent effects of the combination of caffeine and alcohol in a two-bottle choice intermittent access voluntary paradigm. The results showed that mice did not have a preference for either low or moderate amounts of caffeine; however, when low caffeine was combined with increasing concentrations of alcohol, the mice consumed larger quantities and developed a strong preference that was significantly different from that seen with either alcohol alone or alcohol combined with a higher concentration of caffeine. Overall, results showed that caffeine was more readily consumed by C57BL/6J mice when it was combined with alcohol, which was also readily consumed by both sexes.

In the current study, the mice showed little interest in consuming large, physiologically relevant amounts of caffeine alone. In both conditions where mice were
presented with only caffeine, they showed no significant preference for the caffeine over water. This finding contrasts with previous studies that found notable caffeine consumption in mice; however, these studies added sucrose to the caffeine (Robins et al., 2016) or the mice did not have a water option (Fritz et al., 2014). These distinctions could be sufficient to explain the difference in caffeine consumption across studies.

The consumption patterns of mice presented with alcohol alone are in agreement with what has been observed in previous studies (Fritz et al., 2014, Hwa et al., 2011). Mice exhibited a significant preference for alcohol over water throughout the experiment, and as alcohol content increased, so did consumption. Additionally, at the highest concentration (20%), female mice consumed significantly more alcohol than male mice, which is in line with previous research both in the current lab and in previous literature (Hwa et al., 2011).

Although the current results showed that mice did not consume large quantities of caffeine alone, when combined with alcohol the consumption increased dramatically. Male mice in the AC15 and AC30 conditions consumed larger amounts of caffeine than male mice in the 0.015% caffeine and 0.03% caffeine conditions, respectively, and this difference was significant after the first two weeks. Female mice exhibited similar patterns, although consumption was more variable between individual mice. Previous research in this lab observed that mice tended to ignore caffeine alone but prefer alcohol to water, therefore it would follow that adding alcohol made caffeine more appealing.

The concentration of caffeine significantly impacted the consumption of the alcohol + caffeine combination, particularly as the concentration of the alcohol rose over the course of the experiment. Male mice in the AC15 condition drank significantly more alcohol than
the mice in the alcohol alone condition (weeks 4-5) and the AC30 condition (week 5). Female mice in the AC15 condition also consumed significantly more alcohol compared to the AC30 condition (weeks 3-5) and the alcohol alone condition (week 5). These findings contrast with previous work done in our lab showing no significant differences between alcohol consumption with or without caffeine. The previous study utilized 0.05% caffeine for nearly the entirety of the study, and in light of the current results, that would suggest 0.05% caffeine is too high to significantly alter alcohol consumption. The results of the current study, however, are more consistent with those found by Kunin et al. (2000a) in rats - an increased preference for alcohol is dependent on the dose of caffeine present. The current findings are unique as this has yet to be shown in a voluntary co-consumption paradigm with a rodent model. Previous studies either used forced caffeine consumption with voluntary alcohol consumption (Kunin et al., 2000a), presented the drugs independently (Robins et al. 2016) or did not see a change in alcohol consumption patterns (Fritz et al., 2014).

The current results varied greatly across sex, as was not completely unexpected. Female mice typically had less consistent consumption patterns, especially when examining their caffeine consumption. As mentioned before, female mice also consumed significantly more alcohol than male mice, which is line with previous research (Hwa et al., 2011). The data was notably different when broken apart by sex, and therefore all of the consumption patterns were examined across sex. However, the EPM maintained the lack of significant findings when the data was separated by sex. This is in contrast with what other studies have seen with regards to sex differences during alcohol withdrawal but is likely due to the overall lack of significance (Butler et al., 2009).
Unfortunately, the current study found no significant differences in regards to the EPM. This lack of significance could indicate a few possible findings. There could be confounding variables that inhibited any significant patterns from emerging. For example, the time between when individual mice last consumed substances varied due to the voluntary nature of the paradigm and the time needed to administer the EPM). Previous research has also suggested that the light present during a behavioral test could interfere with finding results, and this study utilized a bright room during the mice’s typical dark period (Perez & De Biasi, 2015). This lack of significance may instead support the claim made by some previous studies that the EPM may not be the best measure of anxiety in rodents (Begg et al., 2005; Kliethermes, 2004). These results could also suggest that withdrawal from these drugs at the doses examined in the current study does not have a significant impact on anxiety.

This study provided further information about the effects of the combination of alcohol and caffeine, yet many questions surrounding this research remain. Future research could examine other doses of alcohol in combination with either low or moderate amounts of caffeine. Interesting consumption patterns began to emerge when low caffeine was combined with 10% alcohol but these patterns were overwhelmed with 20% alcohol. It may be that low amounts of caffeine augment low levels of alcohol, but at higher concentrations of alcohol the effect of caffeine is minimized. The current study suggests that the alcohol and caffeine content both appear to be important variables in the consumption of combined alcohol+caffeine, and thus could both be examined further.

The age of the mice could also be an important factor and potentially an interesting avenue for future research. In humans, the heavy consumption of alcohol combined with
caffeine has been reported most frequently in young adults (Malinauskas et al., 2007). Previous studies have shown that adolescent caffeine consumption did not alter subsequent adult voluntary alcohol intake or preference in mice (Robins et al., 2016). However, no study to date has examined the effects of the consumption of alcohol and caffeine in adolescent mice and thus there could be illuminating results by presenting mice with both drugs earlier in life or by reversing the order of drug presentation.

Although the current study found no significant group differences in anxiety-like behavior, it is worth noting that only one behavioral task was examined and a single timepoint, thus studies investigating other models of withdrawal-induced anxiety-like behavior are warranted. Previous studies examining alcohol withdrawal in mice have utilized other measures of anxiety-like behavior such as the open field arena with success, and perhaps it would be more effective for the combination of alcohol and caffeine (Perez & De Biasi, 2015). Although the best way to objectively measure anxiety during withdrawal remains unclear, the light/dark box may be another method worth utilizing (Kliethermes, 2004). All three tests (EPM, open field, and light/dark box), however, may not quantify anxiety as well as once thought due to their reliance on voluntary movement, and a confound with overall decreased locomotion during withdrawal can be found (Kliethermes, 2004).

In sum, although extensive research exists on the effects of alcohol in mice, there is much less research regarding the effects of caffeine and even less on the combination of alcohol and caffeine. The current study was designed to address this gap in knowledge. It was the first study to our knowledge to attempt to understand the dose dependent effects of caffeine on alcohol while using a voluntary access paradigm for both drugs. Our results
suggests that low, but not moderate, amounts of caffeine may increase alcohol consumption and preference in both male and female mice. Given the increasing popularity of the combination of alcohol and caffeine, more research is needed to determine why these substances are so often co-abused and any physical or psychological dependence resulting from this combination.
References


Fig 1. Caffeine consumption in the five-week intermittent voluntary access drinking paradigm in C57 mice. Two-Way ANOVA found no significant effects. (A) Consumption by the mice in the Caffeine 0.015% condition for male (n = 8) and female (n = 8) mice. (B) Consumption by the mice in the Caffeine 0.03% condition for male (n = 8) and female (n = 8) mice.
Fig. 2. Consumption patterns for the alcohol only condition in the five-week intermittent voluntary access drinking paradigm in male (n = 8) and female (n = 8) C57 mice. (A) The consumption of the alcohol bottles. Two-way ANOVA found significant concentration (p<0.0001), gender (p<0.005), and interaction (p<0.0001) effects. (B) The preference for alcohol alone over water. Male preference was .62 and female preference was .84.
Fig 3. Consumption of alcohol and caffeine in C57 mice that were presented with a bottle containing both for the five-week intermittent voluntary access drinking paradigm. (A) Average consumption of alcohol and caffeine by male mice presented with 0.015% caffeine (n=4). (B) Average consumption of alcohol and caffeine by female mice presented with 0.015% caffeine (n=4). (C) Average consumption of alcohol and caffeine by male mice presented with 0.03% caffeine (n=4). (D) Average consumption of alcohol and caffeine by female mice presented with 0.03% caffeine (n=4).
Fig 4. Preference for alcohol + caffeine over water during the five-week intermittent voluntary access drinking paradigm in male and female C57 mice. (A) Average preference for the combined drugs over water in the alcohol + caffeine 0.015% condition (n=8). (B) Average preference for the drugs over water in the alcohol + caffeine 0.015% condition (n=8).
Fig 5. Comparison of alcohol consumption between C57 mice placed in three experimental groups that contained alcohol for five weeks. (A) Results for male mice (n=12, 4 per group). (B) Results for female mice (n=12, 4 per group).
Fig 6. Comparison of caffeine consumption between C57 mice placed in four experimental groups that contained caffeine for five weeks. (A) Results for male mice (n=16, 4 per group). (B) Results for female mice (n=16, 4 per group).
Fig 7. Results from placing C57 mice (n=48) in an Elevated Plus Maze after five weeks of an intermittent drinking paradigm. (A) Entries made into open and closed arms of the maze by which condition the mice were in. (B) Time spent in open and closed arms of the maze by which condition the mice were in.