Summer 2023

Genetic Substrates of the Initial Subjective Rewarding Effects of Alcohol in Mice

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By

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A Thesis

Presented to the Faculty of Bucknell University in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Psychology

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Table of Contents

List of Tables

List of Figures

Abstract

Susceptibility to alcohol use disorders (AUDs) arises from a complex interplay of genetics and environmental experiences. While the initial subjective response predicts susceptibility to AUD, genetic variation is responsible for about 50% of an individual's risk. This study used a single-exposure conditioned place preference paradigm (SE-CPP) to identify phenotypic and genetic correlates of the initial subjective rewarding effects of alcohol (EtOH) in diversity outbred (DO) mice. We assessed the relationship between SE-CPP and anxiety-like behaviors using a marble burying test and light-dark box test. Ninety-six male and female diversity outbred mice were tested in a Marble Burying test at 7-8 weeks, followed by the light-dark box at 9-10 weeks of age. Between 11-12 weeks, subjects were assessed for SE-CPP. Animals received 1.5 g/kg EtOH or equivolume saline on either Day 1 and Day 3 in two distinct contexts (counterbalanced) and were tested for a context preference on Day 5. Overall, SE-CPP was evident in both males and females. In females, there was a correlation between the percentage of time spent in the EtOH-paired context and the number of marbles buried four weeks prior. In males, there was a correlation between the distance traveled during EtOH conditioning and the percentage of time spent in the EtOH-paired context. Genotyping will be used to understand the possible link between the observed phenotypes and genetic variation. This research contributes to a better understanding of the influences of sex and genetic variation in AUD.

Introduction

Alcohol Use

Alcohol consumption is a factor for many diseases, injuries, and other health conditions, such as mental and behavioral disorders (WHO, 2022). Alcohol consumption turns into alcohol abuse when alcohol is being consumed in excessive amounts despite negative consequences. More than 28.6 million individuals, eighteen and older have an alcohol use disorder (AUD) (NIH, 2021). According to the DSM-5, individuals with the presence of at least two symptoms indicate an AUD, but the severity is defined based on the number of symptoms (Table 1). A mild AUD is the presence of 2-3 symptoms, moderate is the presence of 4-5 symptoms, and severe is the presence of 6 or more symptoms (NIH, 2021). Alcohol misuse accounts for approximately 5.3% of global deaths due to its contribution to diseases, such as chronic liver disease and stomach, pancreatic, and prostate cancer, as well as car accidents, and violent crimes (WHO, 2022).

Alcohol Use and Genetics

The causes of AUDs are complex, but genetic differences affect individual risk (Edenberg, 2013). Research suggests that heritable factors are responsible for about 50% of the risk for AUDs, with the other 50% of the risk stemming from a variety of things such as stress, trauma, family dynamics, drug access, developmental factors, and random chance (Tawa, 2016). Evidence to support

genetic contributions in the risk for AUD have come from adoption studies and twin studies. Adoption studies have shown that alcoholism in adoptees correlates with their biological parents more strongly than with their adoptive parents, and this genetic influence appears strong in both women and men (Edenberg, 2013; Heath, 1995). In addition, twin studies revealed that concordance for alcohol dependence is higher in monozygotic twins, suggesting that genetics plays a role in AUD (Oei, 2013). Twin studies suggest that approximately 45-65% of the risk is due to genetic factors (Heath, 1995).

Despite challenges, the largest portion of the variance in risk has been identified by genome-wide association studies. These studies have identified single nucleotide polymorphisms (SNPs) in alcohol metabolism enzyme genes which have been protective against the risk of developing AUD (Tawa, 2016) For example, individuals with genetic variations in the aldehyde dehydrogenase isoform ALDH2, metabolize acetaldehyde at a much slower rate, which causes an unpleasant alcohol flushing syndrome (Luczak, 2006; Hurley and Edenberg, 2012; Li et al., 2012). In addition, individuals with genetic variations in the alcohol dehydrogenase isoform ADH1B, metabolize alcohol at a much faster rate, which has been associated with a decrease in risk of developing an AUD (Luczak, 2006; Edenberg, 2007; Li et al., 2012). Taken together, due to the unpleasant side effects and quick alcohol metabolism, variations in aldehyde dehydrogenase and alcohol dehydrogenase contribute to the risk of developing AUD (Tawa, 2016).

While enzymatic variants have been identified as protective against the development of AUD, identifying genetic variants associated with the risk of developing AUD has been gaining interest. Recently, one genetic variant has been identified from a genome-wide association meta-analysis and replication study that might increase the risk of AUD. Beta-Klotho (KLB) was identified as a locus associated with alcohol consumption. KLB is a receptor component for the endocrine fibroblast growth factors (FGFs), FGF19 and FGF21, which is secreted from the liver and implicated in macronutrient preference in humans. To better understand this gene, researchers used KLB knockout mice, and they determined that these animals had an increased alcohol preference and FGF21 inhibits alcohol drinking. Humans with a variation in the beta-Klotho gene seem to be able to control their drinking by only consuming one or two drinks and then stopping. These results suggest that the liver-brain endocrine axis may play a role in the regulation of alcohol consumption, and this mechanism could be a pharmacological target for reducing alcohol intake and preventing the development of AUD (Schumann et al., 2016). Variation in the beta-Klotho gene, FGF21, might be associated with risk of developing AUD based on the role it plays in regulating alcohol consumption.

Alcohol Reinforcement

Both positive and negative reinforcement have been associated with alcohol use and misuse (Brown et al., 1980, Cooper et al., 1995; Kuntsche et al.,

2005). Positive reinforcement refers to the pleasant effects that alcohol provides, and negative reinforcement involves relief from negative affective states like stress or anxiety (Cho et al., 2019). The Reinforcement Sensitivity Theory considers that behaviors consist of two motivational systems that underlie the sensitivity to reward and sensitivity to punishment, the Behavioral Activation System (BAS) and the Behavioral Inhibition System (BIS) (Gray, 1970, 1982). BAS is sensitive to signals of reward and relief of punishment and is thought to control appetitive motivation towards reward. BIS is sensitive to the signals of punishment and the termination of reward and is thought to control aversive motivation (Corr, 2008). Individuals with strong BAS may be more attentive to the pleasurable effects of alcohol compared to those with low BAS due to their high sensitivity to the signals of reward. BAS sensitivity may increase the use of alcohol because of factors associated with positive reinforcement and that may become a risk factor for AUD (Studer et al., 2016). On the other hand, BIS is associated with enhanced anxiety and stress which are associated with problematic drinking, and BIS sensitivity might cause individuals to drink in order to relieve that heightened anxiety and stress (O'Connor and Colder, 2005), High BIS might be an indirect risk factor for problematic drinking because of heightened anxiety and stress that can drive individuals to drink (Studer et al., 2016).

Positive Reinforcement and the Initial Subjective Response

The subjective response to alcohol has been proposed to represent a biologically based and informative marker of AUD risk. Shuckit (1995,2000), investigated whether a low response to alcohol's sedative effects, in both men and women, predict greater alcohol consumption in individuals with a family history of AUD. A low response to alcohol's effects was determined based on the changes in body sway in relation to blood alcohol concentrations (BAC) at subsequent time points after alcohol consumption (Shuckit, 1995; Shuckit, 2000). In males, those that had a lower response to alcohol during peak BAC, sixty minutes after consumption, later developed alcohol abuse or dependence (Shuckit, 1995). In females, a low response was only seen for the first ninety minutes after consumption and researchers believe this might be due a biphasic alcohol response in those with a positive family history. It's possible that they exhibit a lower response during rising and peak BAC, and an enhanced response as alcohol levels fall (Shuckit, 2000). The results of these studies determined that men and women with a family history of AUD differ in their response to alcohol, and these differences were associated with having an increased risk of developing alcohol abuse and dependence (Shuckit, 1995; Shucki, 2000). In general, lower sensitivity to the aversive effects of alcohol confers a higher risk for the development of AUDs, and individuals that were more sensitive to the stimulant and rewarding effects of alcohol were more likely to develop an AUD. These results suggest that the subjective response to alcohol is a translational

phenotype for alcohol use disorder (Ray, 2014). Therefore, the initial subjective response to alcohol is one important aspect of vulnerability to AUD.

One of the best ways to better understand the initial subjective response to alcohol is through the use of animal models. An animal model that can be used to study the initial subjective response to alcohol is the single-exposure conditioned place preference (SE-CPP) paradigm. In this paradigm, animals are only ever given one injection of EtOH that is paired with a specific context (Grisel, 2014). Therefore, the pharmacological effects of the drug are associated with environmental cues paired with the drug, and the behavioral preference for an alcohol-paired context over one associated with a placebo (saline) is used to understand the initial subjective effects of alcohol (Cunningham et al., 2006). The main differences between SE-CPP and conditioned place preference (CPP) is that animals in this paradigm only receive a single drug exposure whereas conventional CPP employs multiple drug exposures. Conventional EtOH CPP is typically used to evaluate the consequences of a repeated alcohol exposure, whereas SE-CPP assesses the initial subjective experience to better understand the potential underlying risk factors of AUD. This paradigm is especially helpful in understanding the biologically vulnerable phenotype that can predict future disordered drinking (Grisel, 2014).

Negative Reinforcement as the Removal of Stress and Anxiety

Anxiety disorders and AUD have a high rate of comorbidity, and research suggests that heritable traits associated with individual differences in the neurobiological and subjective-stress response are associated with the development and persistence of AUD and anxiety disorders (Anker, 2019). Interestingly, research indicates that anxiety disorders precede alcohol misuse in upwards of three-quarters of individuals who had both conditions (Kushner, 1990). Because of the robust positive correlation between anxiety and the risk of AUD, it is highly likely that the two disorders share a genetic linkage that could be causing an increased risk for both conditions (Crum et al., 2013).

The comorbidity rates of anxiety disorders and AUD is different in males compared with females. Based on a National Comorbidity Survey, 35.8% of alcohol dependent men had a co-occurring anxiety disorder, compared to 60.7% in alcohol-dependent women. Females in this study had an increased likelihood of having an anxiety disorder compared to men, and prior anxiety disorders were more strongly predictive of future AUD in women compared to men (Kessler et al., 1997). A potential explanation for this difference is that women might be more prone to self-medicate for mood problems which can be further supported by women reporting higher levels of stress and a stronger link between stress and drinking when compared to men (Brady and Randall, 1999; Rice and Van Arsdale, 2010; Timko et al., 2005).

Another anxiety-related disorder that may be linked to AUD is obsessive-compulsive disorder (OCD). Problem drinking is common among individuals with obsessive-compulsive psychopathology (Osland et al., 2018; Virtanen et al., 2020). Similar to anxiety disorders, females have a higher comorbidity rate of OCD and AUD compared with males, and these results might also be tied to the notion that females tend to self-medicate for mood problems with alcohol (Goh, 2022; Brady and Randall, 1999).

Stress and anxiety disorders have characteristic behavioral and physiological indicators. Stress activates the hypothalamic-pituitary-adrenal (HPA) axis which raises cortisol levels and leads to a higher cortisol awakening response (CAR). For example, individuals, ages 18-65, with anxiety disorders have higher levels of cortisol (or CAR) as well as total cortisol secretion (Vreeburg et al., 2010; Mantella et al., 2008). Since there is a link between anxiety disorders and the stress response, and a potential link between OCD and the stress response, determining cortisol levels or corticosterone levels in rodents in relation to an exposure of alcohol could be used to better understand if the levels of these chemicals can be used as a predictor of AUD. Ethanol activates the HPA axis and it has been seen that administration of ethanol increases blood plasma corticosterone levels in rodents (Barney et al., 2022; Seely, 1996).

One way to better understand sensitivity to negative reinforcement as an initial or baseline trait to predict risk for alcohol use disorder, is to use animal

models that assess anxiety-like or impulsive-like behaviors. A behavioral model that is a validated test for unconditioned anxiety-like behaviors in rodents is the light-dark box (Crawley and Goodwin, 1980). There is a conflict between exploration and the avoidance of unfamiliar surroundings, therefore the amount of time spent in a dark portion of the apparatus reflects stress and anxiety-like behaviors (Bourin, 2003). Another behavioral model that is used to test for anxiety-like behaviors in marble burying. Marble burying was originally proposed as a test to assess anxiety-like behavior in mice because the behavior is readily blocked by anxiolytic drugs (Hoffman, 2016). However, some research suggests that marble burying is more reflective of compulsive-like behavior than anxiety-like behavior (Londei, 1998). For example, Thomas et al, (2009) found that marble burying in mice is genetically regulated, and it is related to digging behavior, which is also associated with obsessive-compulsive behaviors (Thomas et al, 2009).

Diversity Outbred Mice

Diversity outbred mice (DO) are helpful to model individual risk factors in humans. Each DO mouse is a unique individual and this population has an effectively unlimited source of allele combinations, which resembles what is seen in the human population, and they can be used to model susceptibility or resistance to a variety of human diseases, such as AUD (Churchill, 2012). The DO population was recently used to investigate genetic associations and ethanol

sensitivity, which is the first study using alcohol and the DO mice. Parker et al. (2022), characterized ethanol sensitivity (ataxia, hypothermia, and loss of righting response), genotyped the animals, and determined genetic linkages and single nucleotide polymorphism (SNP) associations. The results of this study identified significant quantitative trail loci (QTL) on chromosome 16 for ethanol-induced ataxia and significant QTL on chromosome 1 for ethanol-induced hypothermia. There were also suggestive QTL on chromosome 2 for ethanol-induced loss of righting response. In addition to the QTL analysis, there were various SNPs associated with their characterization of ethanol sensitivity, the chromosomes with significant QTLs, and the genetics of the eight founder strains used to create the DO population. The results of this study may be used to identify alleles associated with AUD in humans or assist in the development of therapeutic interventions (Parker et al., 2022).

We examined marble burying and light-dark box behaviors in DO mice to assess individual differences in basal anxiety and stress levels, which are associated with negative reinforcement. We also examined SE-CPP to assess for the initial subjective response of alcohol which is associated with positive reinforcement. This study aimed to address the following questions: Do the negative and positive reinforcement behavioral tests covary within individuals? Would individual differences in these behavioral tests represent different genes and risk factors associated with AUD? Does the DO mouse line exhibit sex differences in these behavioral paradigms?

Methods

Subjects

96 Diversity Outbred (DO) mice were shipped to Bucknell from The Jackson Laboratories at age 4-5 weeks. These animals were housed in same-sex groups of three, in Plexiglas cages filled with corn cob bedding in a colony with a 12-h reverse light:dark cycle (lights off at 0930) maintained at 21 ± 2 °C. During their first few days of acclimation, cages were numbered, and each mouse received an approximately 0.75-1 cm cut in their ear to designate subject number. Subjects were given free access to mouse chow and tap water at all times before, and during, the study. Due to fighting, all males were singly housed on the second day of acclimation. Females were single housed nine days before they were tested in the SE-CPP test, and both males and females were given a cardboard enrichment tube.

Marble Burying

At 7-8 weeks old, marble burying was assessed in all subjects. Animals were placed into a novel Plexiglas chamber (9cm by 42 cm), in a behavioral suite across the hall from the colony room, filled with 4cm of sawdust and containing 8 1cm glass marbles that were evenly dispersed. Subjects were undisturbed during the 30-minute burying test. The number of marbles buried by each subject are scored, and marbles were considered buried if they are ⅔ covered with sawdust.

Light-Dark Box

Between 9-10 weeks of age, mice were tested in the light-dark box, in a behavioral suite across the hall from the colony room. The light-dark box (27 x 45 cm and 35 cm high) is divided into two chambers. The black-walled, dark chamber is 27 x 15 cm and has an 8-cm-wide and 9-cm-high opening in the middle of the wall adjoining the 27 x 30-cm white-walled lighted chamber. Animals were gently placed in the dark chamber (a red plastic lid closed over the dark side of the apparatus) and behavior was assessed for twenty minutes. The latency to emerge from the dark component and time spent in the light side was recorded by cameras mounted on the ceiling, and subsequently analyzed by Any-maze (San Diego) and then SPSS 28.0.

Single Exposure Conditioned Place Preference (SE-CPP)

Between 11-12 weeks old, mice were conditioned and tested in the single exposure conditioned place preference paradigm. Our apparatus has three chambers, differentiated by tile floor patterns. The center chamber is smaller than the others (11.5 × 24) and has a smooth black floor, and the two outer chambers are the same size (42 × 24 cm); one side has a floor made of tile circles of various sizes and the other has a floor of uniformed square tiles; both floors are painted red. The SE-CPP assay takes place across 5 days. On day 1, animals were given an intraperitoneal injection of 1.5 g/kg 20% EtOH in saline, or equivolume saline and placed on one side of the apparatus. They received the

opposite injection and exposure to the alternate chamber (counterbalanced) on day 3. On days 2 and 4 all subjects were left undisturbed in their home cage. On Day 5 subjects received a second saline injection followed by the opportunity to access all portions of the apparatus in a 30-minute preference test. After completion of the place preference test on Day 5, animals were anesthetized by vaporized isoflurane and immediately euthanized by decapitation.

Tissue Collection

Brains and tail tips were dissected on dry ice and stored at -80° F for subsequent RNA transcription by RT-PCR and genotyping. Blood was collected and blood corticosterone levels were analyzed using an ELISA (Enzo Life Sciences).

Genotyping

DNA from each animal was collected and sent to Neogen for genotyping. Once it has been determined what genetic variants each animal has, a quantitative trait locus analysis (QTL) will be done to determine any linkage between our phenotypic data and genetic data. QTL analysis is a statistical method that links phenotypic data (trait measurements) and genotypic data to try and explain the genetic basis of variation in complex traits (Falconer & Mackay, 1996; Kearsey, 1998; Lynch & Walsh, 1998). This QTL analysis will allow us to link certain phenotypes, such as marble burying, time spent in the light side of

the light-dark box, and percentage of time spent in the alcohol-paired context, to specific regions of chromosomes (Miles & Wayne, 2008).

ELISA

Plasma was collected from the blood samples, obtained immediately after the final day of SE-CPP, was analyzed to determine corticosterone levels using a colorimetric competitive immunoassay corticosterone ELISA kit (Enzo Life Sciences) according to manufacturer's instructions. The plasma was diluted 1:40 with assay buffer. All samples were run in duplicates, and the average of the duplicates were used for the analysis. The absorbance was read at 405 nm using an iMark microplate reader (Biorad, Hercules, CA). Sample concentrations for corticosterone were calculated from a standard curve using GraphPad Prism software (GraphPad, La Jolla). The sensitivity of the assay was 27 pg/mL with a range of detection up to 20,000 pg/mL. Determining corticosterone levels in the blood plasma could be used to better understand the stress response of these animals and the potential role stress and anxiety play in their preference for the EtOH-paired context.

Data Analysis

Behavioral data were analyzed blind to condition using SPSS 28.0.1.0. Conditioned place preference conditioning was assessed using one sample, two-tailed t-tests, as a measure of drug preference by evaluating %time in the EtOH-

paired context = (Time in EtOH Context/((Time in EtOH Context + Time in Saline Context))*100, and to determine locomotor effects of EtOH by subtracting distance traveled during the two conditioning days (saline distance subtracted from EtOH distance). The Spearman's Rank Correlation (GraphPad Prism) was used to assess correlations between measures of anxiety-like behavior, measures of anxiety-like behavior and SE-CPP, measures of anxiety-like behavior and corticosterone levels, and SE-CPP and corticosterone levels. A two-tailed t-test was used to determine regression line slope differences (GraphPadPrism (9.4.1)), and the unequal variance Welch's t-test was used to determine whether variances differed based on sex, also in GraphPadPrism.

Results

Summary

The primary interest of this study was to better understand the substrates of initial subjective reward to alcohol. We assessed correlations between measures of anxiety-like behaviors, between measures of anxiety-like behaviors and SE-CPP, and between behavioral measures and genetic variation in the DO mice.

Results show that both male and female DO mice demonstrated preference for a context paired one time with alcohol but suggest that the underpinnings behind the SE-CPP may be sex-dependent. The number of marbles buried in females, but not males, predicted the percentage of time spent in the alcohol-paired context as well as the locomotor distance traveled following a saline injection. The distance traveled during EtOH conditioning predicted the percentage of time spent in the alcohol-paired context in males, but not females. There were no significant correlations between the light-dark box activity and SE-CPP in either sex.

Among males, there was one high alcohol preferring subject (86.4%) who was a statistical outlier. In females, there was one statistical outlier high corticosterone level (354.6 pg/mL). Because each of the DO mice is genetically distinct, we analyzed behavioral data with and without these two subjects in anticipation that genotyping might shed light on these extreme cases.

Single exposure conditioned place preference in both male and female DO mice (Figure 1)

Overall, the 96 subjects showed a SE-CPP, by preference for the alcohol-paired context greater than 50% by a one-sample t-test ($t_{(1,95)} = 3.925$, $p < 0.001$). Separate one-sample, two-tailed t-tests to assess place preference scores in males and females showed a significant place preference for the ethanol paired chamber in males ($M = 53.31$, $t_{(47)} = 2.44$, $p = 0.009$) and in females ($M = 54.21$, $t_{(48)} = 3.085$, $p = 0.002$), and one-way ANOVA showed that these did not differ ($F_{(1,95)} = 0.213$, $p = 0.645$).

Injection order during conditioning did affect SE-CPP (Figure 2)

A 2 -way ANOVA to look at injection order by sex found no effect of sex ($F_{(1,96)} = 0.123$, $p = 0.726$), though there was an effect of EtOH day ($F_{(1,96)} = 9.504$, $p = 0.003$), but no interaction between sex and day ($F_{(1,96)} = 0.019$, $p = 0.890$) on the percentage of time spent in the alcohol-paired context. There was no effect of sex ($F_{(1,96)} = 0.586$, $p = 0.446$), but again, there was an effect of EtOH day ($F_{(1,96)} = 10.449$, $p = 0.002$) in that the SE-CPP was evident in subjects that received EtOH on day 1 but not day 3. There was no interaction between sex and day ($F_{(1,96)} = 0.131$, $p = 0.718$) on the amount of time spent in the alcohol-paired context. There was no effect of sex ($F_{(1,96)} = 3.444$, $p = 0.067$), no effect of EtOH day ($F_{(1,96)} = 3.390$, $p = 0.069$), and no interaction between sex and day ($F_{(1,96)} = 0.341$, $p = 0.561$) on the amount of time spent in the saline-paired

context. There was no effect of sex ($F_{(1,95)}$ = 0.243, p = 0.623), no effect of EtOH day ($F_{(1,95)}$ = 3.667, p = 0.059), and no interaction between sex and day ($F_{(1,95)}$ = 1.441, p = 0.233) on saline distance. There was no effect of sex ($F_{(1,95)}$ = 0.156, p = 0.694), no effect of EtOH day ($F_{(1,95)}$ = 2.111, p = 0.150), and no interaction between sex and day ($F_{(1,95)}$ = 0.079, p = 0.780) on EtOH distance. There was no effect of sex ($F_{(1,95)}$ = 0.004, p = 0.948), but there was an effect of EtOH day ($F_{(1,95)}$ = 6.545, p = 0.012), and no interaction between sex and day ($F_{(1,95)}$ = 0.231, p = 0.632) on the difference in distance traveled on saline and EtOH conditioning days. However, using separate two-sample t-tests, animals that received EtOH on day 1 were more significantly stimulated ($t_{(1,47)}$ = -1.990, p = 0.05) than animals that received EtOH on day 3 ($t_{(1,46)}$ = 1.661, p = 0.103), contributing to the effect of EtOH day on the distance difference.

The number of marbles buried predicted SE-CPP in females, but not males (Figure 3)

There was a significant correlation between number of marbles buried and percentage of time spent in the alcohol-paired context in females (r = 0.342, p = 0.017, N = 48), but not males (r = -0.112, p = 0.448, N = 48), and removing one male outlier did not change the results (r = 0.134, p = 0.368, N = 47). There was also a significant difference in the slopes of the regression lines from males and females with the male outlier included (p = 0.021, $F_{(1,48)}$ = 5.491), but not when the outlier was excluded (p = 0.196, $F_{(1,47)}$ = 1.700).

The distance traveled during EtOH conditioning predicted SE-CPP in males, but not females (Figure 4)

. There was a correlation between EtOH distance, and the percentage of time spent in the alcohol-paired context in males ($r = -0.315$, $p = 0.029$, $N = 48$), but not in females ($r = 0.081$, $p = 0.588$, $NN = 47$), showing that males that had a lower distance traveled in the EtOH conditioning context had a higher percentage of time spent in the alcohol-paired context.

There were no correlations between light-dark box behaviors and marble burying (Figures 5-6)

There was no correlation between the number of marbles buried and time spent in the light side of the light-dark box in males ($r = 0.115$, $p = 0.438$, $N = 48$) or females ($r = 0.005$, $p = 0.972$, $N = 48$), and there was no correlation between the number of marbles buried and the latency to enter the light compartment in males ($r = -0.057$, $p = 0.699$, $N = 48$) or females ($r = 0.048$, $p = 0.746$, $N = 48$) suggesting that the light-dark box and marble test are not assessing the same construct.

There was a correlation between the number of marbles buried and saline distance (Figure 7), but no other correlations between marble burying and locomotor assessments during conditioning (Figures 8-11)

The number of marbles buried predicted distance traveled following the saline injection in females ($r = 0.308$, $p = 0.035$, $N = 47$), but not in males ($r = 0.048$, $p = 0.746$, $N = 48$). There were no correlations between the marbles buried and EtOH distance (Males: $r = 0.018$, $p = 0.903$, $N = 48$; Females: $r = 0.101$, $p = 0.498$, $N = 47$), or distance difference (Males: $r = 0.007$, $p = 0.960$, $N = 48$; Females: $r = 0.141$, $p = 0.345$, $N = 47$). There were no correlations between marbles buried and the time spent in the saline-paired context (Males: $r = 0.086$, $p = 0.559$, $N = 48$; Females: $r = -0.262$, $p = 0.072$, $N = 38$), or alcohol-paired context (Males: $r = -0.162$, $p = 0.272$, $N = 48$; Females: $r = 0.227$, $p = 0.121$, $N = 48$).

There were no correlations between light-dark box behaviors and SE-CPP (Figures 12-23)

There was no correlation between light time and percentage of time spent in the alcohol-paired context (Males: $r = -0.049$, $p = 0.742$, $N = 48$; $r = 0.039$, Females: $p = 0.790$, $N = 48$), even with the outlier removed (Males: $r = -0.020$, $p = 0.892$, $N = 47$). There was no correlation between light time and time spent in alcohol-paired context (Males: $r = -0.089$, $p = 0.545$, $N = 48$; Females: $r = 0.172$, $p = 0.243$, $N = 48$), or time spent in the saline-paired context (Males: $r = 0.000$, p

= 0.998, N = 48; Females: r = -0.070, p = 0.636, N = 48). There was no correlation between light time and EtOH distance (Males: r = -0.120, p = 0.416, N = 48; Females: r = 0.274, p = 0.062, N = 47), light time and saline distance (Males: r = 0.071, p = 0.633, N = 48; Females: r = 0.138, p = 0.354, N = 47), or light time and distance difference (Males: r = 0.150, p = 0.309, N = 48; Females: r = -0.137, p = 0.357, N = 47).

Moreover, there was no relationship between light latency and percentage of time spent in the alcohol-paired context (Males: r = 0.126, p = 0.393, N = 48; Females: r = -0.182, p = 0.216, N = 48), even with the outlier removed (Males: r = 0.138, p = 0.355, N = 47). There was no correlation between light latency and time spent in the alcohol-Paired context (Males: r = 0.183, p = 0.214, N = 48; Females: r = -0.233, p = 0.111, N = 48), or light latency and time spent in the saline-paired context (Males: r = -0.071, p = 0.629, N = 48; Females: r = -0.028, p = 0.848, N = 48). There was no correlation between light latency and EtOH distance (Males: r = -0.120, p = 0.416, N = 48; Females: r = 0.036, p = 0.808, N = 47), light latency and saline distance (Males: r = 0.111, p = 0.452, N = 48; Females: r = -0.038, p = 0.801, N = 47), or light latency and distance difference (Males: r = 0.129, p = 0.384, N = 48; Females: r = -0.060, p = 0.688, N = 47). The variance in light time was significantly different in male and female DO mice (p = 0.012, F = 2.115).

There were no correlations between blood corticosterone levels and marble burying, light-dark box behaviors or SE-CPP (Figures 24-32)

There were no correlations between blood corticosterone levels and marble burying (Males: r = 0.059, p = 0.700, N = 45; Females: r = -0.201, p = 0.175, N = 47). There were no correlations between blood corticosterone and light latency (Males: r = -0.118, p = 0.438, N = 45; Females: r = -0.110, p = 0.462, N = 47), or between blood corticosterone and light time (Males: r = -0.123, p = 0.421, N = 45; Females: r = 0.260, p = 0.077, N = 47). There were no correlations between blood corticosterone and saline distance (Males: r = 0.108, p = 0.480; N = 45; Females: r = -0.193, p = 0.198, N = 46), EtOH distance (Males: r = -0.062, p = 0.685, N = 45; Females: r = 0.137, p = 0.365, N = 46), or distance difference (Males: r = 0.111, p = 0.470, N = 45; Females: r = -0.264, p = 0.076, N = 46). There were no correlations between blood corticosterone and the amount of time spent in the saline-paired context (Males: r = -0.185, p = 0.224, N = 45; Females: r = 0.222, p = 0.133, N = 47), or the amount of time spent in the alcohol-paired context (Males: r = 0.218, p = 0.151, N = 45; Females: r = -0.127, p = 0.396, N = 47). There were no correlations between blood corticosterone and the percentage of time spent in the alcohol-paired context (Males: r = 0.226, p = 0.136, N = 45; Females: r = -0.173, p = 0.246, N = 47).

Blood corticosterone differed between males and females, but there was no effect of sex on marble burying, light-dark box behaviors, or SE-CPP

There was an effect of sex on corticosterone levels ($F_{(1,92)} = 10.775$, $p = 0.001$), and there was significant difference in the variability of corticosterone levels in male and female mice when one female outlier was removed ($F_{(1,91)} = 31.73$, $p < 0.001$). There was no effect of sex on marble burying ($F_{(1,96)} = 1.424$, $p = 0.236$). There was no effect of sex on the amount of time spent on the light side of the light-dark box ($F_{(1,96)} = 0.232$, $p = 0.631$), and no effect of sex on light latency ($F_{(1,96)} = 0.605$, $p = 0.438$). There was no effect of sex on the distance traveled during saline conditioning ($F_{(1,95)} = 0.139$, $p = 0.710$), on the distance traveled during EtOH conditioning ($F_{(1,95)} = 0.219$, $p = 0.641$), or on the distance difference ($F_{(1,95)} = 0.041$, $p = 0.841$). There was no effect of sex on the time spent in the saline-paired context ($F_{(1,96)} = 3.674$, $p = 0.058$), on the time spent in the alcohol-paired context ($F_{(1,96)} = 0.365$, $p = 0.547$), or on the percentage of time spent in the alcohol-paired context ($F_{(1,96)} = 0.213$, $p = 0.645$).

Discussion

The purpose of the study was to see if there were correlations between measures of anxiety-like behavior and SE-CPP across a genetically diverse population of male and female mice. We wanted to see if there were sex differences in these behavioral paradigms, and if individual differences in each animal's behavior represent genetic variants associated with AUD. Because there is a high comorbidity between anxiety disorders and AUD (Anker, 2019), we hypothesized that measures of basal anxiety and stress, marble burying and light-dark box behavior, would correlate with SE-CPP.

We hypothesized that animals with higher basal anxiety-like behaviors would prefer the alcohol-paired context more than animals with lower basal anxiety-like behaviors due to the positive correlation between anxiety and the risk of AUD (Crum et al, 2013). While we expected to see a significant correlation between measures of basal anxiety and stress and SE-CPP, we did not. However, in females we found a significant correlation between the number of marbles buried and the percentage of time spent in the alcohol-paired context four weeks later, but this relationship was not evident in males. One possibility is that compulsive-like behaviors predict a positive subjective experience from alcohol in females, a hypothesis that would require more testing, especially employing other measures of compulsive behavior. There were no significant correlations between light-dark box behavior and SE-CPP in either sex, suggesting that anxiety-like behaviors might not influence the initial subjective

response to alcohol in males or females, or that the light-dark box was not sufficient to assess anxiety states. We also hypothesized that specific genetic variation in the DO mice would explain the differences in SE-CPP, but due to sex differences, we did not have a sufficient number of animals to conduct the genetic mapping. Perhaps a replication of this study will provide information on the sex-dependent genetic factors that underlie these differences.

In the present study, there was a significant correlation between the number of marbles buried and the percentage of time spent in the alcohol-paired context but only in females, and there were no correlations between the number of marbles buried and any measure in males. Prior research suggest that marble burying is an effective measure of anxiety-like behaviors, other research suggests that it may actually be assessing for compulsive-like behaviors, making this test much more applicable to studies on obsessive compulsive disorder (OCD) (Londei, 1998; Thomas et al., 2009). In humans, a higher prevalence of OCD and AUD comorbidity was seen in females than males (Goh, 2022). Mitra et al (2017), used control mice, mice that exhibited high compulsive-like behaviors, and mice that exhibited low compulsive-like behaviors to investigate marble burying. They found that mice with high compulsive-like behaviors buried more marbles than those with low compulsive-like behaviors, but males with high compulsive-like behaviors buried more marbles than their female counterparts (Mitra et al., 2017). These results suggest that there are influences of genetics and sex on marble burying. We did not see an effect of sex on marble burying,

but it is possible that compulsive-like behavior could be a driving force for the preference that was seen in these female animals but not males.

Animal models that are used to assess novelty seeking typically rely on the evident expression of a behavior that is often described by the degree to which an animal explores a novel stimulus or demonstrates a preference for a novel stimulus compared to a familiar one (Piazza, 1991; Palanza, 2001). In marble burying, it is possible that mice are burying marbles due to the novelty of them (Thomas et al., 2009). In SE-CPP, animals receive an injection of either EtOH or saline, and they are placed into one designated side of the apparatus. There is no habituation to the apparatus, therefore the behavioral changes that are seen in SE-CPP could be due to the novelty of the contexts associated with EtOH and saline (Grisel, 2014).

In humans, females have an increased likelihood of having an anxiety disorder compared to men, and prior anxiety disorders are more strongly predictive of future AUD in women compared to men. Also, 60.7% of females that were alcohol dependent had a co-occurring anxiety disorder compared to only 35.8% in males (Kessler et al., 1997). It has been said that women drink in order to relieve stress and anxiety, and research shows that women report higher levels of stress and a stronger link between stress and drinking compared to men (Brady and Randall, 1999; Rice and Van Arsdale, 2010; Timko et al., 2005). In the present study, the light-dark box was used as a validated measure of anxiety-like behaviors, and there were no correlations between light-dark box behaviors

and SE-CPP in either males or females. We had hypothesized that individuals with higher basal-anxiety-like behaviors will have higher preference for the alcohol-paired context, but that was not what was observed. It is possible that anxiety might not be the driving force behind the preference that was seen, especially in females, and in males as well.

In this study, male and female DO mice preferred the alcohol-paired context in the SE-CPP test, but there was not a significant sex difference in this behavior, congruent with other published data in other mouse models, including C57BL/6, DBA/2, and Swiss Webster mice (Grisel, 2014). The present study evaluated baseline first exposure behaviors, and since females are more susceptible to stress it is possible that if these animals had been exposed to some sort of stressor prior to SE-CPP that sex differences might have been observed. SE-CPP was being used as a measure of positive reinforcement as SE-CPP measures the initial subjective response to alcohol, and it is possible that positive reinforcement is the main factor behind males' preference to the alcohol-paired context, but in females' negative reinforcement is the main factor behind their preference to the alcohol-paired context. We found no correlations between any of our measures associated with negative reinforcement in males, there was only a preference to the alcohol-paired context, but in females, there was a significant correlation between the number of marbles buried and the percentage of time spent in the alcohol-paired context. These results suggest

that negative reinforcement is associated with female preference, and positive reinforcement is associated with male preference.

In addition to male and female DO mice preferring the alcohol-paired context, there was an effect of drug day on that preference. Animals that received alcohol on day 1 had a higher preference for the alcohol-paired side compared to the animals that received alcohol on day 3. There was also an effect of drug day on the distance difference, and this effect was due to the animals that received alcohol on day 1 being more stimulated than the animals that received alcohol on day 3. In previous studies using SE-CPP, there was no effect of drug day seen in C57BL/6, DBA/2, or Swiss Webster mice (Grisel, 2014), but this group did find a similar interaction between drug day and sex in rats (Nentwig, 2017) as was seen in the present study. Female rats that received alcohol on day 3 had a significant preference for the alcohol-paired context which was not evident when they received alcohol on the first day of conditioning. Male rats showed a SE-CPP when EtOH was conditioned on day 1, but not if they received alcohol on day 3 (Nentwig, 2017). It is possible that the effect of drug day seen in the DO mice is due to an association between alcohol and the novelty of the alcohol-paired context. Grisel (2014) found that when there is a habituation period included in SE-CPP, the preference for the alcohol-paired context is attenuated, suggesting that the changes in behavior expressing the initial subjective response to alcohol are dependent on the novelty of the context (Grisel, 2014). Therefore, it is possible that for DO mice the effect of drug day

and increase in stimulation is dependent on the novelty of the context associated with alcohol.

Overall, the main objective of the study was to identify factors involved in vulnerability to AUD. We sought to determine whether the initial subjective response to alcohol correlated with measures of anxiety-like behavior, and whether there were genetic factors that would influence AUD susceptibility. Our observations showed that there was genetic variation in the initial subjective response to alcohol, suggesting that SE-CPP may be a useful animal model for studying risk factors for AUD.

We expected to see that animals with a higher basal anxiety-like behavior would have higher basal levels of corticosterone, and these would correlate with behavior in the SE-CPP test. We did not find a correlation between light-dark box behaviors, which is a validated measure of anxiety-like behavior in rodents, and SE-CPP in either sex, and we did not find a correlation between corticosterone levels and SE-CPP in either sex. A limitation is that Typically, corticosterone levels are higher in female mice compared to male mice, but in my study, corticosterone levels were higher in males compared to females. This may have been caused by males exhibiting hyper-aggressive behavior shortly before sacrifice, which most likely impacted the reliability of the corticosterone data. Because this animal model is relatively new, and there is no data available on the baseline corticosterone levels or stress-induced corticosterone levels in these animals, there is no existing data set to compare our results to.

Summary and Future Directions

The purpose of this study was to see if individual animal differences in behavioral tests, like SE-CPP, can be used to find genetic factors that might increase or decrease susceptibility to AUD, with the hopes of better understanding the factors that contribute to AUD vulnerability. In the present study, there are promising phenotypic results, suggesting that future, sufficiently powered, genetic analyses to identify genetic variants associated with the initial subjective response may be fruitful. Studies such as this will provide better understanding of the role that genetic variations play in AUD and what specific genes might be involved. Determining genes that might be involved in AUD will help prevent someone from participating in behaviors that could lead them to develop an AUD, and also may point the way to more targeted treatments. Also, individuals that have these associated genes, and a family history of AUD should especially be informed to avoid alcohol. Understanding individual risk for AUD can help prevent the negative physical and emotional damage that comes with disordered use. Alcohol consumption is a factor for many diseases, injuries, and other mental and behavioral disorders, accounting for 5.3% of global deaths (WHO, 2022). Identifying predisposing factors for AUD will be useful in designing specific prevention and intervention strategies, reducing the consequences of this devastating illness.

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World Health Organization (WHO)

Appendix

Table 1: The DSM-5 diagnostic criteria for AUD. Individuals meeting 2 of the 11 criteria during the same 12-month period would receive a diagnosis of AUD. The severity of AUD is based on the number of criteria met. Mild is the presence of 2-3 symptoms, moderate is the presence of 4-5 symptoms, and severe is the presence of 6 or more symptoms.

The presence of at least 2 of these symptoms indicates **Alcohol Use Disorder (AUD)** In the past year, have you:
Had times when you ended up drinking more, or longer, than you intended?
More than once wanted to cut down or stop drinking, or tried to, but couldn't?
Spent a lot of time drinking? Or being sick or getting over the aftereffects?
Wanted a drink so badly you couldn't think of anything else?
Found that drinking – or being sick from drinking – often interfered with taking care of your home or family? Or caused job troubles? Or school problems?
Continued to drink even though it was causing trouble with your family and friends?
Given up or cut back on activities that were important or interesting to you, or gave you pleasure, in order to drink?
More than once gotten into situations while or after drinking that increased your chances of getting hurt (such as driving, swimming, using machinery, walking in a dangerous area, or having unsafe sex)?
Continued to drink even though it was making you feel depressed or anxious or adding to another health problem? Or after having had a memory blackout?
Had to drink much more than you once did to get the effect you want? Or found that your usual number of drinks had much less effect than before?
Found that when the effects of alcohol were wearing off, you had withdrawal symptoms, such as trouble sleeping, shakiness, restlessness, nausea, sweating, a racing heart, or a seizure? Or sensed things that were not there?

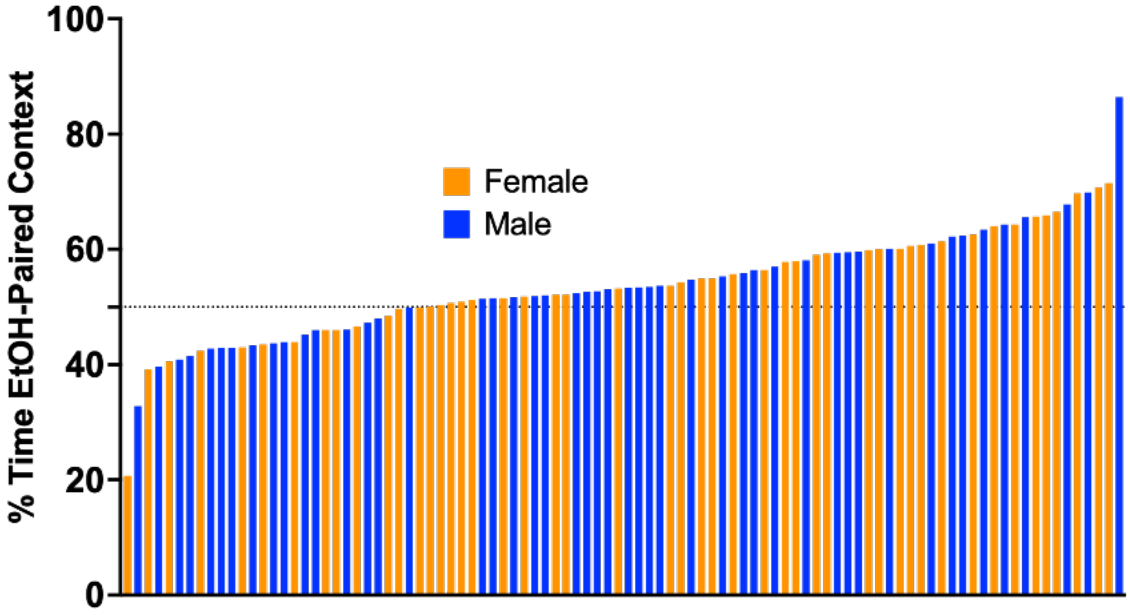

Figure 1: Male and female DO mice demonstrated SE-CPP. Both males and females showed a significant place preference for the ethanol-paired chamber, and the preference was not significantly different.

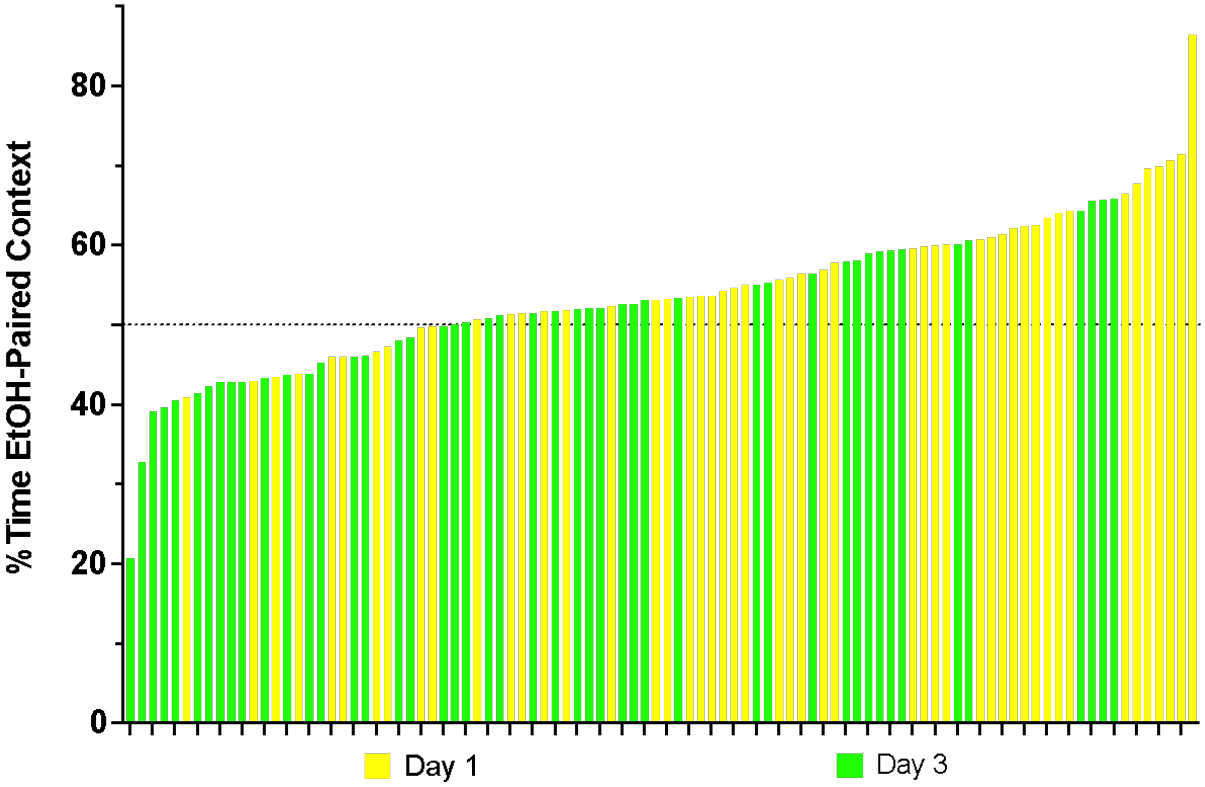

Figure 2: Percentage of time spent in the EtOH-paired context separated by injection order. Animals that received EtOH on day 1 were more significantly stimulated than animals that received EtOH on day 3.

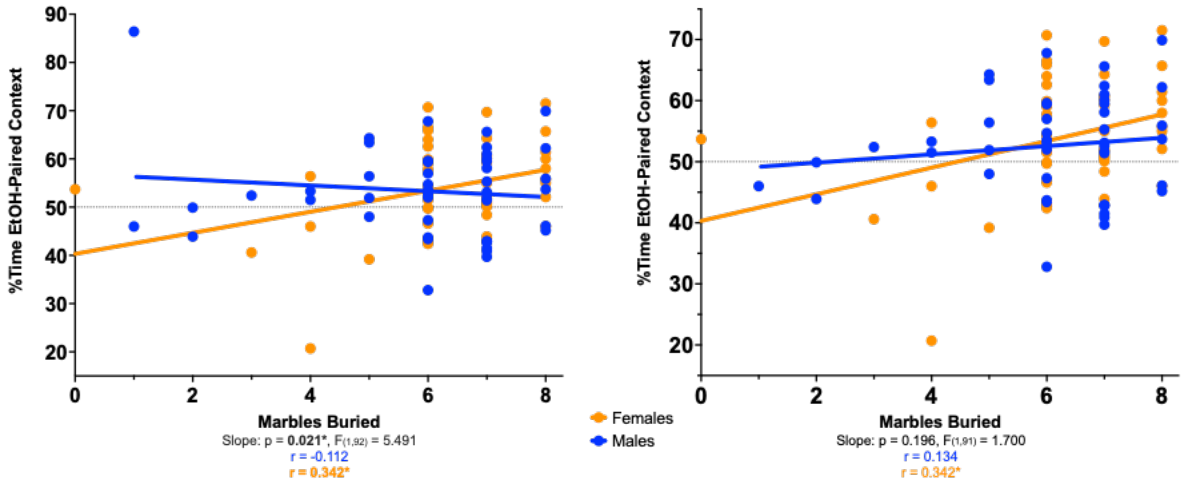

Figure 3: Correlation Between Marbles Buried and SE-CPP. In females, there was a significant correlation between marbles buried and SE-CPP, but not in males. There was a significant difference in the slope of the regression line, but only when the male outlier was included.

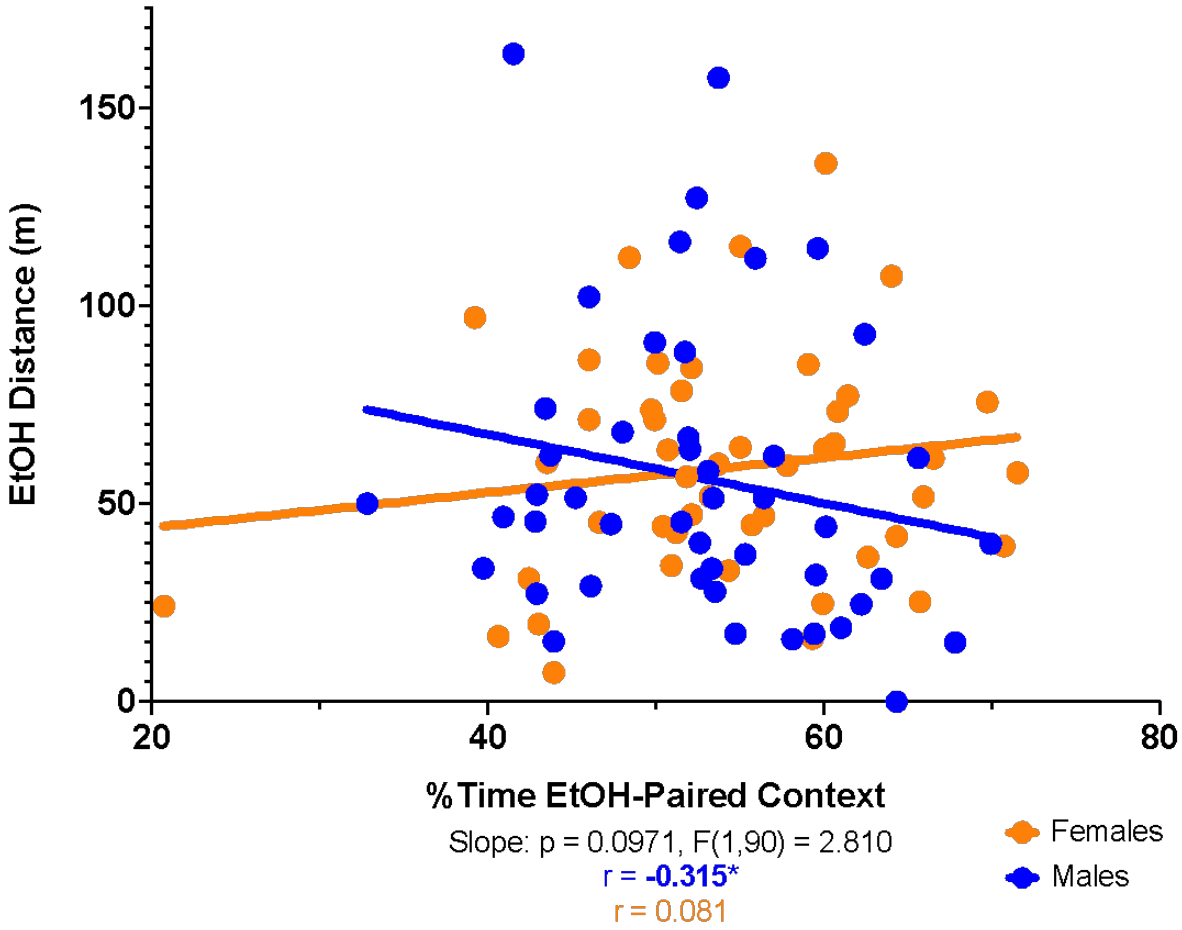

Figure 4: Correlation between the distance traveled during EtOH conditioning and SE-CPP. In males, here was a significant correlation between the distance traveled during EtOH conditioning and the percentage of time spent in the EtOH-paired context, but not in females. The difference between the slopes was not quite significant.

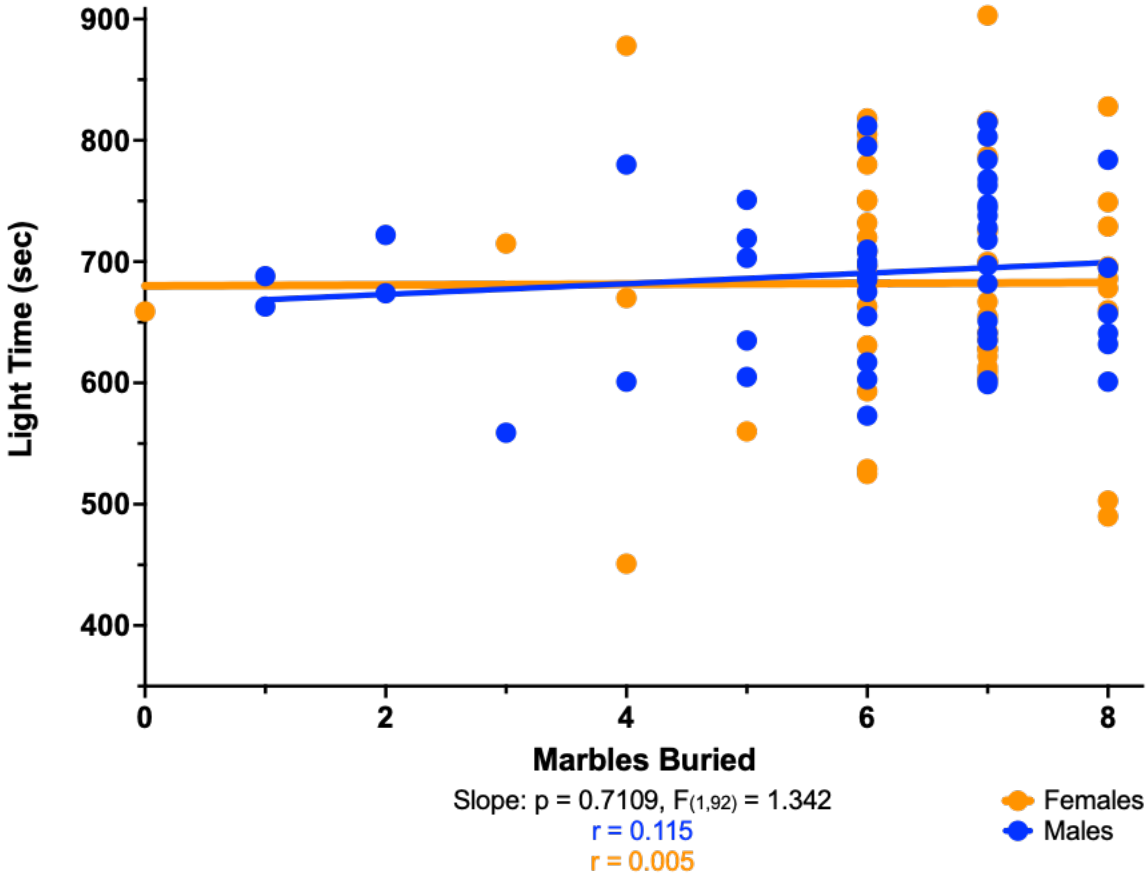

Figure 5: Correlation between marble burying and light time. There was no significant correlation between the number of marbles buried and light time in the light-dark box. The difference between the slopes was not significant.

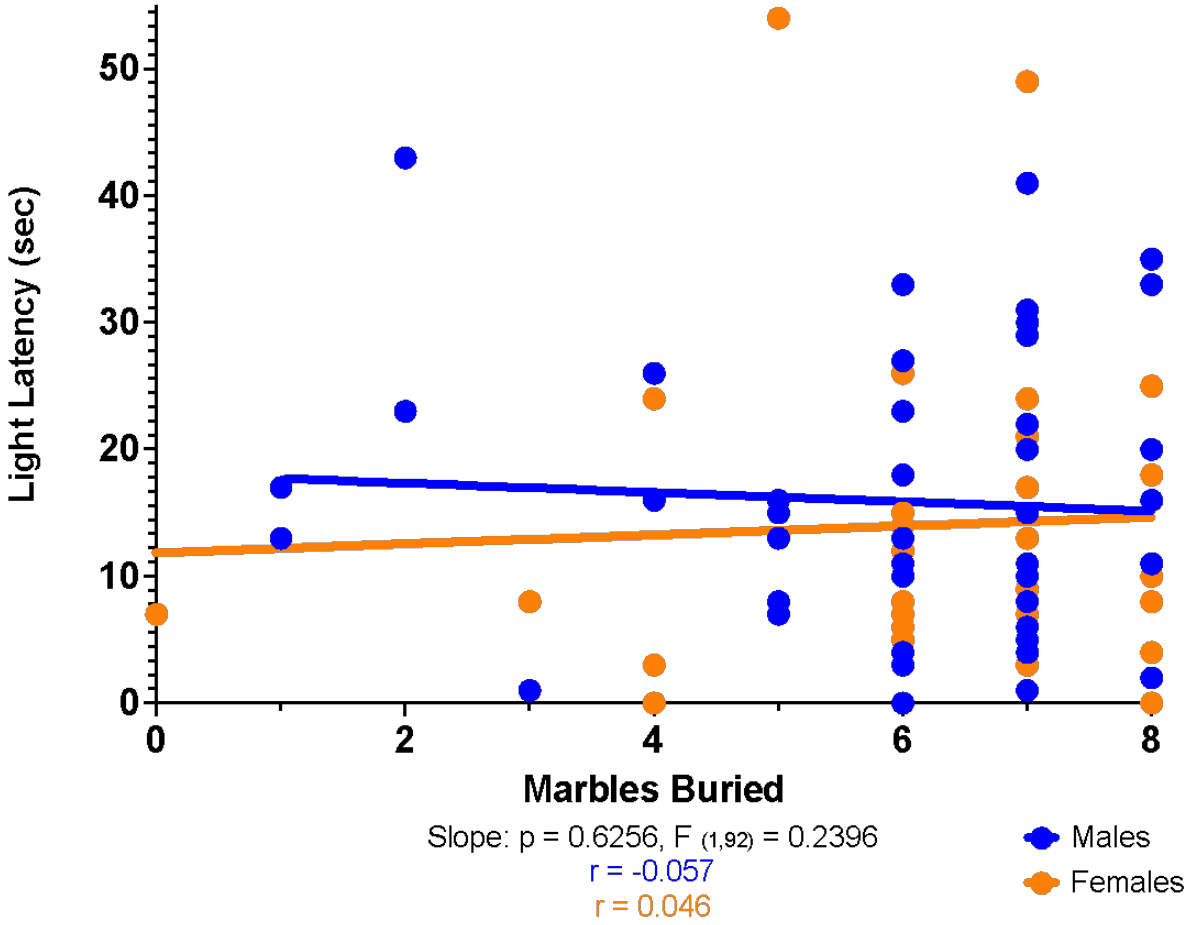

Figure 6: Correlation between marble burying and light latency. There was no correlation between the number of marbles buried and the light latency. The difference between the slopes was not significant.

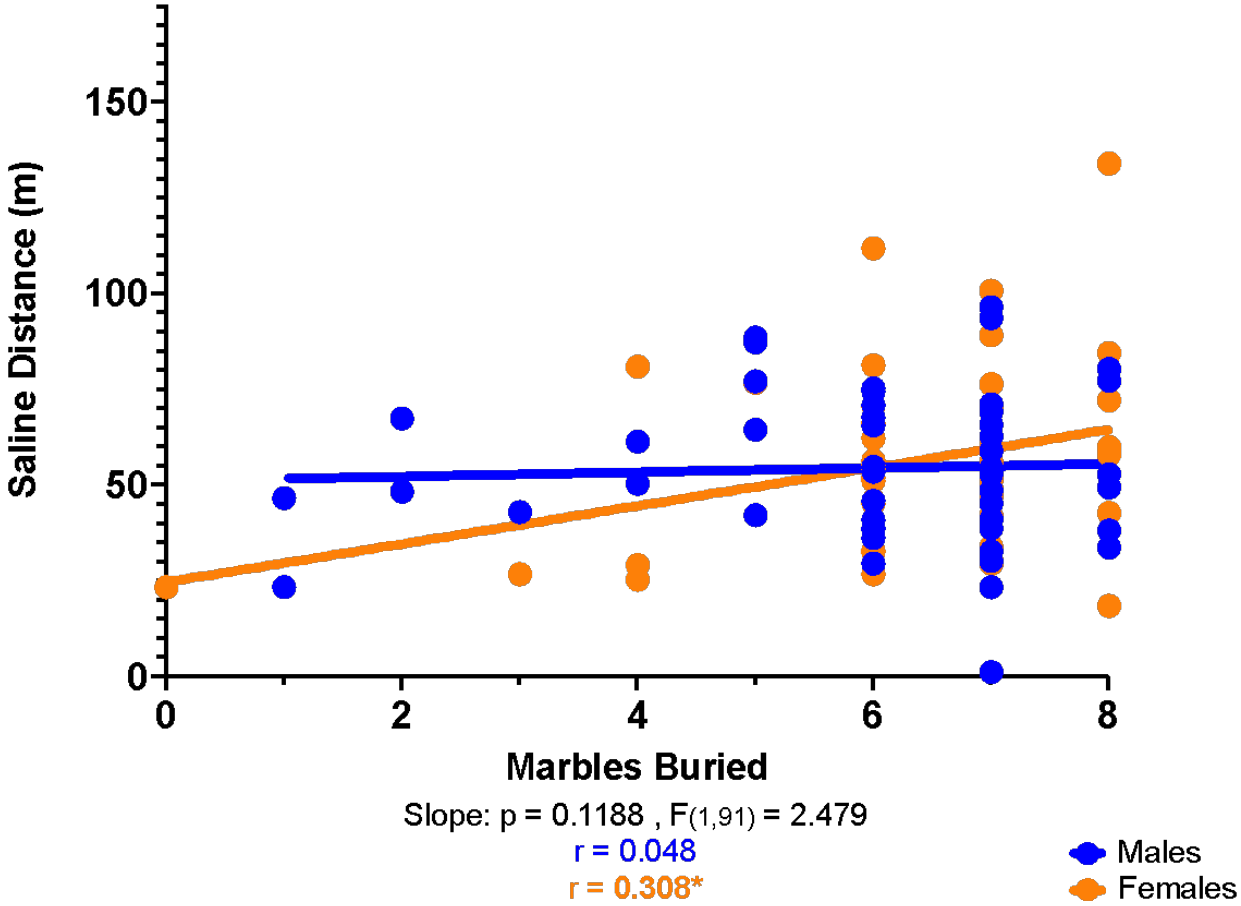

Figure 7: Correlation between then number of marbles buried, and the distance traveled during saline. In females, there was a significant correlation between the number of marbles buried and the distance traveled during saline conditioning days, but not in males. The difference between the slopes was not significant.

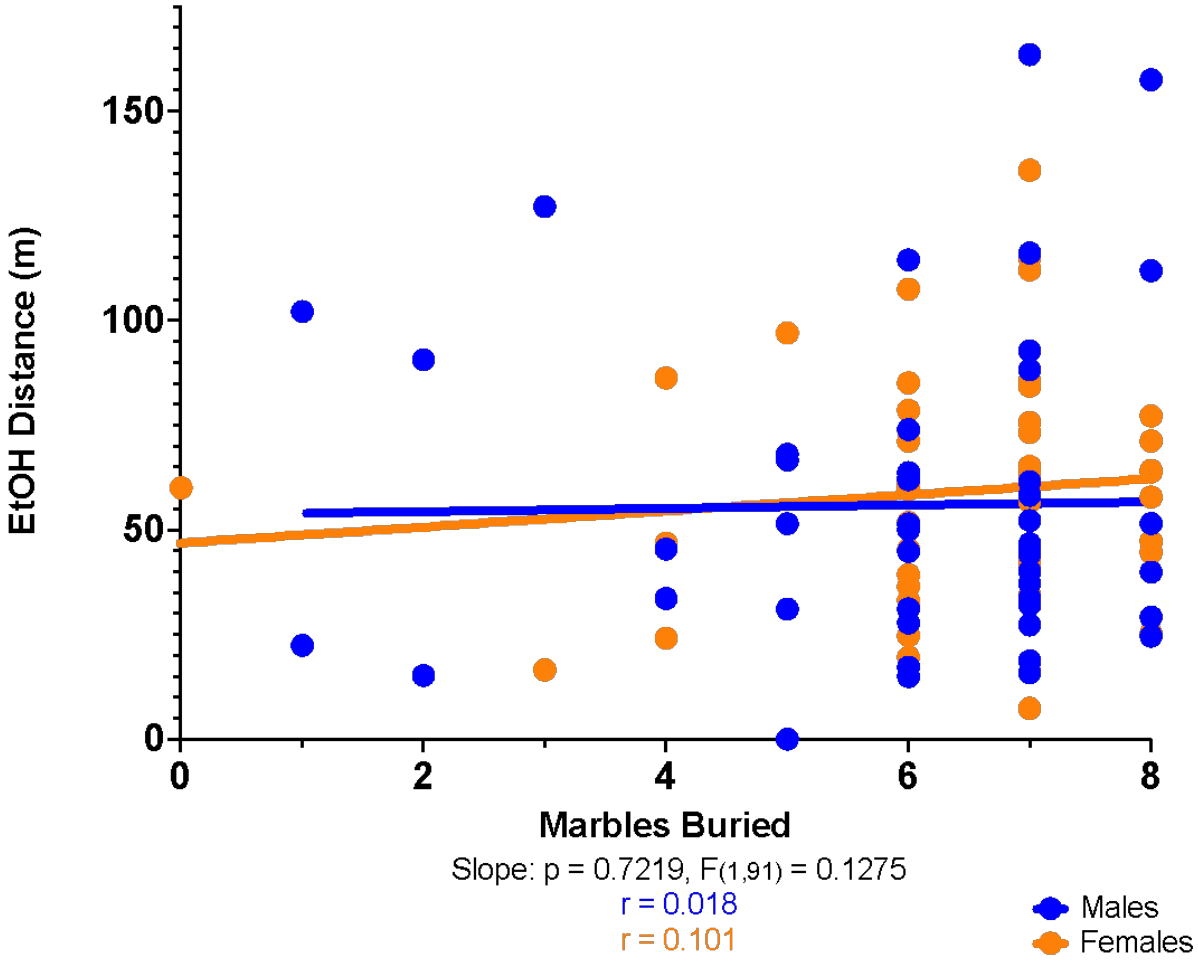

Figure 8: Correlation between the number of marbles buried and the distance traveled during EtOH conditioning. There was no correlation between the number of marbles buried and the distance traveled during EtOH conditioning. The difference between the slopes was not significant.

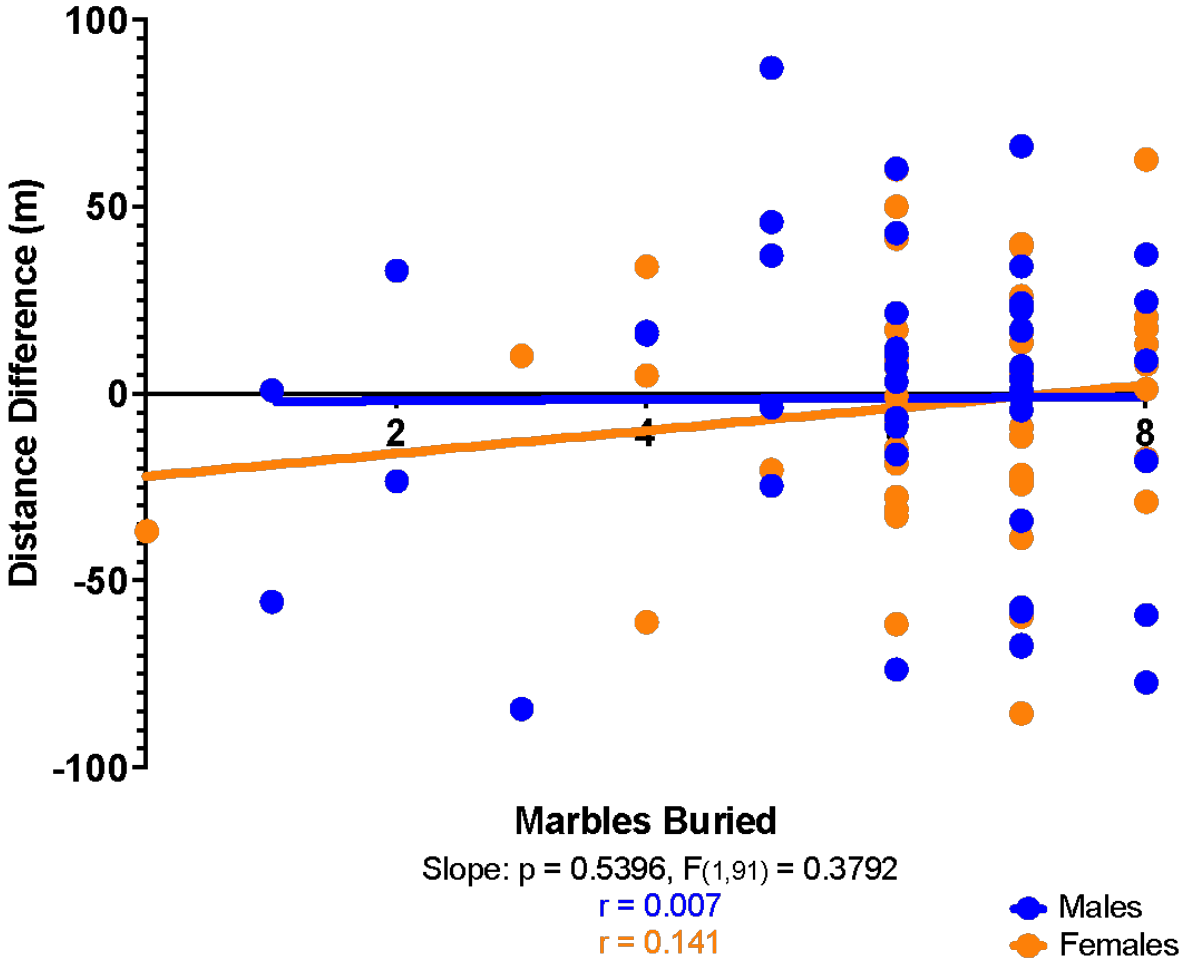

Figure 9: Correlation between the number of marbles buried and the distance difference. There was no correlation between the number of marbles buried and the distance difference. The difference between the slopes was not significant.

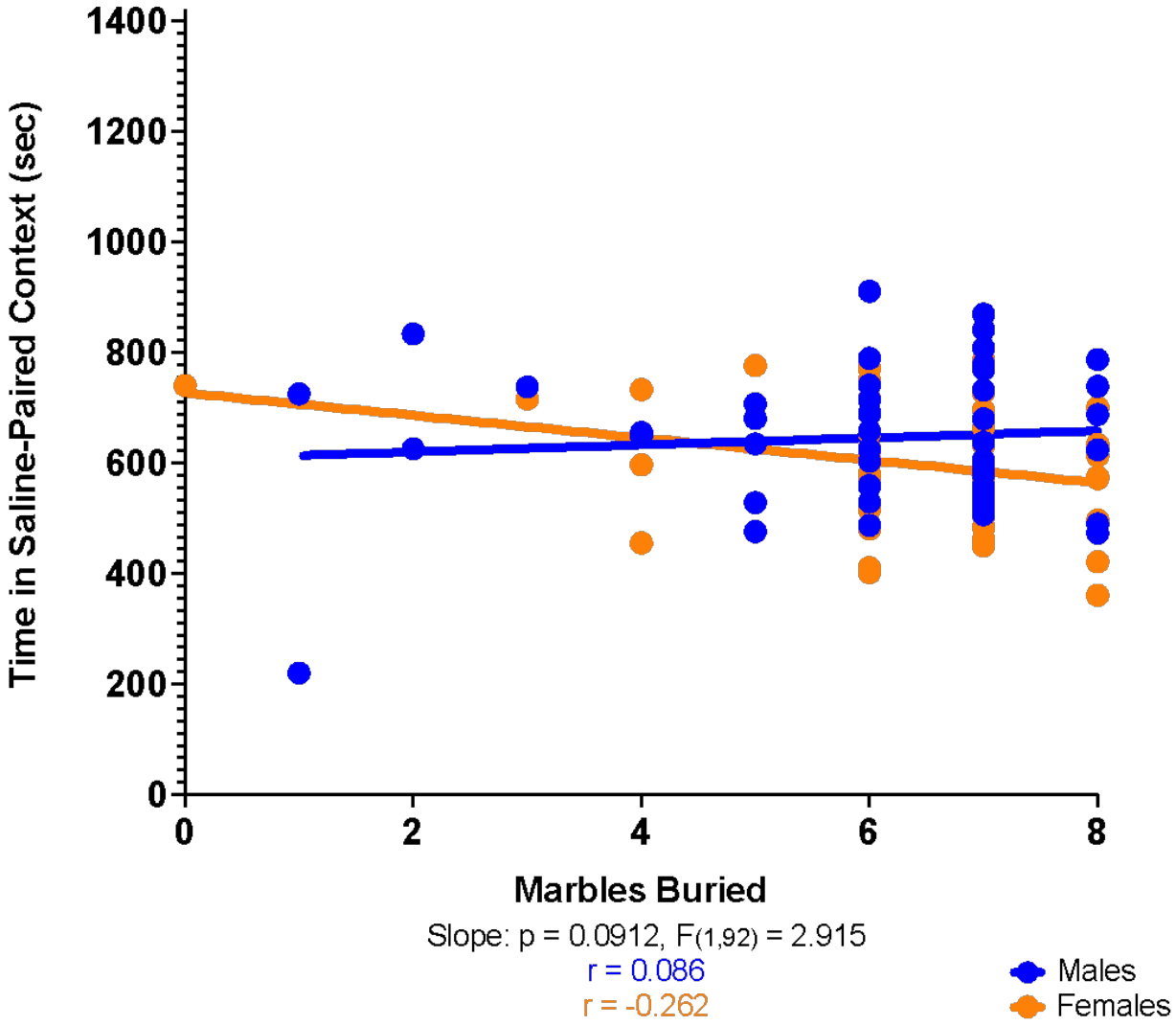

Figure 10: Correlation between marble burying and the time spent in the saline-paired context and the number of marbles buried. There was no correlation between the number of marbles buried and the time spent in the saline-paired context. The difference between the slopes was not quite significant.

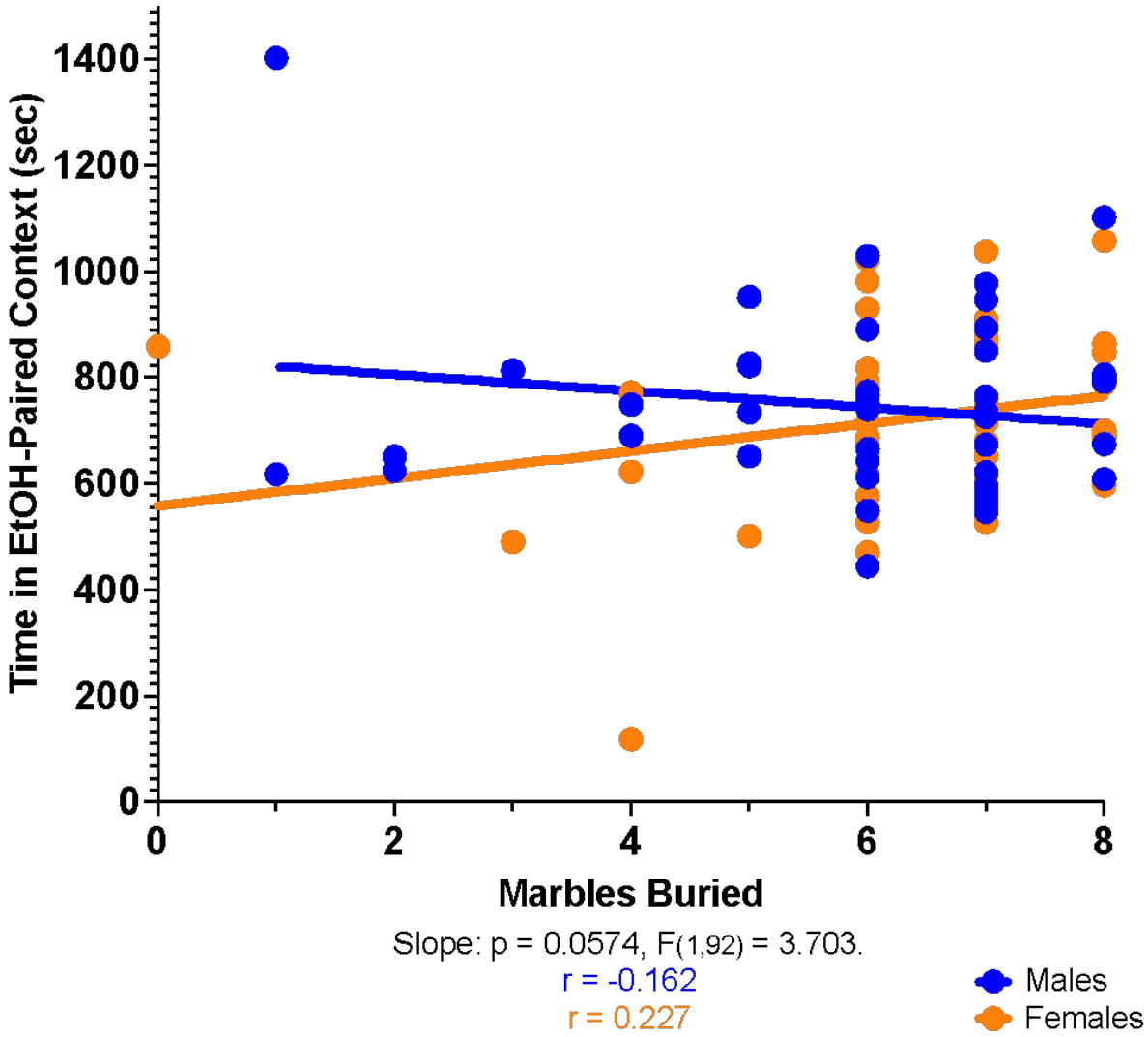

Figure 11: Correlation between the number of marbles buried and the time spent in the EtOH-paired context. There was no correlation between the number of marbles buried and the time spent in the EtOH-paired context. The difference between the slopes was not quite significant.

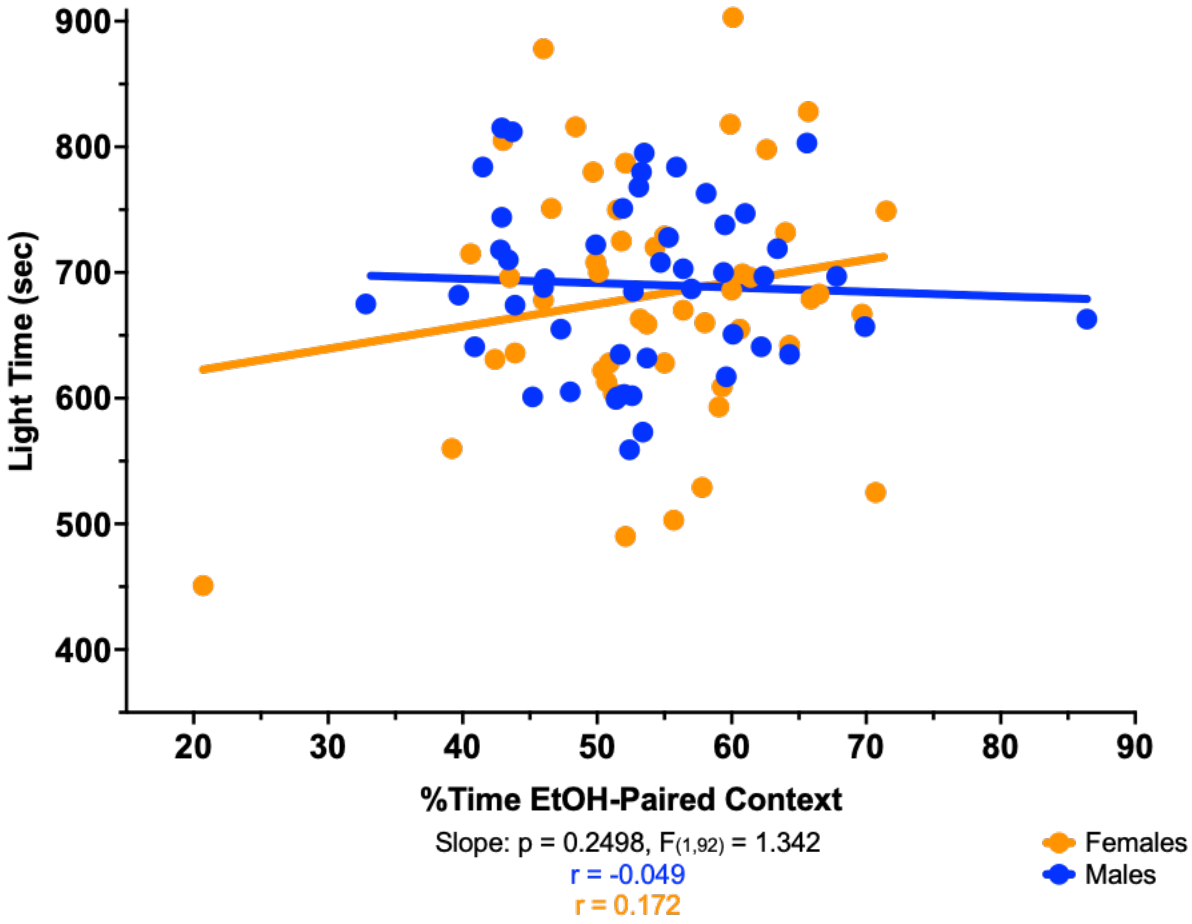

Figure 12: Correlation between light time and SE-CPP. There was no significant correlation between light time in the light-dark box and SE-CPP. The difference between the slopes was not significant.

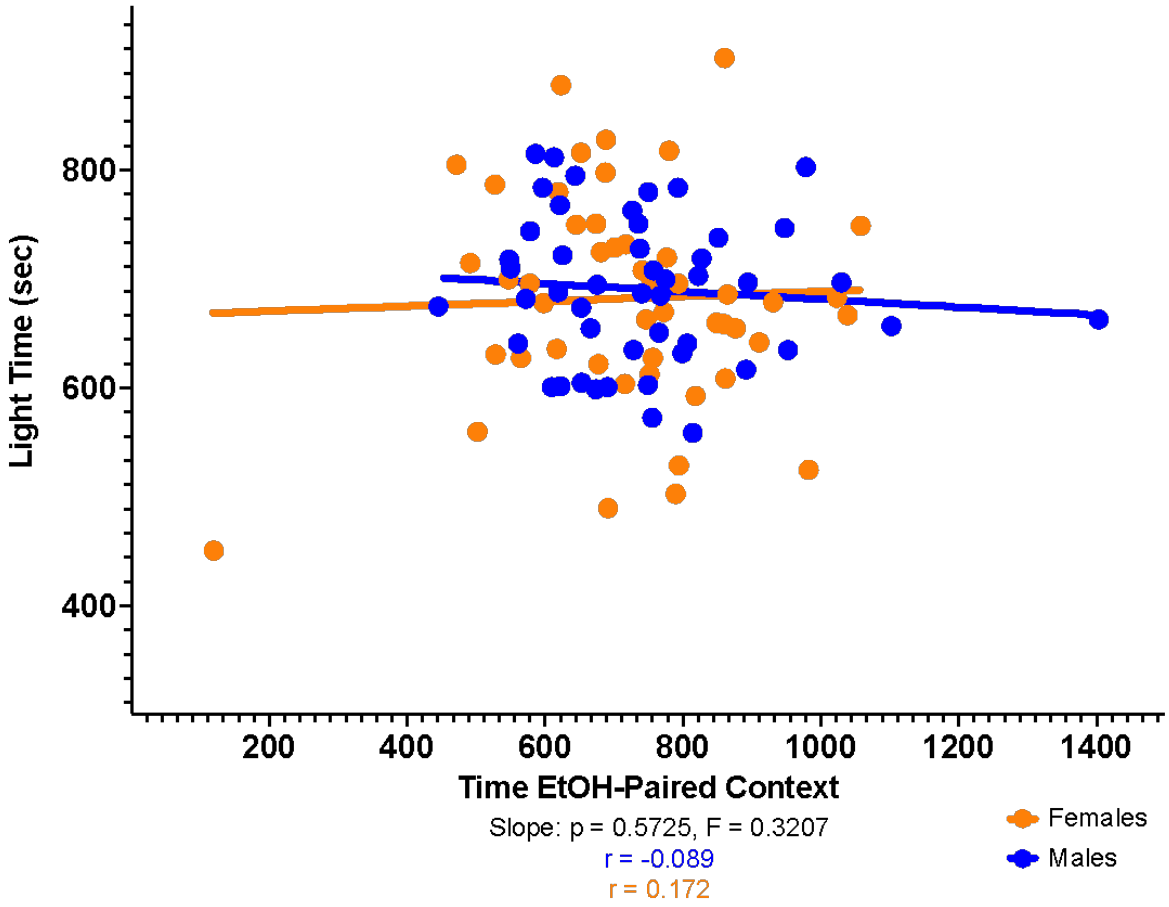

Figure 13: Correlation between time spent in the light side of the light-dark box and time spent in the EtOH-paired context. There was no correlation between the amount of time spent in the light side of the light-dark box and the amount of time spent in the EtOH-paired context. The difference between the slopes was not significant.

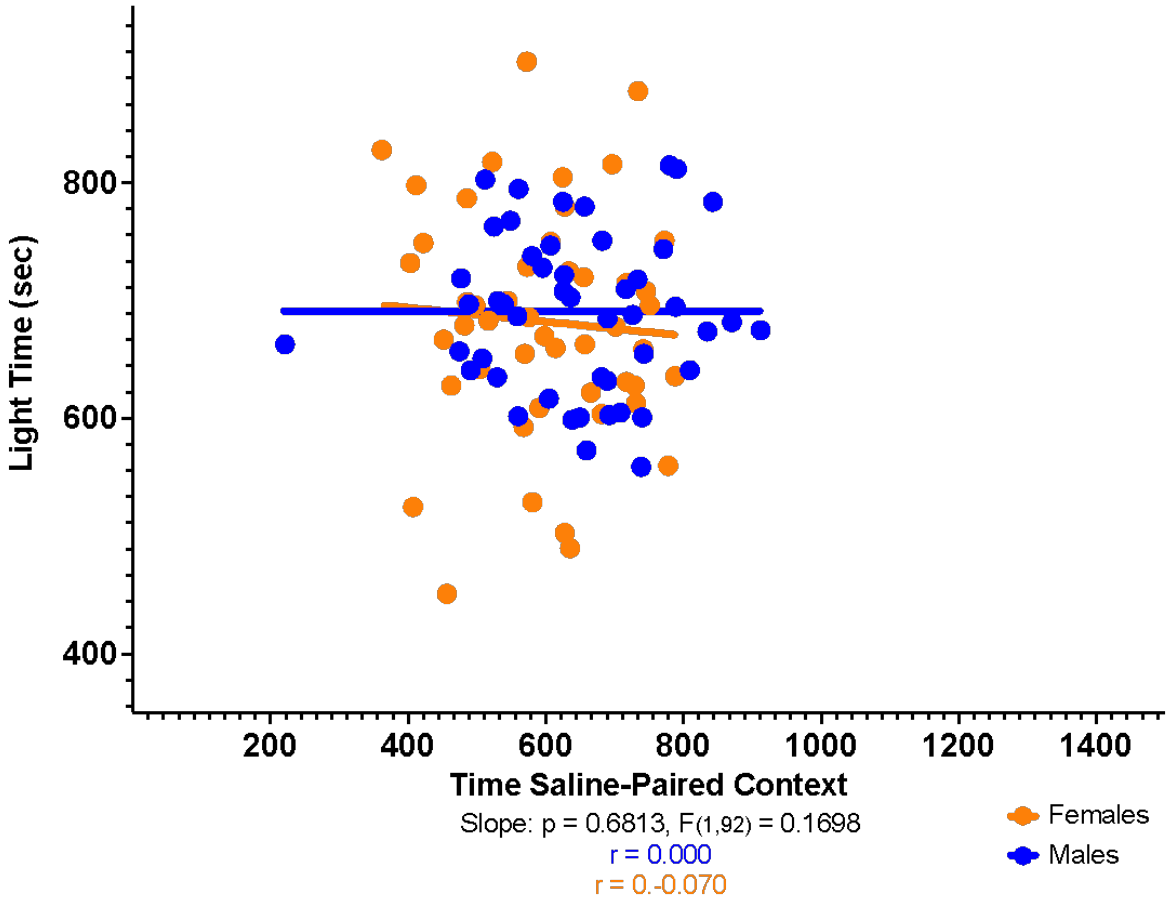

Figure 14: Correlation between the time spent in the light side of the light-dark box and the time spent in the saline-paired context. There was no correlation between the amount of time spent in the light side of the light-dark box and the amount of time spent in the saline-paired context. The difference between the slopes was not significant.

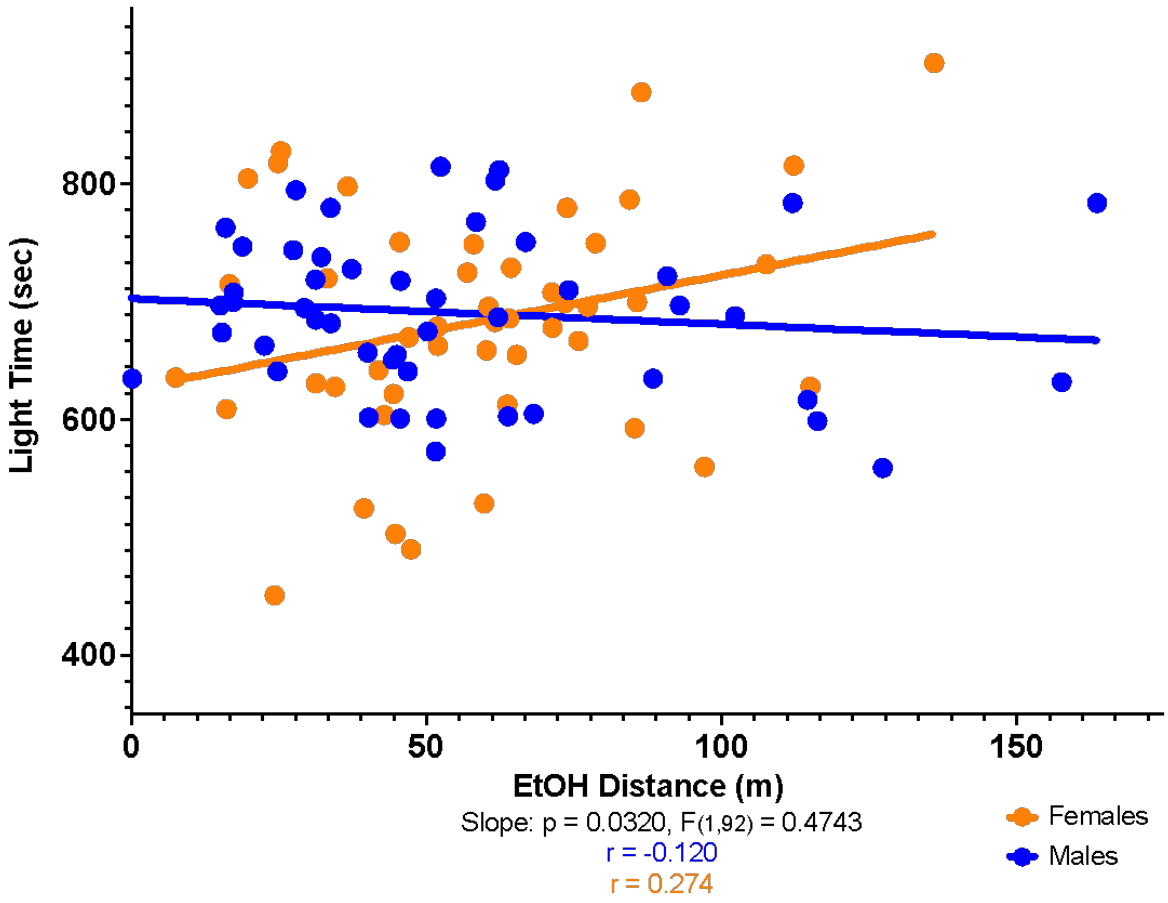

Figure 15: Correlation between the time spent in the light side of the light-dark box and the distance traveled during EtOH conditioning. There was no correlation between the amount of time spent in the light side of the light-dark box and the distance traveled during EtOH conditioning. The difference between the slopes was significant.

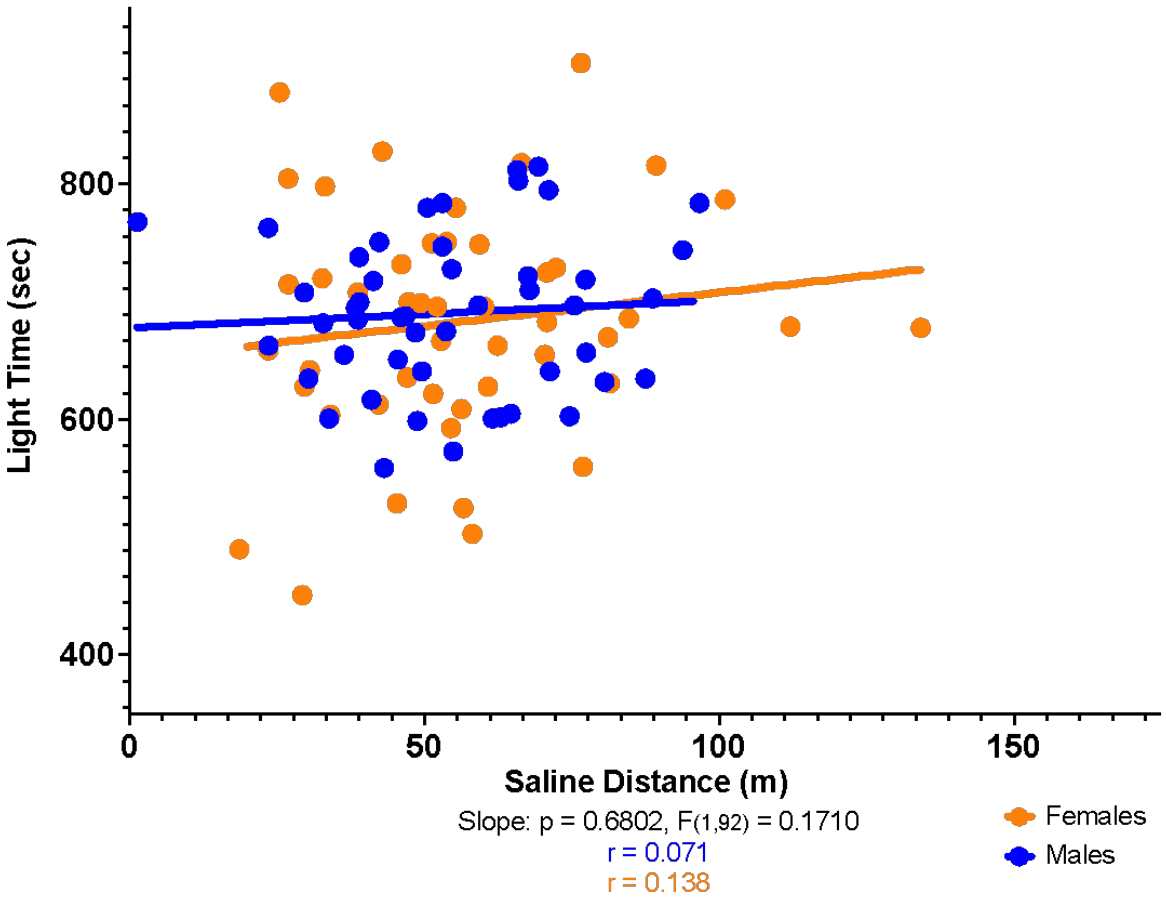

Figure 16: Correlation between the time spent in the light side of the light-dark box and the distance traveled during saline conditioning. There was no correlation between the amount of time spent in the light side of the light-dark box and the distance traveled during saline conditioning. The difference between the slopes was not significant.

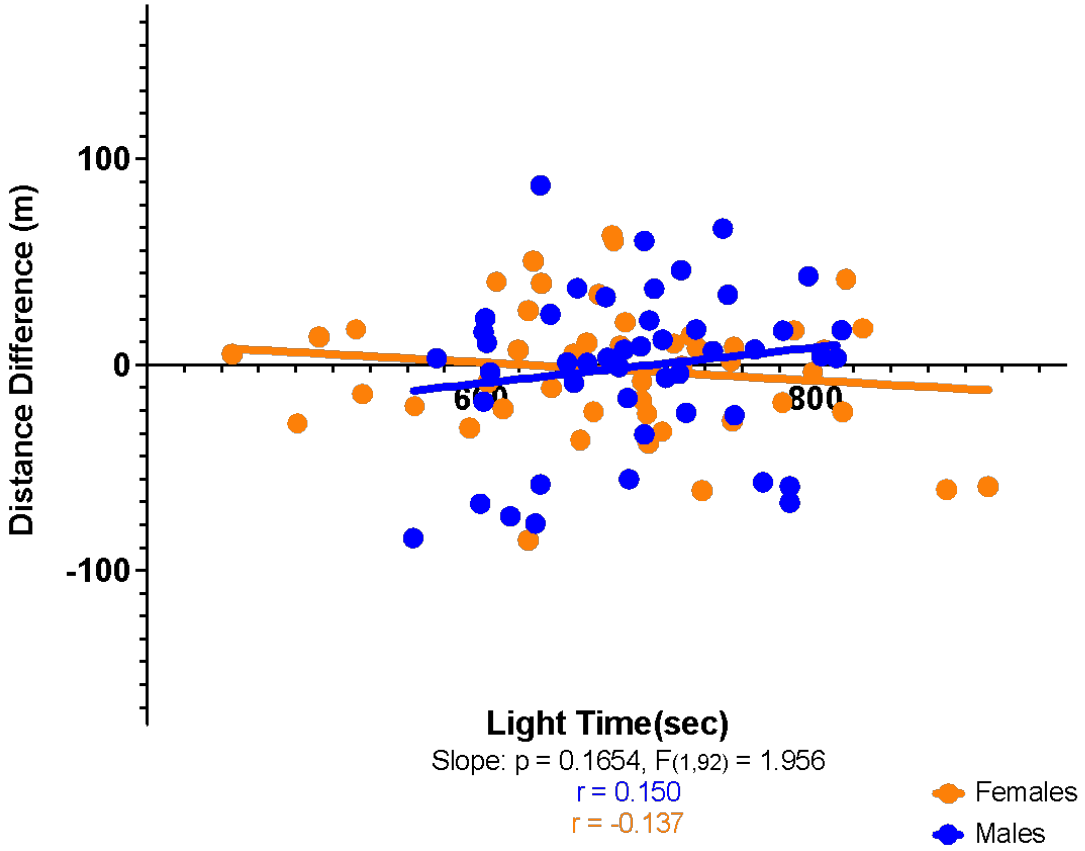

Figure 17: Correlation between the time spent in the light side of the light-dark box and the distance difference. There was no correlation between the amount of time spent in the light side of the light-dark box and the distance difference. The difference between the slopes was not significant.

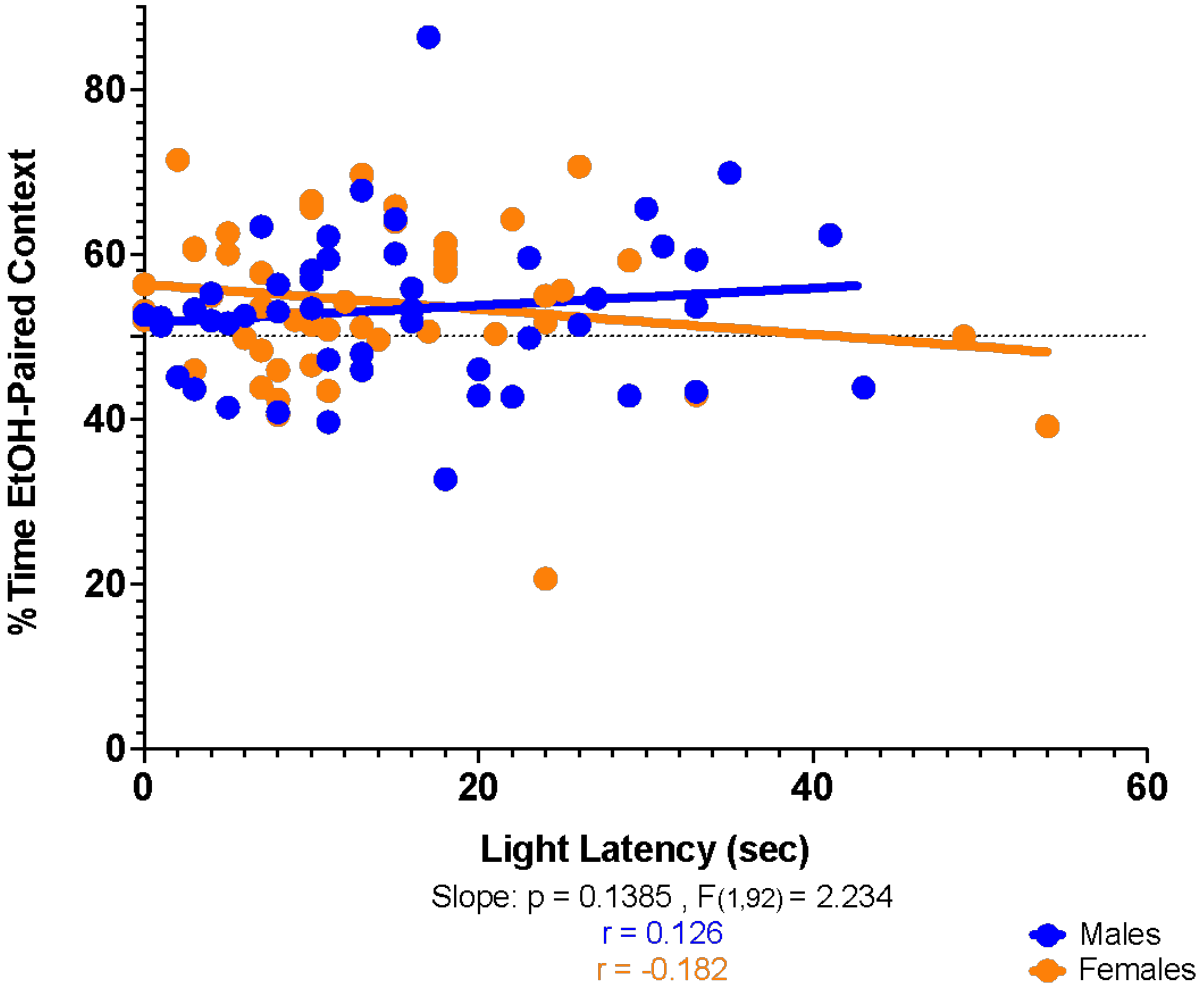

Figure 18: Correlation between light latency and the percentage of time spent in the EtOH-paired context. There was no correlation between light latency and the percentage of time spent in the EtOH-paired context. The difference between the slopes was not significant.

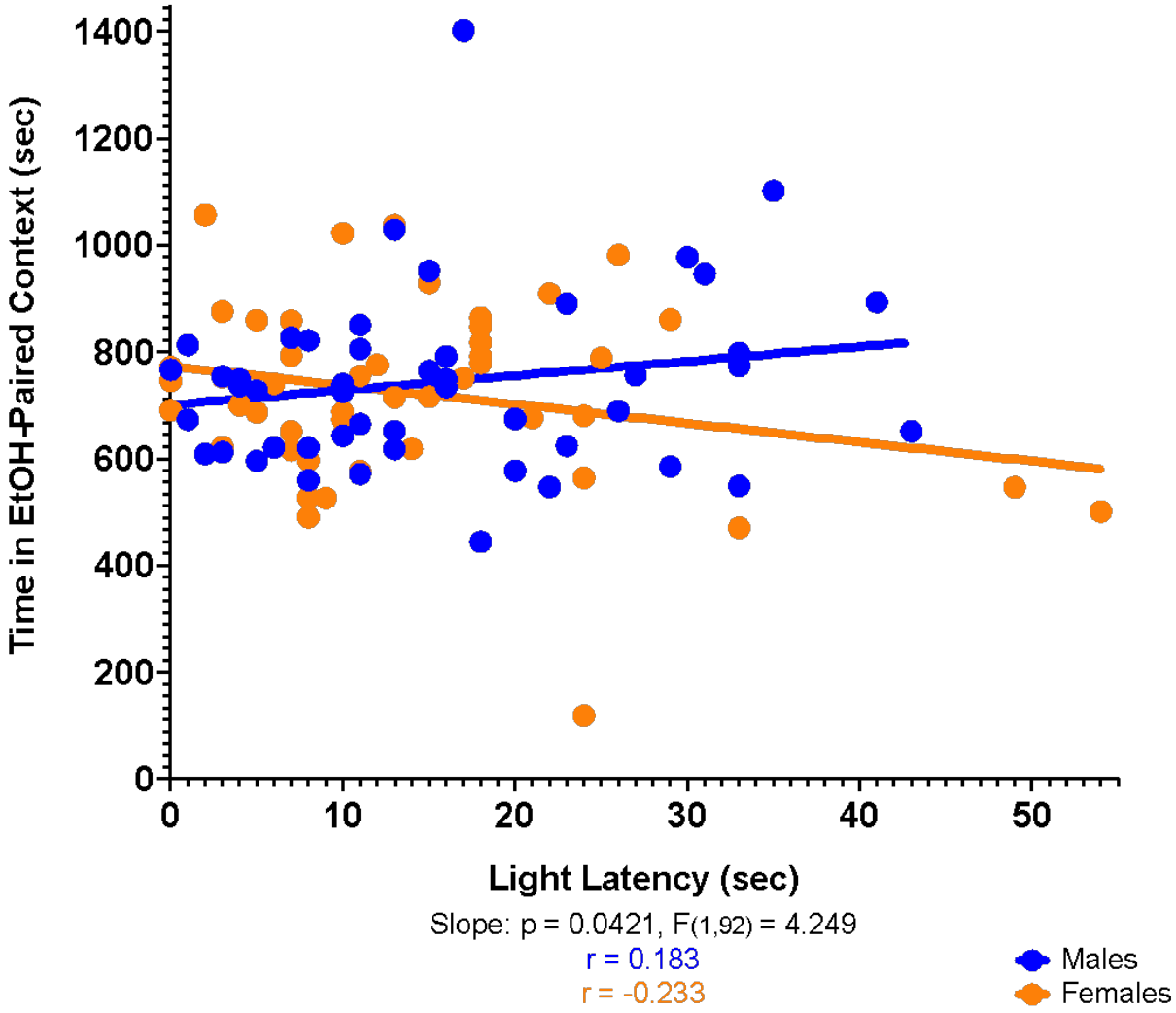

Figure 19: Correlation between light latency and the time spent in the EtOH-paired context. There was no correlation between light latency and the time spent in the EtOH-paired context. The difference between the slopes was significant.

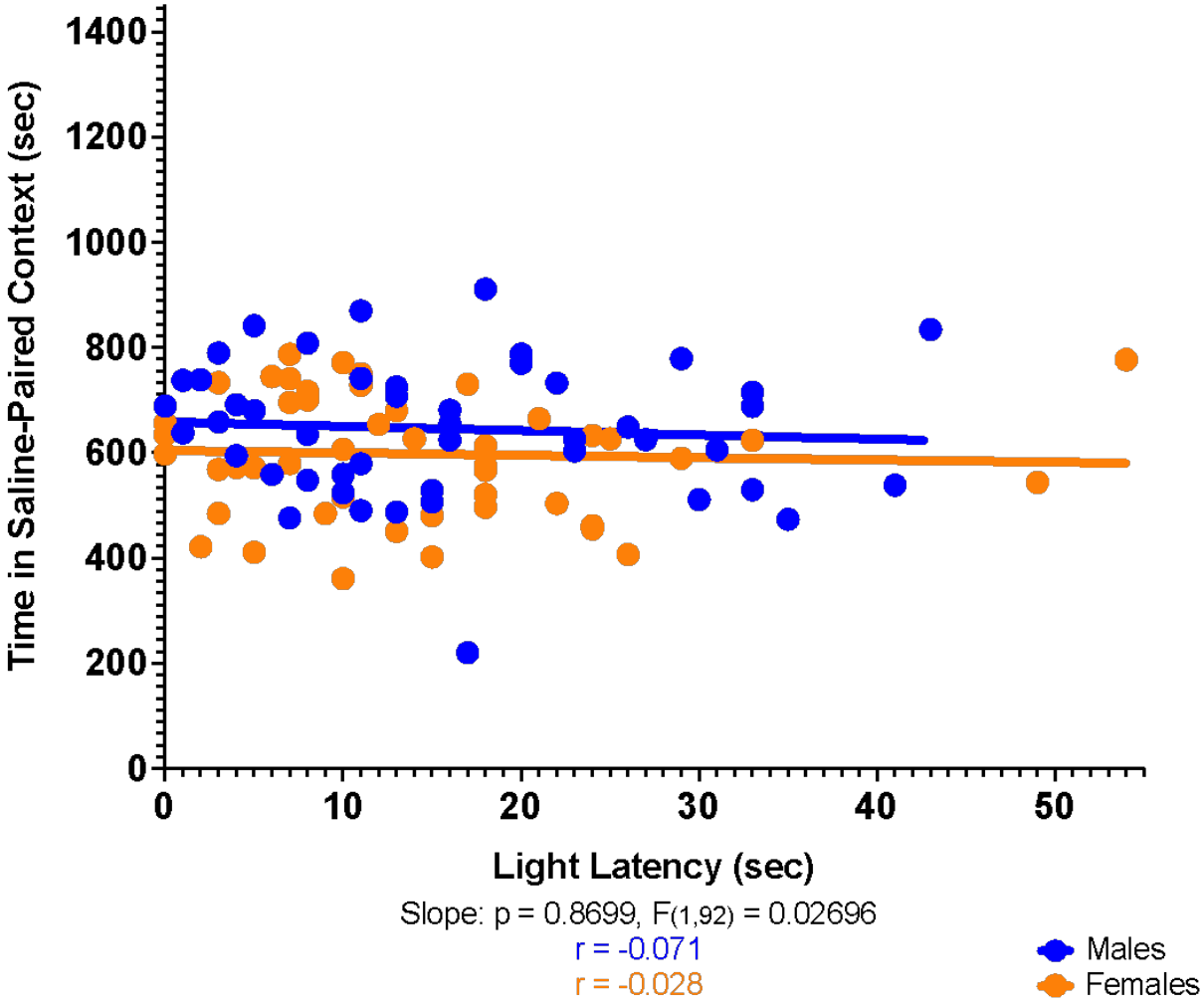

Figure 20: Correlation between light latency and the time spent in the saline-paired context. There was no correlation between light latency and the time spent in the saline-paired context. The difference between the slopes was not significant.

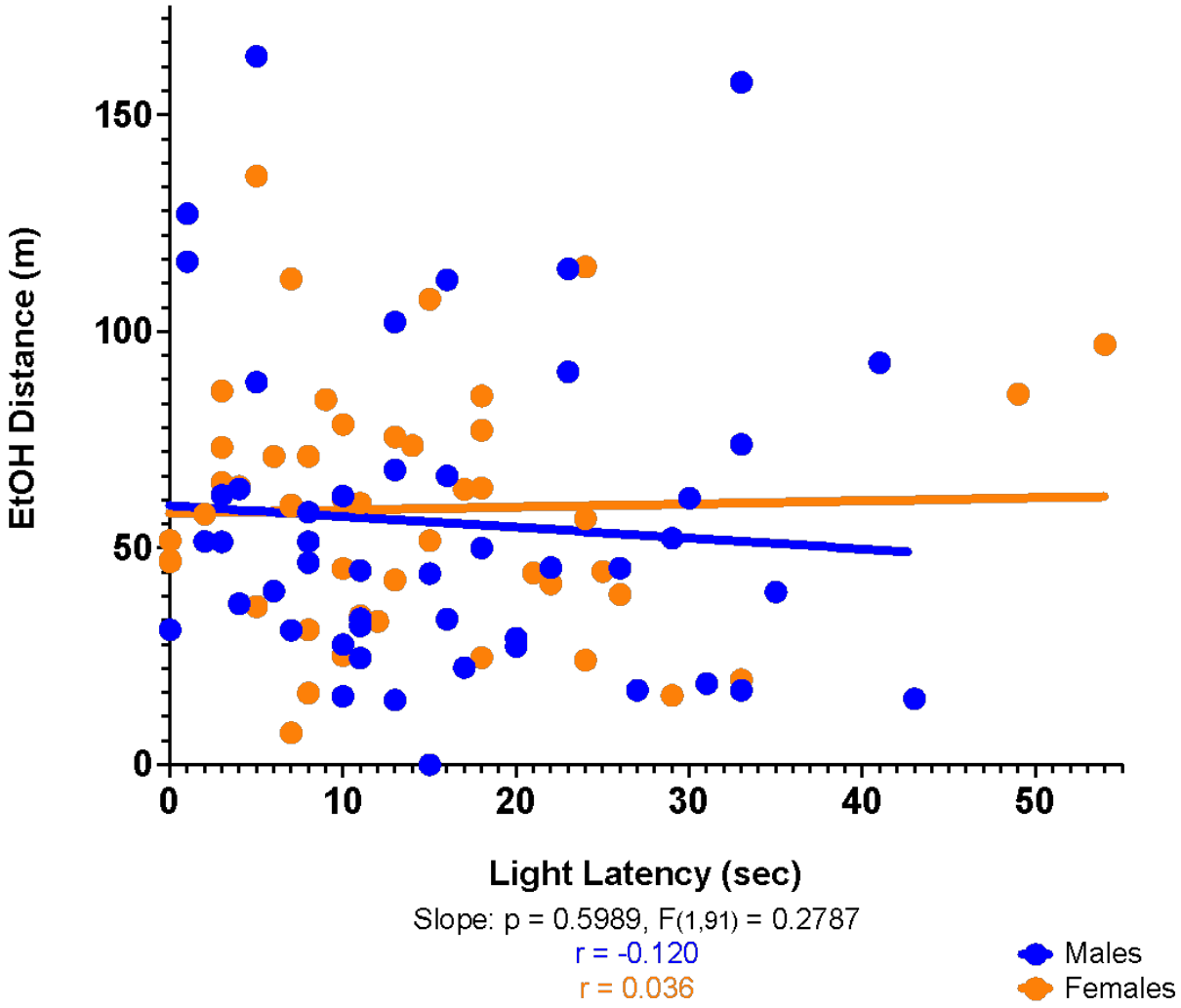

Figure 21: Correlation between light latency and the distance traveled during EtOH conditioning. There was no correlation between light latency and the distance traveled during EtOH conditioning. The difference between the slopes was not significant.

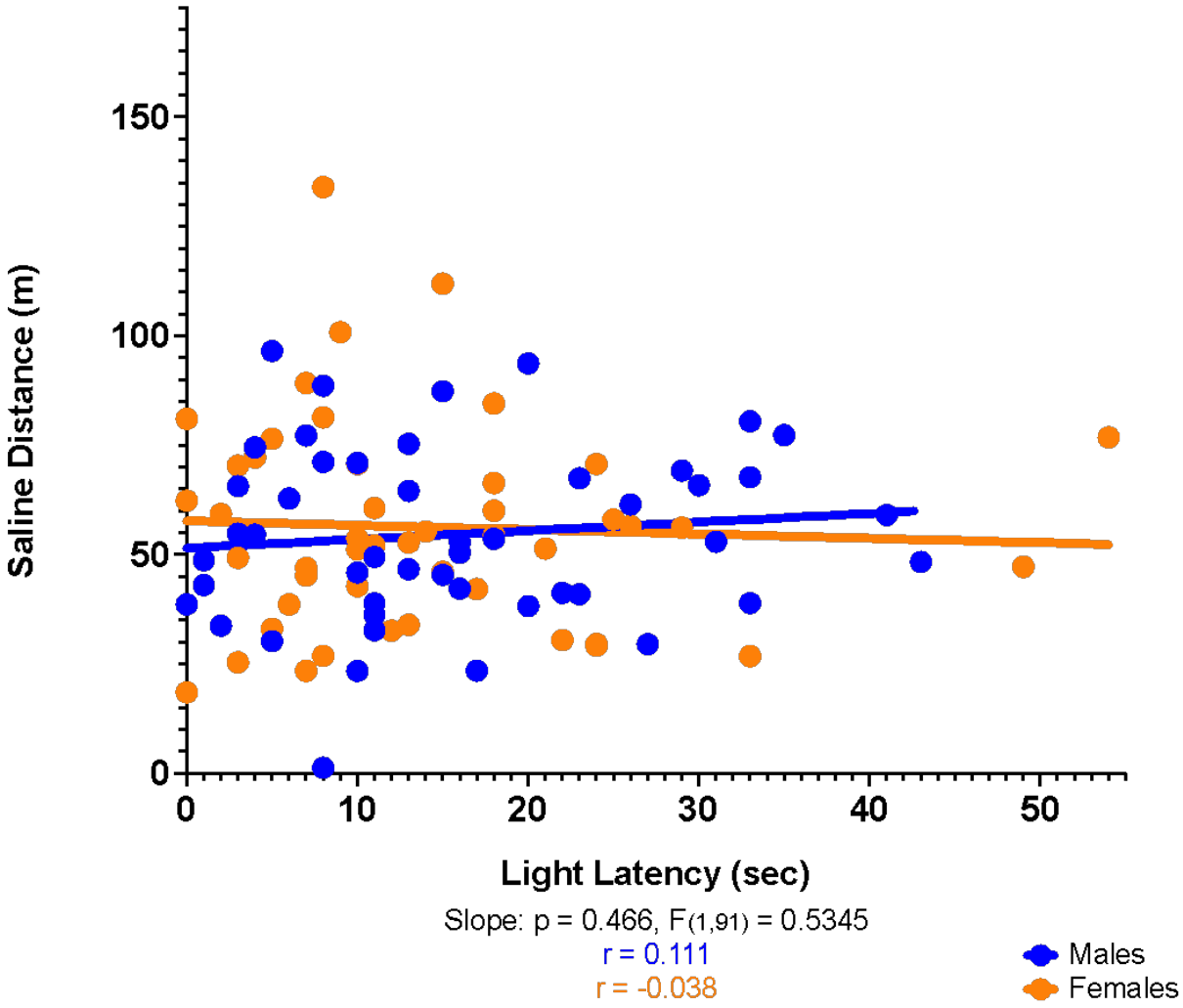

Figure 22: Correlation between light latency and the distance traveled during saline conditioning. There was no correlation between light latency and the distance traveled during saline conditioning. The difference between the slopes was not significant.

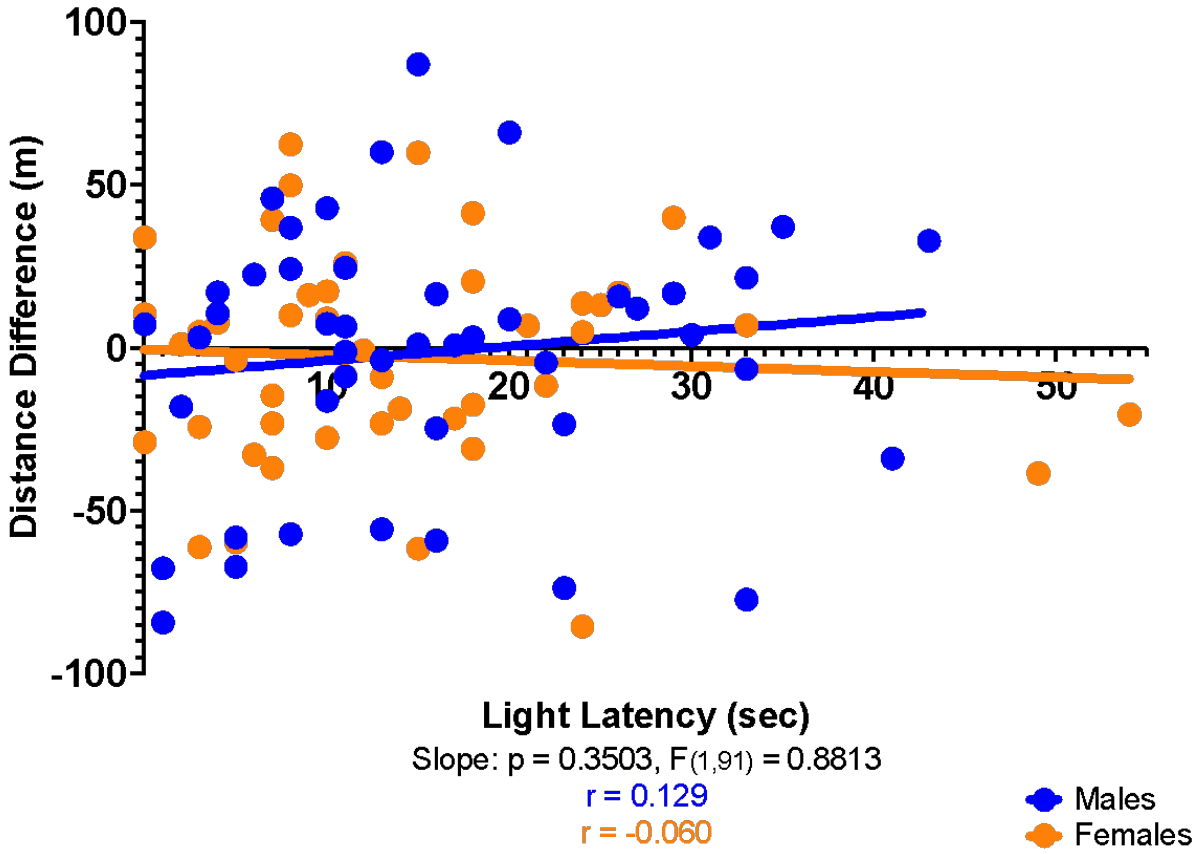

Figure 23: Correlation between light latency and distance difference. There was no correlation between light latency and the distance difference. The difference between the slopes was not significant.

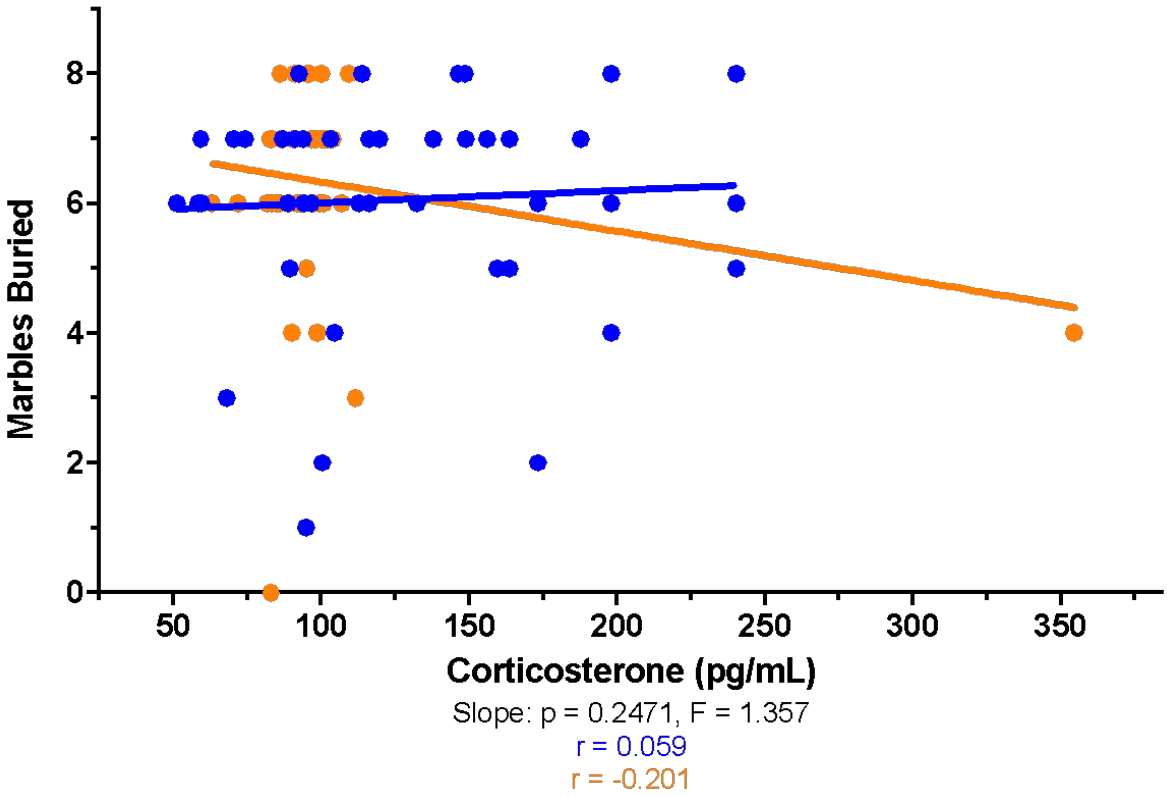

Figure 24: Correlation between blood corticosterone levels and the number of marbles buried. There was no significant correlation between blood corticosterone levels and the number of marbles buried. The difference between the slopes was not significant.

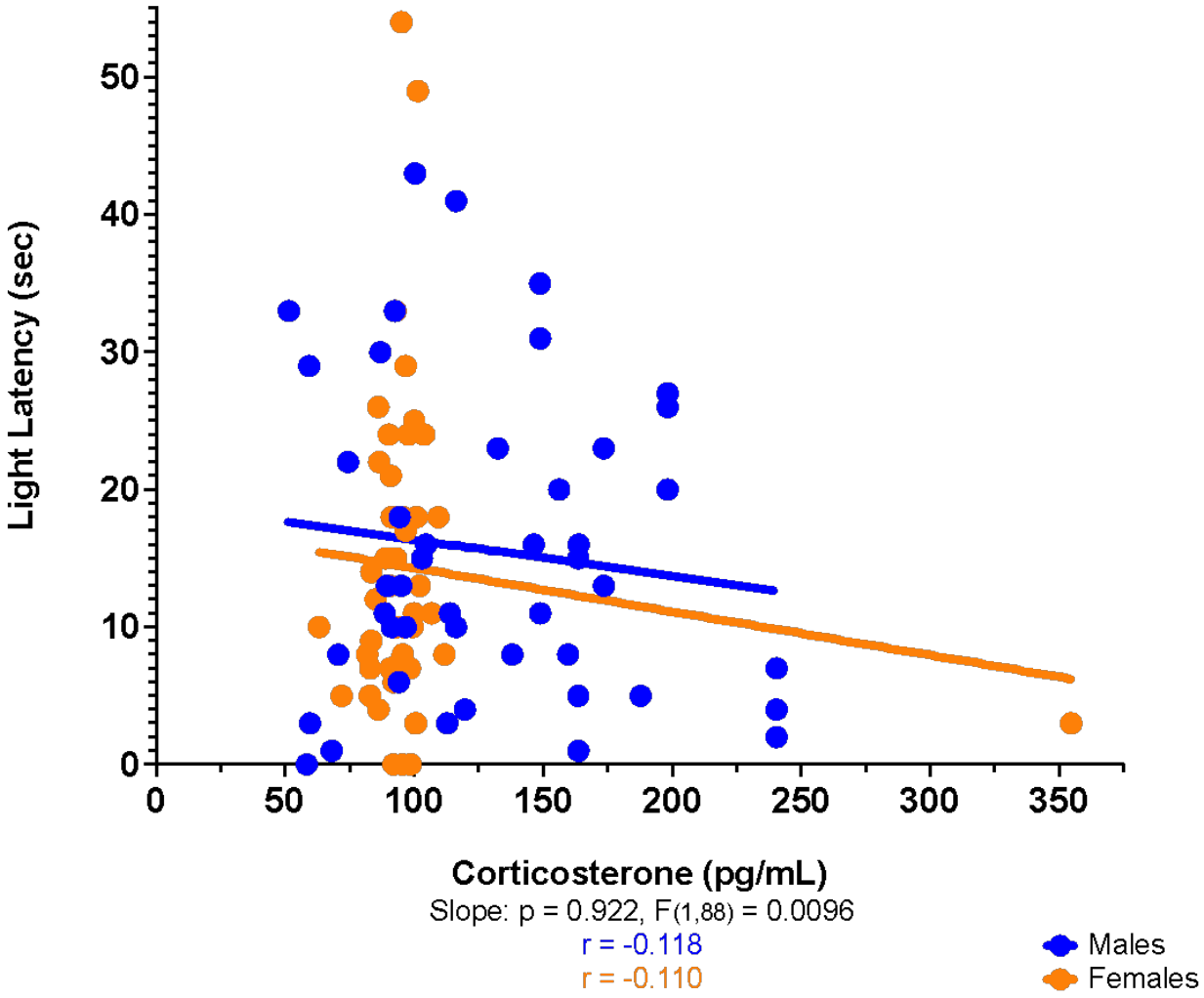

Figure 25: Correlation between blood corticosterone levels and light latency. There was no correlation between blood corticosterone levels and light latency. The difference between the slopes was not significant.

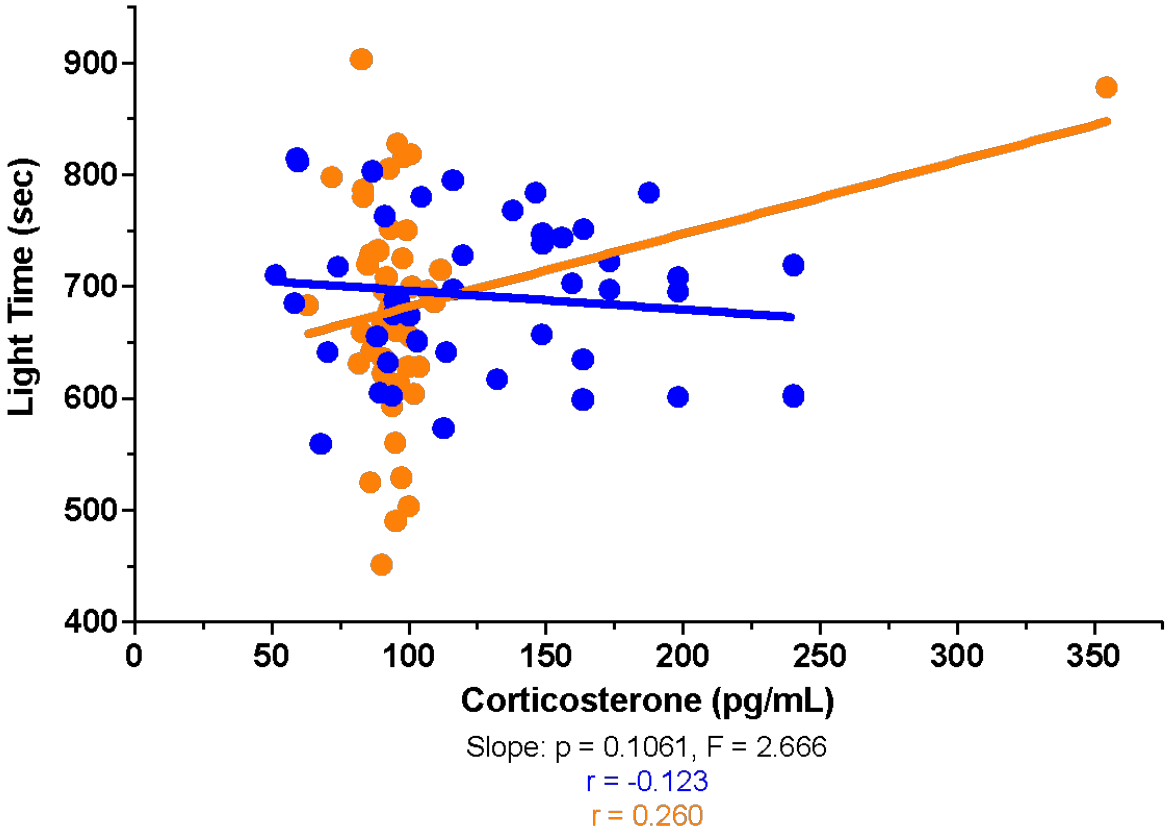

Figure 26: Correlation between blood corticosterone levels and light time. There was no significant correlation between blood corticosterone levels and the amount of time spent in the light side of the light-dark box. The difference between the slopes was significant.

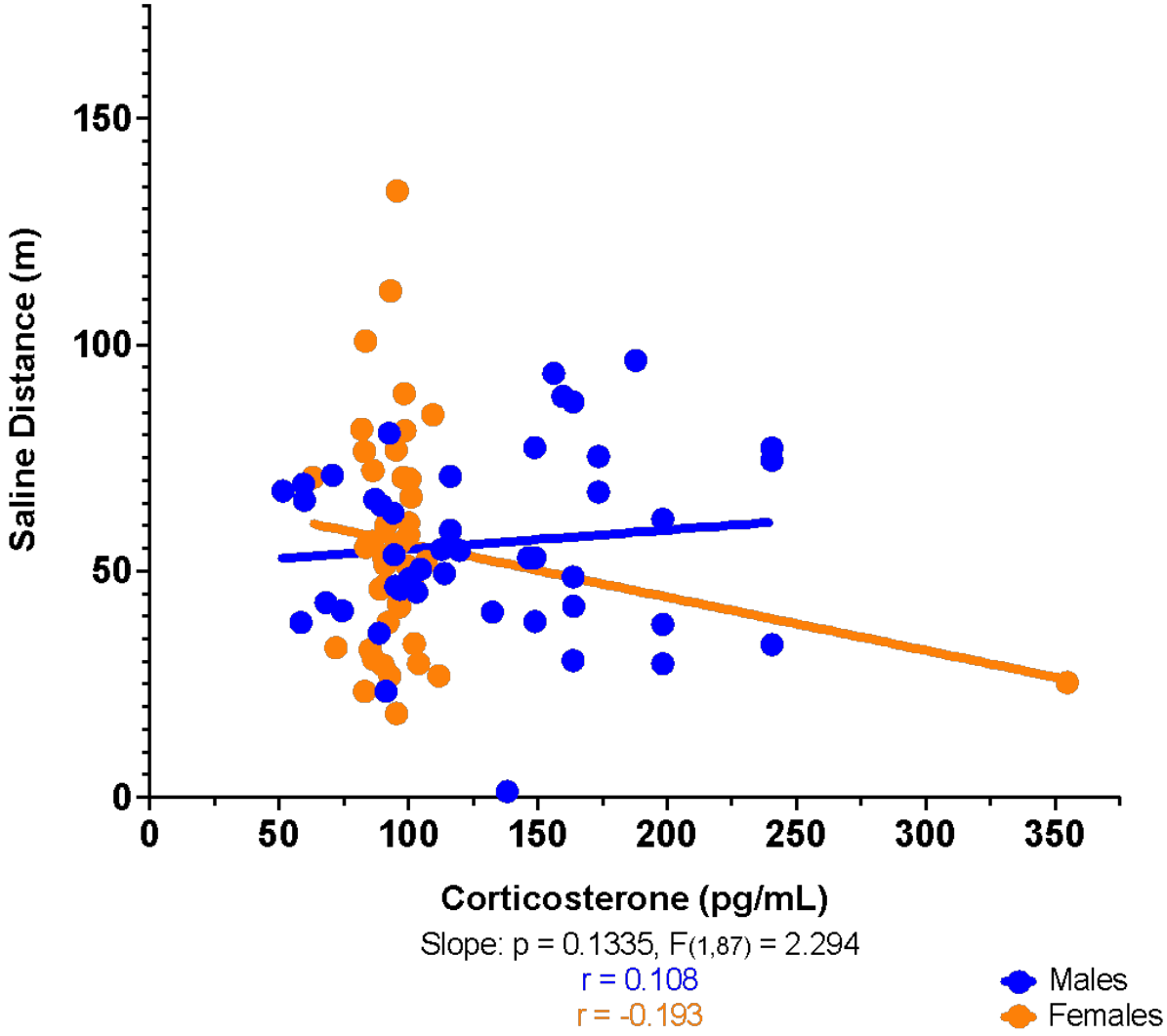

Figure 27: Correlation between blood corticosterone levels and saline distance. There was no correlation between blood corticosterone levels and the distance traveled during saline conditioning. The difference between the slopes was not significant.

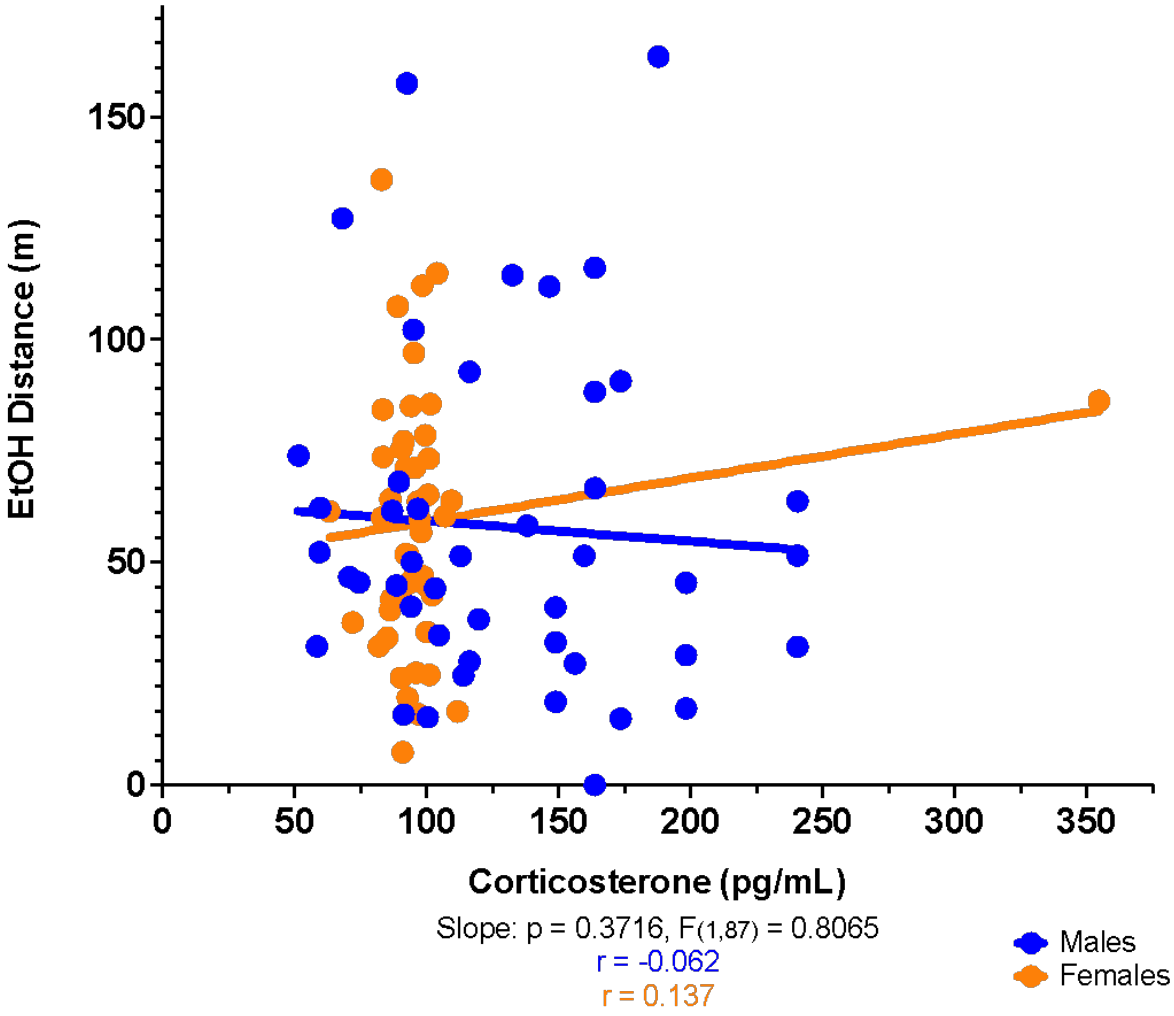

Figure 28: Correlation between blood corticosterone levels and EtOH distance. There was no correlation between blood corticosterone levels and the distance traveled during EtOH conditioning. The difference between the slopes was not significant.

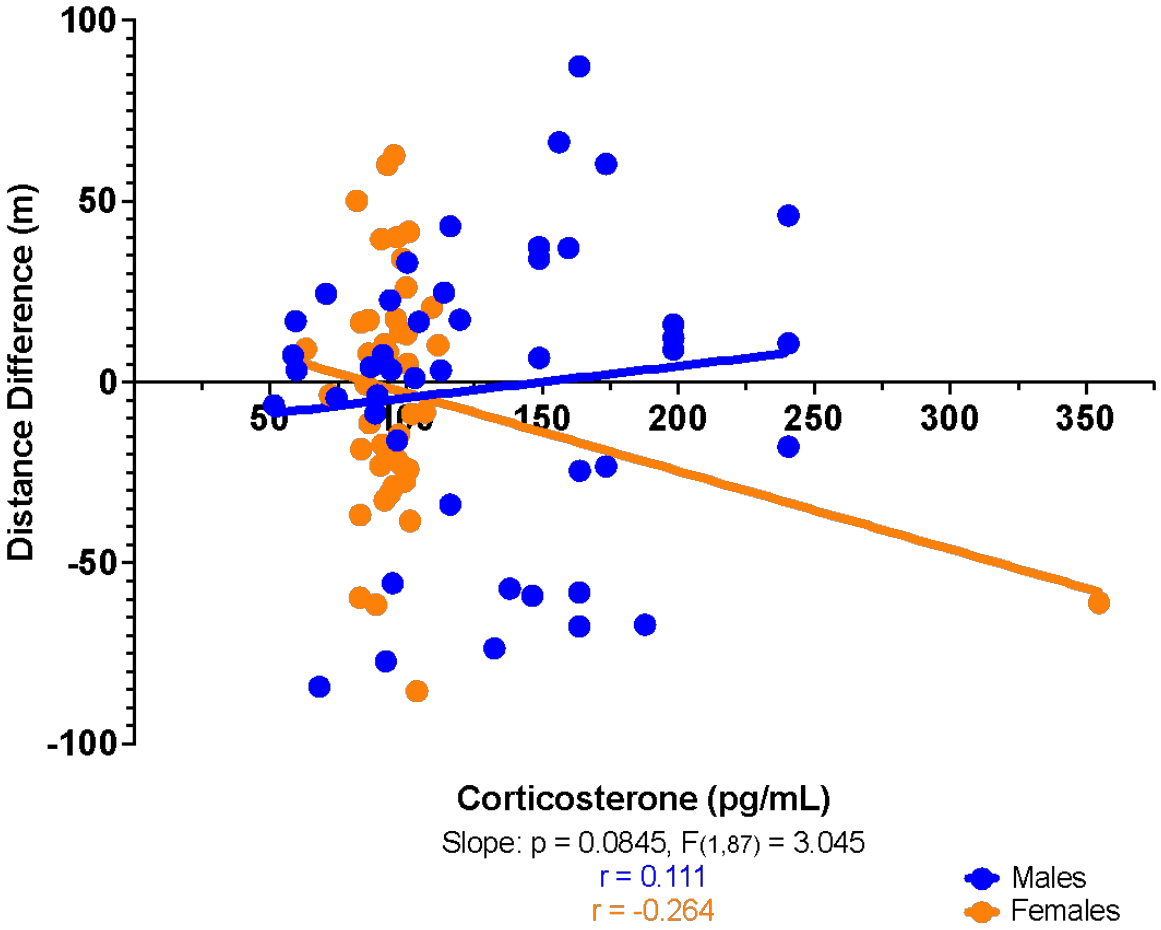

Figure 29: Correlation between blood corticosterone levels and distance difference. There was no correlation between blood corticosterone levels and the distance difference. The difference between the slopes was not quite significant.

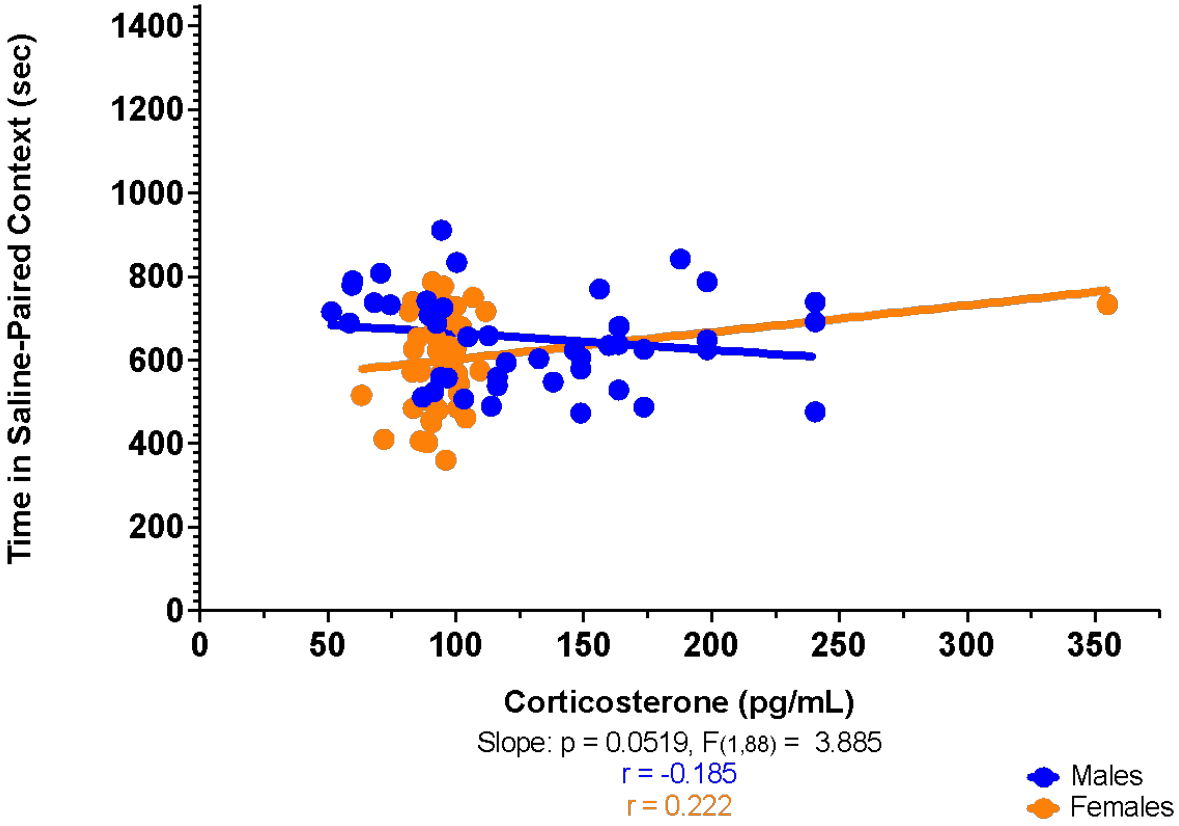

Figure 30: Correlation between blood corticosterone levels and saline time. There was no correlation between blood corticosterone levels and the amount of time spent in the saline-paired context. The difference between the slopes was not significant.

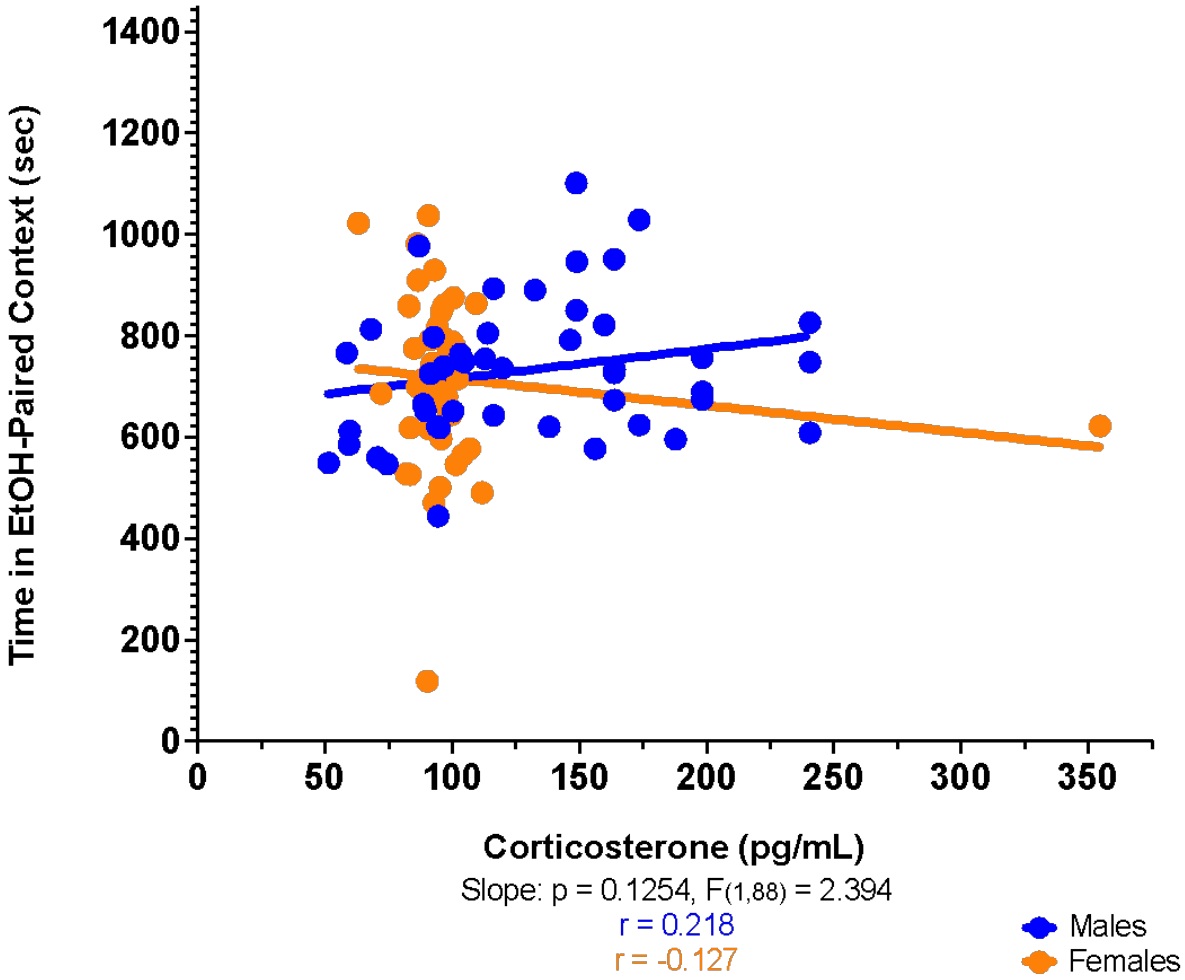

Figure 31: Correlation between blood corticosterone levels and EtOH time. There was no correlation between blood corticosterone levels and the amount of time spent in the EtOH-paired context. The difference between the slopes was not significant.

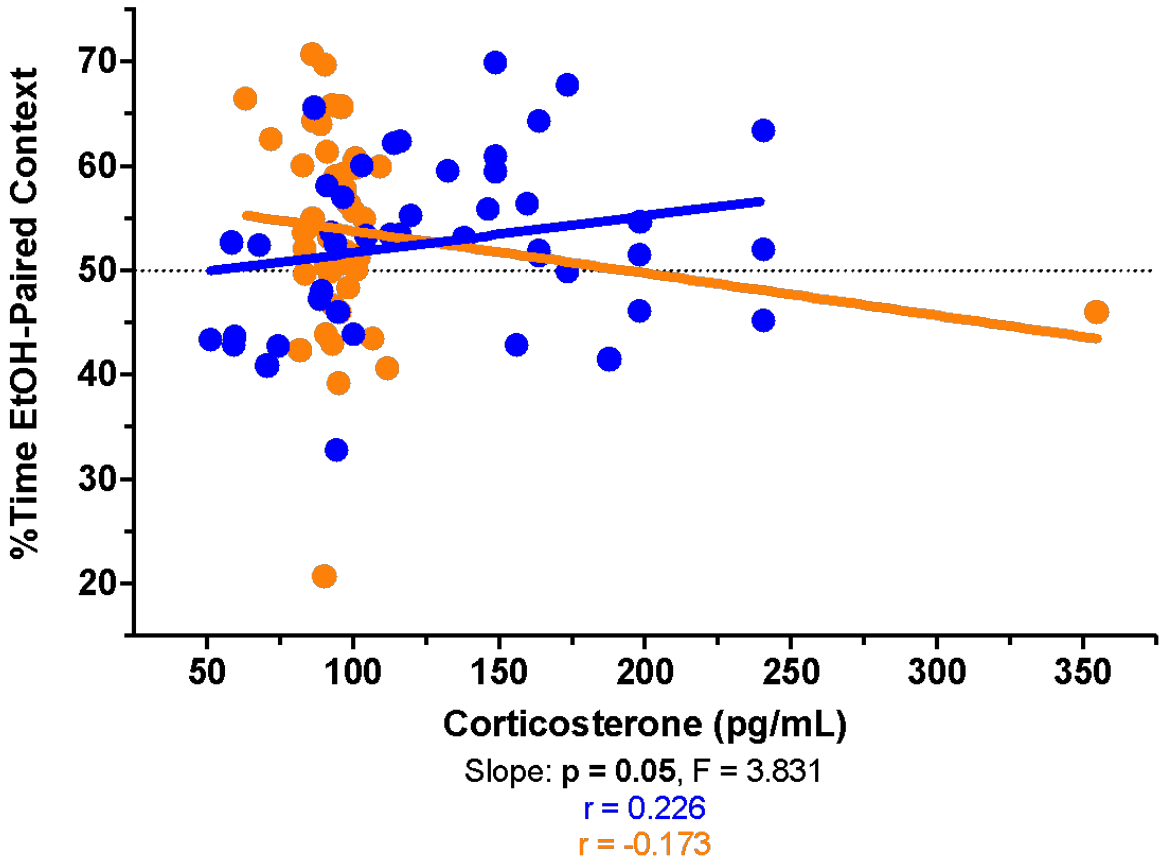

Figure 32: Correlation between blood corticosterone levels and SE-CPP. There was no significant correlation between blood corticosterone levels and the percentage of time spent in the EtOH-paired context. The difference between the slopes was not significant.