A RUDIMENTARY STUDY ON AGING AND ALCOHOL SENSITIVITY IN A MOUSE MODEL

By

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ABSTRACT

Aging is a natural process that generates physiological and psychological changes. Although aspects of aging are defined by genetic programs, age can interact with environmental exposures to produce variation in phenotypes. Specifically, data suggest that aging people are differentially affected by alcohol compared to their younger counterparts (Adamson et al., 2017). Thus, elderly people, who can be at greater risk for lifestyle-related health issues, are advised to moderate alcohol intake. An interesting question is whether age plays a role in alcohol's effect on behavior. We understand that blood alcohol concentrations are linearly correlated with behavioral change, but is age a moderating factor? What about sex? Does being male or female or being of more advanced age provide some protection against abnormal behavior related to consumption? This study examines potential age-dependent behavioral changes from alcohol consumption in mice at two distinct ages. Young and old, male, and female, mice (3 and 9 months) will be provided ad lib food, along with water (control group) or 25% ethanol in water (experimental group) via self-administration for one week, with consumption monitored daily. All control and experimental mice will be assessed for anxiety/depressive-like behaviors, hyperactivity, risk-taking, and sensory-motor integration on the last day of dosing using the following behavioral assays: Sociability Chamber, Elevated Plus Maze, Rotarod and Forced Swim (Conner et al., 2020; Bottom et al., 2022). Data will reveal the impact of age and sex on behavior during alcohol consumption. We hypothesize that female older mice will be the most affected and young males, the least.

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INTRODUCTION

Alcohol has been around for thousands of years, with its uses having undergone many changes throughout history (Huang, 2020). In ancient times, alcohol served multiple purposes, from medicinal use to its inclusion in religious and social ceremonies. In the Middle Ages, alcohol was utilized as a form of payment and commonly used as a trade commodity. During the Industrial Revolution, the world witnessed an all-time high in alcohol consumption due to its newfound affordability. In the present day, alcohol is consumed for a wide variety of reasons such as socializing, relaxing, and stress relief (Al-Ameri et al., 2022).

Alcohol consumption has been associated with various physical health risks, including liver disease, cardiovascular disease, and respiratory distress syndrome (Norton, 2021; Thakur et al., 2009). In a recent study by Huang et al., heavy drinking, defined as long-term habitual alcohol consumption of more than 40 g/d in males and 20 g/d in females, or a history of binging on alcoholic beverages within the past 2 weeks, has been linked to poor outcomes in COVID-19 patients (2020). Research also shows that alcohol use disorders and tobacco use contribute a significant amount of risk to the global burden of disease, each of which is a major public health concern (Verplaetse et al., 2017). More specifically, in the last decade, the use of alcohol by adolescents has become a growing problem and an important research topic in the etiology of alcohol use disorders (Schwandt et al., 2010).

In addition to physical health concerns, alcohol use has been linked to mental health issues such as anxiety and depression. Family history of alcoholism has been related to patient reports of premenstrual alcohol consumption and severity of anxiety-related symptoms (McLeod et al., 1994). Despite its historical and cultural significance, alcohol consumption carries a plethora of potential physical and mental health risks. As such, it is crucial to acknowledge and

understand these risks in order to promote responsible drinking habits and prevent negative outcomes.

As commonly known, the inhibitory range of effects caused by ethanol consumption in humans are dependent largely upon dose. Due to social convention, alcohol is commonly ingested by humans intermittently. Because of this social practice, the ethanol found in these alcoholic drinks is metabolized slowly, causing a rapid intake of ethanol to result in the feeling of "drunkenness". This feeling of drunkenness is widely attributed to be caused by the effects of ethanol on the central nervous system, as it is a psychoactive substance that can affect various neurotransmitter systems in the brain, including the GABA, glutamate, and dopamine systems (Bjork et al., 2014). Ethanol enhances the inhibitory effects of GABA, which leads to a decrease in brain activity and a feeling of relaxation and sedation. At the same time, ethanol inhibits the excitatory effects of glutamate, which can impair cognitive and motor function. The release of dopamine in the brain's reward system is also increased by ethanol, which can contribute to the pleasurable effects of alcohol consumption (Gilman et al., 2008). While the average individual can metabolize one drink in an hour (Cedarbaum, 2012), it is important to note that this rule of thumb is widely inaccurate, as it fails to consider variables such as the individual's age, sex, weight, and other factors (Crabbe et al., 2008).

Although rates of both illicit and prescription drug abuse have increased since 1998, alcohol has consistently remained the most used substance among adults over the age of 65 (Mende, 2016). This capstone project focuses on the effect age plays on alcohol metabolism and inhibition. This is significant because although there are many laws that take blood alcohol content into consideration, none of them take age into account. With further research, more

effective and safer laws can be enacted to ensure automobile safety, healthier communities, and increased education and awareness.

Upon initial review, there are very few studies that compare the effects of alcohol use and age using mouse models. Of those few, many include other variables such as other types of drugs and different methods of alcohol intake. They also focus more on the biochemical and physiological effects of being inebriated (Mende, 2019). The last relevant study on this topic which was conducted in a similar fashion as this experiment was published in 1976, and although that project aimed to study the same topic using similar behavioral tests, the author acknowledged that there were some problems with the control group, and the age ranges used (Wood, 1976). Considering that this was 47 years ago, another study using more recent behavioral assays and methods of data collection would greatly benefit the field of alcohol interactions. For this project, the effects of intoxication between mice consisting of two age groups (three and nine months) will be compared to see if there is a link between aging and alcohol metabolism.

METHODS AND APPROACH

Subjects

44 outbred males and 32 outbred females CD-1 mice (*Mus musculus*) were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in an animal care facility at the University of California, Riverside under Professor Kelly Huffman's research laboratory and animal use protocol. While under the experimental paradigm, data was collected on both ethanol exposure and control mice's weight, food intake, and liquid intake every twenty-four hours, spanning our 5 day exposure model. This model consisted of giving mice ethanol in their

drinking water at a concentration of 25%. The control mice were given water without ethanol. Mice were kept separately in a secure mouse cage measuring 10 x 6 x 5 inches, or 300 square inches of space. The mouse was provided with adequate bedding, food, and liquid *ad libitum*. All mice were housed in a room with a 12-hour light and a 12-hour dark cycle. Treatment of these mice was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Riverside.

Male and female mice at 3 months of age (n=33) and 9 months of age (n=43) were randomly assigned to either the control or experimental (ethanol) group, resulting in eight groups of mice: 3-month male control, 3-month male experimental, 9-month male control, 9-month male experimental, 3-month female control, 3-month female experimental, 9-month female control, and 9-month female experimental.

Measurement of Variables

During the 5-day experimental paradigm, weight change, food intake, and liquid consumption was measured daily for each mouse. To avoid disturbance and get accurate data, these measurements were taken at the same time of day (e.g. 9am) every day.

Behavioral Assays

Behavioral Assays were conducted on mice after they were exposed to their respective experimental and control treatments for a period of 5 days. For both the experimental and control mice, subjects were isolated with enough mouse chow to feed them for 5 days. The only difference between these two groups was the twenty-five percent (25%) ethanol solution in water that the experimental group was given, in contrast to the one hundred percent (100%) water

solution that was given to the control group. To help minimize the potential impact of unfamiliar surroundings on the behavior of the mice and ensure the accuracy of the experimental results, both the experimental and control groups were moved from the animal facilities to our laboratory's behavioral testing room at least thirty minutes prior to testing in order to give the mice time to acclimate to the new environment.

For the purposes of this project, behavioral assays such as the elevated plus maze (EPM), the sociability chamber (SOC), the Forced swim test (FS), and the Accelerated Rotarod (AR) were used. Mice used for this project were subjected to a maximum of two behavioral tests during the testing period with the Accelerated Rotarod and Forced Swim always performed last due to the intensive and stress-inducing nature of both of these tests. Typically, mice were subjected to the EPM test or the SOC test first, followed by either the FS test or AR after a period of at least thirty minutes. All of the behavioral analyses and scoring were performed and analyzed by members of the Huffman Lab. All testing apparatuses were cleaned using Virkon before and after each test in order to ensure a consistent level of cleanliness and safety for mice and to eliminate olfactory cues.

Elevated Plus Maze

The elevated plus maze is one of the most commonly used behavioral assays for rodents to assess anxiety-related behaviors (Lister, 1987). The apparatus used for this assay consists of four arms arranged in a plus shape, with two of these arms closed on three sides, and the other two being open. The mouse is placed in the center junction of these four arms, facing an open arm. The rodent is recorded by a camera for the duration of the experiment, with this video

recording then rewatched and scored to track the entries and time the rodent spent on each arm during its time in the elevated-plus apparatus.

The time spent in the open arms is considered an index of risk-taking behavior, and the time spent in the closed arms is considered to be an index of anxiety-like behavior in rodent studies. By analyzing the time spent in the open and closed arms, researchers can gain a more detailed understanding of whether the mouse is more prone to anxiety-like or risk-taking behaviors, and use the number of entries into each arm to provide information about the exploratory behavior of the animal

Three-Chambered Sociability Test

Originally developed by Crawley and colleagues, this test has several advantages that improve data reliability for examination of sociability, social affiliation, social memory, and performance using similar procedures. Over the years, this test has become a standard paradigm used to evaluate social preference and social novelty preference in mice (Kaidanovich-Beilin et al., 2011).

The test is conducted in a three-chambered apparatus, with a wire cage on the two most lateral sides of the chamber. The test mouse is placed in the center chamber to habituate to its new environment for the first phase of the test. During the second phase of the test, the mouse is allowed to explore all three chambers. Time spent in each chamber is recorded for analysis in a "sidedness test", where researchers can see if the mouse prefers one side of the chamber over another. For the third and final phase of the test, a novel mouse is introduced into one of the wire cages on either side of the chamber. The mouse is allowed to move around and explore the chamber once again, while time spent in each chamber is recorded to evaluate for "sociability".

Accelerated Rotarod

The Accelerated Rotarod test is a widely used behavioral assay used to measure levels of physical activity, specifically motor coordination, balance, and endurance in rodents. In the past, it has been used to evaluate the effects of various interventions on motor coordination and balance, such as calorie restriction, the plant micronutrient resveratrol (Bordone et al., 2007), immunotherapy, and for our purposes – ethanol (Perez et al., 2023).

The apparatus required for this test consists of a rotating rod, usually made of plastic or metal, which is divided into individual lanes. This rod is suspended horizontally and can either rotate at a fixed speed or accelerate at a uniform rate. The subject mouse is placed on the rotating rod and the amount of time it takes for the animal to fall off is recorded. This test is typically performed over several trials with set inter-trial intervals being allotted for subject recuperation.

Although this test is highly regarded because of its sensitivity to subtle changes in motor function and it being a relatively quick and easy assay to perform, it does have some limitations. The data obtained from each trial is highly dependent on the animal's motivation to remain on the rod, and results can be affected by other variables such as anxiety or fear.

While it is important to use the accelerated rotarod in conjunction with other tests to obtain a more comprehensive assessment of motor function, it is suitable for our project as our focus is not exclusively on the motor effects of ethanol, but rather on the effects of ethanol across multiple assays and ages.

Forced Swim Test

The forced swim test is used for assessing antidepressant-like behavior in rodents, specifically for testing experimental antidepressant drugs (Cryan, 2012). The test involves placing the subject mouse in a cylinder filled with water from which there is no escape. The mouse is given a certain amount of time to acclimate, and then time spent immobile in the cylinder is recorded. For the purposes of our project, immobility is defined as the absence of active movements, such as swimming, struggling, or climbing. Common discourse considers this to reflect a state of despair or hopelessness.

Even though this test is very popular for measuring antidepressant-like behavior, forced swim has some limitations, due to the varied nature of chamber construction, water temperature, time of day, etc. Therefore, we will evaluate our FS results as between active and passive coping strategies (Amario et al., 2021; Perez et al., 2023). Active behaviors such as swimming and attempting to climb are considered adaptive (or active) coping strategies, whereas immobility is considered a maladaptive (or passive) coping strategy. This “coping type” theory proposes that the immobility behavior recorded during forced swim tests reflect a coping strategy used by the animal to conserve energy and minimize stress. This may reflect a learned helplessness response, in which the animal learns that its actions are inevitably futile and that it cannot control its environment. Mice with different coping strategies could be attributed to different underlying neural mechanisms and may respond differently to certain treatments.

Scoring of Assays

Recorded videos of the elevated plus maze, three-chambered sociability test, and forced swim were scored by a trained observer using a custom Python program. The scorer pressed certain

keys on a computer keyboard to record the location of the mouse during the EPM and sociability assays, or whether or not the mouse was immobile during the forced swim tests. Observer was unaware of the experimental condition, to reduce experimenter bias.

Data Analysis

Data collected from the measurements in weight change, food intake, water intake, and all behavioral assays except accelerated rotarod were analyzed using unpaired t-tests. For the accelerated rotarod, either a mixed effects analysis or a 2-way ANOVA was used. All statistical analyses were performed using Graphpad Prism (Version 9.0).

RESULTS

Food, Liquid, and Weight Comparisons Between Control Mice at 3 and 9 Months

In order to establish baselines and account for the potential confounding factors solely associated with aging, we conducted data analysis of food intake, water consumption, and weight changes in control mice at 3 and 9 months.

There was a slightly significant difference in weight change (in grams) between male control mice ($t(21) = 2.408$, $p < 0.0253$) at 3 and 9 months **(Figure 1e)**, but no significant differences between female control mice ($t(15) = 0.4220$, $p < 0.6790$) at 3 and 9 months **(Figure 1f)**. The male control mice at 9 months (n= 14, M= -0.8571, SD= 0.9493) lost less weight compared to their 3 month counterparts (n= 9, M= 0.1111, SD= 0.9280), while the female control mice had no significant changes in weight at 3 (n= 7, M= -0.7143, SD= 1.380) and 9 months (n= 10, M= -0.500, SD= 0.7071).

We found no significant differences in food intake (in grams) for both the control males **(Figure 1a)** ($t(15) = 0.3944$, $p < 0.6988$) and the control females at 3 and 9 months **(Figure 1b)** ($t(14) = 0.08320$, $p < 0.9349$). Food intake was fairly similar between control males at age 3 (n= 8, M= 10.44, SD= 2.939) and age 9 (n= 9, M= 10.44, SD= 1.039), and control females at age 3 (n= 6, M= 8.792, SD= 3.352) and age 9 (n= 10, M= 8.650, SD= 3.266).

We also found no significant differences between liquid intake (in mLs) for male **(Figure 1c)** ($t(21) = 0.6558$, $p < 0.5191$) and female **(Figure 1d)** ($t(15) = 0.6558$, $p < 0.5219$) control mice at 3 and 9 months. Male mice at 3 (n= 9, M= 11.94, SD= 2.732) and 9 months (n= 14, M= 11.16, SD= 2.836) drank roughly the same amount of water, as did the female mice at 3 (n= 7, M= 11.431, SD= 2.095) and 9 (n= 10, M= 10.50, SD= 3.291) months.

Behavioral Metric Comparisons for Control Mice at 3 and 9 months

We also compared performance on behavioral assays for control mice at 3 and 9 months in order to establish clear baselines for our data, as well as account for any confounds caused by aging.

We found no significant differences in performance on the Elevated Plus Maze (EPM) for both control males **(Figure 2a)** ($t(10) = 0.9211$, $p < 0.3787$) and females **(Figure 2b)** ($t(9) = 1.277$, $p < 0.2336$) at the two age points while comparing time (in seconds) spent on open arms. Times were similar for male control mice at 3 (n= 5, M= 2.402, SD= 0.7798) and 9 (n= 7, M= 3.691, SD= 1.142) months and for female controls at 3 (n= 4, M= 4.097, SD= 2.535) and 9 (n= 7, M= 8.438, SD= 6.397) months.

There were also no significant differences in male **(Figure 2c)** ($t(6) = 0.7987$, $p < 0.4549$) and female **(Figure 2d)** ($t(7) = 0.8276$, $p < 0.4352$) control mice at 3 and 9 months while

analyzing the data for time (in seconds) the mice spent with novel mice using the 3-Chambered Sociability Test. Though it wasn't significant, control males at 9 months (n= 4, M= 48.24, SD= 5.407) were slightly more social than controls at 3 months (n= 4, M= 42.72, SD= 12.72) and control females at 9 months (n= 5, M= 51.96, SD= 10.28) were also slightly more social than controls at 3 months (n= 4, M= 54.57, SD= 12.98).

We did not find any significant differences between the male **(Figure 2e)** ($t(8) = 0.09055$, $p< 0.9301$) and female **(Figure 2f)** ($t(5) = 0.7856$, $p< 0.4677$) controls while comparing the percentage of time the mice spent immobile for the Forced Swim Test. The time the control males spent immobile at 3 months (n= 5, M= 30.86, SD= 24.53) versus 9 months (n= 5, M= 31.94, SD= 10.59) was a lot more similar compared to the amount of time the female mice spent immobile at 3 (n= 3, M= 25.94, SD= 14.10) and 9 (n= 4, M= 31.79, SD= 5.090) months.

A 2-way ANOVA was performed to compare mouse performance on the Accelerated Rotarod at 3 and 9 months for both control males and females. We did not find any significant differences between the time spent on the rod by males **(Figure 2g)** ($F(1, 9) = 1.215$, $p= 0.2990$) and females **(Figure 2h)** ($F(1, 7) = 0.0009669$, $p= 0.9761$) due to differences in age. Post-hoc analysis was performed using Sidak's planned comparisons, where we observed no statistical differences between the two age groups for both sexes.

Food, Liquid, and Weight Comparisons for Control vs. Experimental Mice at 3 Months

Subject weight, average food intake, and average liquid intake during the paradigm were all measured and analyzed. While analyzing these changes during our five day experimental period, and we report significant decreases ($t(16) = 6.903$, $p<0.0001$) in weight for 3 month male mice exposed to ethanol (n= 9, M= -4.889, SD= 1.965) compared to 3 month control mice (n= 9,

M= 0.111, SD= 0.9280) **(Figure 3e)**. We also reported a significant decrease (t(13) = 3.803, p<0.0022) in weight for female mice exposed to ethanol during the experimental period (n= 8, M= -3.750, SD= 1.669) versus the 3 month female control mice (n= 7, M= -0.7143, SD= 1.380) **(Figure 3f)**.

We found no significant differences comparing average daily food intake between the experimental group and the control group for male **(Figure 3a)** (t(14) = 0.4670, p<0.6477) and female **(Figure 3b)** (t(11) = 1.391, p<0.1917) mice at 3 months of age. Therefore, there were no significant differences for male food intake while comparing the control (n=8, M= 10.44, SD= 2.939) and the experimental (n=8, M= 9.750, SD= 2.949) group. Similarly, there was also no significance between food intake for age 3 females from the control group (n= 6, M= 8.792, SD= 3.352) compared to the experimental group (n=7, M= 6.643, SD = 2.184).

There were also no significant differences when comparing average daily liquid (either water or 25% ethanol) consumption between the experimental group and control group for males **(Figure 3c)** (t(16) = 0.8219) and females **(Figure 3d)** (t(13) = 1.508) at 3 months of age. Due to this, there were no significant differences for male liquid consumption while comparing the control group (n= 9, M= 11.94, SD= 2.732) to the experimental group (n= 9, M= 10.83, SD= 2.997). There was also no significance found while comparing the female liquid consumption between the control (n= 7, M= 11.43, SD= 2.095) and experimental treatments (n= 8, M= 9.219, SD= 3.337).

Behavioral Metric Comparisons for Control vs. Experimental Mice at 3 Months

However, we did find significant differences when comparing mouse performance on behavioral assays. While analyzing data from the Elevated Plus Maze, we found significant

differences between time spent on the open arms of the apparatus for both the male **(Figure 4a)** ($t(8) = 4.242$, $p<0.0028$) and female **(Figure 4b)** ($t(6) = 2.699$, $p<0.0356$) groups on mice 3 months of age. For male mice in this age group, control mice (n= 5, M= 2.402, SD= 0.7798) spent significantly less time on the open arms compared to our mice who were treated with ethanol (n= 5, M= 14.66, SD= 6.413). For female mice in the same age group, we also saw control mice (n= 4, M= 4.097, SD= 2.535) spend a significantly less amount of time in the open arms compared to our experimental group (n=4, M= 34.10, SD= 11.05).

We did not find any significant differences between either the male **(Figure 4c)** ($t(7) = 0.1754$, $p<0.8657$) or female **(Figure 4d)** ($t(7) = 0.8081$, $p< 0.4456$) groups while analyzing the data from the Sociability Chamber experiment. When comparing the time that the control (n= 4, M= 42.72, SD= 12.72) and experimental (n= 5, M= 41.61, SD= 5.678) groups of 3 month males spent with the novel mouse for this experiment, we did not find any significant differences. We also did not find any significant differences for time spent with the novel mouse between the control (n= 4, M= 45.57, SD= 12.98) and experimental (n=5, M= 40.16, SD= 6.911) groups in the age 3 female category.

While comparing the time the 3 month mice spent idle in the Forced Swim test, we did not find significant differences **(Figure 4e)** ($t(7) = 1.240$, $p<0.2551$) for the males or the **(Figure 4f)** females ($t(4) = 1.470$, $p< 0.2156$). We see that the time the average male experimental mouse (n= 4, M=48.50, SD= 15.76) spent idle during the course of this test is slightly higher than that of the average control mouse (n=5, M= 30.86, SD= 24.53). The time the average female experimental mouse (n=3, M= 44.32, SD= 16.42) spent idle is also higher than that of the average female control (n=3, M= 25.94, SD= 14.10), but the differences between these two groups was not significant.

For the Accelerated Rotarod, a mixed effects analysis was performed to compare the effects of the male's experimental condition on time spent on the rotarod. There was a statistically significant difference for the amount of time spent on the rotarod between the male experimental condition (control versus ethanol) at 3 months ($F(1, 3) = 40.86$, $p=0.0078$).

Post-hoc analysis was performed using Sidak's planned comparisons. We observed statistical differences between the two conditions at trial 3 ((control n=3, ethanol n=4), p= 0.003) and trial 4 ((control n=3, ethanol n=4), p= 0.003) **(Figure 4g)**.

A 2-way Repeated Measures Analysis of Variance (ANOVA) was performed to compare the effects of the female's experimental condition to time spent on the accelerated rotarod. We did not find a significant difference in time spent on the rotarod between the control and experimental conditions in 3 month females ($F(1, 3) = 6.557$, $p= 0.0832$) **(Figure 4h)**. No significant differences were found on the additional post hoc analysis was performed using Sidak's multiple comparisons test.

Food, Liquid, and Weight Comparisons for Control vs. Experimental Mice at 9 Months

The same data analysis was also done for mice that were 9 months old. In this age group, we also found significant decreases in weight for both male **(Figure 5e)** ($t(24) = 6.142$, $p< 0.0001$) and female mice **(Figure 5f)** ($t(15) = 3.621$, $p< 0.0025$). The male control group (n=14, M= -0.8571, SD= 0.9493) maintained a relatively uniform weight, while the male experimental group l(n=12, M= -3.50, SD= 1.243) lost much more weight. This same effect can be observed between the control females (n= 10, M= -0.500, SD= 0.7071) and the experimental females (n=7, M=-2.571, SD= 1.618) for the same age group.

In addition to weight, we also found a decrease in food intake for both male **(Figure 5a)** ($t(18) = 4.847$, $p < 0.0001$) and female **(Figure 5b)** ($t(14) = 2.133$, $p < 0.0511$) mice at 9 months. The experimental males (n=10, M= 4.875, SD= 1.976) for this age group ate a significantly lower amount of food than the control males (n=10, M= 11.16, SD= 3.592). Although the experimental females (n=6, M= 5.375, SD= 2.355) in this age group ate less food than the control group (n=10, M= 8.650, 3.266), the difference was not large enough to be statistically significant.

We found no significant differences between liquid intake in both the male **(Figure 5c)** ($t(24) = 1.548$, $p < 0.1348$) and female **(Figure 5d)** ($t(15) = 0.5535$, $p < 0.5881$) experimental groups. Therefore, there was no significant difference between experimental males (n=12, M=13.33, SD= 4.275) and control males (n=14, M= 11.16, SD= 2.836), as well as experimental females (n= 8, M= 9.219, SD= 3.337) and control females (n=7, M= 11.43, SD= 2.095).

Behavioral Metric Comparisons for Control vs. Experimental Mice at 9 Months

Similar to our findings in 3 month mice, we found a significant difference in male **(Figure 6a)** ($t(12) = 2.939$, $p < 0.0124$) and female **(Figure 6b)** ($t(9) = 2.378$, $p < 0.0414$) mouse behavior using the elevated plus maze. The amount of time the experimental males (n=7, M= 13.18, SD= 7.990) spent in the open arms of the elevated plus maze apparatus was significantly higher than that of the control males (n=7, M= 3.691, SD= 3.020). We see similar results for experimental females (n=4, M= 18.09, SD= 6.628) and control females (n=7, M= 8.438, SD= 6.397) in this age group as well.

While analyzing data for the three-chambered sociability test, we found significant differences in both the male **(Figure 6c)** ($t(7) = 3.232$, $p < 0.0144$) and female **(Figure 6d)** ($t(6) =$

4.121, p< 0.0062) groups of this age group. The experimental males (n=5, M= 31.90, SD= 8.801) spent a lot less time in the same chamber as the novel mouse than the control group (n=4, M= 48.24, SD= 2.704). Similarly, we see a decrease in the time the experimental females (n=3, M= 25.67, SD= 4.191) spent with novel mice compared to the control females (n=5, M= 51.96, SD= 10.28).

We also found significant differences while testing the mice using the forced swim test for both the male **(Figure 6e)** (t(9)= 2.487, p< 0.0346) and female **(Figure 6f)** (t(5)= 7.172, p< 0.0008) groups. The experimental males (n=6, M= 61.92, SD= 24.97) were immobile for a significantly higher fraction of the test compared to the control males (n= 5, M= 31.94, SD= 10.59). Similarly, the experimental females (n=3, M= 72.04, SD= 9.804) were immobile for a significantly longer time compared to the control females (n=4, M= 31.79, SD= 5.090).

A mixed effects analysis was performed on both the male and female groups to compare effects of experimental conditions on time spent on the accelerated rotarod.

There was a statistically significant difference in time spent on rotarod between control and ethanol males **(Figure 6g)** (F(1, 8)= 5.858, p= 0.0418), as well as the control as ethanol females **(Figure 6h)** (F(1, 4)= 19, p= 0.0121) at 9 months.

Additional post hoc analysis was performed using Sidak's multiple comparisons test. We observed statistically significant differences in both experimental conditions at trial 4 for both the males ((control n= 9 , ethanol n= 7), p= 0.0087) and the females ((control n= 5, ethanol n=4), p= 0.0350).

Food, Liquid, and Weight Comparisons Between Experimental Mice at 3 and 9 Months

Following the comparison of control mice at our two different time points and comparing the differences between the ethanol group and the control group within each age, we proceeded to conduct unpaired t-tests exclusively on mice exposed to ethanol at either 3 or 9 months. The purpose of these tests were to determine whether any statistically significant changes occurred as a result of ethanol administration over the six month age difference.

We found trends towards significance in both our experimental male **(Figure 7e)** ($t(19)= 1.984$, $p< 0.0619$) and female **(Figure 7f)** ($t(13)= 1.384$, $p< 0.1898$) groups when comparing weight change. The 9 month experimental males ($n=12$, $M= -3.500$, $SD= 1.243$) lost less weight compared to the 3 month experimental males ($n=9$, $M= -4.889$, $SD= 1.965$), and our 9 month experimental females ($n=7$, $M= -2.571$, $SD= 1.618$) also lost less weight compared to our 3 month experimental females ($n=8$, $M= -3.750$, $SD= 1.669$).

There was no significance found between the liquid consumption in both our male **(Figure 7c)** ($t(19)= 1.496$, $p< 0.1511$) and female **(Figure 7d)** ($t(13)= 1.239$, $p< 0.2371$) experimental groups when comparing between 3 months and 9 months. However, we did see a slightly higher intake of liquid in males at 9 months ($n=12$, $M= 13.33$, $SD= 4.275$) compared to males at 3 months ($n= 9$, $M= 10.83$, $SD= 2.997$). We also saw a similar trend occur within the experimental females that are 9 months ($n= 7$, $M= 11.43$, $SD= 3.567$) compared to 3 months ($n= 8$, $M= 9.219$, $SD= 1.180$).

We observed a significant difference in food intake between experimental males **(Figure 7a)** ($t(15)= 3.903$, $p< 0.0014$) at 3 ($n= 8$, $M= 9.750$, $SD= 2.949$) and 9 months ($n= 9$, $M= 4.972$, $SD= 2.071$), with males eating less under the effects of ethanol as they get older. We did not see a significant difference between experimental females **(Figure 7b)** ($t(11)= 1.007$, $p< 0.3355$)

when comparing food intake at 3 (n= 7, M= 6.643, SD= 2.184) and 9 months (n= 6, M= 5.375, SD= 2.355), but we still see a decrease in the average of 9 month females compared to the 3 month group.

Behavioral Metric Comparisons Between Experimental Mice at 3 and 9 Months

There were no significant differences in performance on the Elevated Plus Maze when comparing the percent of time spent in the open arms for both experimental males **(Figure 8a)** ($t(10)= 0.3408$, $p< 0.0014$) and females **(Figure 8b)** ($t(15)= 3.903$, $p< 0.0014$) in consideration with our two age points. Percent of time in the open arms was similar for experimental males at 3 (n= 5, M= 14.66, SD= 6.413) and 9 (n= 7, M= 13.18, SD= 7.990) months, as well as the experimental females at 3 (n= 4, M= 34.10, SD= 22.09) and 9 months (n= 4, M= 18.09, SD= 6.628).

While comparing percent of time the mice spent with novel mice on the 3-Chambered Sociability Test, we found significant differences in both our experimental male **(Figure 8c)** ($t(8)= 2.513$, $p< 0.0362$) and female groups **(Figure 8d)** ($t(6)= 3.232$, $p< 0.0179$). We see a decrease in social time in male (n= 5, M= 30.90, SD= 7.662) and female mice (n= 3, M= 25.67, SD= 4.191) at 9 months of age compared to male (n= 5, M= 41.61, SD= 5.678) and female mice (n= 5, M= 40.16, SD= 6.911) at 3 months of age.

Although we see no significant differences between experimental males **(Figure 8e)** ($t(8)= 0.9460$, $p< 0.03718$) and females **(Figure 8f)** ($t(4)= 2.512$, $p< 0.0659$) when comparing time the mice were immobile during the Forced Swim Test, we can see a trend emerging where the 9 month males (n= 6, M= 61.92, SD= 24.97) and females (n= 3, M= 72.04, SD= 9.804)

spend an increased amount of time immobile compared to the 3 month male (n= 4, M= 48.50, SD= 15.76) and females (n= 3, M= 44.31, SD= 16.42).

A 2-way ANOVA was performed to compare mouse performance on the Accelerated Rotarod at 3 and 9 months for both experimental males and females. We did not find any significant differences between the time spent on the rod by males **(Figure 8g)** ($F(1, 9) = 2.181$, $p= 0.1739$) and females **(Figure 8h)** ($F(1, 6) = 0.739$, $p= 0.4229$) due to differences in age. Post-hoc analysis was performed using Sidak's planned comparisons, where we observed no statistical differences between the two age groups for both sexes. We did see a slight decrease in average time on the rod in the older mice however, as evidenced by the figures.

DISCUSSION

Previous research has established that alcohol affects humans differently through time due to various factors – one of which may be sex hormones. According to Lenz and colleagues (2012), sex hormones exert both organizational (permanent) and activational (transient) effects on the human brain, with the sensitive period for these effects lasting throughout human life. A sex difference such as being male is a crucial risk factor for the onset of mental conditions such as Alcohol Use Disorder (AUD), with testosterone playing a modifying role in alcohol addiction-related behavior. On the other hand, females are more susceptible to the negative effects of alcohol due to their lower body weight and water content, leading to higher blood alcohol concentrations (Lancaster et al., 1994).

Factors such as age, genetics, and metabolism also contribute to how alcohol affects humans differently throughout time. Geriatric populations have a decreased ability to metabolize alcohol, leading to higher blood alcohol concentrations and increased susceptibility to the

negative effects of alcohol (Bengtsson et al., 1993). Research has shown that Alcohol Dehydrogenase (ADH) activity, which is a crucial enzyme in the metabolism of alcohol in humans, has been found to vary with age. For example, Bhatt et al. found that hepatic cytosolic ADH activity decreases with age in humans (2017). This age-dependent decrease in ADH activity may contribute to the increased susceptibility of older individuals to the negative effects of alcohol.

In our current study, we evaluated changes in food and liquid consumption, body weight, and performed a battery of behavioral tasks in mice to evaluate whether alcohol plays a role in affecting any of these variables at different time points in life.

Our study evaluated the effects of acute ethanol exposure on male and female mice at two different ages, 3 months and 9 months. Our results showed sustained reductions in weight for male and female mice treated with ethanol at 3 months and 9 months. To make sure this decrease was due solely because of the aging process, we also compared weight changes in control mice at 3 and 9 months. In doing so, we found a significant, but negligible difference of around 0.75 grams between the averages of these two groups. We also see that the average weight loss decreases in our 9 month experimental male and female mice, even though they consumed less food than the 3 month experimental mice. This could be due to the additional calories provided by the alcohol exposure, as well as the higher fat reserves present in older mice, particularly males. Reynolds et al. (2019) supports this hypothesis by demonstrating that in their study, male C57BL/6J mice tend to accumulate significantly more fat mass with advancing age compared to female mice.

Notably, there is no clear evidence that male and female mice lose weight after consuming ethanol for multiple days; however, some studies have shown that chronic ethanol

consumption can lead to changes in body weight and metabolism in mice. Specifically, a study found that chronic ethanol consumption lessens the gain of body weight, liver triglycerides, and diabetes in obese ob/ob mice (Fromentry et al., 2009).

In our study, liquid consumption was not significantly different between age, sex, and treatment groups. However, we did observe a substantial reduction in food intake for both male and female mice treated with ethanol at 9 months compared to their control counterparts. We can confirm that this reduction in food intake is not completely attributable to the aging process, as there were no significant differences in food intake when comparing our 3 month control mice with our 9 month control mice. Although the amount of food consumed at 9 months is lower in both the male and female experimental groups in comparison to the 3 month data, we only see a significant difference between the two in the males.

This decrease in appetite may have been due to the distinct and unpleasant taste of ethanol, which could have led to a conditioned taste aversion to the food when consumed with ethanol (Crabbe et al., 2019). While we suggest that taste aversion may have played a role in the observed reduction in food intake in mice treated with ethanol, further research would be required to confirm this theory and the mechanism behind this effect and to determine whether other factors, such as changes in metabolism or satiety signaling, may also contribute to the observed reduction in food intake. It is well-established that alcohol consumption can lead to reduced food intake in humans. This effect has been observed in real-world settings, where individuals may consume alcohol instead of eating a meal or may simply feel less hungry after consuming alcohol. In sum, our findings suggest that acute ethanol exposure can lead to a reduction in body weight due to an increased aversion to food, more commonly seen at older ages.

Our study showed a significant increase in the amount of time spent on the open arms in the EPM for the mice that were put on the ethanol paradigm compared to the control group at both 3 and 9 months of age. When comparing times for our control 3 month mice with our control 9 month mice, we found no significant differences between the two. We also see no significant difference between the two experimental groups at both age points, suggesting that risk-taking and anxiety-like behaviors are minimally affected by ethanol, even when aging. These findings are consistent with previous research done by our lab, where we measured behavioral differences in mice exposed to ethanol via Lacational Ethanol Exposure (LEE) (Perez et al., 2023). Although Perez and colleagues (2023), did not observe any significant differences in the EPM, they did see trends towards significance, with LEE mice spending higher amounts of time on the open arms compared to controls. The similarities between these results and our findings suggest that ethanol exposure may have an impact on risk-taking behaviors in mice, but again, further research is needed to determine the underlying mechanisms and potential age-related effects.

As for the 3-chambered sociability test, we observed little to no differences between the control and ethanol groups at 3 months of age. However, at 9 months of age, we found significant decreases in sociability for both male and female mice in the ethanol group compared to controls. When comparing sociability between our experimental groups at both time points, we found significant decreases at 9 months compared to 3 months. This pattern of significant decreases in sociability for the 9-month old ethanol-exposed mice compared the controls suggests that some developmental processes may be involved in the observed changes, as is supported by Demarque and colleagues (2020) in their study on Swiss mice, which investigated

the effects of ethanol exposure during brain growth spurts. Our data suggests that ethanol leads to a decrease in social behaviors in aging mice.

Our results from the forced swim test (FST) suggest that ethanol exposure may lead to a more passive coping style, indicated by the increased immobility seen in both male and female mice at 9 months. At 3 months, we only saw trends towards significance for both male and female ethanol-exposed groups spending a longer amount of time immobile compared to control mice. This finding is also consistent with a previous study in our lab, which also used the FST to assess passive coping behavior in mice exposed to ethanol through breastmilk (Perez et al., 2023). When comparing the control groups at both age groups, we found no significant differences, which could mean that aging did not affect the results of this behavioral assay. When comparing out experimental groups at both age points, we see strong trends towards significance, suggesting that the effects of ethanol exposure on passive coping behavior may vary depending on developmental stage. Coping style may be a strategic approach to dealing with stressful situations which may change with age, as suggested in Oh et al., 2018. Oh and colleagues found that proactive coping strategies (such as time spent mobile in the FST) tend to decline with age, while stress responsiveness was the highest in young mice and decreased with age. Several studies support the idea that ethanol exposure during a critical developmental period could lead to a more passive coping style and changes in stress responsiveness, impacting fear memory consolidation and maintenance (Perez et al., 2023; Oh et al., 2018). Further studies are needed to investigate the potential mechanisms underlying these changes. By using data collected from the Forced Swim test, we can see a sustained alteration in coping style from 3 to 9 months, with a trend towards more detrimental effects at later ages for our experimental groups.

A sustained and significant reduction in time spent on the accelerated rotarod (AR) due to the experimental condition can be observed in males at age 3 and both sexes at age 9, with a strong trend towards significance in females at age 3. It is likely that the decrease in time spent on the rotarod is a result of the mice being exposed to the ethanol treatment, as ethanol is widely known to impair motor coordination and balance. Even though we saw these time reductions in the experimental group, we still saw a main effect of trial in all of the four groups we tested, indicating that both our control and experimental mice were capable of learning and adapting to the AR task, something that we can see in many of the previous studies done using the AR task (Shiotsuki et al., 2010). Although there is no significance when comparing the control groups at both age points for this test, we can see that the 9 month control mice do slightly better compared to the 3 month control mice. We can also see this when comparing our experimental groups at the two different ages. Although there were no significant differences, we can see a slight increase in the performance in the older age group. These slight differences could disappear if the sample size for this test was increased, or it could be indicative of an older age possibly having a modest positive effect on AR performance, as supported by the testing we had done when comparing the control mice at 3 and 9 months of age.

In summary, our study on the effects of ethanol exposure on CD-1 mice at different developmental stages revealed several sensory processing deficiencies such as weight changes, decreased social interaction, and a shift towards more passive coping styles compared to the control group. Our findings suggest that the natural decline in sensory processing is exacerbated due to our acute alcohol exposure paradigm. Future longitudinal studies that focus on mouse behavior after our ethanol treatments or studies that examine changes in brain structure or function could also help us better determine the effect ethanol plays on the process of aging.

There are some limitations in our current study that are potentially hindering our ability to fully characterize the impacts of acute ethanol exposure and age. For example, we could increase the sample size for all age, sex, and treatment groups to allow for a more robust statistical analysis and a better understanding of the effects of ethanol on sensory processing across developmental time points. Creating another testing group at age 12, or beyond, would help provide a more comprehensive picture of the long-term effects of ethanol exposure as well. The last thing we can do to improve our research is use a different data analysis test, such as a 3-way ANOVA. This test would be better for analyzing the factors that age, sex, and experimental conditions play in relation to each other.

The findings from our study have implications for future research involving alcohol exposure at different ages. Our study found significant differences in both behavior, as well as physiology in mice at 3 months of age and 9 months of age. Researchers can use the data as a comparison point for testing mice with ethanol at different ages. Furthermore, these findings have implications for individuals who have experienced alcohol exposure and could inform interventions aimed at improving sensory processing and motor functions in those affected with AUD or those who engage in binge drinking.

FIGURES

Figure 1

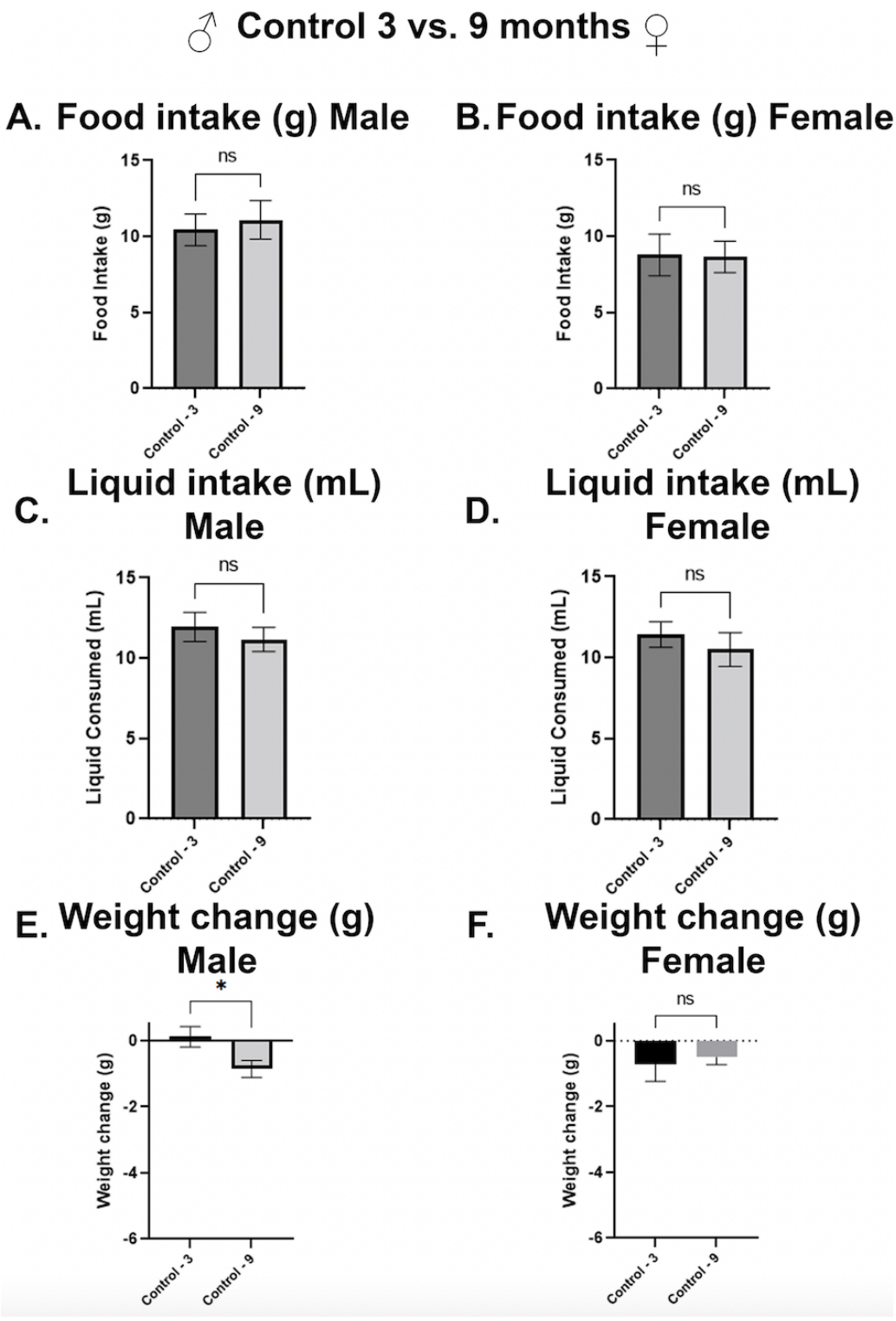


Figure 2

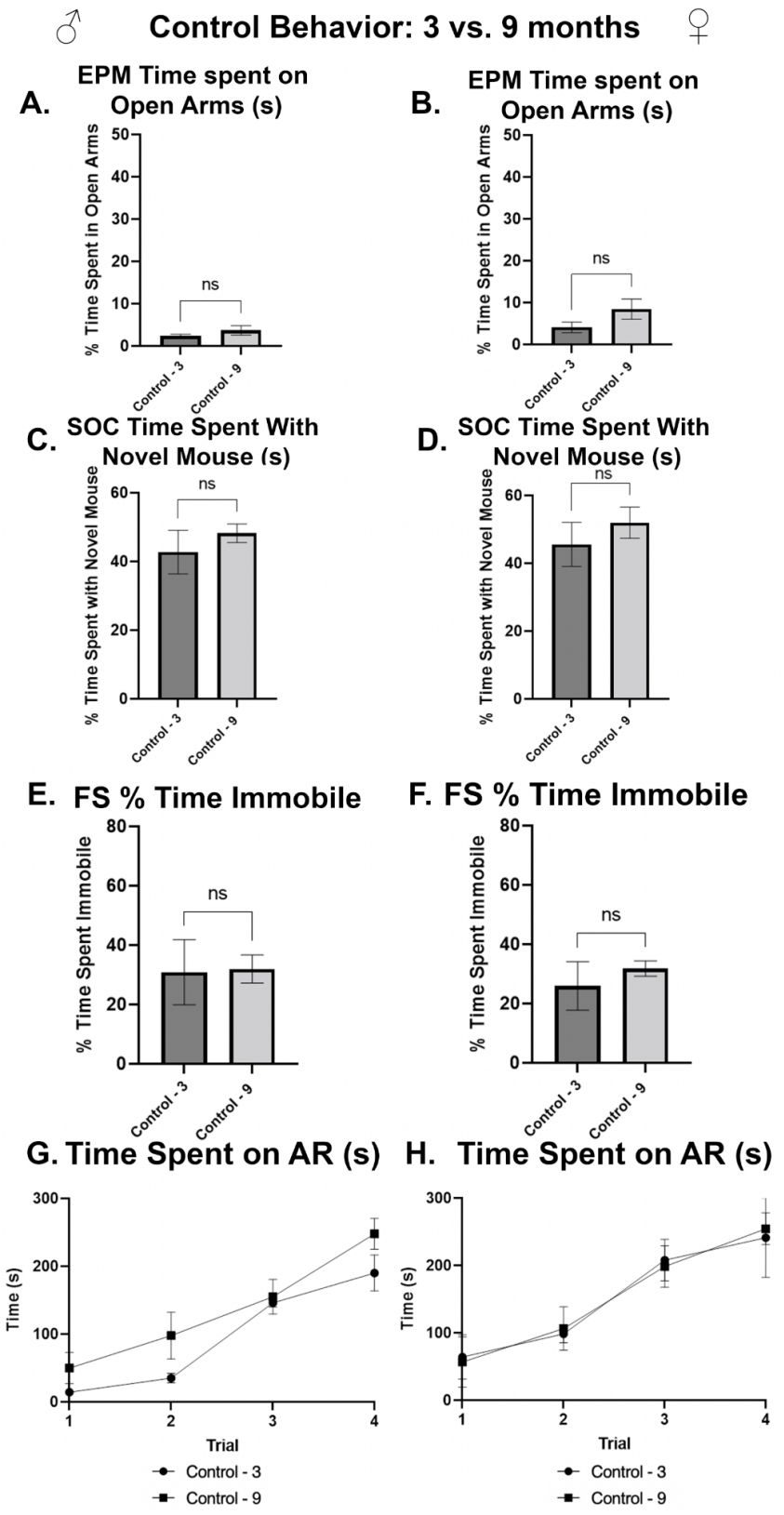

♂
Control Behavior: 3 vs. 9 months
♀
A.
EPM Time spent on Open Arms (s)
% Time Spent in Open Arms
ns
Control - 3
Control - 9
B.
EPM Time spent on Open Arms (s)
% Time Spent in Open Arms
ns
Control - 3
Control - 9
C.
SOC Time Spent With Novel Mouse (s)
% Time Spent with Novel Mouse
ns
Control - 3
Control - 9
D.
SOC Time Spent With Novel Mouse (s)
% Time Spent with Novel Mouse
ns
Control - 3
Control - 9
E. FS % Time Immobile
% Time Spent Immobile
ns
Control - 3
Control - 9
F. FS % Time Immobile
% Time Spent Immobile
ns
Control - 3
Control - 9
G. Time Spent on AR (s)
Time (s)
Trial
Control - 3
Control - 9
H. Time Spent on AR (s)
Time (s)
Trial
Control - 3
Control - 9

Figure 3

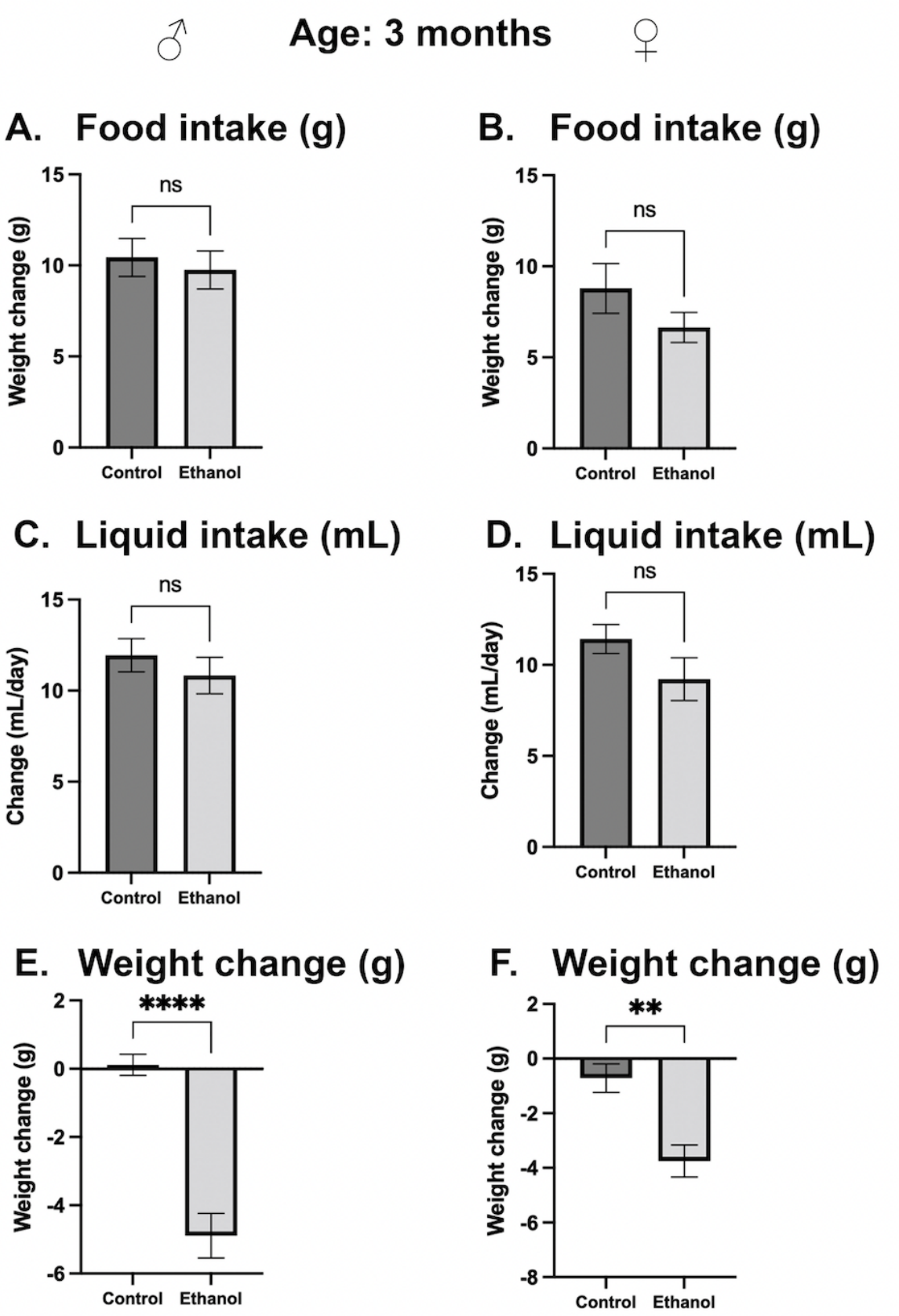

♂
Age: 3 months
♀
A. Food intake (g)
B. Food intake (g)
C. Liquid intake (mL)
D. Liquid intake (mL)
E. Weight change (g)
F. Weight change (g)
Weight change (g)
Change (mL/day)
ns
****
**
Control
Ethanol
15
10
5
0
2
-2
-4
-6
-8

Figure 4

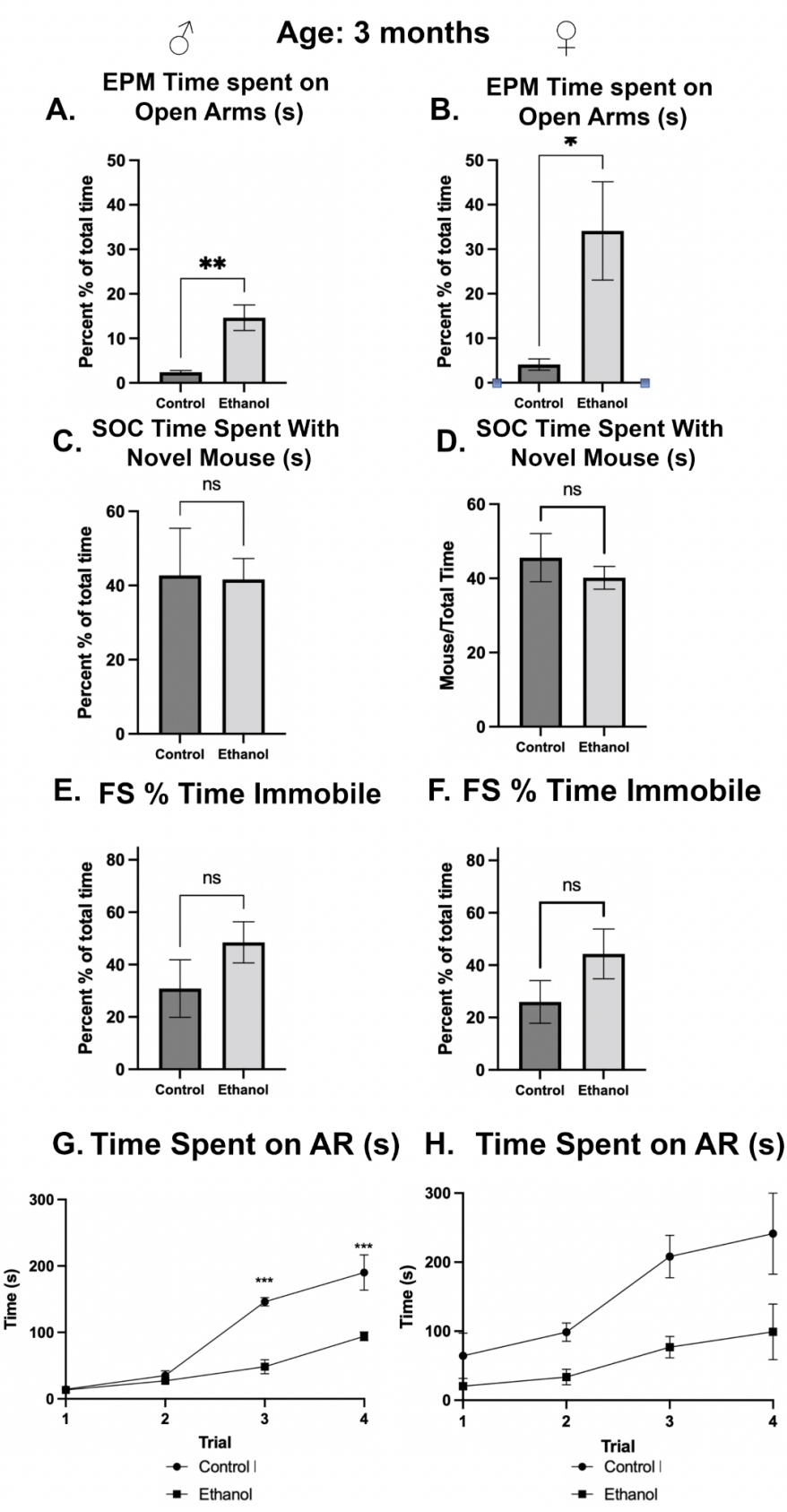

♂
Age: 3 months
♀
A.
EPM Time spent on Open Arms (s)
Percent % of total time
**
Control
Ethanol
B.
EPM Time spent on Open Arms (s)
*
C.
SOC Time Spent With Novel Mouse (s)
ns
D.
SOC Time Spent With Novel Mouse (s)
Mouse/Total Time
E.
FS % Time Immobile
F.
FS % Time Immobile
G.
Time Spent on AR (s)
Time (s)
***
Trial
Control
Ethanol
H.
Time Spent on AR (s)

Figure 5

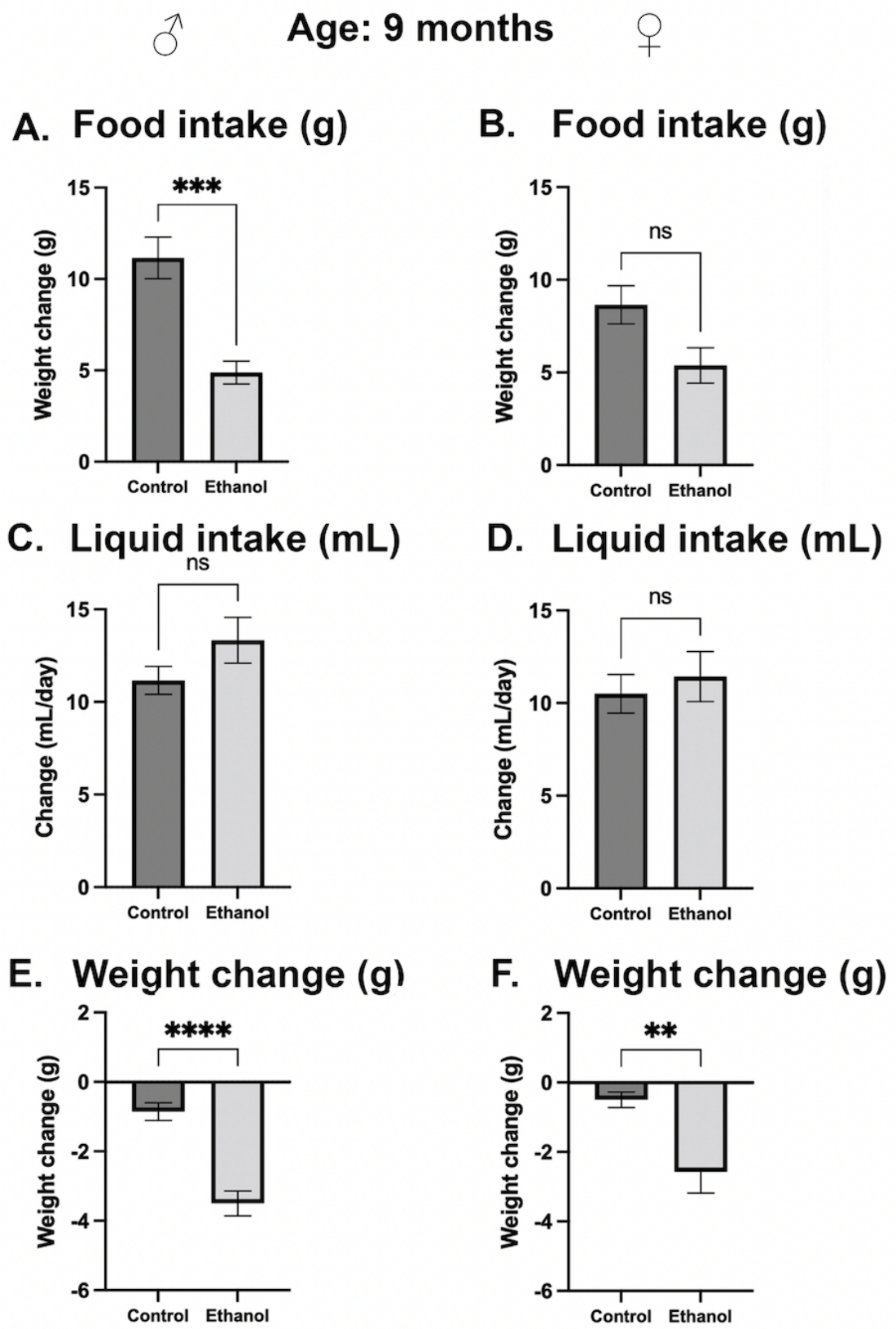

♂
Age: 9 months
♀
A. Food intake (g)
B. Food intake (g)
C. Liquid intake (mL)
D. Liquid intake (mL)
E. Weight change (g)
F. Weight change (g)
Weight change (g)
Change (mL/day)
***
ns
****
**
Control
Ethanol
15
10
5
0
2
-2
-4
-6

Figure 6

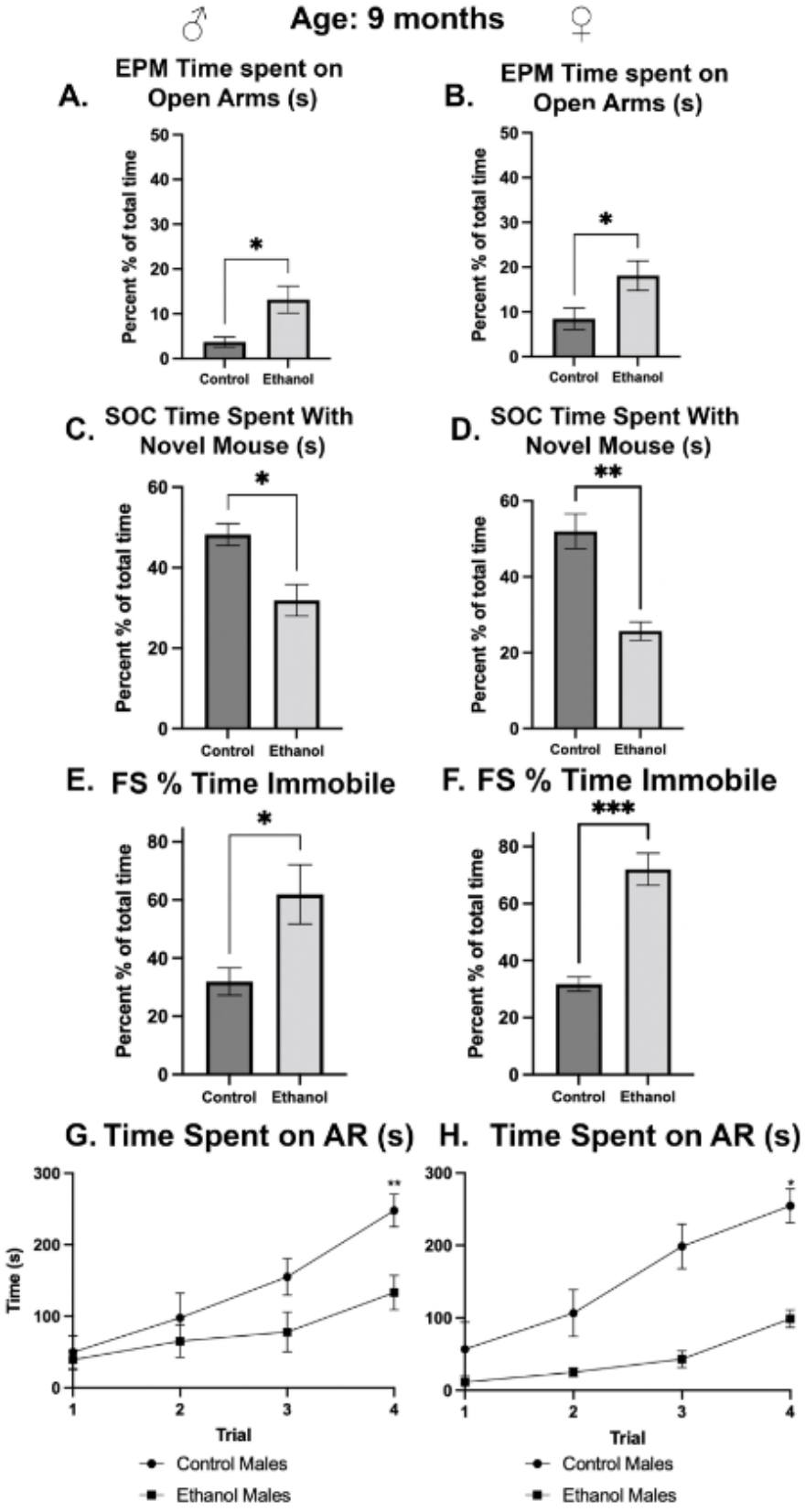

♂
Age: 9 months
♀
A.
EPM Time spent on Open Arms (s)
B.
EPM Time spent on Open Arms (s)
Percent % of total time
Control
Ethanol
C.
SOC Time Spent With Novel Mouse (s)
D.
SOC Time Spent With Novel Mouse (s)
E.
FS % Time Immobile
F.
FS % Time Immobile
G. Time Spent on AR (s)
H. Time Spent on AR (s)
Time (s)
Trial
Control Males
Ethanol Males

Figure 7

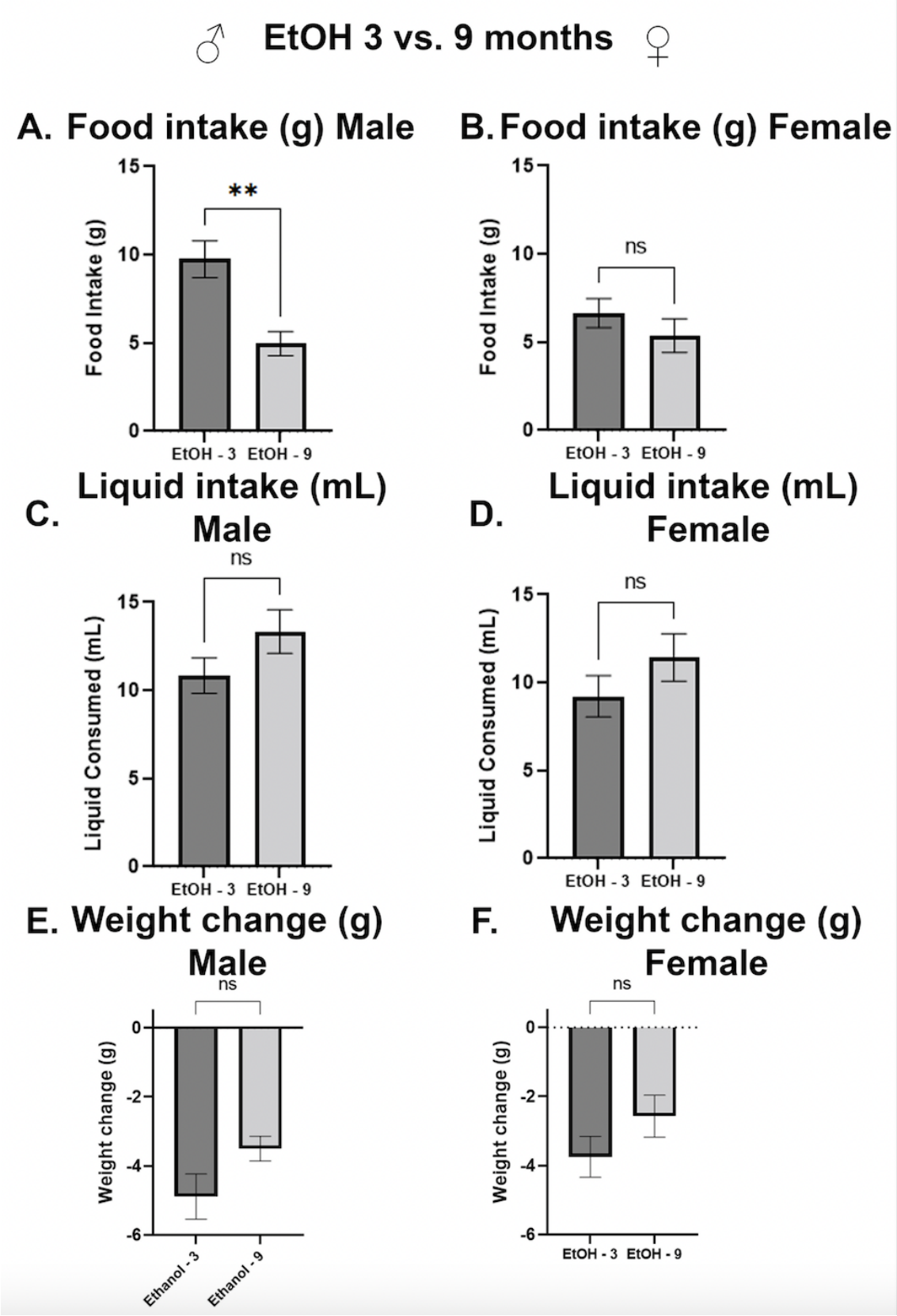
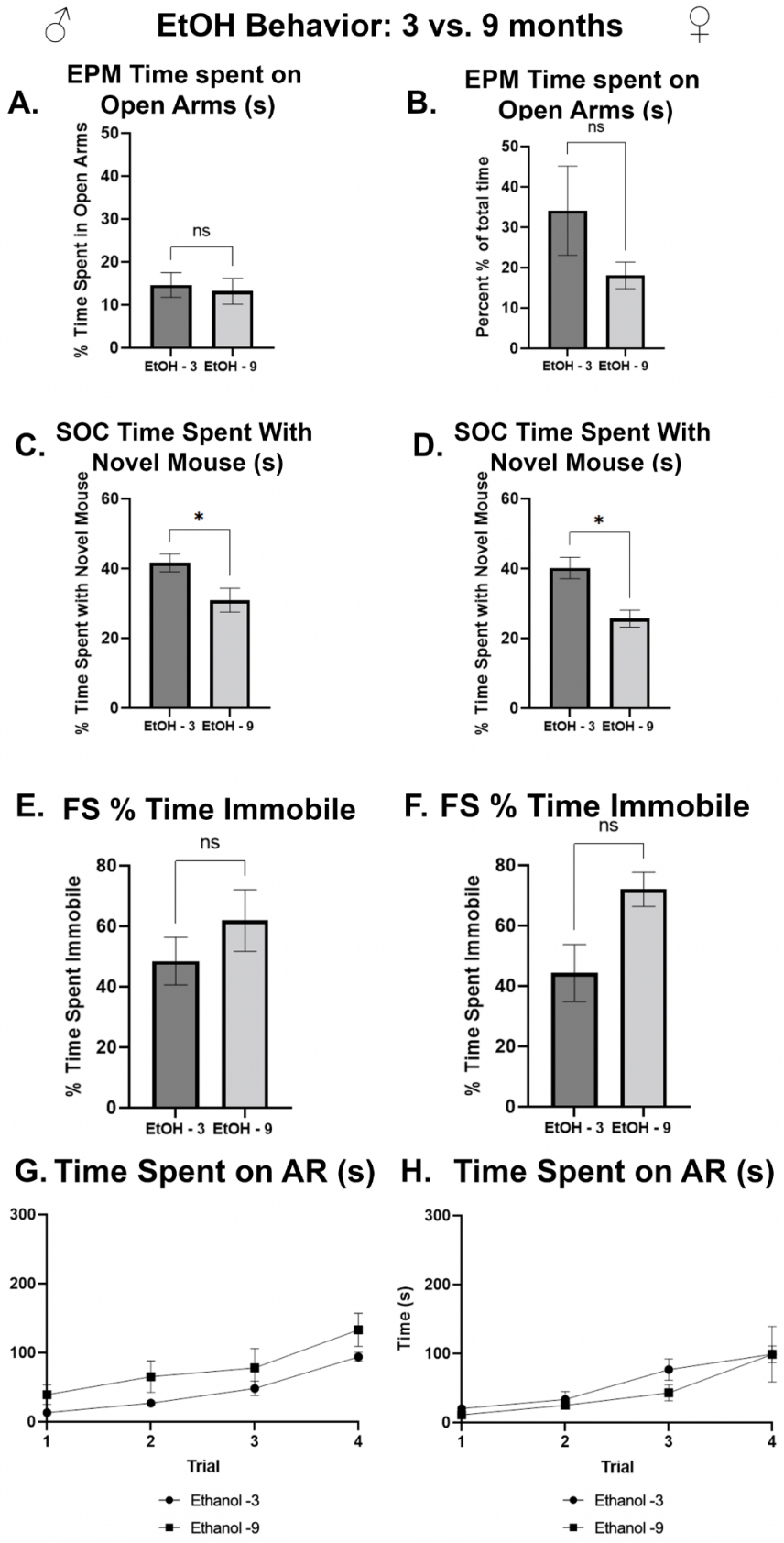

♂ EtOH 3 vs. 9 months ♀
A. Food intake (g) Male
Food Intake (g)
15
10
5
0
**
EtOH - 3
EtOH - 9
B. Food intake (g) Female
Food Intake (g)
15
10
5
0
ns
EtOH - 3
EtOH - 9
C. Liquid intake (mL) Male
Liquid Consumed (mL)
15
10
5
0
ns
EtOH - 3
EtOH - 9
D. Liquid intake (mL) Female
Liquid Consumed (mL)
15
10
5
0
ns
EtOH - 3
EtOH - 9
E. Weight change (g) Male
Weight change (g)
0
-2
-4
-6
ns
Ethanol - 3
Ethanol - 9
F. Weight change (g) Female
Weight change (g)
0
-2
-4
-6
ns
EtOH - 3
EtOH - 9

Figure 8


♂
EtOH Behavior: 3 vs. 9 months
♀
A.
EPM Time spent on Open Arms (s)
% Time Spent in Open Arms
ns
EtOH - 3
EtOH - 9
B.
EPM Time spent on Open Arms (s)
Percent % of total time
ns
EtOH - 3
EtOH - 9
C.
SOC Time Spent With Novel Mouse (s)
% Time Spent with Novel Mouse
*
EtOH - 3
EtOH - 9
D.
SOC Time Spent With Novel Mouse (s)
% Time Spent with Novel Mouse
*
EtOH - 3
EtOH - 9
E.
FS % Time Immobile
% Time Spent Immobile
ns
EtOH - 3
EtOH - 9
F.
FS % Time Immobile
% Time Spent Immobile
ns
EtOH - 3
EtOH - 9
G.
Time Spent on AR (s)
Trial
Ethanol -3
Ethanol -9
H.
Time Spent on AR (s)
Time (s)
Trial
Ethanol -3
Ethanol -9

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