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Three Weeks of Binge Alcohol Drinking Generates Increased Alcohol Front-Loading and Robust Compulsive-Like Alcohol Drinking in Male and Female C57BL/6J Mice

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Abstract

Background: Current models of compulsive-like quinine-adulterated alcohol (QuA) drinking in mice, if improved, could be more useful for uncovering the neural mechanisms of compulsive-like alcohol drinking. The purpose of these experiments was to further characterize and improve the validity of a model of compulsive-like QuA drinking in C57BL/6J mice. We sought to determine whether compulsive-like alcohol drinking could be achieved following 2 or 3 weeks of Drinking-in-the-Dark (DID), whether it provides evidence for a robust model of compulsive-like alcohol drinking by inclusion of a water control group and use of a highly concentrated QuA solution, whether repeated QuA exposures alter compulsive-like drinking, and whether there are sex differences in compulsive-like alcohol drinking.

Methods: Male and Female C57BL/6J mice were allowed free access to either 20% alcohol or tap water for 2 hours each day for approximately 3 weeks. After 2 or 3 weeks, the mice were given QuA (500 μ M) and the effect of repeated QuA drinking sessions on compulsive-like alcohol drinking was assessed. 3-minute front-loading, 2 hour binge-drinking, and blood alcohol concentrations were determined.

Results: Compulsive-like QuA drinking was achieved after 3 weeks, but not 2 weeks, of daily alcohol access as determined by alcohol history mice consuming significantly more QuA than water history mice and drinking statistically nondifferent amounts of QuA than nonadulterated alcohol at baseline. Thirty-minute front-loading of QuA revealed that alcohol history mice front-loaded significantly more QuA than water history mice, but still found the QuA solution aversive. Repeated QuA exposures did not alter these patterns, compulsive-like drinking did not differ by sex, and BACs for QuA drinking were at the level of a binge.

Conclusions: These data suggest that compulsive-like QuA drinking can be robustly achieved following 3 weeks of DID and male and female C57BL/6J mice do not differ in compulsive-like alcohol drinking.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

Keywords

Alcohol; Binge; Compulsion; Quinine; Aversion-Resistant

A defining feature of alcohol use disorder (AUD) is compulsive alcohol drinking, characterized by drinking alcohol despite the knowledge of negative consequences (American Psychiatric Association, 2013). Quinine-adulterated alcohol (QuA) is a commonly used rodent model of compulsive-like alcohol taking and has been used to assess molecular and circuit-based mechanisms of AUD (Hopf and Lesscher, 2014; Patton et al., 2020; Siciliano, et al., 2019; Wolffgramm and Heyne, 1991). While this model affords promise, the complete characterization and validity for a compulsive-like QuA-drinking phenotype in mouse models has not been fully established, and the detriment that compulsive alcohol drinking serves for treatment and prevention of AUD has not diminished. Because of this, further characterization and increased validity of the model are warranted to improve the discovery of the biological underpinnings of compulsive alcohol drinking.

Compulsive alcohol drinking is exemplified by making decisions to consume alcohol with clear knowledge of a negative consequence. For instance, a compulsive alcohol drinker may choose to drink alcohol during work hours being fully aware that this behavior may result in the loss of income. Additionally, a person may compulsively drink a nonbeverage or surrogate alcohol solution (e.g. methanol) being fully aware that this may result in an aversive taste and poisoning (Egbert et al., 1985; Green et al., 2018; Lachenmeier et al., 2007; Leon et al., 2007). QuA is a commonly used rodent model of compulsive-like alcohol taking which leverages alcohol drinking paradigms where rodents freely consume alcohol for some period prior to adulteration with bitter tasting quinine. In doing this, the animal now faces the disgusting and aversive tasting solution in order to consume the alcohol, thus creating a negative consequence associated with alcohol drinking. This model was first employed in rats (Wolffgramm and Heyne, 1991) but has been adapted and utilized in mice (Fachin-Scheit et al., 2006). In the current mouse models using QuA, large discrepancies exist in terms of alcohol drinking history, quinine concentration used, use of an alcohol naïve control, and investigation into sex differences (please see Table 1 for a summary).

Previous research using QuA drinking models in mice primarily uses either 2-bottle choice (2BC), Drinking-in-the-Dark (DID), or limited daily access procedures (LDA). The general procedure involves mice drinking in one of these models for a set amount of time, and then having their alcohol solution adulterated with quinine with concentrations varying from 100 to 1,000 μM , though most commonly 100 to 250 μM has been used. This has primarily been done in male mice with no investigation into sex differences, though more recently female mice have been included in QuA experiments (Bocarsly et al., 2019; Houck et al., 2019; Sneddon et al., 2020) with some directly testing sex difference hypotheses (Sneddon et al., 2019; Top of Form Fulenwider, et al., 2019; Shaw et al., 2020). Compulsive-like QuA drinking is then determined by the definition wherein compulsive mice will consume the same amount of QuA as nonadulterated alcohol, whereas noncompulsive mice would consume significantly less QuA than nonadulterated alcohol.

Additionally, compulsive-like QuA drinking should theoretically differ in alcohol history mice compared with alcohol naïve mice, though most experiments lack this water control group. Inclusion of the water (alcohol naïve) control group is further necessitated by the aforementioned differing QuA concentrations used to produce aversion because if the QuA concentration used does not reduce QuA drinking in naïve mice, it may not be adequately producing an aversion in alcohol history mice, reducing the overall validity of the model. While differing experimental models of compulsive-like QuA drinking are necessary due to differences in hypotheses and research designs, and have provided valuable information on the neurocircuitry of compulsive-like QuA drinking, we argue that current models could be further legitimized through extended characterization and improved validation.

In attempts to further characterize and improve the validity of current mouse models of compulsive-like QuA drinking, we conducted the following experiments using a limited-access model of alcohol drinking, DID (Thiele et al., 2014) in C57BL/6J mice. In experiment 1, we assessed whether 2 or 3 weeks of daily DID produce compulsive-like QuA drinking using a within-subjects design. In experiment 2, we assessed whether repeated QuA exposures alters the pattern of compulsive-like QuA drinking after 3 weeks of daily DID, because we utilized a within-subjects design in experiment 1 which resulted in 2 QuA exposures. Since alcohol front-loading is thought to represent motivation to drink alcohol because the consumption occurs prior to the absorptive effects of alcohol (Ardinger et al., 2020; Linsenhardt and Boehm, 2014, 2015; Wilcox et al., 2014), we also sought to determine the pattern of front-loading in QuA-drinking mice. Finally, we determined BACs following QuA drinking. In these experiments, we used a relatively high concentration of QuA (500 μ M) in attempts to improve the external validity of this model and inclusion of both male and female mice to allow the opportunity to further characterize sex differences in the compulsive-like QuA drinking phenotype. Most notably, we offer improvements to the model of compulsive-like QuA drinking by demonstrating compulsive-like QuA drinking with 2 defining criteria. First, alcohol history mice must consume significantly more QuA than water history (alcohol naïve) mice on QuA Test Day. Second, alcohol history mice must not differ in their QuA intakes as compared to their nonadulterated baseline drinking (i.e. the day prior to the QuA Test Day). Using our 2-criterion model and definition of compulsive-like QuA drinking, we determined that 3 weeks of daily DID, but not 2, robustly produces compulsive-like QuA drinking in male and female C57BL/6J mice.

MATERIALS AND METHODS

Animals

Forty naïve adult male and 40 naïve adult female C57BL/6J mice (PND 63-68 at drinking start) were acquired from The Jackson Laboratory (Bar Harbor, ME). Animals were individually housed in a vivarium with 12 hour:12 hour reverse light-dark cycle for 1 week prior to the start of experiments. Mice were housed in nonfiltered wired top standard shoe box mouse cages (18.4 cm wide, 29.2 cm long, 116 12.7 cm tall) and were given food (Lab Diet 5001, Rodent Diet) and tap water ad libitum with the exception of water bottle removal during the DID sessions. Procedures were approved by the IUPUI School of

Science Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (The National Academic Press, 2003).

Solutions

One hundred and ninety proof alcohol was purchased from Pharmco, Inc (Brookfield, CT) and was added to tap water to create a 20% v/v alcohol solution for use in the DID experiments. QuA was made by adding quinine hemisulfate (500 μ M; 0.1957 g/L) to 20% alcohol solutions. Quinine hemisulfate was purchased from Millipore Sigma (St Louis, MO).

DID

DID is a limited-access model of binge-like alcohol consumption which has been described in depth elsewhere (Bauer et al., 2020; Thiele et al., 2014). Briefly, mice received one 10 ml ball-bearing sipper tube of 20% v/v alcohol in tap water into their home cages in place of the regular water bottle 3 hours into the dark cycle for 2 hours each day. In experiment 1, mice underwent DID for 14 consecutive days, were given QuA on day 15, underwent DID for an additionally 7 days, and were again given QuA on day 22. In experiment 2, mice underwent 26 total days of DID where days 22, 24, and 26, the alcohol was adulterated with QuA. Consumption was measured by reading the sipper tubes to the nearest 0.025 ml, and volumes were adjusted for leak based on remaining fluid measured from sipper tubes in empty cages on the same drinking rack. Sipper tubes were read at 30 minutes and 2 hours on baseline and QuA Test Days to determine front-loading and 2-hour binge intakes, respectively. Body weights were taken the day prior to day 1 of DID and weekly thereafter to determine g/kg consumption.

Blood Alcohol Concentrations (BACs)

In experiment 2, on day 26, immediately following DID mice had retro-orbital sinus bloods taken. Blood plasma was spun down on the centrifuge at 470 *g* for 5 minutes, plasma was pipetted out, and bloods were stored at -20° F. BACs were determined using an Analox EtOH Analyzer (Analox Instruments, Lunenburg, MA). The Analox was calibrated with a 5 μ l injection of 100 mg/dl EtOH standard. Following calibration, blood plasma was then briefly vortexed and approximately 5 μ l were pipetted into the analyzer. BACs were immediately displayed and cataloged. The Analox was recalibrated every 5 to 10 samples to ensure accurate readouts.

Statistical Analyses

Statistical analyses were conducted for alcohol drinking, QuA drinking, and BACs in experiment 1 and 2. Baseline consumption (days 1 to 14 and days 1 to 21 for experiment 1, days 1 to 21 for experiment 2) was analyzed by 2-way repeated measures ANOVA (day \times sex for alcohol history animals only; RM by day). Compulsive-like alcohol drinking was assessed by testing whether alcohol history mice drank significantly more QuA than water history mice and whether QuA intakes were statistically nondifferent from baseline in the alcohol history mice. In experiment 1, the effect of drinking history on QuA intake was assessed with 2-way ANOVA (drinking history \times sex) after 2 weeks DID and after 3 weeks DID. The effect of day (i.e., baseline vs. QuA) was assessed with 2-way RM ANOVA (day

× sex; RM by day) after 2 weeks DID and after 3 weeks DID. Assessment of whether sex affected the percent baseline consumption of QuA was assessed with unpaired *t*-tests (male vs female on QuA Test Day 1 and QuA Test Day 2). In experiment 2, 2-hour and 30-minute front-loading was assessed with separate ANOVAs as follows. The effect of drinking history and repeated QuA exposures on QuA intake was assessed with 3-way RM ANOVA (sex × drinking history × QuA Test Day; RM by QuA Test Day). The effect of day (i.e. respective baseline vs. QuA Test Day) was assessed with 3-way RM ANOVA (sex × day × QuA Test Day; RM by QuA Test day). Assessment of whether sex affected the percent baseline consumption of QuA was assessed with 2-way RM ANOVA (sex × QuA Test Day; RM by QuA Test Day). BACs on QuA Test Day 3 were assessed between the drinking history groups with 2-way ANOVA (sex × drinking history). BACs were correlated using Pearson's correlation with 30 minute front-loading and 2-hour QuA intakes on QuA Test Day 3. Greenhouse Geisser corrections were applied as necessary. Differences were considered significant at $p < 0.05$. Data were analyzed using GraphPad Prism 8 and R Studio (www.r-project.org).

RESULTS

Experiment 1

Baseline alcohol and water drinking (days 1 to 14) for male and female C57BL/6J mice ($n = 7$ to 9/group) are shown in Fig. 1. Alcohol consumption is displayed in grams consumed per kilogram of body weight. Water consumption is displayed in milliliters consumed per kilogram of body weight per 2 hours. Two-way RM ANOVA of baseline drinking in the alcohol history mice by sex revealed a significant main effect of day, $F(13, 195) = 4.78$, $p < 0.0001$, $\eta_G^2 = 0.20$, sex, $F(1, 15) = 8.49$, $p = 0.01$, $\eta_G^2 = 0.10$, and no interactions. While the daily pattern of alcohol consumption differed across day, female alcohol history mice consumed significantly more alcohol than male mice across the 14 baseline days (Fig. 1A).

On day 15, all groups were given QuA during DID in place of their regular alcohol DID bottle to assess compulsive-like QuA drinking after 2 weeks of daily DID. Compulsive-like QuA drinking was defined by alcohol history mice drinking significantly more QuA than water history mice on QuA test day and alcohol history mice drinking statistically nondifferent amounts of QuA than nonadulterated alcohol at baseline (i.e., the day prior to QuA). The effect of a 14 day drinking history on compulsive-like QuA consumption is shown in Fig. 1. Two-way ANOVA of drinking history and sex on QuA drinking revealed a main effect of sex, $F(1, 28) = 11.28$, $p = 0.002$, $\eta_G^2 = 0.29$, a trend toward a main effect of drinking history, $F(1, 28) = 4.181$, $p = 0.0504$, $\eta_G^2 = 0.13$, and no interactions; Fig.

1B. This demonstrates that 2-week DID does not produce compulsive-like QuA drinking because alcohol history and water history mice do not differ in QuA intake. Next, we tested whether alcohol history mice drank statistically nondifferent amounts of QuA on day 15 than nonadulterated alcohol at baseline (i.e. day 14). Two-way RM ANOVA of sex and day revealed a main effect of sex, $F(1, 15) = 21.96$, $p < 0.001$, $\eta_G^2 = 0.41$, a main effect of day, $F(1, 15) = 8.88$, $p = 0.009$, $\eta_G^2 = 0.24$, and no interactions; Fig. 1C. We next tested whether male and female alcohol history mice differed in the percent baseline intakes (amount of

QuA consumed/amounts of nonadulterated alcohol consumed at baseline). Unpaired *t*-test determined that male and female mice did not differ in the percent baseline consumption (Fig. 1D). These data demonstrate that QuA suppressed consumption as compared to baseline after 2 weeks of DID and that male and female mice do not differ in their QuA drinking. Together, these data demonstrate that 14-day alcohol history is not sufficient to produce robust compulsive-like QuA drinking and suggests that while female alcohol history mice drink more alcohol than male alcohol history mice, their QuA intakes do not differ.

To establish whether an additional week of binge-like alcohol drinking would produce compulsive-like alcohol drinking, we returned the mice to their regular DID solution (either alcohol or water) for an additional 6 days. Baseline alcohol and water drinking (days 1 to 21) for male and female C57BL/6J mice ($n = 7$ to 9 /group) are shown in Fig. 2. Alcohol consumption is displayed in grams consumed per kilogram of body weight. Water consumption is displayed in milliliters consumed per kilogram of body weight per 2 hours. Two-way RM ANOVA of baseline drinking by sex in the alcohol history mice revealed a significant main effect of day, $F(19, 285) = 4.4$, $p < 0.0001$, $\eta_G^2 = 0.20$, sex, $F(1, 15) = 12.21$, $p = 0.003$, $\eta_G^2 = 0.14$, and no other interactions. While the daily pattern of alcohol consumption differed across day, female alcohol history mice consumed significantly more alcohol than male mice across the 21 baseline days (Fig. 2A).

On day 22, all groups were again given QuA during DID in place of their regular alcohol DID bottle to assess compulsive-like QuA drinking after 3 weeks of daily DID. Compulsive-like QuA drinking was defined by alcohol history mice drinking significantly more QuA than water history mice on QuA test day and alcohol history mice drinking statistically nondifferent amounts of QuA than nonadulterated alcohol at baseline (i.e. the day prior to QuA). The effect of a 21-day drinking history on compulsive-like QuA consumption is shown in Fig. 2. Two-way ANOVA of sex and drinking history on QuA drinking revealed a main effect of drinking history, $F(1, 28) = 12.27$, $p = 0.002$, $\eta_G^2 = 0.30$, no effect of sex, and no interaction; Fig. 2B. This demonstrates that 3-week DID produces compulsive-like QuA drinking by our first measure because alcohol history mice consumed significantly more QuA than water history mice. Next, we tested whether alcohol history mice drank statistically nondifferent amounts of QuA on day 22 than nonadulterated alcohol at baseline (i.e. day 21). Two-way RM ANOVA of sex and day on consumption in the alcohol history mice revealed a main effect of sex, $F(1, 15) = 4.572$, $p = 0.0494$, $\eta_G^2 = 0.15$, and no effect of day or interaction; Fig. 2C. We next tested whether male and female alcohol history mice differed in the percent baseline intakes (amount of QuA consumed/amounts of nonadulterated alcohol consumed at baseline). Unpaired *t*-test determined that male and female mice did not differ in the percent baseline consumption (Fig. 2D). These data demonstrate that QuA did not suppress consumption as compared to baseline after 3-week DID and that male and female mice do not differ in their QuA drinking. Together, these data demonstrate that 21-day alcohol history is sufficient to produce robust compulsive-like QuA drinking and suggests that while female alcohol history mice drink more alcohol than male alcohol history mice, their compulsive-like drinking does not differ.

Experiment 2

Because we utilized a within-subjects design in experiment 1 and found that after 3-week DID, not 2, alcohol history mice displayed compulsive-like QuA drinking, we sought to determine 3 things. First, we wanted to know whether repeated QuA exposures alter compulsive-like QuA drinking. Second, we wanted to determine the relationship between alcohol drinking history, sex, and repeated QuA exposures on motivational and the preabsorbative effects of alcohol and QuA drinking by assessing front-loading. And third, we sought to determine whether the BACs differed between compulsive-like alcohol history mice and non-compulsive-like water history mice.

Baseline alcohol and water drinking (days 1 to 21) for male and female C57BL/6J mice ($n = 12/\text{group}$) are shown in Fig. 3. Alcohol consumption is displayed in grams consumed per kilogram of body weight. Water consumption is displayed in milliliters consumed per kilogram of body weight per 2 hours. Two-way RM ANOVA of baseline drinking in the alcohol history mice by sex revealed a significant main effect of day, $F(20, 440) = 12.50$, $p < 0.0001$, $\eta_G^2 = 0.27$, sex, $F(1, 22) = 5.24$, $p = 0.032$, $\eta_G^2 = 0.07$, and no interactions. While the daily pattern of alcohol consumption differed across day, female alcohol history mice consumed significantly more alcohol than male mice across the 21 baseline days (Fig. 3A).

On days 22, 24, and 26, all groups were given QuA during DID in place of their regular alcohol DID bottle to assess compulsive-like QuA drinking after 3 weeks of daily DID. Compulsive-like QuA drinking was defined by alcohol history mice drinking significantly more QuA than water history mice on QuA test day and alcohol history mice drinking statistically nondifferent amounts of QuA than nonadulterated alcohol at baseline (i.e. the day prior to each respective QuA Test Day). The effect of a 21-day alcohol history on compulsive-like QuA consumption is shown in Fig. 3. Three-way RM ANOVA of drinking history, sex, and QuA Test Day revealed a main effect of drinking history, $F(1, 44) = 15.97$, $p = 0.0002$, $\eta_G^2 = 0.18$, a main effect of sex, $F(1, 44) = 18.07$, $p = 0.0001$, $\eta_G^2 = 0.20$, QuA Test Day, $F(2, 88) = 8.60$, $p = 0.0004$, $\eta_G^2 = 0.07$, and no interactions; Fig. 3B)

This demonstrates that 3 weeks of DID produces compulsive-like QuA drinking and that repeated QuA exposures did not alter compulsive-like drinking. Next, we tested whether alcohol history mice drank statistically nondifferent amounts of QuA across QuA Test Days than nonadulterated alcohol at baseline (i.e. days 21, 23, 25). Three-way RM ANOVA of sex, Test Day, and day (i.e. baseline vs QuA Test Day) revealed a main effect of sex, $F(1, 44) = 17.30$, $p = 0.0001$, $\eta_G^2 = 0.21$, and no other main effects or interactions; Fig.

3C) We next tested whether male and female alcohol history mice differed in the percent baseline intakes (amount of QuA consumed/amounts of nonadulterated alcohol consumed at baseline). Unpaired t -test determined that male and female mice did not differ in the percent baseline consumption (Fig. 3D). These data demonstrate that QuA did not suppress consumption as compared to baseline after 3 weeks of DID and that repeated QuA exposures did not alter this pattern. Together, these data demonstrate that 3-week binge-like alcohol history produces robust compulsive-like QuA drinking and that multiple QuA exposures does not alter the compulsive-like phenotype. Additionally, while female mice drink more alcohol and QuA, they do not differ from males in their compulsive-like QuA drinking.

We also assessed 30 minute front-loading during the 3 baseline and 3 QuA Test sessions; Fig. 4. Alcohol consumption is displayed in grams consumed per kilogram of body weight. We assessed the same measures of compulsive-like QuA drinking as outlined for the 2-hour drinking but adapted it to reflect 30 minute front-loading. For example, compulsive-like QuA drinking was defined by alcohol history mice front-loading significantly more QuA than water history mice across QuA Test Days and alcohol history mice front-loading statistically nondifferent amounts of QuA than nonadulterated alcohol across baselines (i.e. the day prior to each respective QuA Test Day). Three-way RM ANOVA of compulsive-like QuA front-loading on drinking history, sex, and QuA Test Day revealed a main effect of drinking history, $F(1, 44) = 21.50, p < 0.0001, \eta_G^2 = 0.22$, a main effect of sex, $F(1, 44) = 11.57, p = 0.0014, \eta_G^2 = 0.13$, QuA Test Day, $F(2, 88) = 35.59, p < 0.0001, \eta_G^2 = 0.25$, and no interactions; Fig. 4A) This demonstrates that alcohol history mice front-load significantly more of the aversive tasting QuA than water history mice, which may reflect increased motivation for alcohol, and that repeated QuA exposures did not alter this pattern. Next, we tested whether alcohol history mice front-loaded statistically nondifferent amounts of QuA across QuA Test Days than nonadulterated alcohol across baselines (i.e. days 21, 23, 25). Three-way RM ANOVA of 30-minute front-loading on sex, Test Day, and day (i.e. baselines vs QuA Test Days) revealed a main effect of sex, $F(1, 44) = 7.40, p = 0.009, \eta_G^2 = 0.08$, a main effect of solution, $F(1, 44) = 61.16, p < 0.0001, \eta_G^2 = 0.43$, a main effect of test day, $F(2, 88) = 18.40, p < 0.0001, \eta_G^2 = 0.16$, and no other main effects or interactions; Fig. 4B) We next tested whether male and female alcohol history mice differed in the percent baseline front-loading intakes (amount of QuA consumed/amounts of nonadulterated alcohol consumed at baseline). Two-way RM ANOVA of QuA Test Day and sex displayed as percent baseline consumption for each QuA Test Day revealed no significant main effects or interactions; Fig. 4C. These data demonstrate that alcohol history mice front-load significantly less across QuA test days as compared to baseline and this pattern does not change across test days. Together, these data indicate that while alcohol history mice will front-load significantly more QuA than water history mice suggesting increased motivation to consume the QuA solution, alcohol history mice still front-load significantly less QuA than nonadulterated alcohol, which indicates a decreased interest in the preabsorptive and motivational effects of QuA.

At the completion of the 2 hour DID session on day 26, all mice underwent retro-orbital sinus blood collection for BAC calculations; Fig. 5. To determine whether QuA consumption on day 26 was reflected in BACs, we assessed the effect of sex and drinking history on BACs. Two-way RM ANOVA on BACs of sex and drinking history revealed a significant main effect of drinking history, $F(1, 44) = 6.00, p = 0.018, \eta_G^2 = 0.12$, and no other main effects or interactions; Fig. 5A. Next, we sought to determine whether BACs predicted bottle readings by correlating 30-minute and 2-hour bottle readings with BACs. Pearson's correlation of 30-minute front-loading of QuA on Test Day 3 revealed no significant relationship with front-loading consumption and BACs. Pearson's correlation of 2-hour front-loading of QuA on Test Day 3 revealed a significant positive relationship with front-loading consumption and BACs, $r(46) = 0.7052, p < 0.0001, 95\% \text{ CI } [0.53, 0.82]$; Fig. 5B. Together, these data demonstrate that alcohol history mice had significantly higher BACs

than water history mice, 2-hour consumption predicted BACs, and alcohol history mice consumed alcohol at or above the level of a binge (i.e. ~80 mg/dl in 2 hours).

DISCUSSION

The goal of the present experiment was to further characterize and improve the validity of current mouse models of compulsive-like QuA drinking using binge-like alcohol drinking C57BL/6J mice. The primary findings from this experiment demonstrate that compulsive-like QuA drinking is achieved after 3 weeks, but not 2 weeks, daily DID, and male and female mice do not differ in compulsive-like QuA drinking though female mice consume more alcohol and QuA than male mice. After 3 weeks of DID, alcohol history mice consumed significantly more QuA than water history control mice and the alcohol history mice achieved at or above the level of a binge whereas the water history control mice did not achieve binge-like BACs, likely due to the aversiveness of the QuA. Furthermore, the compulsive-like drinking was accompanied by greater front-loading of the QuA solution by the alcohol history mice compared with water history mice, suggesting greater motivation for alcohol. However, the compulsive-like QuA drinking displayed in the alcohol history mice following 3 weeks of DID does not appear to be due to an inability to detect the aversive taste of the QuA, as exemplified in the front-loading data showing that compulsive-like mice front-load significantly less QuA compared with baseline consumption of nonadulterated alcohol. Additionally, we were able to replicate our main finding from experiment 1 in experiment 2, where 3 weeks of DID produced robust compulsive-like QuA drinking.

Our drinking data findings are in-line with previous research demonstrating an alcohol drinking history in C57BL/6J mice can result in compulsive-like QuA drinking and that this drinking differs from water history controls (Lesscher et al., 2010). Although Lesscher and colleagues (2010) found that 250 μ M QuA produced compulsive-like QuA drinking after 2 weeks, they failed to demonstrate the development of quinine resistance to 500 μ M QuA (the concentration used in our experiment) even after 8 weeks of alcohol exposure. The difference in compulsive-like drinking observed in our findings is likely due to differences in alcohol consumption model. While both our experiment and Lesscher and colleagues (2010) used a limited-access consumption model for 2 hours a day, 3 hours into the dark cycle, we used a single alcohol bottle, rather than a 2BC approach. This resulted in our alcohol consumption being around 4 (males) to 6 (females) g/kg/2 h, whereas Lesscher and colleagues (2010) found intakes around 2.5 g/kg/2 h. The differences in alcohol consumption model and thus, alcohol consumption, also yield differences in BAC where we found BACs at or above a binge (~80 mg/dl) and Lesscher and colleagues (2010) BACs would correlate to less than a binge (~50 mg/dl; Lesscher, et al., 2009; Lopez and Becker, 2005). This provides evidence that higher BACs and/or the daily binge-model of DID used here may cause an increase in the rate of development or degree of compulsive-like QuA drinking. The same factors could be at play in Sneddon and colleagues (2019), where the same 2-bottle LDA approach was used as in Lesscher and colleagues (2010) and found compulsive-like QuA drinking using 100 μ M QuA, but not 250 μ M QuA, after 2 weeks of alcohol exposure. In support of this drinking and alcohol consumption model hypothesis, Blegen and colleagues (2018) found compulsive-like QuA drinking in male and female

C57BL/6J mice using 500 and 1,000 μ M QuA after 6 to 7 weeks of DID-like exposure (3 weeks DID then 3 to 4 weeks operant self-administration) in mice that were post hoc determined to be high drinkers, but not mice that were low drinkers throughout the experiment. These data all together suggest that the consumption model chosen impacts the development of the compulsive-like phenotype. Notwithstanding, however, remains the importance of adequate control groups in assessment of this compulsive-like phenotype.

We also investigated whether male and female mice differ in their compulsive-like QuA drinking. We found female mice drink significantly more alcohol and QuA than male mice, but this effect does not likely represent increased compulsive-like drinking for female mice as the sexes did not differ in QuA intake as compared to baseline nonadulterated alcohol consumption. That female C57BL/6J mice binge-drink more alcohol than male mice is well established (Nentwig et al., 2019; Sneddon et al., 2019) and is consistent with findings from our laboratory (Bauer et al., 2020). Furthermore, female drinking does not fluctuate with the estrous cycle (Satta et al., 2018), mitigating concerns that our findings in female mice may differ across estrous cycle. To date, 3 QuA papers directly assessing sex differences in compulsive-like alcohol drinking using C57BL/6J mice have been published. Of these, one found no effect of sex on QuA drinking using binge-like drinking (Sneddon et al., 2019), one found that female mice drink more QuA than male mice using intermittent access 2BC (Shaw et al., 2020), and one found that female mice require higher concentrations of QuA to suppress drinking using a 2BC 24-hour consumption model with no effect of estrous cycle (Fulenwider et al., 2019). Shaw and colleagues (2020) found that females consumed more nonadulterated alcohol than males suggesting the increased QuA drinking may be more related to overall heightened alcohol consumption patterns and not an increased compulsive-like phenotype in the female mice. Fulenwider and colleagues (2019) found no baseline difference in aversion to QuA in alcohol naïve mice. Additionally, because Fulenwider and colleagues (2019) used a 24-hour 2BC paradigm, it is not possible to know whether sex differences did occur in a binge-like fashion because bottles were read only at 24 hours. Our findings add to this literature and suggest that while male and female mice differ in level of binge-drinking and QuA drinking, they do not differ in the overall quinine aversion-resistant compulsive-like phenotype.

Our front-loading findings are the first of their kind to assess this behavior in compulsive-like QuA drinking. Here, we found that alcohol history mice front-load significantly more QuA than water history (alcohol naïve) mice which is in line with the overall 2-hour compulsive-like QuA drinking, and also suggests that alcohol history mice may be more motivated for QuA than water history mice as front-loading is thought to measure interest in preabsorptive effects of alcohol, and the motivation for alcohol drinking. It has been demonstrated before that C57BL/6J mice front-load alcohol in the DID procedure after repeated DID sessions (Linsenhardt and Boehm, 2014, 2015; Wilcox et al., 2014), meaning our finding could also be interpreted as the addition of quinine to the alcohol solution did not prevent front-loading, or motivation for alcohol, in the alcohol history mice. This finding is in line with other measures of motivation (e.g. licking bout, time-course of consumption) where others have demonstrated that compulsive-like QuA drinking is accompanied by consummatory behaviors associated with motivation for alcohol drinking (Darevsky et al., 2019; Darevsky and Hopf, 2020). Additionally, when we compared front-loading of

QuA to nonadulterated alcohol at baseline in the alcohol history mice we found that QuA significantly reduced front-loading relative to baseline. This suggests that while alcohol history mice are significantly more motivated for QuA than water history mice, they still prefer or are more interested in the preabsorptive effects of alcohol when it is nonadulterated as compared to adulterated with quinine. This finding is important because it demonstrates the alcohol history mice are still capable of detecting the difference in taste between alcohol and QuA solutions, mitigating any concerns that alcohol drinking history would alter ability to taste the bitter quinine. While we interpret the front-loading as an increased motivation for alcohol, interpretation of this finding as a measure of motivation should be cautioned as we do not explicitly test motivation for alcohol with an operant procedure to parse out appetitive versus consummatory behavior. Future research should directly test whether a compulsive-like QuA drinking phenotype is associated with increased motivation for alcohol using an operant paradigm.

Our findings are limited in the following ways. First, while we offer a model that may be improved in validity relative to other options, we also acknowledge that, as described earlier in our discussion, different consumption models may require different QuA concentrations. Our model is directly useful for DID and C57BL/6J mice, although the importance of improved validity, inclusion of females, use of alcohol naïve controls, and comparison to baseline for 2-factor confirmation criteria is still withstanding, and the 2-factor confirmation criteria can be easily applied to other models. Future research should work to further characterize and validate compulsive-like QuA drinking across consumption models. On a similar note, we observe our data across a single genetic background (i.e. C57BL/6J mice). If validity is to be improved, then assessment of compulsive-like QuA drinking across genetically different models is warranted. Recent work has assessed compulsive-like QuA drinking across rodents at genetic risk for high alcohol consumption. For example, Timme and colleagues (2020) found that P rats (selectively bred for high alcohol consumption) with an alcohol history displayed compulsive-like QuA drinking, while still finding the QuA aversive when given a choice between nonadulterated alcohol and QuA. Additionally, replicate lines of crossed high alcohol preferring (cHAP) (selectively bred for high alcohol consumption) with or without an alcohol drinking history demonstrated innate compulsive-like QuA drinking at ~250 μ M QuA concentrations (Houck et al., 2019). Interestingly, and in further validation of the need for an alcohol naïve control group, Houck and colleagues (2019) found that alcohol history mice significantly differed from naïve mice after 2 weeks 2BC drinking history only when the QuA concentrations were as high as ~800 and 1,870 μ M. Finally, although neophobia is not an issue in our model since we did not observe differences in QuA drinking across test sessions regardless of drinking history, if this model were to be applied elsewhere, a repeated QuA exposure approach may be necessary to overcome neophobia of the QuA solution. Future research should continue exploring other models of consumption or genetic background for compulsive-like QuA drinking to further improve the construct validity of this model.

Previous research using mice and QuA drinking to model compulsive-like alcohol drinking behavior has yielded relevant findings for the neurobiology of AUD (Lei et al., 2016a; Patton et al., 2020; Siciliano, et al., 2019). While these previously used models have provided vital information to the field, our findings demonstrate that current models of

compulsive-like QuA drinking can be improved, further characterized, and extended upon to provide greater validity as a model. For example, Siciliano and colleagues (2019) gave their mice an alcohol drinking history and introduced their mice to increasing concentrations of QuA (250 to 1,000 μ M) to post hoc classify mice as either high drinkers, compulsive drinkers, or low drinkers. Siciliano and colleagues (2019) used relatively high concentrations of QuA which adds strength to their interpretation; however, they did not use a water control group. Because they did not use a water control group, it is not possible to determine whether the mice are actually compulsive (i.e. drinking more QuA than water history mice and not differing from baseline). Additionally, if the mice are compulsive, the lack of water control group means we cannot determine whether an alcohol drinking history or high alcohol drinking history causes compulsive-like alcohol drinking. Inclusion of a water control group in Siciliano and colleagues (2019) would have increased the validity of the compulsive-like QuA drinking model, making the interpretation of their findings clearer. Increasing the overall validity of this model would ideally lead to increases in predictive validity, which is key to any animal model being successful. As summarized in Table 1, many different models have been employed to study compulsive-like QuA drinking. To improve current models, we suggest future research includes alcohol naïve control groups to assess both how alcohol history mice consume QuA relative to baseline and relative to the naïve controls (i.e. 2-factor confirmation of compulsive-like drinking). We also suggest using highly concentrated QuA to improve face validity and to assess how females and males may differ in their compulsive-like phenotype to allow for further characterization of the model. In doing so, an inexpensive and relatively simple model of compulsive-like alcohol drinking can be more applicable.

In summary, we have shown that robust compulsive-like QuA drinking can be established after 3-week DID and that males and females do not differ in their compulsive-like QuA drinking. Additionally, we extend on previous research to provide evidence that use of relatively highly concentrated QuA (500 μ M; 0.1957 g/L) and use of our novel definition of compulsive-like QuA drinking where alcohol history mice must consume significantly more QuA than water history mice, and alcohol history mice must consume statistically nondifferent amounts of QuA than nonadulterated alcohol at baseline, allows for a more robust definition and subsequent phenotype that provides greater face validity as a model. To our knowledge, this is the first experiment which explicitly seeks to increase face validity of current mouse models of compulsive-like QuA drinking in male and female C57BL/6J, demonstrates increased front-loading for QuA in alcohol history mice, and is the first to demonstrate 2-hour binge-like (>80 mg/dl) BACs for compulsive-like QuA drinking.

FUNDING

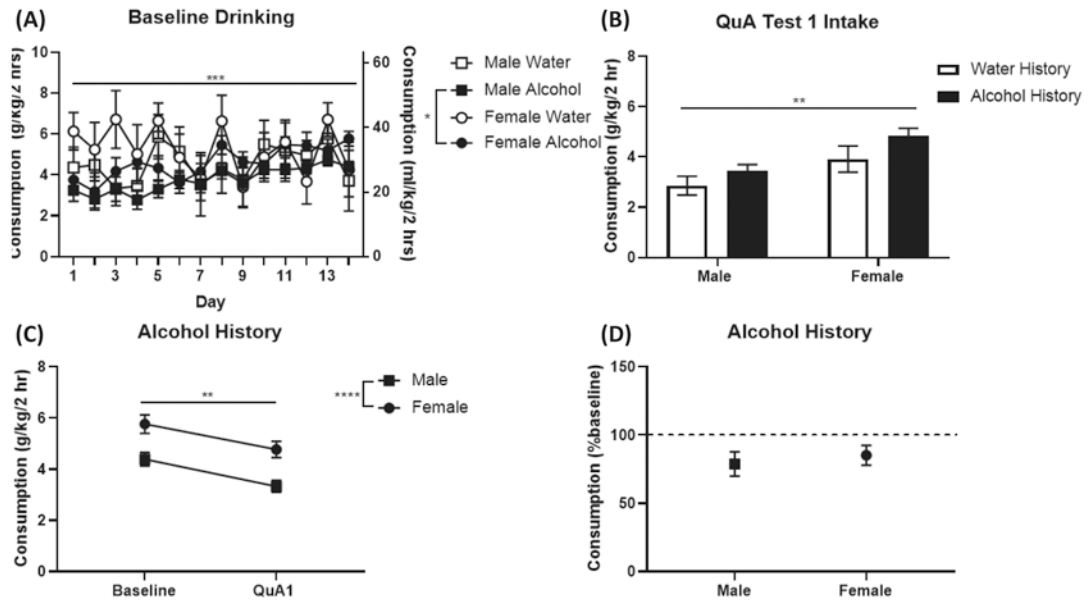
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**Fig. 1.**

Absence of compulsive-like alcohol drinking phenotype after 2 weeks of DID. Male and female C57BL/6J mice consumed either alcohol or water for 14 days. On day 15, all mice were given QuA (QuA Test 1; $n = 7$ to 9 /group). **(A)** Average daily consumption of either alcohol or water across the 14 days prior to QuA1 in male and female mice. Two-way RM ANOVA of day and sex in the alcohol history mice revealed a significant main effect of day ($***p = 0.0006$) and sex ($*p = 0.01$). **(B)** QuA consumption in male and female water and alcohol history mice on QuA Test 1. Two-way ANOVA of sex and drinking history on QuA Test 1 revealed a main effect of sex ($**p = 0.0023$). **(C)** Alcohol consumption at baseline and QuA consumption at QuA Test 1 for male and female alcohol history mice. Two-way RM ANOVA of day (baseline vs. QuA Test 1) and sex in alcohol history mice on consumption revealed a significant main effect of sex ($****p = 0.0003$) and day ($**p = 0.0045$). **(D)** Unpaired t -test of QuA consumption on Test 1 displayed as percent baseline between male and female alcohol history mice did not reveal a significant difference. Note the change in y -axis between Figures **(A)** and **(B-C)**. Data are displayed as mean \pm SEM.

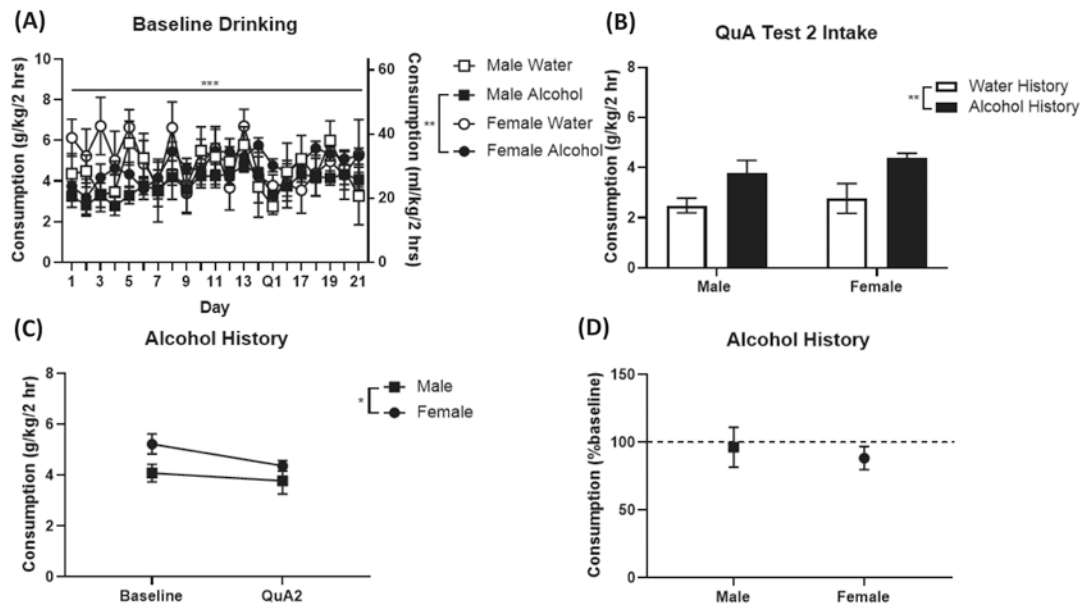
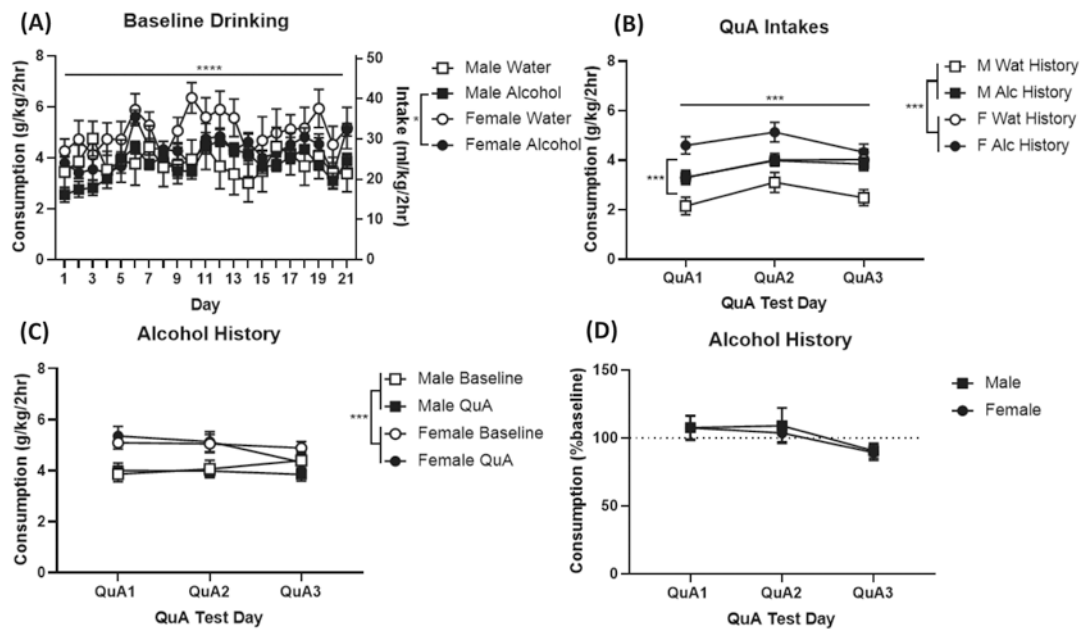
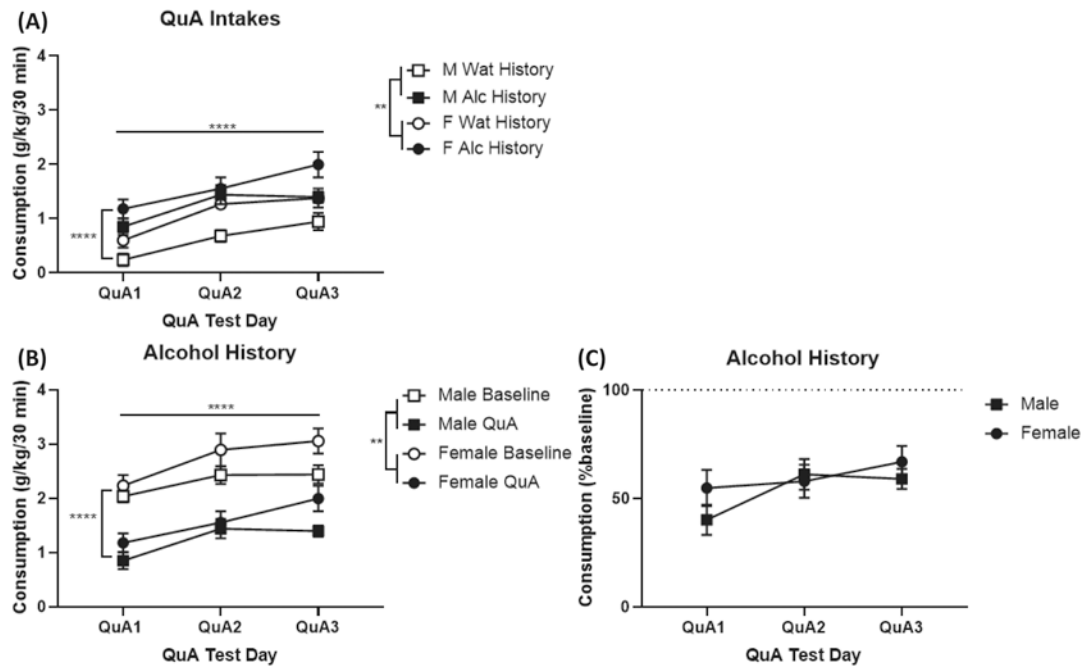


Fig. 2.

Presence of compulsive-like alcohol drinking phenotype after 3 weeks of DID. The same male and female C57BL/6J mice from Fig. 1 consumed either alcohol or water for an additional 7 days. On day 22, all mice were given QuA (QuA Test 2; $n = 7$ to 9 /group). **(A)** Average daily consumption of either alcohol or water across the 21 days prior to QuA Test 2 in male and female mice. Two-way RM ANOVA of day and sex in the alcohol history mice revealed a significant main effect of day ($***p = 0.0004$) and sex ($**p = 0.0033$). **(B)** QuA consumption in male and female water and alcohol history mice on QuA Test 2. Two-way ANOVA of sex and drinking history on QuA Test 2 revealed a main effect of drinking history ($**p = 0.0016$). **(C)** Alcohol consumption at baseline and QuA consumption at QuA Test 2 for male and female alcohol history mice. Two-way RM ANOVA of day (baseline vs. QuA Test 2) and sex in alcohol history mice on consumption revealed a significant main effect of sex ($*p = 0.0495$). **(D)** Unpaired t -test of QuA consumption on Test 2 displayed as percent baseline between male and female alcohol history mice did not reveal a significant difference. Note the change in y -axis between Figures **(A)** and **(B-C)**. Data are displayed as mean \pm SEM.

**Fig. 3.**

Repeated QuA exposures do not alter compulsive-like QuA drinking. A separate group of male and female C57BL/6J mice consumed either alcohol or water for 21 days. On days 22, 24, and 26, all mice were given QuA (QuA Test 1 to 3; $n = 12/\text{group}$). **(A)** Average daily consumption of either alcohol or water across the 21 days prior to QuA Test Days 1 to 3 in male and female mice. Two-way RM ANOVA of day and sex in the alcohol history mice revealed a significant main effect of day (**** $p < 0.0001$) and sex ($*p = 0.032$). **(B)** QuA drinking in male and female water and alcohol history mice across QuA Test Days. Three-way RM ANOVA of sex, drinking history, and QuA Test Day on QuA consumption revealed a significant main effect of QuA Test Day (*** $p = 0.0004$), sex (*** $p = 0.0001$), and drinking history (*** $p = 0.0002$). **(C)** Consumption on baseline and QuA drinking sessions across QuA Test Days in male and female alcohol history mice. Three-way RM ANOVA of QuA Test Day, sex, and day (respective baseline vs. QuA Test Day) on consumption revealed a significant main effect sex (*** $p = 0.0001$). **(D)** QuA consumption as percent baseline in male and female alcohol history mice. Two-way RM ANOVA of QuA Test Day and sex displayed as percent baseline consumption for each QuA Test Day revealed no significant main effects or interactions. Data are displayed as mean \pm SEM.

**Fig. 4.**

Repeated QuA exposures alter front-loading of compulsive-like QuA drinking. 30-minute front-loading of QuA in the same group of male and female C57BL/6J mice as in Fig. 3. On days 22, 24, and 26, all mice were given QuA and 30-minute bottle readings were taken (QuA Test 1 to 3; $n=12$ /group). **(A)** Three-way RM ANOVA of QuA Test Day, sex, and drinking history on 30-minute QuA consumption revealed a significant main effect of test day ($****p < 0.0001$), sex ($**p = 0.0067$), and drinking history ($****p < 0.0001$). **(B)** Three-way RM ANOVA of QuA test day, sex, and day (i.e. respective baseline vs. QuA Test Day) on 30-minute QuA front-loading revealed a significant effect sex ($**p = 0.009$), QuA test day ($****p < 0.0001$), and day (respective baseline vs. QuA Test Day) ($****p < 0.0001$). **(C)** Two-way RM ANOVA of QuA Test Day and sex displayed as percent baseline consumption for each QuA Test Day revealed no significant main effects or interactions. Data are displayed as mean \pm SEM.

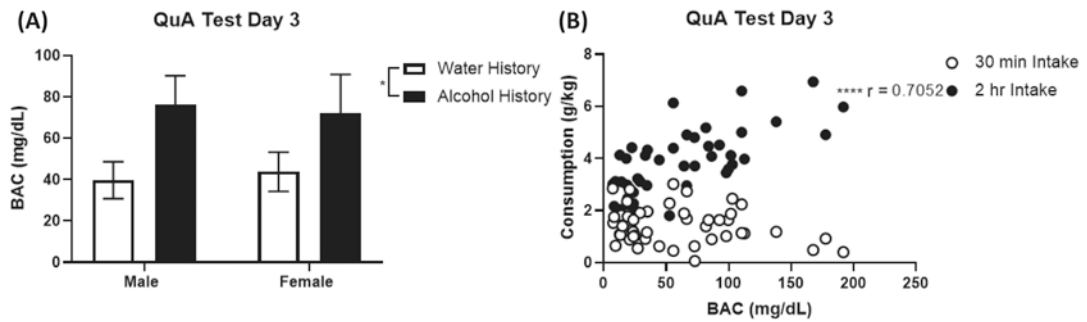


Fig. 5.

Blood Alcohol Concentrations on QuA Test Day 3. BACs were taken immediately after DID on QuA Test Day 3 ($n = 12/\text{group}$). **(A)** Two-way RM ANOVA of sex and drinking history on BACs revealed a significant main effect of drinking history ($*p = 0.0183$). Data are displayed as mean \pm SEM. **(B)** Pearson's correlation of 30-minute front-loading of QuA on Test Day 3 and BACs taken immediately after the 2-hour DID session did not reveal a significant relationship. Pearson's correlation of 2-hour QuA consumption on Test Day 3 and BACs taken immediately after the 2-hour DID revealed a significant relationship ($****p < 0.0001$).

Table 1.
Summary of PubMed Search of Compulsive-Like QuA Publications in C57BL/6J Mice

References	Quinine concentration (μM)	Alcohol history and consumption model	use of alcohol naive control	Sexes used
Blegen and colleagues (2018)	500, 1,000	3 weeks DID + 4 weeks SA	No	Male and female
Bocarsly and colleagues (2019)	500, 1,000	3 weeks DID + 3 weeks SA	No	Male and female
Chen and Lasek (2019)	100	0 days 2BC	No	Male
Hartog and colleagues (2016)	60	8 weeks IA 2BC with increasing alcohol concentrations (3 to 40%)	No	Male
Darcq and colleagues (2014)	60	2 weeks 2BC + 2 weeks IA 2BC	No	Male
Fulenwider and colleagues (2019)	30, 100, 300	6 weeks 2BC with increasing alcohol concentrations (3 to 24% v/v)	No	Male and Female
Lei and colleagues (2016a)	100	24 hours 2BC + 2 weeks LDA	No	Male
Lei and colleagues (2016b)	100	24 hours 2BC + 2 to 6 weeks LDA	Yes	Male
Lesscher and colleagues (2010)	25, 50, 100, 250, 500	2 to 8 weeks LDA	Yes	Male
Lesscher and colleagues (2012)	100, 250, 350, 500, 750	3 to 4 weeks LDA	No	Male
Olney and colleagues (2018)	30, 100	1 to 6 weeks DID	Yes	Male
Patton and colleagues (2020)	300	4 weeks DID	No	Male and Female
Shaw and colleagues (2020)	15, 25, 125, 250, 375, 500	4 weeks IA 2BC	No	Male and Female
Siciliano and colleagues (2019)	250, 500, 750, 1,000	5 days SA + 2 weeks LDA	No	Male
Sneddon and colleagues (2019)	100, 250	3 weeks LDA	No	Male and Female
Sneddon and colleagues (2020)	100, 250	2 weeks SA	No	Male and Female

Results are from [PubMed.gov](https://pubmed.gov) search criteria: quinine, compulsive, alcohol, aversion-resistant, and mice. The purpose of this table is to highlight the vast differences between models and to summarize the literature surrounding QuA using C57BL/6J mice. The most frequent quinine concentration used is 100 μM but ranged from 15 to 1,000 μM . Of the 16 publications, only 3 used a water control group for comparison to other groups with an alcohol drinking history. Seven of the publications included both males and females, one of which concluded that there are sex differences in compulsive-like QuA alcohol drinking.

DID, Drinking-in-the-Dark; LDA, Limited Daily Access; SA, Operant Self-Administration; 2BC, Continuous Access 2-Bottle Choice; IA 2BC, Intermittent Access 2BC.