# EFFECT OF DRINKING HISTORY ON REINFORCED AND EXTINCTION RESPONDING IN CROSSED HIGH ALCOHOL-PREFERRING MICE

by

**Garrett Winkler**

**A Thesis**

*Submitted to the Faculty of Purdue University*

*In Partial Fulfillment of the Requirements for the degree of*

**Master of Science**


Department of Psychology at IUPUI

Indianapolis, Indiana

December 2022

# THE PURDUE UNIVERSITY GRADUATE SCHOOL
# STATEMENT OF COMMITTEE APPROVAL

**Dr. Christopher Lapish, Co-Chair**

Department of Psychology

**Dr. Nicholas Grahame, Co-Chair**

Department of Psychology

**Dr. Marian Logrip**

Department of Psychology

**Approved by:**

Dr. Stephen Boehm

## ACKNOWLEDGMENTS

I would like to thank my mentors for their guidance with this project. I would also like to thank members of the Grahame and Lapish labs who helped me iron out technical issues throughout the project.

# TABLE OF CONTENTS

# LIST OF FIGURES

# ABSTRACT

Tolerance is a diagnostic criterion for alcohol use disorder (AUD) and dependence and is often measured metabolically or behaviorally by comparing blood ethanol concentrations (BEC) or locomotor performance to an ethanol (EtOH) challenge before and after a drinking history, respectively. To explore another aspect of chronic behavioral tolerance in a family history positive (FH+) model of AUD, crossed High Alcohol Preferring (cHAP) mice were allowed to respond instrumentally for an EtOH reinforcer after either a five-week history of continuous home cage two-bottle choice (2BC) drinking or a concurrent five-week water-drinking period. Additionally, some of these animals were placed back into the operant box after home cage drinking histories to respond in extinction, allowing for the quantification of alcohol-motivated seeking alone in the absence of EtOH taking and its intoxicating effects. The results demonstrate that an alcohol history does not lead to a subsequent increase in active lever responding or inactive lever responding when compared to water-drinking controls. However, female cHAP mice with an EtOH-drinking history respond more on the inactive lever in extinction compared to water controls, suggesting that home cage EtOH history potentiates variation in responding in extinction. Overall, female mice responded more on the active lever and drank more alcohol in the reinforced condition, but again, there was not an effect of drinking history on this sex-specific effect. Together these results suggest that while female cHAPs, regardless of drinking history, are more motivated to work to drink EtOH, reinforced and non-reinforced instrumental responding are not reliable readouts for tolerance in cHAP mice compared to other endpoints such as drinking in the dark (DID) assays.

# INTRODUCTION

## Forms of Alcohol Tolerance: An Overview

One diagnostic component of alcohol use disorder (AUD) in humans is the development of alcohol tolerance whereby repeated doses have diminishing effects (American Psychological Association, 2013). Acute tolerance can develop after just one experience with ethanol in healthy individuals as recently recruited metabolic and neurobiological pathways are reactivated (Kalant, 1996). This type of tolerance describes reduced impairment in the descending limb of the blood ethanol concentration (BEC) curve after a single exposure: participants may self-report less intoxication, even at the same BEC. (Fillmore & Weafer, 2011). Repeated alcohol drinking sessions can then induce metabolic tolerance via increased efficiency to catabolize alcohol; an individual with such tolerance will thus have a lower BEC than a naïve individual after imbibing the same amount of ethanol (EtOH) (Tabakoff et al., 1986). Finally, chronic EtOH drinking can induce a chronic type of tolerance as measured via behavioral output. For example humans and animals can become tolerant to the ataxic effects of EtOH (Bennett et al., 1993); in rodent pre-clinical models specifically, this can be measured by footslips on a balance beam or rotarod test (Pohorecky et al., 1986). Human subjects can also become functionally tolerant to the negative cognitive and attentional deficits of alcohol consumption (Fadda & Rossetti, 1998). These assays explore how behavioral tolerance emerges to various negative effects of alcohol intoxication, but they do not describe behavioral tolerance to the positive effects of alcohol, a subtype of tolerance that can explain why an individual continues to escalate drinking to achieve a level of intoxication that feels as rewarding as it had previously. Tolerance to the rewarding effects of EtOH could be observed in tasks that require motivated operant behavior whereby animals with variant degrees of alcohol experience toggle specific manipulanda to receive alcohol as a reinforcer. This requires that (1) subjects are sufficiently familiar with task contingencies, and (2) subjects find EtOH rewarding enough to continue responding. The first requirement is met via sufficient training and shaping while the second is achievable with a more specific set of subjects.

## Measuring Tolerance in Crossed High Alcohol Preferring Mice

Selectively bred lines of mice like the High Alcohol Preferring mice (HAP) and the crossed High Alcohol Preferring mice (cHAP) satisfy the second requirement (O'Tousa et al., 2015) but also may be more representative of individuals diagnosed with AUD. These lines show elevated two-bottle choice (2BC) preference and intake of EtOH when compared to inbred strains such as C57BL/6J (B6) mice (Matson & Grahame, 2011), which are commonly used in alcohol research because they outdrink other inbred strains (Grahame et al. 1999; McClearn et al. 1959). the HAP and cHAP mice may not only represent a family history positive model for increased alcohol consumption and/or AUD, but are generated from a heterogeneous stock of progenitors, resulting in increased heterozygosity at loci unrelated to the high drinking phenotype (Grahame et al. 1999). This is in contrast to B6 mice where all loci are homozygous, and the genetic N = 1 (apart from sex). Additionally, the cHAPs have exhibited evidence of metabolic tolerance (Matson et al., 2013) and behavioral tolerance as measured by ataxia (Matson et al., 2014). Though they respond for EtOH after instrumental conditioning in operant self-administration (OSA) (O'Tousa et al., 2015, Houck & Grahame, 2018), behavioral tolerance to the positively reinforcing effects of EtOH has not been shown in the cHAPs.

EtOH history effects have been examined in rodents; however, these studies often utilize chronic intermittent ethanol (CIE) vapor inhalation as the source of EtOH exposure. In B6 mice, for example, the general CIE procedure involves exposure to EtOH vapor for four days, 16h a day, leading to BECs in the range of 175-225mg/dL (Becker & Lopez, 2004). Compared to room air controls, CIE-exposed mice and rats have higher response rates in EtOH OSA (Lopez & Becker, 2014; Vendruscolo & Roberts, 2014; Chu et al. 2007; O'Dell et al., 2004) Additionally, following CIE exposure, animals exhibit signs of acute and protracted withdrawal measured by handling-induced convulsions or affective disturbance, respectively (Kliethermes et al., 2006; Becker & Hale, 1993). These withdrawal responses, along with the increase in EtOH OSA are said to be indicative of physiological dependence, a hallmark of human AUD, whereby an individual uncontrollably drinks to stave off withdrawal (APA, 2013). Additionally, in line with other DSM-V AUD criteria, CIE causes tolerance to alcohol's aversive effects (Lopez et al., 2012), neuroinhibitory effects (Nimitvilai et al., 2016), and ataxic effects (Daut et al., 2016), among others (Becker & Baros et al., 2006). While these repeated, vapor-induced spikes in BECs reliably induce a "dependent" and tolerant phenotype, there are several criticisms of this

paradigm: CIE's face validity and predictive validity have been called into question as experimenter-controlled exposure does not mimic the human drinking experience, and most neuromodulator systems that have been implicated in dependent animals do not translate to clinical models (Spanagel, 2017; Kwako et al., 2015). Additionally, the stress induced by involuntary EtOH vapor inhalation can be difficult to untangle from the effects of EtOH exposure alone. Finally, high drinking selected lines like the HAP2 mice (a progenitor of cHAP mice) do not show a withdrawal component or a consistently increased EtOH OSA response following CIE when compared to their parallel low-drinking analogs the LAP2s (Lopez et al., 2011). Additionally, cHAPs show no signs of affective disturbance following a 5-week alcohol history (Winkler & Grahame, unpublished). In other words, there is evidence indicating that the selected lines show behavioral EtOH tolerance but do not show dependence.

## Extinction Responding

Measuring the rewarding effects of EtOH in an operant task where EtOH is the reinforcer can complicate interpretation as animals become more and more intoxicated: an animal that is less intoxicated because it has ataxic tolerance will be able to respond for more EtOH. An extinction test can measure EtOH seeking behavior separate from tolerance effects to understand how motivation to obtain EtOH reward differs by drinking history. Extinction responding has been used profusely in EtOH literature, especially to study drug reinstatement as a model of relapse (Katner et al., 1999, Keistler et al., 2017). Since interpretation can be confounded by intoxication, extinction trials on their own, however, can be used as a measure of alcohol seeking in animals that have only been trained to understand the contingency between the instrumental response of lever pressing and the operant outcome of EtOH access (Samson & Chappell, 2006; Gass et al., 2014; Cannady et al., 2017; Czachowski et al., 2018). Comparing extinction responding between animals with and without EtOH history may reveal a difference in history-induced sensitivity to cues that signal the availability of alcohol via a measurement of seeking in the absence of taking, especially in animals who have not yet experienced an extinction test.

## Rationale and Hypotheses

In summary, CIE vapor procedures lead to a reliable dependent and tolerant phenotype. Also, it is known that cHAP mice show chronic tolerance but do not exhibit protracted withdrawal after 5 weeks of drinking. A progenitor of cHAPs, the HAP2s, also show no acute withdrawal following CIE. This leaves open the question of whether increased responding for an alcohol reinforcer following an EtOH history is reliant on dependence alone (as in CIE) or involves some component of behavioral tolerance to EtOH's positively reinforcing effects – a question that could be succinctly answered by the use of cHAPs due to their observed behavioral tolerance and propensity to drink to CIE-level BECs ( > 175 mg/dL, especially 6–10h into the dark part of the light cycle). (Matson et al., 2013) Thus, to answer this question, the current study compared reinforced and non-reinforced (extinction) responding between animals with or without a 5-week history of continuous 2BC access to EtOH. We hypothesized that (1) compared to water-drinking controls, male and female cHAP mice with an alcohol history will respond more on the active lever in reinforced and non-reinforced testing sessions, and (2) females will consume more alcohol than males in 2BC and in the reinforced task.

# METHODS

## Subjects

This experiment was performed in two balanced replications, each with 36 EtOH-naïve cHAP mice (18M, 18F) that were 65-87 days old were single-housed one week before operant training on a 12h:12h reverse light:dark cycle with lights off at 10pm. For operant training, animals were counterbalanced into 3 squads of 12 by sex, parents, and active lever assignment. Throughout the experiment, animals received *ad libitum* food and water in the home cage, except for deprivation periods described below. Cages were changed by the experimenter biweekly, and mice were weighed weekly. Procedures were approved by the IUPUI School of Science Institutional Animal Care and Use Committee and conformed to the Guide for Care and Use of Laboratory Animals (The National Academic Press, 2003).

## Apparatus

Twelve operant chambers (Med-Associates) were run simultaneously for each squad with dimensions of 21.6 × 19.7 × 12.7 cm. Each chamber was placed in its own sound- and light-attenuating box with a fan situated in the center of the right-facing wall relative to the front door of the box to promote air flow during sessions. A retractable sipper tube attached to a 10mL graduated pipette could provide a 10% EtOH solution to serve as a reinforcer. On either side of the sipper and on the same wall, two non-retractable levers (active versus inactive) were available to manipulate. Manipulation of the levers (lever presses) and delivery of the sipper (rewards) were tracked by Med-PC IV software (Med-Associates). For training and testing, no house lights or lever lights were utilized. "Yesterday's News" bedding covered the bottom of the compartment under the grid floor of each chamber and was changed weekly. Operant chambers were cleaned with 70% EtOH before each squad each day.

## Water Deprivation

Animals were fluid deprived for 22 hours before training to encourage drinking behavior in early operant training. Water bottles were returned to the home cage for two hours before

being removed again before the following session. Water deprivation occurred only for the fixed time 120s (FT120) sessions and the first fixed ratio 1 (FR1) session for each animal, described below; otherwise, water was available *ad libitum* in the home cage, but not during operant sessions.

## Operant Training

Training began with one 30-minute FT120 magazine training session where all active lever presses were reinforced with 20s of access to the sipper containing 10% EtOH. The sipper was also presented every 120 seconds regardless of the subject's input. Animals that did not consume at least 0.2mL of fluid received additional FT120 sessions until they reached criterion.

Animals then moved to FR1 training sessions for 60 minutes where each lever press on the active lever was reinforced by the presentation of the EtOH sipper for 5 seconds while inactive lever presses resulted in nothing. Levers were not retracted during sipper access, but after the sipper retracted, response-contingent reinforcement was available immediately without a timeout. Lever presses occurring while the sipper was extended were not recorded. Mice had a minimum of three FR1 training sessions and were moved up to FR3 when they consumed at least 0.2mL on average over three sessions.

During the 60-minute FR3 sessions, mice had 12s of sipper access when the active lever was pressed 3 times, regardless of the length of time between presses or number of inactive lever presses between active lever presses. When animals consumed an average of at least 0.2mL over at least three sessions, they advanced to FR6 sessions.

FR6 sessions were also 60 minutes in length but required 6 presses on the active lever to lower the alcohol sipper for 12 seconds. Once reaching the same threshold requirement of 0.2mL over at least three sessions, animals advanced finally to FR8 sessions.

FR8 sessions lasted 60 minutes and required 8 presses on the active lever to lower the sipper for 12 seconds. Mice moved to the 2BC portion of the experiment once they reached the 0.2mL intake benchmark and when variability in responding on the active lever in the three most recent sessions was less than 20%.

Once an animal reached criteria in FR8 training, they transitioned to 2BC. If an animal became stuck in FR1 or FR3 training due to low intake, water deprivation as described above

was used to encourage drinking in the following training session. If an animal was held up in FR6 or FR8 training, those specific subjects were run on back-to-back sessions that same day until they reached criterion, and then training continued on the next day without additional fluid deprivation. If these retraining methods failed to produce a lasting pattern of drinking behavior in the operant context, the animal was excluded from the study.

### Two-Bottle Choice EtOH History

Mice were split into counterbalanced groups by sex and baseline FR8 responding (the average active lever presses for the last three FR8 sessions) for continuous 2BC water/water or water/10% (v/v) EtOH in the home cage. EtOH was provided in 50mL graduated cylinders while water was provided in 25mL graduated cylinders both affixed with sipper tubes of appropriate width. Water/water animals had matching cylinder sizes but both tubes were filled with tap water. Fluid volume was measured every Monday, Wednesday, and Friday and tubes were topped off as needed on those days; cylinders were swapped on these days as well to control for any positional preferences. Mice were weighed every Monday and cages were changed every other Monday.

### Operant Self-Administration and Extinction Testing

Three hours before testing, 2BC cylinders were removed from the home cage and replaced with standard glass water bottles. Mice were again split into two counterbalanced groups based on sex, family, and drinking history (W or E). One cohort received reinforced FR8 on the first day of testing, then FR8 extinction the next day. The other cohort received the tests in the opposite order. Reinforced sessions were 2 hours in length while extinction sessions were 1 hour. Longer reinforced sessions were used here with the hope of increasing the likelihood of detecting an alcohol history effect on persistence of responding during longer sessions, which may yield higher BECs. During extinction testing, 8 lever presses on the active lever caused an empty sipper to descend into the operant chamber.

There were only two days of testing; therefore, every animal received both tests, though order was dependent on cohort. After testing, 2BC bottles were not reintroduced to the home cage. Blood samples were not obtained after either session for the following reasons: (1) blood

collection after Day 1 may interfere with Day 2 testing and (2) blood collection after Day 2 would be hard to interpret because the animals who received EtOH in reinforced testing have had 28 hours of abstinence and (3) their first test day was an extinction session, which may change their understanding of the contingencies of lever pressing. Fig 1 shows the Experimental Timeline for this Aim.

## Statistics

Data showing instrumental outcomes (intake and lever pressing) are collapsed across both replicates because no interactions were found on any measure on any day via three-way analysis of variance test (ANOVA) with factors of drinking history, sex, and replicate. Patterns in 2BC intake and preference between sexes were analyzed via mixed effects analyses due to a few incidents of tube leakage that caused missing data points for some animals. Operant behavioral endpoints were analyzed via two-way ANOVAs where the factors analyzed included drinking history and sex; order cohort was not examined as a third factor because receiving reinforced sessions after extinction or extinction after reinforced sessions complicates interpretation of differences between groups. For data examined over the operant session in 5-minute bins, a repeated measures ANOVA was used with factors of time and alcohol history. Interactions were further examined post-hoc via Tukey's multiple comparisons and reported as p-values alone.

# RESULTS

## Two-Bottle Choice Drinking and Preference

Alcohol intake in grams per kilogram of body weight over the 5 weeks of 2BC is shown in Fig 2A. “Sessions” on the x-axis refers to fluid volume measurement days on MWF. To account for differences in length of time between readings, g/kg intake is divided by number of days between measurement sessions; thus data in Fig 1A reflects g/kg/24h. Mixed effects analysis of EtOH intake revealed a significant main effect of session [$F$ (4.221, 62.41) = 6.880, $p < 0.0001$] and sex [$F$ (1, 15) = 8.219, $p = 0.0118$].

Alcohol preference (Fig 2B) over water was measured by dividing total 10% EtOH volume consumed between measurement sessions by total fluid volume consumed (10% EtOH + water), yielding a preference score between 0 and 1. Mixed effects analysis of EtOH preference revealed a significant main effect of session [$F$ (3.374, 49.88) = 14.35, $p < 0.0001$], but not sex. It should be noted that Fig 2 only displays data from EtOH-drinking 2BC groups, not water/water controls.

## Day 1 Reinforced FR8

Endpoints of OSA (reinforced) FR8 on Day 1 are shown in Fig 3. Fig 3A shows active lever pressing behavior across drinking history and sex. A two-way ANOVA reveals a main effect of sex [$F$ (1, 30) = 5.093, $p = 0.0315$] but no main effect of drinking history or significant interaction. Inactive lever pressing is shown in Fig 3B and a two-way ANOVA revealed no main effects or interaction. Fig 3C shows g/kg EtOH intake over the 2-hour session, and a two-way ANOVA revealed a main effect of sex [$F(1, 30) = 12.57$, $p < 0.01$] but again no main effect of drinking history or significant interaction.

## Day 1 Non-reinforced FR8

Endpoints of EXT (non-reinforced) FR8 on Day 1 are shown in Fig 4. Again,. Fig 4A shows active lever pressing behavior; a two-way ANOVA revealed no main effects or

interactions. Fig 4B shows inactive lever pressing, and a two-way ANOVA revealed a significant main effect of drinking history [$F$ (1, 28) = 5.773, $p$ = 0.023] and a significant interaction [$F$ (1, 28) = 4.889, $p$ = 0.0354], but no main effect of sex. Tukey's multiple comparison post-hoc analysis revealed that the main effect of drinking history was driven almost entirely by the female mice ($p$ = 0.018).

### Day 1 Active Lever Pressing over Time

For day 1 only, responses on the active lever were grouped into 5-minute bins and examined across the 2-hour reinforced session and the 1-hour extinction session (data not shown) to probe for any possible behavioral frontloading differences between EtOH and water history groups in responding. Repeated measures ANOVAs were employed for this analysis and revealed no main effect of time or EtOH history nor significant interactions in either reinforced or extinction cohorts. This reveals that in cHAPs, responding for EtOH over a 2-h period and responding in extinction in a 1-h period is stable over time and is not influenced by drinking history.

### Day 2 Reinforced FR8

Endpoints of reinforced FR8 on Day 2 are shown in Fig 5. These were the same subjects from Fig 4 who received non-reinforced FR8 on Day 1. Fig 5A shows active lever presses; a two-way ANOVA did not reveal any main effects or interactions. Fig 5B shows inactive lever pressing behavior, and a two-way ANOVA did not reveal any main effects or interactions. Fig 5C shows g/kg intake over the two-hour session. According to a two-way ANOVA, there was a main effect of sex [$F$ (1, 28) = 8.734, $p < 0.01$] but not drinking history and no interaction.

### Day 2 Non-reinforced FR8

Endpoints of non-reinforced FR8 on Day 2 are shown in Fig 6. These were the same subjects from Fig 3 who received reinforced FR8 on Day 1. Fig 6A shows active lever presses in extinction; a two-way ANOVA revealed a significant main effect of sex [F(1, 30) = 4.812, p =

0.036] but not of alcohol history or an interaction. Fig 6B shows inactive lever presses in extinction, and a two-way ANOVA revealed no main effects or significant interactions.

# DISCUSSION

Following an EtOH history of continuous home cage 2BC, cHAP mice, regardless of sex, did not respond more on the active lever in a reinforced or non-reinforced instrumental task than water-drinking controls on either day of testing. Female mice drank more than males regardless of drinking history in 2BC and also drank more during the reinforced tests; they also responded more on the active lever on Day 1 during reinforced testing and on Day 2 during non-reinforced testing. Finally, an interaction in Day 1 extinction lever pressing revealed that female mice press more than male mice on the inactive lever. This effect does not carry over to inactive responding on Day 2 reinforced testing.

The 2BC procedure used to provide an alcohol history in the current study has previously been sufficient to generate ataxic (Matson et al., 2014) and metabolic tolerance in cHAPs (Matson et al., 2013). Home cage 2BC measures of intake and preference also match existing data using these animals, so aberrant 2BC drinking cannot explain the lack of separation between history groups in reinforced and non-reinforced responding or intake in the operant chamber. Furthermore, escalation, which emerges along with ataxic tolerance, in cHAPs (Matson et al., 2013) can be clearly seen in Fig 2A as cHAPs drink more and more over the 5-week drinking period, but again, this did not translate to high responding or intake during reinforced FR8 over water/water controls. A recent pilot study from our lab examined effects of a similar (albeit shorter) 2BC drinking history on intake in the binge drinking model known as drinking in the dark (DID) in which animals have access to a single sipper of 20% EtOH (v/v) for 2-hour period three hours into their dark cycle. Contrary to the results of the current study, a 2-week drinking history in cHAPs led to increased DID intake compared to water/water controls, consistend with the idea that cHAP mice do show multiple forms of EtOH tolerance after a drinking history. (Winkler & Grahame, personal communication)

The question remains as to why cHAP mice with an EtOH drinking history did not show tolerance to the rewarding effects of EtOH as measured by either reinforced or nonreinforced operant responding. While one could purport that cHAPs do not acquire reward tolerance or that such tolerance cannot be accurately measured by reinforced or non-reinforced FR8, some aspects of the experimental design may help explain why drinking history did not have an effect. Firstly, as discussed, EtOH was used as the reinforcer throughout training weeks so as to not cause any

positive or negative contrast effects when the reinforcer was presented again in the testing phase. This means that water history animals were not necessarily alcohol naïve; therefore, though EtOH history animals had an additional 5 weeks of home cage drinking, water history animals cannot be assumed to have zero tolerance based on previous experience with EtOH. Additionally, water deprivation prior to the FT120 session caused quite high drinking in all animals, possibly inducing some aversive effects which potentially extended training as animals became comfortable with drinking sufficiently again, and unintentionally allowing water animals even more experience with EtOH. The goal of the FT120 session is not to promote rapid intoxication, but rather to familiarize the animals with the operant chamber, the location of the levers and the sipper, and the differential contingencies of the active and inactive lever. If replicated, FT120 sessions may be monitored more closely, and animals may be promptly removed once the drinking criterion is reached. Finally, intake at baseline 1-h FR8 during training (average 1.95g/kg), on Day 1 2-h reinforced testing (average 1.99g/kg), and Day 2 2-h reinforced testing (average 2.02g/kg) are not as high as seen in DID where average cHAP intakes can reach 3-4g/kg in 2-h, yielding average BECs of 130mg/dL (Ardinger et al., 2021). The lower intakes seen here would most likely not lead to this level of intoxication, either due to the required responses to access EtOH, thus lowering the rate of intake, and/or because the concentration of EtOH in the sipper was 10% instead of 20%. If animals cannot reach intoxicating BECs in the operant chamber like they can in the home cage (Matson et al., 2013), detection of a history effect may be complicated by the fact that animals cannot even reach a rewarding level of intake. Taken together, drinking history animals may not have shown any effect in either test due to water/water control EtOH exposure and/or low intakes within the operant chamber itself.

Our second, sex-specific hypothesis was supported. Consistent with extensive previous work with cHAPs, female mice outdrank male mice in 2BC, but this difference in intake also carried over into reinforced FR8 sessions. Female cHAPs also made more active lever responses (regardless of drinking history) in several tests in this study; for example, females pressed more on the active lever on Day 1 reinforced FR8 and Day 2 non-reinforced FR8. These response data and reflect enhanced reinforcing effects of EtOH on female measures of EtOH-seeking and taking or an enhanced memory of the operant chamber than in males, especially since this increased responding matched female increased intake of EtOH over their male equivalents.

One interesting sex-specific finding came from the Day 1 non-reinforced lever pressing behaviors of female cHAPs. As shown in Fig 4B, EtOH-history females drove the difference in inactive lever pressing between EtOH and water-history groups by pressing on average at least twice as much on the inactive lever as their female water-history counterparts. These mice then went on to reinforced FR8 on Day 2, and this difference in inactive responding disappeared, suggesting that these females only increased their inactive lever pressing in the non-reinforced session in which an empty sipper descended following eight active lever responses. The simplest explanation for this increase is that extinction often results in an increase in behavioral variability, as demonstrated by Neuringer et. al (2000). Thus, while overall responding decreases in an extinction curve, the types of emitted behaviors tend to become more varied in order to maximize any possible reinforcement from a source that had previously been reliable. Since only two types of behaviors were recorded while the animals were in the operant box – active lever pressing and inactive lever pressing – an increase in behavioral variability would manifest as increases in inactive lever pressing. The fact that this increase was only observed in female mice with an EtOH history is most likely connected to the findings that female mice, overall, seek and take alcohol at a higher rate than male cHAPs. EtOH may be more reinforcing for female cHAPs than males and having an alcohol history enhances the reinforcing qualities of EtOH for females only. A downshift in reward magnitude as seen in extinction lowers responding, but increases behavioral variability (Carlton, 1962), and this variability was increased in EtOH drinking females only without changing overall responding, suggesting that the downshift from FR8 to extinction is greater for EtOH history females compared to water history females . However, this increase in inactive lever pressing during Day 1 extinction testing is the only effect of history found in this study and is, at best, only an indirect measure of the reinforcing qualities of EtOH in these animals.

Failure to reject the null hypothesis in these experiments does not allow us to make definitive conclusions about dependence and behavioral tolerance in the cHAPs. These animals achieve BECs in home cage 2BC that are comparable to vapor exposure and cyclical in nature when animals wean off the sipper as the end of their dark cycle approaches; these attributes of cHAP drinking seem congruent with CIE procedures, but clearly, they lack the aspect of involuntary exposure which has been a strike against the model's face validity. Though, even in CIE, HAP2 mice do not show typical withdrawal responses, indicating that the dependence

phenotype may not be inducible in high-drinking populations (Lopez et al., 2012; Metten et al. 1998). If dependence alone is responsible for the reliable increase in OSA responding compared to room air controls, that could explain why we saw no effects in the current study. On the other hand, these results do not allow for the conclusion that there is no such thing as tolerance to the rewarding effects of alcohol: either the use of EtOH in training confounded our ability to detect this tolerance, or an FR8 schedule of reinforcement does not allow sufficient intoxication for the measurement of this tolerance. Since it is difficult to avoid contrast effects if another reinforcer is used in training besides EtOH (e.g. sucrose), adjusting parameters of the operant task to allow for actual intoxication in the operant chamber may allow for detection of tolerance to EtOH's effects.

# REFERENCES


American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders* (5th ed.). https://doi.org/10.1176/appi.books.9780890425596

Ardinger, C. E., Winkler, G., Lapish, C. C., & Grahame, N. J. (2021). Effect of ketamine on binge drinking patterns in crossed high alcohol-preferring (cHAP) mice. *Alcohol (Fayetteville, N.Y.)*, *97*, 31–39. https://doi.org/10.1016/j.alcohol.2021.09.004

Becker, H. C., & Lopez, M. F. (2004). Increased ethanol drinking after repeated chronic ethanol exposure and withdrawal experience in C57BL/6 mice. *Alcoholism: Clinical and Experimental Research*, *28*(12), 1829-1838.

Becker, H. C., & Hale, R. L. (1993). Repeated episodes of ethanol withdrawal potentiate the severity of subsequent withdrawal seizures: an animal model of alcohol withdrawal "kindling". *Alcoholism, clinical and experimental research*, *17*(1), 94–98. https://doi.org/10.1111/j.1530-0277.1993.tb00731.x

Becker, H. C., & Baros, A. M. (2006). Effect of duration and pattern of chronic ethanol exposure on tolerance to the discriminative stimulus effects of ethanol in C57BL/6J mice. *The Journal of pharmacology and experimental therapeutics*, *319*(2), 871–878. https://doi.org/10.1124/jpet.106.108795

Cannady, R., McGonigal, J. T., Newsom, R. J., Woodward, J. J., Mulholland, P. J., & Gass, J. T. (2017). Prefrontal Cortex $K_{Ca}2$ Channels Regulate $mGlu_5$-Dependent Plasticity and Extinction of Alcohol-Seeking Behavior. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *37*(16), 4359–4369.

Carlton P. L. (1962). Effects on deprivation and reinforcement-magnitude of response variability. *Journal of the experimental analysis of behavior*, *5*(4), 481–486. https://doi.org/10.1901/jeab.1962.5-481

Chu, K., Koob, G. F., Cole, M., Zorrilla, E. P., & Roberts, A. J. (2007). Dependence- induced increases in ethanol self-administration in mice are blocked by the CRF1 receptor antagonist antalarmin and by CRF1 receptor knockout. Pharma- cology, Biochemistry, and Behavior, 86, 813-821.

Czachowski, C. L., Froehlich, J. C., & DeLory, M. (2018). The Effects of Long-Term Varenicline Administration on Ethanol and Sucrose Seeking and Self-Administration in Male P Rats. *Alcoholism, clinical and experimental research*, *42*(2), 453–460. https://doi.org/10.1111/acer.13562

Daut, R. A., Busch, E. F., Ihne, J., Fisher, D., Mishina, M., Grant, S. G., Camp, M., & Holmes, A. (2015). Tolerance to ethanol intoxication after chronic ethanol: role of GluN2A and PSD-95. *Addiction biology*, *20*(2), 259–262. https://doi.org/10.1111/adb.12110

Fadda, F., & Rossetti, Z. L. (1998). Chronic ethanol consumption: from neuroadaptation to neurodegeneration. *Progress in neurobiology*, *56*(4), 385–431. https://doi.org/10.1016/s0301-0082(98)00032-x

Fillmore, M. T., & Weafer, J. (2012). Acute tolerance to alcohol in at-risk binge drinkers. *Psychology of addictive behaviors : journal of the Society of Psychologists in Addictive Behaviors*, *26*(4), 693–702. https://doi.org/10.1037/a0026110

Gass, J. T., Trantham-Davidson, H., Kassab, A. S., Glen, W. B., Jr, Olive, M. F., & Chandler, L. J. (2014). Enhancement of extinction learning attenuates ethanol-seeking behavior and alters plasticity in the prefrontal cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *34*(22), 7562–7574.

Grahame, N. J., Li, T. K., & Lumeng, L. (1999). Selective breeding for high and low alcohol preference in mice. *Behavior genetics*, *29*(1), 47–57.

Houck, C. A., & Grahame, N. J. (2018). Acute drug effects on habitual and non-habitual responding in crossed high alcohol preferring mice. *Psychopharmacology*, *235*(7), 2167–2175. https://doi.org/10.1007/s00213-018-4914-8

Kalant H. (1996). Current state of knowledge about the mechanisms of alcohol tolerance. *Addiction Biology*. 1: 133-41.

Katner, S., Magalong, J. & Weiss, F. Reinstatement of Alcohol-Seeking Behavior by Drug-Associated Discriminative Stimuli after Prolonged Extinction in the Rat. *Neuropsychopharmacol* **20,** 471–479 (1999). https://doi.org/10.1016/S0893-133X(98)00084-0

Keistler, C. R., Hammarlund, E., Barker, J. M., Bond, C. W., DiLeone, R. J., Pittenger, C., & Taylor, J. R. (2017). Regulation of Alcohol Extinction and Cue-Induced Reinstatement by Specific Projections among Medial Prefrontal Cortex, Nucleus Accumbens, and Basolateral Amygdala. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *37*(17), 4462–4471. https://doi.org/10.1523/JNEUROSCI.3383-16.2017

Kliethermes, C. L., Cronise, K., & Crabbe, J. C. (2004). Anxiety-like behavior in mice in two apparatuses during withdrawal from chronic ethanol vapor inhalation. *Alcoholism, clinical and experimental research*, *28*(7), 1012–1019. https://doi.org/10.1097/01.alc.0000131976.40428.8f

Kwako, L. E., Spagnolo, P. A., Schwandt, M. L., Thorsell, A., George, D. T., Momenan, R., Rio, D. E., Huestis, M., Anizan, S., Concheiro, M., Sinha, R., & Heilig, M. (2015). The corticotropin releasing hormone-1 (CRH1) receptor antagonist pexacerfont in alcohol dependence: a randomized controlled experimental medicine study. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *40*(5), 1053–1063. https://doi.org/10.1038/npp.2014.306

Lopez, M. F., Griffin, W. C., 3rd, Melendez, R. I., & Becker, H. C. (2012). Repeated cycles of chronic intermittent ethanol exposure leads to the development of tolerance to aversive effects of ethanol in C57BL/6J mice. *Alcoholism, clinical and experimental research*, *36*(7), 1180–1187. https://doi.org/10.1111/j.1530-0277.2011.01717.x

Lopez, M. F., Grahame, N. J., & Becker, H. C. (2011). Development of ethanol withdrawal-related sensitization and relapse drinking in mice selected for high- or low-ethanol preference. *Alcoholism, clinical and experimental research*, *35*(5), 953–962. https://doi.org/10.1111/j.1530-0277.2010.01426.x

Lopez, M. F., & Becker, H. C. (2014). Operant ethanol self-administration in ethanol dependent mice. *Alcohol (Fayetteville, N.Y.)*, *48*(3), 295–299.

Matson, L. M., Kasten, C. R., Boehm, S. L., 2nd, & Grahame, N. J. (2014). Selectively bred crossed high-alcohol-preferring mice drink to intoxication and develop functional tolerance, but not locomotor sensitization during free-choice ethanol access. *Alcoholism, clinical and experimental research*, *38*(1), 267–274.

Matson, L., Liangpunsakul, S., Crabb, D., Buckingham, A., Ross, R. A., Halcomb, M., & Grahame, N. (2013). Chronic free-choice drinking in crossed high alcohol preferring mice leads to sustained blood ethanol levels and metabolic tolerance without evidence of liver damage. *Alcoholism, clinical and experimental research*, *37*(2), 194–201.

Matson, L. M., & Grahame, N. J. (2013). Pharmacologically relevant intake during chronic, free-choice drinking rhythms in selectively bred high alcohol-preferring mice. *Addiction biology*, *18*(6), 921–929. https://doi.org/10.1111/j.1369-1600.2011.00412.x

McClearn, G. E., & Rodgers, D. A. (1959). Differences in alcohol preference among inbred strains of mice. *Quarterly Journal of Studies on Alcohol*, *20*(4), 691-695.

Metten, P., Phillips, T. J., Crabbe, J. C., Tarantino, L. M., McClearn, G. E., Plomin, R., Erwin, V. G., & Belknap, J. K. (1998). High genetic susceptibility to ethanol withdrawal predicts low ethanol consumption. *Mammalian genome : official journal of the International Mammalian Genome Society*, *9*(12), 983–990. https://doi.org/10.1007/s003359900911

Neuringer, A., Kornell, N., & Olufs, M. (2001). Stability and variability in extinction. *Journal of experimental psychology. Animal behavior processes*, *27*(1), 79–94.

Nimitvilai, S., Lopez, M. F., Mulholland, P. J., & Woodward, J. J. (2016). Chronic Intermittent Ethanol Exposure Enhances the Excitability and Synaptic Plasticity of Lateral Orbitofrontal Cortex Neurons and Induces a Tolerance to the Acute Inhibitory Actions of Ethanol. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *41*(4), 1112–1127. https://doi.org/10.1038/npp.2015.250

O'Dell, L. E., Roberts, A. J., Smith, R. T., & Koob, G. F. (2004). Enhanced alcohol self-administration after intermittent versus continuous alcohol vapor exposure. *Alcoholism, clinical and experimental research*, *28*(11), 1676–1682. https://doi.org/10.1097/01.alc.0000145781.11923.4e

O'Tousa, D. S., Warnock, K. T., Matson, L. M., Namjoshi, O. A., Linn, M. V., Tiruveedhula, V. V., Halcomb, M. E., Cook, J., Grahame, N. J., & June, H. L. (2015). Triple monoamine uptake inhibitors demonstrate a pharmacologic association between excessive drinking and impulsivity in high-alcohol-preferring (HAP) mice. *Addiction biology*, *20*(2), 236–247. https://doi.org/10.1111/adb.12100

Pohorecky, L. A., Brick, J., & Carpenter, J. A. (1986). Assessment of the development of tolerance to ethanol using multiple measures. *Alcoholism: Clinical and Experimental Research*, *10*(6), 616-622.

Samson, H. H., & Chappell, A. M. (2004). Effects of raclopride in the core of the nucleus accumbens on ethanol seeking and consumption: the use of extinction trials to measure seeking. *Alcoholism, clinical and experimental research*, *28*(4), 544–549. https://doi.org/10.1097/01.alc.0000121649.81642.3f

Spanagel R. (2017). Animal models of addiction. *Dialogues in clinical neuroscience*, *19*(3), 247–258.

Tabakoff, B., Cornell, N., & Hoffman, P. L. (1986). Alcohol tolerance. *Annals of emergency medicine*, *15*(9), 1005–1012. https://doi.org/10.1016/s0196-0644(86)80119-6

Vendruscolo, L. F., & Roberts, A. J. (2014). Operant alcohol self-administration in dependent rats: focus on the vapor model. *Alcohol (Fayetteville, N.Y.)*, *48*(3), 277–286. https://doi.org/10.1016/j.alcohol.2013.08.006

Winkler, G. A., & Grahame, N.J. (2022). Crossed High Alcohol Preferring mice with a drinking history show increased drinking in the dark intake. *Personal communiation*. IUPUI

Winkler, G. A., & Grahame, N. J. (2020). Crossed High Alcohol Preferring mice do not show protracted affective disturbance following a drinking history. *Unpublished manuscript.* IUPUI

# FIGURES

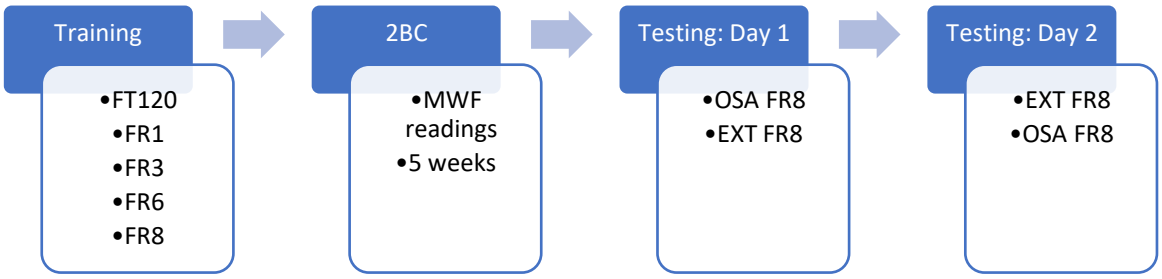


Figure 1. Experimental Timeline

Operant training followed by 5 weeks of 2BC before bottles were removed for either OSA or EXT testing based on cohort. Bottles remained off between Day 1 and Day 2 where cohorts received the opposite test.

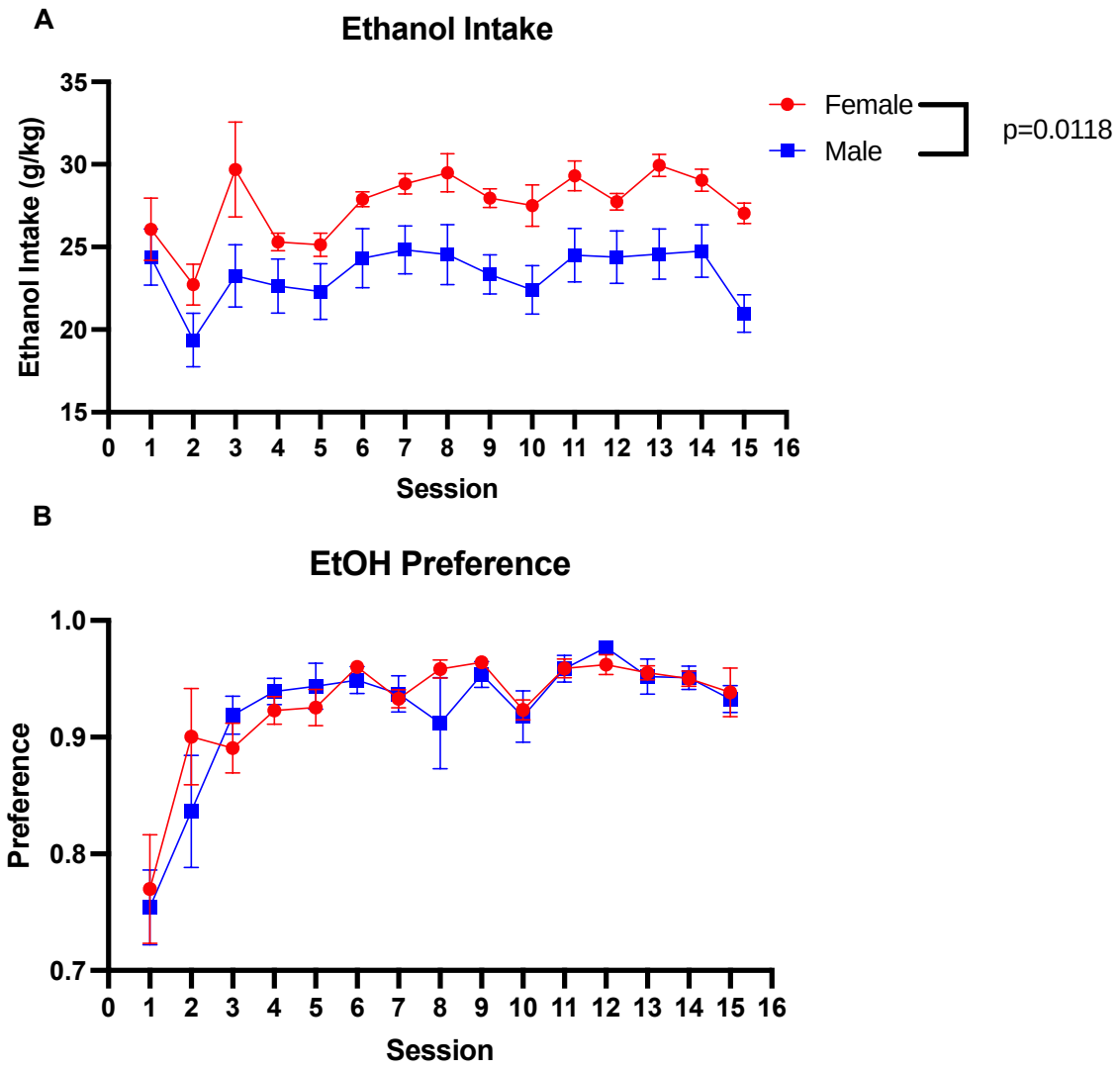


Figure 2. Two-Bottle Choice Intake and Preference

**A.** Average EtOH intake in g/kg/24h across measurement sessions, split by sex. Mixed effects analysis of sex and session revealed a main effect of session (****$p < 0.0001$) and sex (*$p < 0.05$). **B.** Average EtOH preference score across measurement sessions, split by sex. Mixed effects analysis of sex and session revealed a main effect of session (****$p < 0.0001$) but not sex ($p > 0.05$). Data are shown as the mean +/- standard error of the mean.

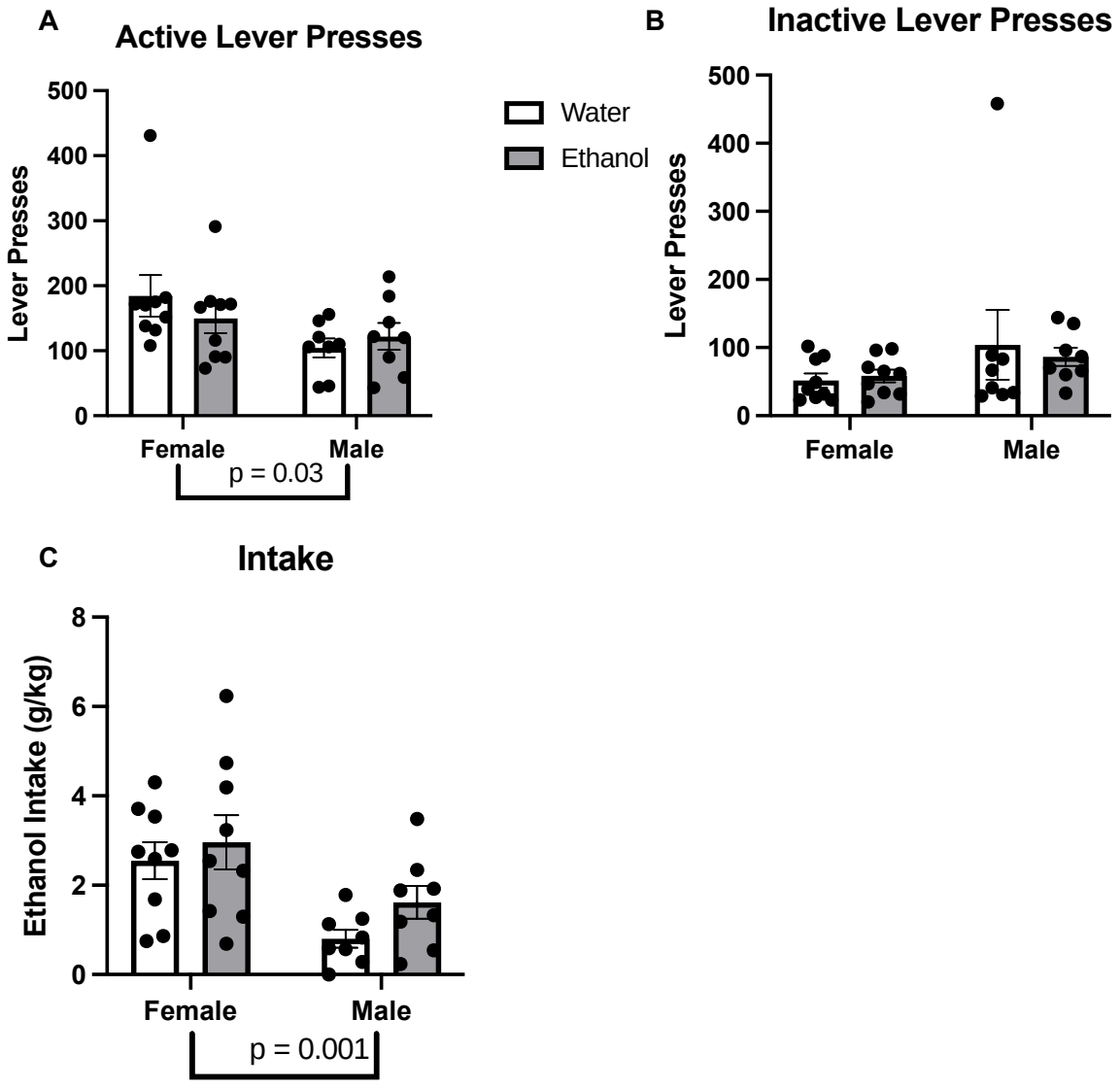


Figure 3. Day 1 Reinforced Responding

**A.** Average active lever presses in the reinforced cohort on day 1 of testing. A 2-way ANOVA revealed a significant main effect of sex (*$p < 0.05$) but not of drinking history and no interaction (both $p > 0.05$). **B.** Average inactive lever presses in the reinforced cohort on day 1 of testing. A 2-way ANOVA did not reveal any main effects or significant interactions (all $p > 0.05$) **C.** Average g/kg intake over the two-hour operant session in the reinforced cohort on day 1 of testing. A 2-way ANOVA revealed a significant main effect of sex (**$p < 0.01$) but not of drinking history and no interaction (both $p > 0.05$). All data are shown as mean +/- standard error of the mean.

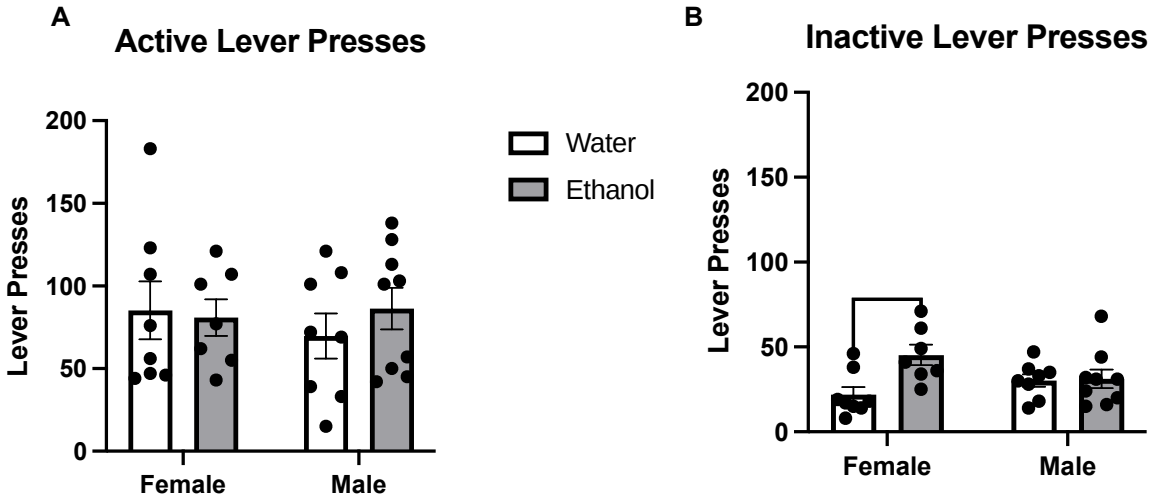


Figure 4. Day 1 Extinction Responding

**A.** Average active lever presses in the non-reinforced cohort on day 1 of testing. A 2-way ANOVA did not reveal significant main effects or interactions (all $p > 0.05$). **B.** Average inactive lever presses in the non-reinforced cohort on day 1 of testing. A 2-way ANOVA revealed a significant main effect of drinking history ($*p < 0.05$) and a significant interaction ($*p < 0.05$) but no main effect of sex ($p > 0.05$). Tukey's post-hoc multiple comparisons reveal that female EtOH history animals pressed the inactive lever significantly more than female water history animals ($*p < 0.05$). Data shown are mean +/- the standard error of the mean.

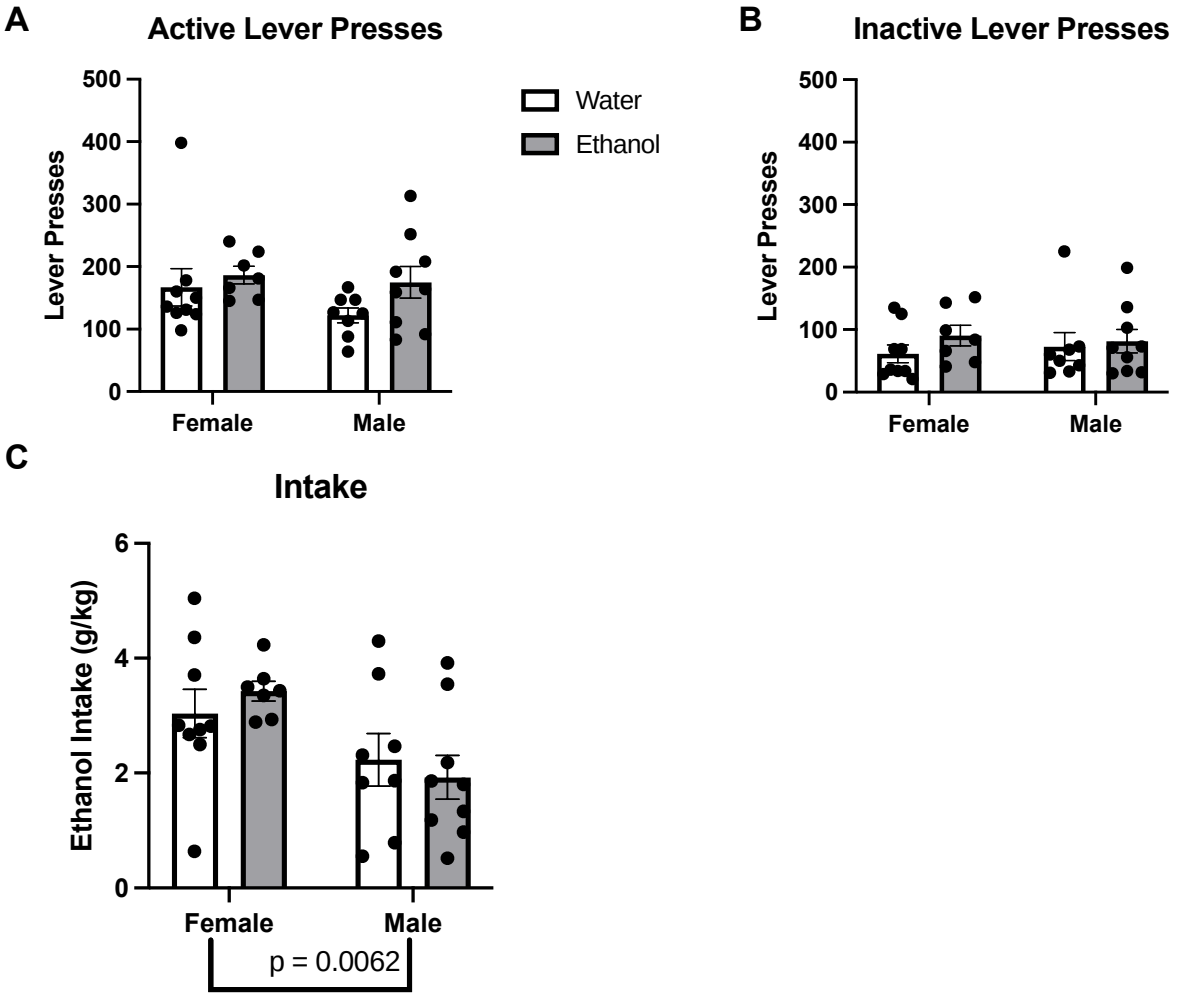


Figure 5. Day 2 Reinforced Responding

**A.** Average active lever presses in the reinforced cohort on day 2 of testing. A 2-way ANOVA did not reveal any significant main effects or interactions (all $p > 0.05$). **B.** Average inactive lever presses in the reinforced cohort on day 2 of testing. A 2-way ANOVA did not reveal any significant main effects or interactions (all $p > 0.05$). **C.** Average g/kg intake over the two-hour reinforced FR8 session on day 2. A 2-way ANOVA revealed a significant main effect of sex (**$p < 0.01$) but not a main effect of drinking history or an interaction (both $p > 0.05$). Data shown as mean +/- standard error of the mean.

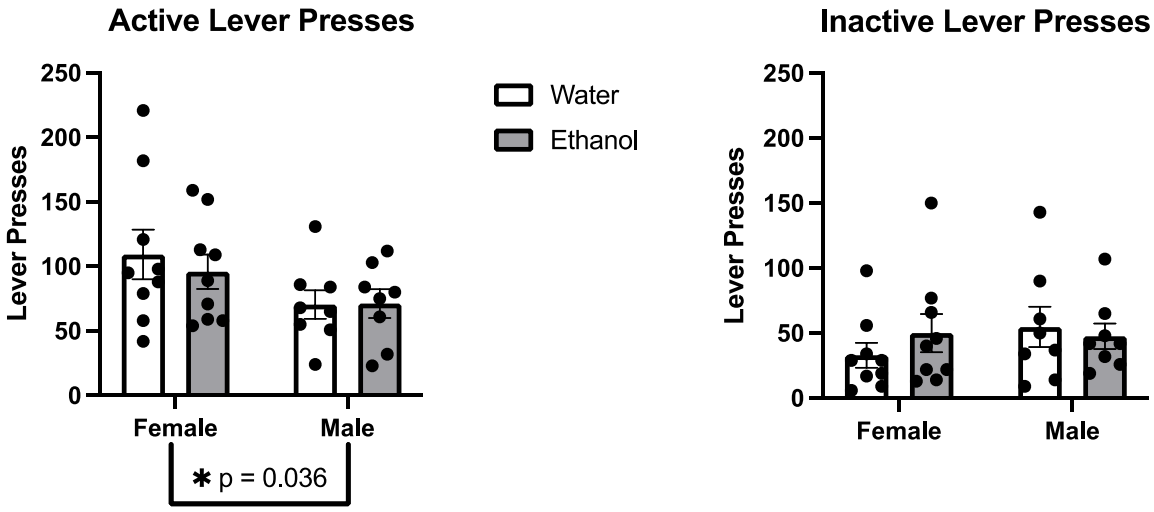


Figure 6. Day 2 Extinction Responding

**A.** Average active lever pressing behavior in the non-reinforced cohort on day 2 of testing. A 2-way ANOVA revealed a main effect of sex (*$p < 0.05$) but not of drinking history and no interaction (both $p > 0.05$). **B.** Average inactive lever pressing behavior in the non-reinforced cohort on day 2 of testing. A 2-way ANOVA did not elucidate any main effects or interactions (all $p > 0.05$). Data shown as mean +/- standard error of the mean.