ANALYZING SEX DIFFERENCES IN ALCOHOL CONSUMPTION USING OVARIECTOMIZED FOUR CORE GENOTYPES MICE

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A Thesis

Presented to the Faculty of Miami University in partial
fulfillment of the requirements
for the degree of

Masters of Arts

Department of Psychology

The Graduate School
Miami University
Oxford, Ohio

2024

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ABSTRACT

Alcohol use disorders (AUDs) are a prevalent and significant public health issue, with hundreds of thousands of people dying from alcohol-related deaths across the United States of America each year. Interestingly, there are sex differences that exist in both human and rodent models of AUD. These sex differences are not well understood, and the influences of sex chromosomes and/or hormones are just beginning to be studied. This project used ovariectomized (OVX) Four Core Genotypes (FCG) mice in a limited access, drinking in the dark (DID) paradigm to assess the separate influences of sex chromosomes and hormones on binge drinking. Mice successfully escalated consumption throughout the paradigm, and decreased consumption and preference in the presence of aversion-resistant quinine, however, there were no significant differences found across sex chromosome (XX vs. XY) or treatment (OVX vs. sham) conditions. The findings from this study may suggest that FCG mice, uniquely, do not differ in EtOH consumption following the surgical removal of ovaries. Additional research needs to be completed in order to better understand why this effect was not found.

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Dedication

I dedicate this project to my mother and her continuous and unwavering support for me throughout my academic career and life. I also dedicate this project to my other family members and friends who keep me going with their positivity.

Acknowledgements

This study was supported by the College of Arts and Sciences, the Department of Psychology, and the Graduate School at Miami University. I would like to thank undergraduate research assistants Delainey Lewis and Yaya Gao for assisting me with the behavioral tests for this study, as well as those that assisted me with ovariectomy surgeries and wound clip removals. I would also like to thank the Laboratory Animal Resources staff for their commitment and diligence to ensuring the health and safety of all animal subjects, particularly Lindy Abrams and her advice with the recovery of mice post-surgery. I also thank my advisor, Dr. Anna Radke, for providing the mentoring, feedback, and training that was necessary to conduct this study and write this thesis. Her guidance was crucial to successfully carrying out this project to its completion. Finally, I would like to thank my committee members Drs. Jennifer Quinn and Haifei Shi for their critique, suggestions, and guidance.

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Introduction

Alcohol use disorders (AUDs) are a persistent issue across the United States and have been for quite some time. In fact, according to the 2016 (US) National Survey on Drug Use and Health, 6.6% of adults in the US reported heavy alcohol use and 26% reported one or more episode of binge drinking in the past month (Center for Behavioral Health Statistics and Quality, 2017). Furthermore, the Centers for Disease Control and Prevention reported about 178,000 alcohol-attributed deaths from 2020-2021 (CDC, 2024).

Sex differences in alcohol drinking: More recently, studies have been conducted to evaluate the differences in alcohol consumption and drinking behaviors between males and females. In general, males have higher rates of AUD than females (Keyes et al., 2008). However, in recent years, females have been closing the gap on men with relative increases in alcohol-related injuries and deaths (White, 2020). Rates of AUD have also increased in females in the last 15 years. A study by Grant et al. (2017) reported an 84% increase in prevalence rates of AUD from 2012-2013, with only a 35% increase for males. Furthermore, the same study pointed to a 58% increase in female high-risk drinking from 2012-2013, but only 16% for males (Grant et al., 2017).

Conversely, preclinical studies typically show that female rodents consume more ethanol than males and are quicker to acquire this self-administration behavior (Becker & Koob, 2016; Radke et al., 2021). In intermittent access (IA) paradigms, female C57BL/6J mice have been shown to consumer higher levels of 20% ethanol than males (Hwa et al., 2011). This finding has also been shown using C57BL/6J mice in a Drinking in the Dark (DID) experiment, where female mice were shown to consume more 10% ethanol than males during the first hour of their active (dark) cycle (Middaugh et al., 1999). Female WSC-1 mice have been shown to consume more 6% ethanol than males in continuous access paradigms across age ranges (Tambour et al., 2009). These findings are also generally seen with rat studies, where female rats tend to consume more (g/kg) and maintain higher preferences for ethanol over time (Almeida et al., 1998, Juárez & De Tomasi, 1999). These differences in rodents suggest a biological vulnerability to alcohol consumption that is driven by sex.

Gonadal vs. chromosomal sex: Biological sex influences physiology and behavior through two primary mechanisms: gonadal hormones and genes located on the sex chromosomes. In typically developing mammals, XX are gonadally females and XY are gonadally males. This is because the Y chromosome contains the *Sry* gene that encodes for the development of testes (National Center for Biotechnology Information, 1998). Since female mice do not typically have a Y chromosome, ovaries develop. Many sex differences in behavior can be attributed to hormones that are released from the gonads. For example, organizational effects of hormones alter the way the brain develops and are crucial to developing animals. Activational effects, on the other hand,

occur throughout life such as regular hormone cycling-related behaviors (Arnold & Breedlove, 1985). For females, estrogen and progesterone are the main hormones cycled. For males, testosterone is the main hormone (Hirschenhauser et al., 2002), however, female hormone involvement will be the main focus of this paper. It is important to mention that although these hormones regularly cycle, this cycling does not increase variability of behaviors in female rodents (Kaluve & Graham, 2022). These hormones can alter behavior through their actions, both direct and indirect, on receptors throughout the brain (Almey et al., 2015). For example, membrane-associated estrogen receptors (ERs) are found in regions important in learning, memory, and motivation, including the prefrontal cortex, hippocampus, and nucleus accumbens (Almey et al., 2015). Furthermore, there is evidence showing that estrogen can affect dopamine-dependent processes, including alcohol consumption (Satta et al., 2018).

There are other biological differences between males and females that are not affected by hormonal influences. These effects of the sex chromosomes are referred to as "direct genetic effects" (Tuck et al., 2011). Although it is still a relatively new area, there have been several genes identified on the sex chromosomes that are expressed differently in males and females. These sex-linked genes can even escape X-chromosome inactivation (XCI), meaning that females (XX) can have an extra copy of a gene when compared to males (XY) (Berletch et al., 2011). X-inactivation is necessary so that chromosomal females (XX) express a comparable number of genes to chromosomal males. This is especially important considering the X chromosome contains somewhere around 1000 protein-coding genes, whereas the Y chromosome contains less than 80 (Ross et al., 2005, Rhie et al., 2023). Without XCI, females would be much more vulnerable to genetic defects and autoimmune diseases (Sun & Wang, 2022).

XCI happens in every stem cell early on in mammalian development (blastocyst stage) (Schulz & Heard, 2013). Each of these cells will randomly (50/50) choose one of the X-chromosomes to inactivate. From here, all daughter-cells that are replicated will contain the chosen X-chromosome. The process of XCI is regulated by the gene X inactive specific transcript (*Xist*), which is located in the X inactivation center (XIC) region of the X chromosome undergoing inactivation (Schulz & Heard, 2013). In human females, an estimated 15%-30% of genes can escape this X-inactivation, referred to as bi-allelic expression, whereas that number is closer to 3% for XX mice (Berletch et al., 2011, Sun & Wang, 2022). The fact that genes can escape X-inactivation likely means that females have sex-specific differences, genetically encoded, that may affect alcohol consuming behaviors.

Mechanisms underlying sex differences in alcohol consumption: This begs the question, is it the chromosomes, the hormones, or both, that are causing the discrepancies in alcohol consumption rates between sexes? One useful model for attempting to unveil these sex differences is the Four Core Genotypes (FCG) mouse model. In FCG males, the *Sry* gene is no longer tied to the Y chromosome (Arnold & Chen, 2009). Here, C57BL/6J female mice can be bred with XY/*Sry*+ (XY mice with testes) to produce different offspring. This mouse model produces four different types of mice: XX/*Sry*-, XX/*Sry*+, XY/*Sry*-, XY/*Sry*+. Previous studies using this mouse line have shown clear differences in drinking habits, with chromosomal complement being the

determining factor (Barker et al., 2010). Other studies show just how complex the interactions between chromosomes and gonadal hormones really are, showing that male mice with testes (XY/*Sry*+) consume more 15% ethanol in a drinking in the dark (DID) paradigm than XX mice with testes (*Sry*+), while XY mice had a higher preference for ethanol (than water) regardless of gonadal type (Sneddon et al., 2023b). However, other paradigms, such as continuous access, show that these effects may be dependent on the drinking task and the concentration of ethanol given (Sneddon et al., 2022). Gonadal influences on alcohol drinking have also been uncovered using FCG mice. For example, Sneddon et al. (2022) found that *Sry*- FCG mice consumed significantly more Ethanol than *Sry*+ mice in 24-hour sessions. However, Sneddon et al. (2023b) found that only mice with testes (*Sry*+) were influenced by sex chromosomes, with no differences in limited-access EtOH consumption seen between XX/*Sry*- and XY/*Sry*- FCG mice.

Another method used to assess the mechanisms underlying sex differences in drinking behaviors is direct manipulation of gonadal hormones. Surgical removal of ovaries, called ovariectomies (OVX), can be completed in order to remove the (activational) effects of gonadal hormones from the equation. This procedure removes any activational effects from the mice once the hormones are flushed, but importantly does not remove hormonal influences that occurred during development (organizational). Furthermore, there are also some hormones in fat cells that can compensate for gonadal hormone removal by upregulating hormone release (Nelson & Bulun, 2001, Hetemäki et al., 2021). Following ovariectomies, mice can be presented with ethanol in various drinking paradigms in order to assess consumption and preference, which may provide insight into mechanisms involved in the development of AUD.

Ovariectomies have similar effects on alcohol drinking independent of the paradigm employed. For example, Sneddon et al. (2023a) found that C57BL/6J mice that have been ovariectomized consume less ethanol in a DID paradigm than mice that have had sham surgeries. Interestingly, the same study showed that removing the ovaries increased preference for ethanol, hinting at some complex influences on drinking behaviors (Sneddon et al., 2023a). A study by Ford et al., 2002, found that female Long-Evans rats that were ovariectomized consumed significantly less ethanol in a continuous access paradigm compared to sham rats and OVX rats that received replacement estradiol. A study by Satta et al. (2018) also showed that OVX C57BL/6J mice consumed significantly less ethanol than gonadally-intact females in a DID paradigm. Furthermore, when OVX mice were given estradiol benzoate, their drinking levels significantly increased when compared to vehicle controls (Satta et al., 2018). Together, these results suggest that the removal of hormonal effects on female mice significantly reduce their ethanol consumption. While there is some research demonstrating the effects of ovariectomies on certain experimental outcomes using FCG mice (Manwani et al., 2015), this approach has yet to be implemented with a drinking paradigm.

A review paper by Finn, 2020, cited papers showing gonadectomies (surgical removal of gonads-ovaries or testes) having different effects on drinking depending on the sex of the animal. They pointed towards self-administration paradigms where OVX females decreased alcohol self-administration, however, orchiectomized (removal of testes) males tended to increase their self-administration of alcohol (Finn, 2020). This finding provides evidence that gonadectomies might

shift the rodent to drink more similarly to the opposite sex (i.e., females consume less, males consume more) in various paradigms (Finn, 2020).

The drinking paradigm that will be used for this study is the DID paradigm. This model of addiction was originally created by Justin Rhodes and colleagues as a way of recreating binge levels of drinking in mice (Rhodes et al., 2005). For this experiment, we used a modified version of the DID procedure previously described by Sneddon et al. (2023a), in which water and 15% ethanol (EtOH) are presented three hours into the active cycle for four hours. Presenting EtOH for a limited period of time during the active cycle produces binge-like drinking behaviors (Thiele & Navarro, 2014). Binge drinking is defined as a pattern of drinking that results in a blood alcohol concentration (BAC) of at least 0.08%. Blood EtOH concentrations (BECs) can be measured using an Analox machine to confirm binge-levels of drinking. Therefore, if the Analox machine returns BECs equivalent to .08% (80 mg/dL), we can assume that the mice drank to intoxicating levels.

Using the DID task, quinine (a bitterant) can be added to EtOH to assess aversion-resistance in mice (Hopf & Lesscher, 2014; Lei et al., 2016; Lesscher et al., 2010, Sneddon et al., 2018). This is a good way of modeling the human behavior of drinking despite negative consequences, which is a core feature of AUD according to DSM-5 criteria. Previous studies on C57BL/6J mice showed that ovariectomies caused a reduction in EtOH consumption and an increase in aversion-resistance (Sneddon et al., 2023a). Sex chromosome complement also appears to play a role in aversion resistant drinking. For example, the previously mentioned Sneddon et al. (2023b) paper showed that XY mice with ovaries displayed complete resistance to aversive quinine, not decreasing consumption at any level added, while their XX counterparts decreased consumption inversely to concentration, as expected. However, a study by Bauer et al. (2021) showed a sex difference in consumption, but not in aversion-resistant drinking. These results seem to suggest that these sex differences in aversion-resistance may only appear following OVX and are not seen through gonadally-intact drinking.

Hypothesis/Objectives: This study aims to determine the effects of ovariectomies on EtOH consumption and aversion resistance in FCG gonadal female mice. More specifically, we hope to determine the role that hormones/sex chromosomes play in EtOH consumption by completing ovariectomies in FCG mice and running them through a DID paradigm. We hypothesize that FCG gonadal female mice require both X chromosomes as well as gonadal hormones (XX/Sry-, sham) in order to retain aversion to quinine (decrease consumption as quinine concentration increases).

Methods

Subjects: 54 gonadal female (*Sry*-) XX and XY FCG mice were selected for this experiment, with an almost-even split (26 and 28 respectively) of XX and XY. These mice were generated from breeding pairs originally obtained from Dr. Art Arnold at UCLA and have since been bred in-house. Male (XY/*Sry*+) FCG mice were paired with C57BL/6J females in harems for breeding. FCG mice (26 XX/*Sry*-, 28 XY/*Sry*-) had either ovariectomies (OVX) or sham

surgeries completed, resulting in 4 groups of mice (12 XX/*Sry*- OVX, 14 XX/*Sry*- sham, 15 XY/*Sry*- OVX, 13 XY/*Sry*- sham). Mice were ran in three separate cohorts consisting of 16 mice, 20 mice, and 18 mice.

Adult mice were housed in a temperature-controlled room with a 12/12-hour reverse light/dark cycle (i.e., lights on at 7PM). Mice were weaned in social housing (2-3 litter-mates) in a standard shoe box rectangular plastic mouse cage (18.4 × 29.2 × 12.7 cm) with 5.08 × 5.08 cm nestlets and Bed-O-Cobb 0.634 cm bedding (Cincinnati Lab Supply, Cincinnati, OH). Mice were all transferred to individual cages before their surgeries in order to supply ibuprofen in their drinking bottles. All mice received standard care and had access to Rodent Diet 5001 chow (Cincinnati Lap Supply, Cincinnati, OH) and reverse-osmosis (RO) drinking water ad libitum. All mice were cared for in agreement with the guidelines set by the National Institutes of Health and all procedures were approved by the Institutional Animal Care Use Committee (IACUC) at Miami University.

Ovariectomies: Mice received 100 mg ibuprofen in their water approximately 24 hours before surgeries. Mice in the ovariectomy and sham groups were put under isoflurane anesthesia. Mice were shaven and then transferred to a stereotaxic instrument to free the surgeon's hands. Mice were cleaned (three times) with betadine and alcohol, applied to the surgery area. A small incision (generally less than 1 mm) was then made slightly temporal of the spine and below the ribs to allow access to the muscle beneath. Next, muscle layers were carefully cut through in order to access ovaries. The ovaries were located and clamped with forceps and removed using a scalpel. This same procedure was repeated on the other side (always right then left). Sham surgeries involved the same process, minus the locating and removal of the ovaries, in order to not accidentally twist or otherwise disturb the ovaries and subsequent hormonal effects. After ovaries were removed (or after the incision was made for sham animals), the muscle layer was stitched back together (usually 2-3 stiches per side), and then the skin was closed with wound clips. Mice were under anesthesia for around 30 minutes. Mice were allowed to recover on a heated pad and were monitored until they were active. Mice were weighed daily for five days following surgery and had their surgery sites monitored. All mice had 100mg ibuprofen in their waters until the end of this monitoring stage. If wound clips (or stitches) were removed by the mice and the wound reopened, they were placed back under anesthesia, cleaned, and new clips (and/or stitches) were applied. After 2 weeks, all wound clips were removed, under light/quick anesthesia (typically <5 minutes).

Drinking solutions: Drinking solutions were prepared fresh every morning before going on the cages. 15% EtOH solutions were created by mixing 100% EtOH with reverse-osmosis water to reach the desired concentration (e.g., 60 mL EtOH + 340 mL H2O). During quinine weeks, various concentrations (100, 250, 500 µM) of quinine hemisulfate salt (Q1250-50G, Millipore-Sigma, St. Louis, MO) in 15% EtOH (or water during control weeks) were prepared. Quinine concentrations were calculated weight per volume (e.g., 100 µM = 19.6 mg/500 mL).

Experiment: 7-Week 4-hour Drinking in the Dark: Following four weeks of recovery, to allow sufficient time to flush hormones, mice were moved to a room with a reverse light cycle (dark room). Mice were allowed one week to acclimate to this new environment before the experiment

commenced (5 total weeks between surgery and experiment start). On day 1 of the experiment, mice were given EtOH in a two-bottle choice, limited access DID paradigm. On drinking sessions, mice received 15% EtOH and water. The side bottles were presented on was alternated each day to prevent possible side-preferences. Dummy bottles were also prepared to account for fluid loss and were placed on the same rack as the mice. Bottles were available for 4 hours, with consumption calculated from bottle weights taken immediately before and after the session, subtracting out dummy bottle loss. Drinking sessions occurred five days (M-F) per week for three weeks. On the final session, mice were allowed to drink for 30 min to assess frontloading and blood was collected so BECs could be measured. The fourth week consisted of increasing levels of quinine (0, 100, 250, 500 µM) added to the EtOH as a measure of aversion resistance. An additional 30-minute measure was added to this week to assess frontloading during aversion-resistant drinking. The mice then had a two-week break, followed by one final week of water/water-quinine control (Experimental Timeline, **Figure 1**). This control was done to assure that the levels of consumption during the aversion-resistance week were due to the presence of EtOH, and not just the addition of quinine.

Blood EtOH concentration analysis: Blood was taken using a tail-bleeding method with capillary tubes and was then transferred to microcentrifuge tubes. Blood was centrifuged to separate plasma from red blood cells, and then plasma was pipetted off the top for BEC analysis. Blood will be analyzed for BEC using an Analox machine at a later date.

Statistical Analysis: Consumption accounting for body weight (g/kg), and preference were used as dependent measures of EtOH intake. Data were expressed as mean ± standard error of the mean and analyzed using a 3-way repeated-measures analysis of variance (RM ANOVA) with session or concentration (for quinine days) as the within-subject factor and chromosome and treatment (OVX or Sham) as the between-subject factors. The alpha level was set at $p < 0.05$. *Post hoc* Dunnett's tests were performed, as appropriate. Analyses were completed using GraphPad Prism 10.2.0 (La Jolla, CA).

Results

Consumption: Consumption of alcohol escalated over sessions but was not affected by sex chromosome complement or ovariectomy. A three-way repeated-measures ANOVA revealed a main effect of session ($F_{(8.662, 433.1)} = 16.74$, $p < 0.0001$) (**Figure 2**) on EtOH consumption (g/kg). There were no significant effects of treatment ($F_{(1, 50)} = 0.09380$, $p = 0.7607$) or chromosome ($F_{(1, 50)} = 0.6348$, $p = 0.4294$) and no significant interactions (**Table 1**).

Preference: Preference of alcohol escalated over sessions but was not affected by sex chromosome complement or ovariectomy. A three-way repeated-measures ANOVA revealed a main effect of session ($F_{(7.815, 390.8)} = 10.64$, $p < 0.0001$) (**Figure 3**) on EtOH preference. There were no significant effects of treatment ($F_{(1, 50)} = 0.3427$, $p = 0.5609$) or chromosome ($F_{(1, 50)} = 0.01167$, $p = 0.9144$) and no significant interactions (**Table 1**).

Water Consumption: Consumption of water decreased over sessions but was not affected by sex chromosome complement or ovariectomy. A three-way repeated-measures ANOVA revealed a main effect of session ($F_{(13, 650)} = 5.372$, $p < 0.0001$) (**Figure 4**) on water consumption. There

were no significant effects of treatment ($F_{(1, 50)} = 0.6683$, $p = 0.4175$) or chromosome ($F_{(1, 50)} = 0.5826$, $p = 0.4489$) and no significant interactions (**Table 1**).

Quinine Consumption: Consumption of alcohol over 4 hours decreased as quinine concentrations increased but was not affected by sex chromosome complement or ovariectomy. A three-way repeated-measures ANOVA revealed a main effect of concentration ($F_{(3, 150)} = 21.45$, $p < 0.0001$) (**Figure 5**) on 4 h EtOH + quinine consumption. There were no significant effects of treatment ($F_{(1, 50)} = 0.8482$, $p = 0.3615$) or chromosome ($F_{(1, 50)} = 0.5468$, $p = 0.4631$) and no significant interactions (**Table 1**).

Follow-up two-way ANOVAs with Dunnett's tests were run to assess aversion resistance in each group. A Dunnett's test found that XX mice in the OVX group consumed significantly less 15% EtOH (vs 0 µM) over 4 hours when 250 µM ($p = 0.0015$) and 500 µM ($p = 0.0017$) quinine was added to the solution, but not at 100 µM. A second Dunnett's test found that XX mice in the sham group consumed significantly less 15% EtOH (vs 0 µM) over 4 hours when 250 µM ($p = 0.0155$) and 500 µM ($p = 0.0035$) quinine was added to the solution, but not at 100 µM. A Dunnett's test found that XY mice in the OVX group consumed significantly less 15% EtOH (vs 0 µM) over 4 hours when 250 µM ($p = 0.0042$) and 500 µM ($p = 0.0473$) quinine was added to the solution, but not at 100 µM. A Dunnett's test found that XY mice in the sham group consumed significantly less 15% EtOH (vs 0 µM) over 4 hours when 250 µM ($p = 0.0040$) and 500 µM ($p = 0.0108$) quinine was added to the solution, but not at 100 µM (**Table 2**).

Consumption of alcohol over 30 minutes decreased as quinine concentrations increased but was not affected by sex chromosome complement or ovariectomy. A three-way repeated-measures ANOVA revealed a main effect of concentration ($F_{(2.820, 141.0)} = 4.272$, $p = 0.0075$) (**Figure 6**) on 30 m EtOH + quinine consumption. There were no significant effects of treatment ($F_{(1, 50)} = 0.05955$, $p = 0.8082$) or chromosome ($F_{(1, 50)} = 0.1765$, $p = 0.6762$) and no significant interactions (**Table 1**).

Follow-up two-way ANOVAs with Dunnett's tests were run to assess aversion resistance in each group. One test found that XX mice in the OVX and sham groups did not reduce consumption of 15% EtOH over 30 minutes when any concentration (100, 250, 500 µM) of quinine was added to the solution. A second Dunnett's test found that XY mice in the OVX group consumed significantly less 15% EtOH (vs 0 µM) over 30 minutes when 100 µM ($p = 0.0314$) and 250 µM ($p = 0.0322$) quinine was added to the solution, but not at 500 µM. A Dunnett's test found that XY mice in the sham group did not reduce consumption of 15% EtOH over 30 minutes when any concentration (100, 250, 500 µM) of quinine was added to the solution (**Table 2**).

Quinine Preference: Preference for alcohol over 4 hours decreased as quinine concentrations increased but was not affected by sex chromosome complement or ovariectomy. A three-way repeated-measures ANOVA revealed a main effect of concentration ($F_{(2.438, 121.9)} = 7.865$, $p = 0.0002$) (**Figure 7**) and an interaction of chromosome x treatment on 4h EtOH + quinine preference ($F_{(1, 50)} = 4.908$, $p = 0.0313$). There were no significant effects of treatment ($F_{(1, 50)} = 0.07139$, $p = 0.7904$) or chromosome ($F_{(1, 50)} = 0.02935$, $p = 0.8647$) and no other significant interactions (**Table 1**).

Follow-up two-way ANOVAs with Dunnett's tests were run to assess aversion resistance in each group. A Dunnett's test found that XX mice in the OVX group did not reduce their preference for 15% EtOH (vs 0 µM) over 4 hours when any concentration (100, 250, 500 µM) of quinine was added to the solution. A Dunnett's test found that XX mice in the sham group reduced their preference for 15% EtOH (vs 0 µM) when 500 µM ($p = 0.0044$) quinine was added to the solution, but not for 100 or 250 µM. A Dunnett's test found that XY mice in the OVX and sham groups did not reduce their preference for 15% EtOH when any concentration (100, 250, 500 µM) of quinine was added to the solution (**Table 2**).

Preference for alcohol over 30 minutes decreased as quinine concentrations increased but was not affected by sex chromosome complement or ovariectomy. A three-way repeated-measures ANOVA revealed a main effect of concentration ($F_{(2.358, 117.9)} = 4.735$, $p = 0.0072$) (**Figure 8**). There were no significant effects of treatment ($F_{(1, 50)} = 1.066$, $p = 0.3068$) or chromosome ($F_{(1, 50)} = 0.006319$, $p = 0.9370$) and no significant interactions (**Table 1**).

Follow-up two-way ANOVAs with Dunnett's tests were run to assess aversion resistance in each group. These tests found that mice in all groups (XX/OVX, XX/Sham, XY/OVX, XY/Sham) did not reduce their preference for 15% EtOH (vs 0 µM) over 30 minutes when any concentration (100, 250, 500 µM) of quinine was added to the solution (**Table 2**).

Water Quinine Consumption: Consumption of water over 4 hours decreased as quinine concentrations increased but was not affected by sex chromosome complement or ovariectomy. A three-way repeated-measures ANOVA revealed a main effect of concentration ($F_{(2.039, 102.0)} = 21.20$, $p < 0.0001$) (**Figure 9**) on 4 h water + quinine consumption. There were no significant effects of treatment ($F_{(1, 50)} = 0.5335$, $p = 0.4685$) or chromosome ($F_{(1, 50)} = 0.2050$, $p = 0.6527$) and no significant interactions (**Table 1**).

Follow-up two-way ANOVAs with Dunnett's tests were run to assess aversion resistance in each group. A Dunnett's test found that XX mice in the OVX group consumed significantly less water (vs 0 µM) over 4 hours when 500 µM ($p = 0.0101$) quinine was added to the solution, but not at 100 or 250 µM. A second Dunnett's test found that XX mice in the sham group consumed significantly less water (vs 0 µM) over 4 hours when 500 µM ($p = 0.0297$) quinine was added to the solution, but not at 100 or 250 µM. A Dunnett's test found that XY mice in the OVX group consumed significantly less water (vs 0 µM) over 4 hours when 500 µM ($p = 0.0006$) quinine was added to the solution, but not at 100 OR 250 µM. A Dunnett's test found that XY mice in the sham group consumed significantly less 15% EtOH (vs 0 µM) over 4 hours when 500 µM ($p = 0.0162$) quinine was added to the solution, but not at 100 or 250 µM (**Table 2**).

Water Quinine Preference: Preference for water over 4 hours decreased as quinine concentrations increased but was not affected by sex chromosome complement or ovariectomy. A three-way repeated-measures ANOVA revealed a main effect of concentration ($F_{(2.727, 136.4)} = 3.376$, $p = 0.0239$) (**Figure 10**). There were no significant effects of treatment ($F_{(1, 50)} = 0.06349$, $p = 0.8021$) or chromosome ($F_{(1, 50)} = 2.876$, $p = 0.0961$) and no significant interactions (**Table 1**).

Follow-up two-way ANOVAs with Dunnett's tests were run to assess aversion resistance in each group. Dunnett's tests found that mice in all groups (XX/OVX, XX/Sham, XY/OVX, XY/Sham)

did not reduce their preference for water (vs 0 µM) over 4 hours when any concentration (100, 250, 500 µM) of quinine was added to the solution (**Table 2**).

Discussion

The main finding of this study is null. All mice drank at expected levels and escalated over sessions, however, there was no effect of chromosome or treatment (OVX) on consumption (**Figure 2**) or preference (**Figure 3**). These results fail to replicate results from Sneddon et al., 2023a, where it was shown that OVX reduced EtOH consumption compared to sham surgeries. It is important to note, however, that while OVX has been shown to decrease alcohol consumption across multiple studies, it has only been shown once before using the current experimental conditions. Additionally, the effect of treatment (OVX) found by Sneddon et al. (2023a) is comparatively less robust than similar OVX studies. We also failed to replicate findings of OVX increasing EtOH preference and reducing water consumption, which were also reported in Sneddon et al. (2023a). These results also fail to replicate findings by Satta et al., 2018, where ovariectomized C57BL/6J mice consumed significantly less 20% EtOH than gonadally-intact female mice. These differences in findings can, perhaps, be attributed to the different mouse lines being used. Both Sneddon et al. (2023a) and Satta et al. (2018) used C57BL/6J mice for their OVX experiments. However, FCG XY/Sry+ mice are bred with female C57BL/6J mice, so their genetic backgrounds are very similar.

In terms of aversion-resistant drinking, the results were also null. Mice decreased consumption at expected concentrations, but there were no effects of treatment or chromosome (**Figure 5**). This suggests that these factors are not involved in aversion-resistant drinking, which is not in line with prior studies completed by Sneddon et al., 2023a and b. As mentioned previously, Sneddon et al, 2023b, found that XY mice with ovaries had complete resistance to the aversive effects of quinine (i.e., they did not decrease consumption at any level). These results were not replicated in the OVX or sham group. Perhaps the stress of surgeries may have modified drinking behaviors previously found in FCG mice. It is important to mention, however, that there were no sex differences seen in aversion-resistant DID drinking by Sneddon et al. (2018), so this particular study's finding was replicated by our findings. Perhaps the DID task is not the best method for examining hormonal vs. chromosomal influences on aversion-resistant drinking.

One interesting finding is the trending main effect of treatment on XY consumption during quinine sessions (**Figure 5**). A 2-way ANOVA was run comparing XY/OVX and XY/Sham mice and found $p = 0.0643$. This non-significant trend shows ovariectomized XY mice tend to consume less EtOH than sham counterparts. This result is in line with previous findings by Satta et al. (2018) where the functioning of estrogen was related to escalated consumption. However, it is important to note that these mice, being XY, do not have the typical chromosomal complement for their gonads (compared to XX/*Sry*- FCG mice). It is therefore surprising that XY mice have this trend, but it is not seen in XX mice. It is also important to note that this finding was during quinine week, whereas Satta et al. (2018) had findings from general consumption. This result does not replicate previous findings reported by Finn, 2020, where XY mice that had gonadectomies increased alcohol self-administration. However, it is relevant to note that the

mice reported on by Finn (2020) had orchiectomies (testes removed), whereas here the XY mice had ovaries removed.

There was also an interaction between chromosome and treatment for preference during the 4-hr EtOH + quinine sessions (**Figure 7**). This chromosome x treatment interaction suggests that OVX might increase preference in XX mice and decrease in XY. These results are consistent with previous findings by Sneddon et al. (2023a), where OVX increased EtOH preference in female C57BL/6J mice (however, this was during alcohol-only sessions).

In order to assess frontloading in aversion-resistant (quinine) drinking, 30-minute measures were taken for the quinine week. This was done in light of findings by Sneddon et al. (2023b) where FCG mice consumed significantly more EtOH in the first 30-minutes of drinking (frontloading) than at 2 hours and 4 hours. However, results from our 30-minute quinine sessions suggest a floor effect that interfered with the ability to assess aversion-resistance at this time point. Peak, unadulterated 30-minute, EtOH consumption (0 µM) was lower in the current study than that reported by Sneddon et al. (2023b) (average of 0.92 g/kg vs. ~1.24 g/kg). As a result, significant decreases in 30 min EtOH consumption in the presence of increasing quinine concentrations were only found in XY OVX mice in this study (**Figure 6**).

An important addition to this study was the quinine sensitivity test, where the same escalating concentrations of quinine as those used to test aversion-resistance for EtOH drinking (100, 250, 500 µM) were added to water. This test showed that FCG mice decrease consumption of water as quinine levels increase (**Figure 9**). This is an excellent control that shows us that any findings from EtOH/quinine week (**Figure 5**), such as the trending main effect of treatment on XY consumption, is likely due to the rewarding effects from EtOH and not effects of hormones or chromosomes on sensitivity to the bitter taste of quinine.

<u>Limitations and Future Directions</u>

There are a few limitations to this set of studies. First off, only ovariectomies were used for gonadectomies. This means that research was only focused on the surgical manipulation of female-associated hormones (estrogen and progesterone). Future research is planned to include orchiectomies in a similar DID paradigm, as work by Finn (2020) has shown that males tend to increase EtOH consumption following orchiectomies.

There were three sets of researchers who were responsible for placing and removing experimental bottles, one of which was male. Some studies have shown that male experimenters may increase stress susceptibility of mice (Georgiou et al., 2022). Since mice were weighed before every drinking session, this could possibly cause higher variation in the results, and may contribute to future replication issues. There are no plans to switch to all-female researchers in the future.

It was recently reported by Art Arnold and colleagues that the FCG Y- chromosome contains a small portion (3.2Mb) of the X chromosome. This report was sent out in December 2023, which was well after the set of experiments here were started. This may complicate findings with the FCG mouse line, as the differences between FCG XX and XY mice may not be as comparable to

chromosomal effects of other lines such as C57BL/6J. Furthermore, there is a possibility that FCG XY mice could have a double dose of some genes that XY mice from other mouse lines wouldn't have. Future studies could use other transgenic mouse lines with sex chromosome manipulation (such as XO).

It is possible that all of the ovaries were not removed from the ovariectomized mice. The ovaries are very small. We are certain that OVX mice had any visible ovaries removed, however, without hormone levels being monitored post-surgery, we are not able to confirm complete hormone eradication. Furthermore, there are still some hormones circulating from other sources (fat cells), as well as the hormonal effects during development (organizational). Given the null findings of this study compared to other OVX studies by Sneddon et al. (2023a) and Satta et al. (2018), it seems likely issues with ovariectomies could be the most plausible answer for failed replication. Future studies should consider taking measures of hormone release/cycling pre- and post-OVX, or perhaps employing other methods to manipulate hormone levels, such as hormone-blockers or perhaps estrogen receptor knockout mice.

Levels of blood ethanol concentrations (BECs) were not measured by the time of writing this. It is possible that differences in BECs could be present even with the null EtOH consumption and preference findings. If found, this could mean that hormones or sex chromosomes are playing a significant role in ethanol metabolism. Sneddon et al. (2023b) did not find any differences in BECs between FCG mice; however, these mice did not receive OVX.

Other future directions include harvesting brains of FCG mice for punches. Using qPCR, these brain punches will be used to analyze levels of gene expression of genes that are likely to escape XCI. For example, *Gprasp1*, which encodes for G-protein-coupled receptor-associated sorting protein (GASP-1) is one of these hypothesized escapees that may be tied to sex differences in alcohol consumption. GASP-1 is implicated in behavioral sensitization to cocaine and other behaviors reliant on the striatum (Boeuf et al. 2009; Mathis et al. 2010). Furthermore, this protein has been shown to regulate a number of G-protein-coupled receptors that are involved in alcohol consumption, such as dopamine type 2 receptors (D2R) (Moser et al., 2010).

Conclusion

Removal of hormonal influence (OVX) in FCG mice did not alter EtOH consumption or preference in a DID paradigm. Quinine-resistant EtOH consumption also did not differ between experimental treatments (OVX or sham). These results suggest that gonadal female (*Sry*-) FCG mice do not require gonadal hormones to consume expected levels of EtOH, although the failure to replicate the known effects of OVX on EtOH consumption is a major caveat to consider. With these results in mind, further studies should investigate these effects on gonadal male (*Sry*+) FCG mice, as well as consider testing this with other drinking paradigms, such as intermittent access (IA).

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Figure Legends

Figure 1: Surgery and experimental timeline. FCG *Sry*- mice underwent either an ovariectomy (OVX) or a sham surgery and were given 4 weeks to recover. Mice were moved to a dark room to acclimate for the 5th week, after which the experiment started. Mice had access to water and 15% EtOH in a 2-bottle-choice DID paradigm for 15 drinking sessions (3 weeks). Session 15 was only 30 min, stopped to allow blood to be taken for later BEC analysis. On sessions 16, 17, 18, and 19, quinine was added in escalating concentrations (0, 100, 250, and 500 µM). Mice then had a 2-week break, followed by the same escalating concentrations of quinine over sessions 20-23, but in water only (quinine control).

Figure 2: Consumption of 15% EtOH in XX and XY female FCG mice treated with ovariectomy. Mice increased EtOH drinking over 14 sessions, but consumption was not affected by chromosomes or hormones. **** $p < 0.0001$ main effect (three-way repeated-measures ANOVA).

Figure 3: Preference for 15% EtOH vs water in XX and XY female FCG mice treated with ovariectomy. Mice increased preference for EtOH over sessions, but it was not affected by chromosomes or hormones. **** $p < 0.0001$ main effect (three-way repeated-measures ANOVA).

Figure 4: Consumption of water in XX and XY female FCG mice treated with ovariectomy. Mice decreased water drinking over sessions, but consumption was not affected by chromosomes or hormones. **** $p < 0.0001$ main effect (three-way repeated-measures ANOVA).

Figure 5: EtOH consumption during 4 hours of quinine-resistant drinking sessions. Mice decreased EtOH drinking as quinine concentrations increased and were not affected by chromosomes or hormones. **** $p < 0.0001$, main effect (three-way repeated-measures ANOVA). # $p < 0.05$, ## $p < 0.01$ vs. 0-µM concentration (Dunnett's test) (**Table 2**).

Figure 6: EtOH consumption during first 30 min of quinine-resistant drinking sessions. Mice decreased EtOH drinking as quinine concentrations increased and consumption was not affected by chromosomes or hormones. ** $p < 0.01$ main effect (three-way repeated-measures ANOVA), # $p < 0.05$ vs. 0-µM concentration (Dunnett's test) (**Table 2**).

Figure 7: EtOH preference during 4 hours of quinine-resistant drinking sessions. Mice decreased preference for EtOH as quinine concentrations increased, though there was an interaction of chromosome x treatment ($F_{(1, 50)} = 4.908$, $p = 0.0313$). ** $p < 0.01$ main effect (three-way repeated-measures ANOVA), ## $p < 0.01$ vs. 0-µM concentration (Dunnett's test) (**Table 2**).

Figure 8: EtOH preference during first 30 minutes of quinine-resistant drinking sessions. Mice decreased preference for EtOH over 30 minutes as quinine concentrations increased and were not affected by chromosomes or hormones. ** $p < 0.01$ main effect (three-way repeated-measures ANOVA).

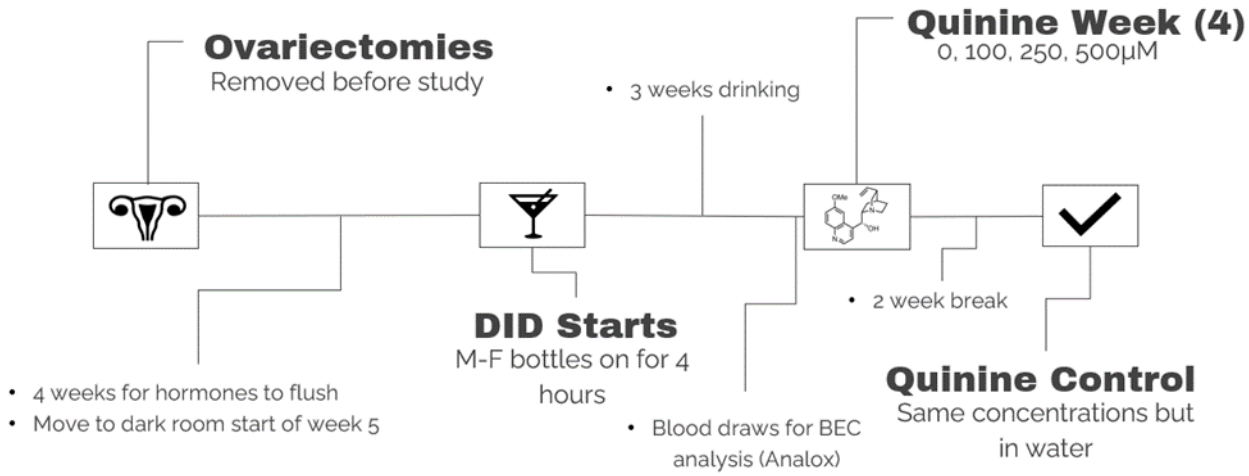
Figure 9: Water consumption during 4 hours of quinine-resistant control drinking sessions. Mice decreased water consumption as quinine concentrations increased and were not affected by chromosomes or hormones. **** $p < 0.0001$ main effect (three-way repeated-measures ANOVA), # $p < 0.05$, ## $p < 0.01$ vs. 0-μM concentration (Dunnett's test) (**Table 2**).

Figure 10: Water preference during 4 hours of quinine-resistant control drinking sessions. Mice decreased preference for water + quinine over 4 hours as quinine concentrations increased and were not affected by chromosomes or hormones. * $p < 0.05$ main effect (three-way repeated-measures ANOVA).

Table Legends

Table 1: Statistical results for all drinking measurements. For EtOH sessions, the main effect of session was always significant, demonstrating escalation across sessions. For EtOH + quinine sessions, the main effect of concentration was always significant, demonstrating that drinking decreased with the addition of quinine. For water + quinine sessions, the main effect of concentration was always significant, demonstrating that water drinking decreased with the addition of quinine. Neither OVX treatment nor sex chromosome complement influenced drinking for any measure.

Table 2: Statistical results of Dunnett's test for quinine drinking sessions. Follow-up two-way repeated-measures ANOVAs with Dunnett's tests were run to assess aversion resistance in each group. Each group was compared to their baseline drinking (0 μM) with 95% Confidence Intervals (CI) of differences and Adjusted P values reported.



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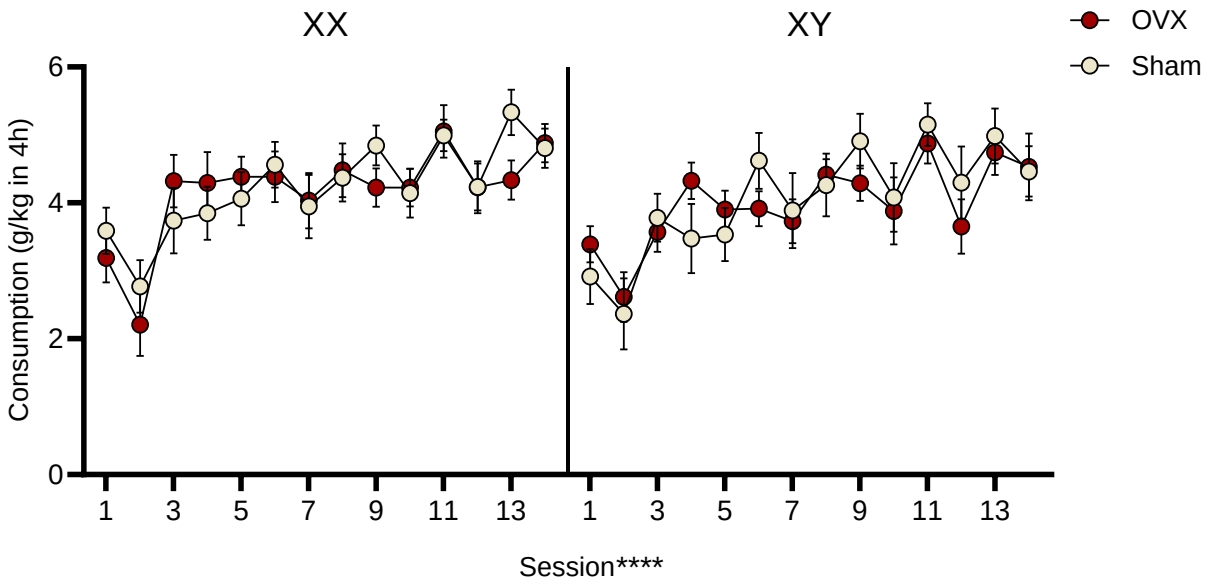

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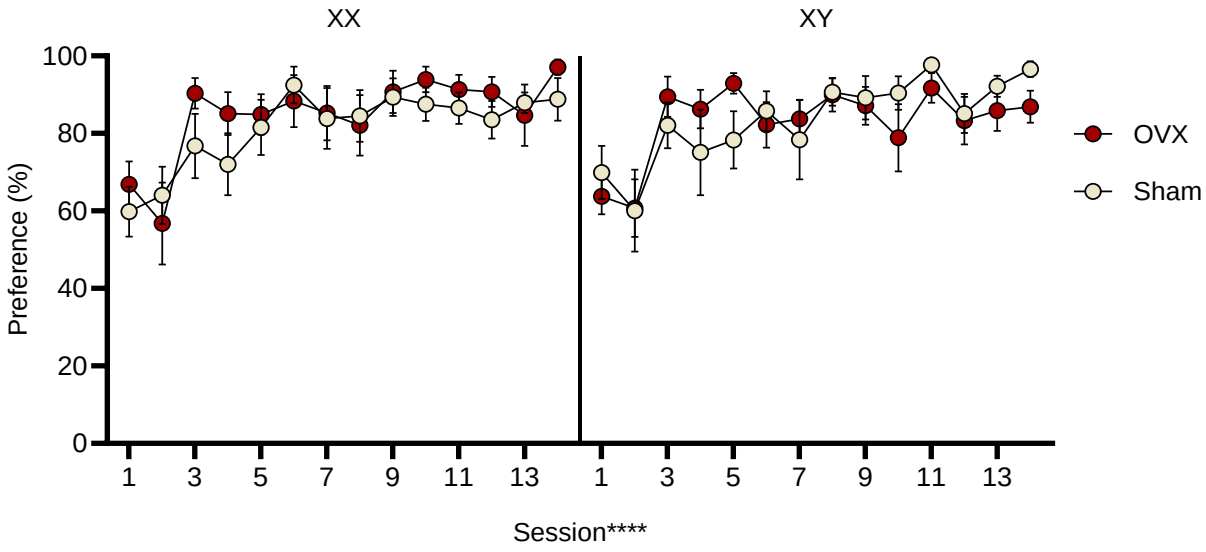

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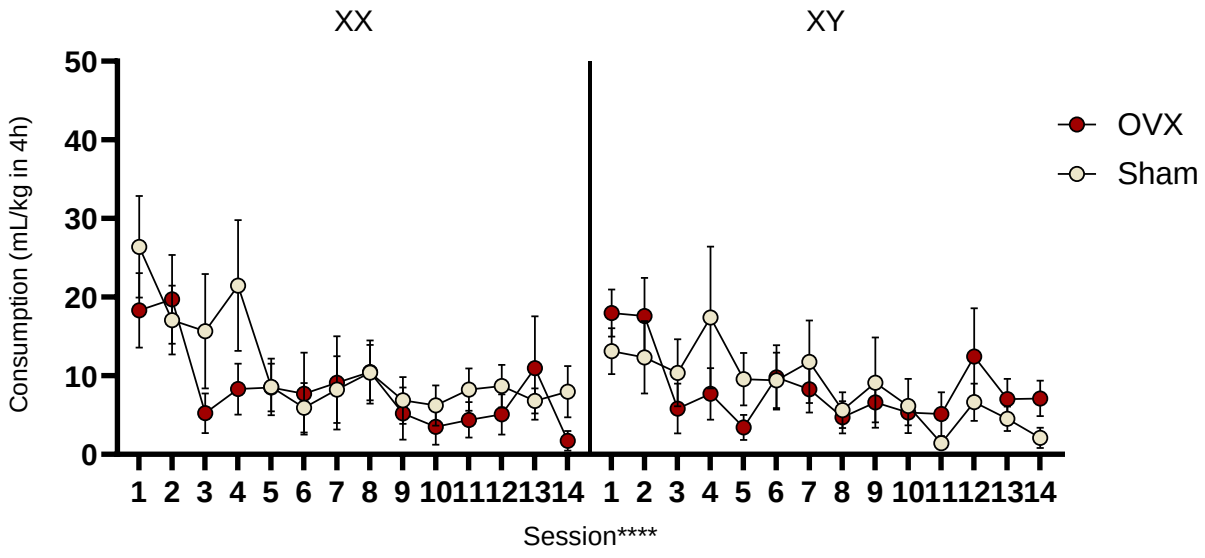

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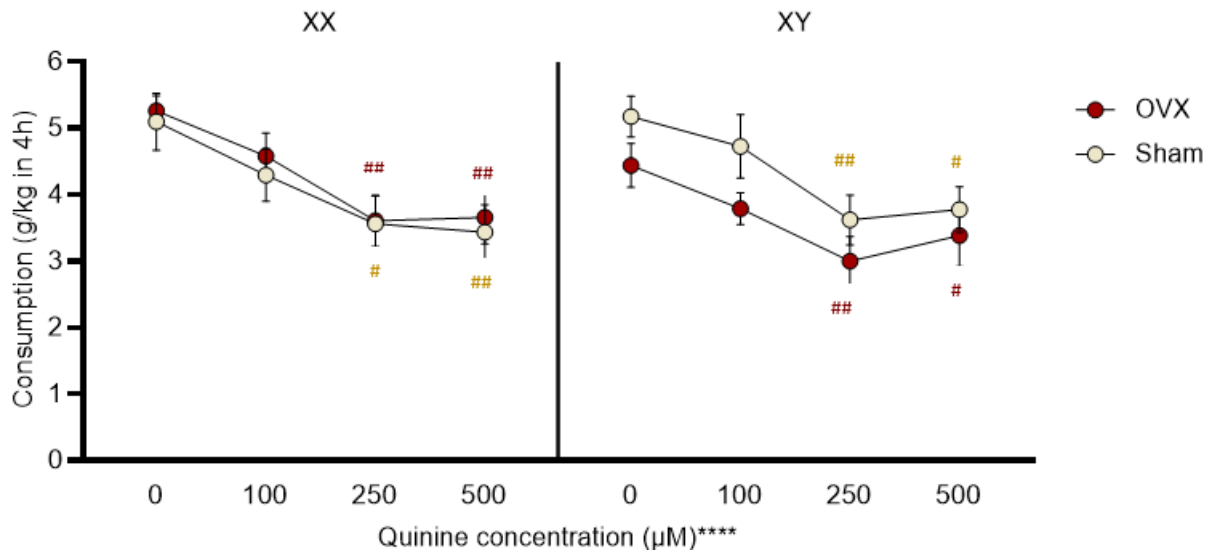

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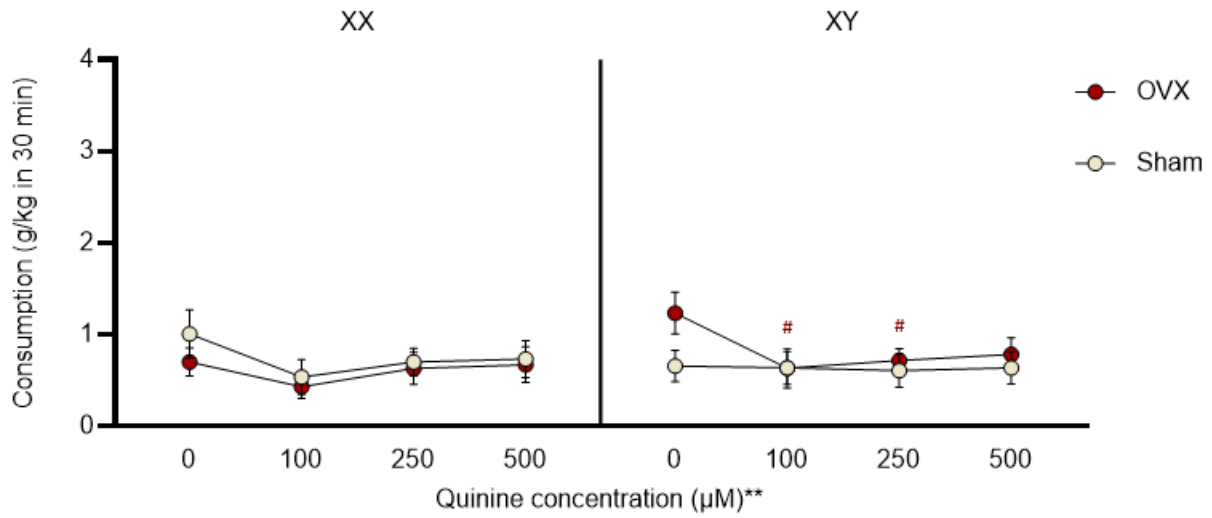

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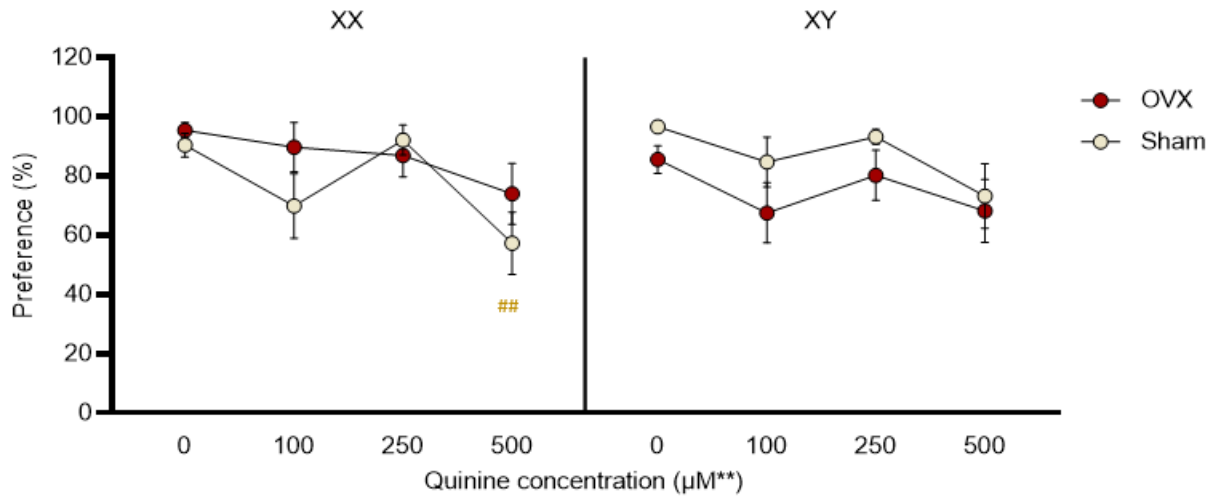

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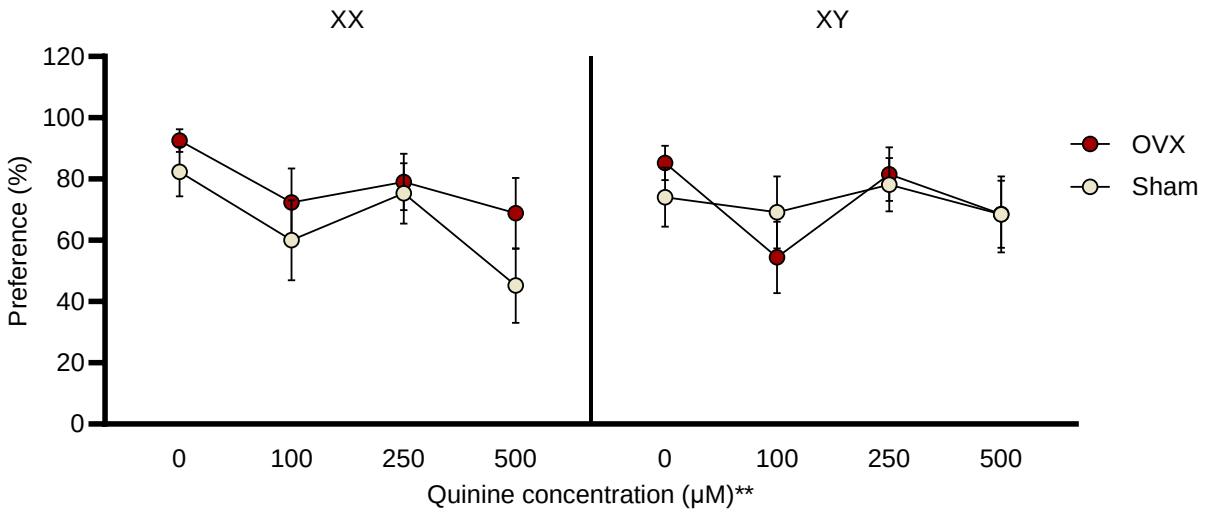

Figure 8: EtOH preference during first 30 minutes of quinine-resistant drinking sessions. Mice decreased preference for EtOH over 30 minutes as quinine concentrations increased and were not affected by chromosomes or hormones. ** $p < 0.01$ main effect (three-way repeated-measures ANOVA).

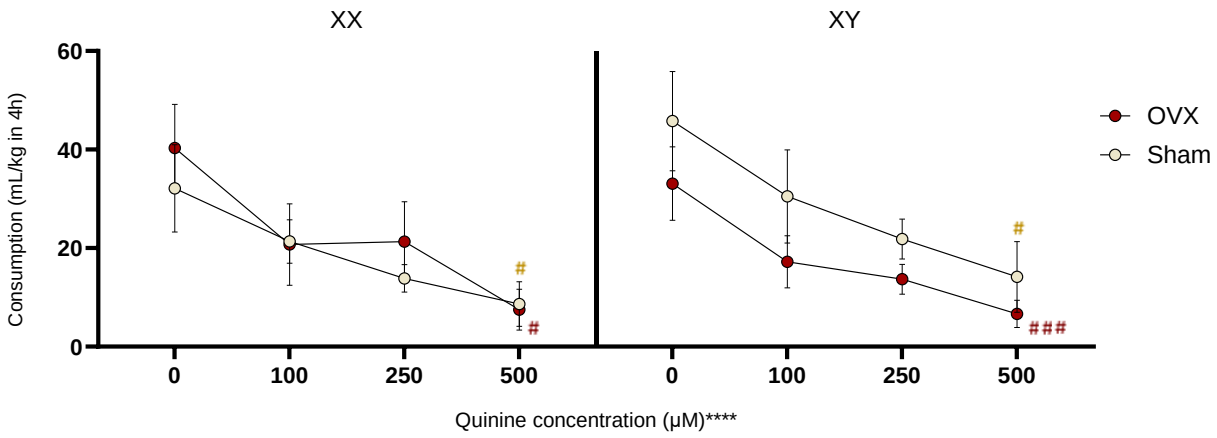

Figure 9: Water consumption during 4 hours of quinine-resistant control drinking sessions. Mice decreased water consumption as quinine concentrations increased and were not affected by chromosomes or hormones. **** $p < 0.0001$ main effect (three-way repeated-measures ANOVA), # $p < 0.05$, ### $p < 0.001$ vs. 0-µM concentration (Dunnett's test).

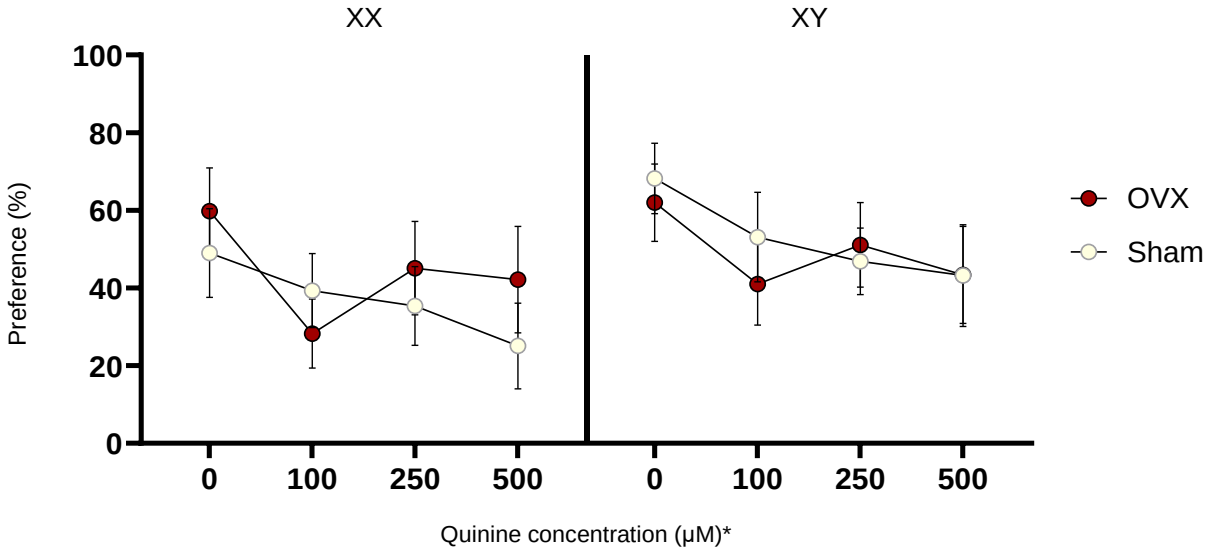

Figure 10: Water preference during 4 hours of quinine-resistant control drinking sessions. Mice decreased preference for water + quinine over 4 hours as quinine concentrations increased and were not affected by chromosomes or hormones. * $p < 0.05$ main effect (three-way repeated-measures ANOVA).

Table 1: Statistical results for all drinking measurements.

Source of Variation	F (DFn, DFd)	P value	Sig.
EtOH Consumption			
Session	F (8.662, 433.1) = 16.74	<0.0001	Yes
Treatment	F (1, 50) = 0.09380	0.7607	No
Chromosome	F (1, 50) = 0.6348	0.4294	No
Session x Treatment	F (13, 650) = 1.250	0.2395	No
Session x Chromosome	F (13, 650) = 0.2624	0.9959	No
Treatment x Chromosome	F (1, 50) = 0.0002678	0.9870	No
Session x Treatment x Chromosome	F (13, 650) = 0.7091	0.7553	No
EtOH Preference			
Session	F (7.815, 390.8) = 10.64	<0.0001	Yes
Treatment	F (1, 50) = 0.3427	0.5609	No
Chromosome	F (1, 50) = 0.01167	0.9144	No
Session x Treatment	F (13, 650) = 0.9032	0.5495	No
Session x Chromosome	F (13, 650) = 0.5164	0.9153	No
Treatment x Chromosome	F (1, 50) = 0.6985	0.4073	No
Session x Treatment x Chromosome	F (13, 650) = 0.6169	0.8411	No
Water Consumption			
Session	F (13, 650) = 5.372	<0.0001	Yes
Treatment	F (1, 50) = 0.6683	0.4175	No
Chromosome	F (1, 50) = 0.5826	0.4489	No
Session x Treatment	F (13, 650) = 1.159	0.3063	No
Session x Chromosome	F (13, 650) = 0.6383	0.8225	No
Treatment x Chromosome	F (1, 50) = 0.6264	0.4324	No
Session x Treatment x Chromosome	F (13, 650) = 0.6217	0.8370	No
4 hr EtOH Quinine Consumption			
Concentration	F (3, 150) = 21.45	<0.0001	Yes

Treatment	F (1, 50) = 0.8482	<0.0001	No
Chromosome	F (1, 50) = 0.5468	0.4631	No
Concentration x Treatment	F (3, 150) = 0.1202	0.9481	No
Concentration x Chromosome	F (3, 150) = 0.3083	0.8194	No
Treatment x Chromosome	F (1, 50) = 2.541	0.1173	No
Concentration x Treatment x Chromosome	F (3, 150) = 0.1978	0.8977	No
30 m EtOH Quinine Consumption			
Concentration	F (2.820, 141.0) = 4.272	0.0075	Yes
Treatment	F (1, 50) = 0.05955	0.8082	No
Chromosome	F (1, 50) = 0.1765	0.6762	No
Concentration x Treatment	F (3, 150) = 0.3250	0.8073	No
Concentration x Chromosome	F (3, 150) = 0.2782	0.8411	No
Treatment x Chromosome	F (1, 50) = 1.364	0.2484	No
Concentration x Treatment x Chromosome	F (3, 150) = 1.729	0.1634	No
4 hr EtOH Quinine Preference			
Concentration	F (2.438, 121.9) = 7.865	0.0002	Yes
Treatment	F (1, 50) = 0.07139	0.7904	No
Chromosome	F (1, 50) = 0.02935	0.8647	No
Concentration x Treatment	F (3, 150) = 0.6927	0.5579	No
Concentration x Chromosome	F (3, 150) = 0.2739	0.8442	No
Treatment x Chromosome	F (1, 50) = 4.908	0.0313	Yes
Concentration x Treatment x Chromosome	F (3, 150) = 0.6446	0.5875	No
30 m EtOH Quinine Preference			
Concentration	F (2.358, 117.9) = 4.735	0.0072	Yes
Treatment	F (1, 50) = 1.066	0.3068	No
Chromosome	F (1, 50) = 0.006319	0.9370	No
Concentration x Treatment	F (3, 150) = 0.4111	0.7453	No
Concentration x Chromosome	F (3, 150) = 0.7894	0.5016	No

Treatment x Chromosome	F (1, 50) = 1.064	0.3073	No
Concentration x Treatment x Chromosome	F (3, 150) = 0.6026	0.6143	No
4 hr Water Quinine Consumption			
Concentration	F (2.039, 102.0) = 21.20	<0.0001	Yes
Treatment	F (1, 50) = 0.5335	0.4685	No
Chromosome	F (1, 50) = 0.2050	0.6527	No
Concentration x Treatment	F (3, 150) = 0.2945	0.8293	No
Concentration x Chromosome	F (3, 150) = 0.06757	0.9771	No
Treatment x Chromosome	F (1, 50) = 2.144	0.1494	No
Concentration x Treatment x Chromosome	F (3, 150) = 0.3358	0.7995	No
4 hr Water Quinine Preference			
Concentration	F (2.727, 136.4) = 3.376	0.0239	Yes
Treatment	F (1, 50) = 0.06349	0.8021	No
Chromosome	F (1, 50) = 2.876	0.0961	No
Concentration x Treatment	F (3, 150) = 0.7658	0.5149	No
Concentration x Chromosome	F (3, 150) = 0.03459	0.9913	No
Treatment x Chromosome	F (1, 50) = 0.6579	0.4211	No
Concentration x Treatment x Chromosome	F (3, 150) = 0.1499	0.9296	No

Table 2: Statistical results of Dunnett's test for quinine drinking sessions.

Source of Variation	95.00% CI of diff.	Adj. P value	Sig.
4 hr EtOH Quinine Consumption			
XX/OVX			
100 µM	-0.1978 to 1.558	0.1396	No
250 µM	0.7186 to 2.589	0.0015	Yes
500 µM	0.6797 to 2.528	0.0017	Yes
XX/Sham			
100 µM	-0.5354 to 2.141	0.2997	No
250 µM	0.2946 to 2.778	0.0155	Yes
500 µM	0.5796 to 2.744	0.0035	Yes
XY/OVX			
100 µM	-0.3932 to 1.700	0.3146	No
250 µM	0.3945 to 2.488	0.0042	Yes
500 µM	0.009880 to 2.103	0.0473	Yes
XY/Sham			
100 µM	-0.6762 to 1.572	0.6580	No
250 µM	0.4311 to 2.680	0.0040	Yes
500 µM	0.2724 to 2.521	0.0108	Yes
30 m EtOH Quinine Consumption			
XX/OVX			
100 µM	-0.1902 to 0.7302	0.3356	No
250 µM	-0.3522 to 0.4906	0.9584	No
500 µM	-0.4910 to 0.5502	0.9981	No
XX/Sham			
100 µM	-0.3191 to 1.256	0.3358	No
250 µM	-0.3881 to 1.003	0.5746	No
500 µM	-0.4230 to 0.9717	0.6586	No

XY/OVX			
100 µM	0.04894 to 1.157	0.0314	Yes
250 µM	0.03999 to 0.9985	0.0322	Yes
500 µM	-0.02463 to 0.9306	0.0653	No
XY/Sham			
100 µM	-0.4001 to 0.4447	0.9985	No
250 µM	-0.4367 to 0.5372	0.9893	No
500 µM	-0.5642 to 0.6078	0.9995	No
4 hr EtOH Quinine Preference			
XX/OVX			
100 µM	-20.34 to 31.82	0.9131	No
250 µM	-17.59 to 34.56	0.7754	No
500 µM	-4.623 to 47.53	0.1296	No
XX/Sham			
100 µM	-3.649 to 44.64	0.1136	No
250 µM	-25.89 to 22.39	0.9963	No
500 µM	8.946 to 57.23	0.0044	Yes
XY/OVX			
100 µM	-12.36 to 48.46	0.3110	No
250 µM	-21.27 to 32.02	0.9110	No
500 µM	-16.88 to 51.66	0.4272	No
XY/Sham			
100 µM	-11.65 to 35.53	0.4190	No
250 µM	-5.782 to 12.55	0.6441	No
500 µM	-2.613 to 49.37	0.0803	No
30 m EtOH Quinine Preference			
XX/OVX			
100 µM	-8.413 to 48.77	0.2029	No

250 µM	-12.61 to 39.51	0.4381	No
500 µM	-6.236 to 53.68	0.1369	No
XX/Sham			
100 µM	-16.54 to 61.21	0.3636	No
250 µM	-17.81 to 31.87	0.8358	No
500 µM	-2.541 to 76.82	0.0693	No
XY/OVX			
100 µM	-3.170 to 64.90	0.0785	No
250 µM	-22.22 to 29.58	0.9651	No
500 µM	-21.64 to 55.15	0.5399	No
XY/Sham			
100 µM	-30.94 to 40.85	0.9658	No
250 µM	-20.15 to 12.01	0.8373	No
500 µM	-44.05 to 55.32	0.9802	No
4 hr Water Quinine Consumption			
XX/OVX			
100 µM	-14.96 to 54.13	0.3295	No
250 µM	-12.01 to 50.03	0.2758	No
500 µM	8.319 to 57.26	0.0101	Yes
XX/Sham			
100 µM	-8.947 to 30.51	0.3672	No
250 µM	-4.565 to 41.11	0.1280	No
500 µM	2.259 to 44.72	0.0297	Yes
XY/OVX			
100 µM	-5.346 to 37.05	0.1636	No
250 µM	-0.6145 to 39.42	0.0581	Yes
500 µM	12.33 to 40.54	0.0006	Yes
XY/Sham			

100 µM	-11.28 to 41.83	0.3249	No
250 µM	-0.4256 to 48.32	0.0543	No
500 µM	5.977 to 57.26	0.0162	Yes
4 hr Water Quinine Preference			
XX/OVX			
100 µM	-12.07 to 75.07	0.1748	No
250 µM	-31.15 to 60.48	0.7230	No
500 µM	-31.47 to 66.66	0.6555	No
XX/Sham			
100 µM	-27.34 to 46.66	0.8295	No
250 µM	-28.06 to 55.30	0.7233	No
500 µM	-18.64 to 66.47	0.3467	No
XY/OVX			
100 µM	-16.11 to 58.07	0.3460	No
250 µM	-25.98 to 47.71	0.7805	No
500 µM	-20.85 to 58.11	0.4822	No
XY/Sham			
100 µM	-19.46 to 49.67	0.5278	No
250 µM	-6.099 to 48.81	0.1398	No
500 µM	-4.648 to 54.67	0.1041	No

This Thesis titled

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by

Roman Anthony Zegarelli

has been approved for publication by

The College of Arts and Sciences

and

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