ANALYZING THE EFFECTS OF GONADAL HORMONES ON ALCOHOL SEEKING AND DRINKING IN ALCOHOL-PREFERRING P RATS

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DEDICATION

To Rose who provided me with love and support throughout my life. I love you and I know you would be so proud.

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Female and male rodents have shown differences in alcohol-seeking and -drinking behaviors, with female rodents typically consuming more alcohol than male rodents. Differences in gonadal hormones may provide one explanation for these sex differences. The current study used selectively bred female and male alcohol-preferring (P) rats to assess sex differences and the possible impacts of circulating gonadal hormones on alcohol seeking and drinking in an operant appetitive/consummatory paradigm. P rats were trained in operant boxes first for water and then 20% alcohol. Rats then underwent ovariectomy (OVX), castration (CAST), or sham surgeries. After recovery from surgery, rats that underwent OVX or CAST surgery then started receiving daily subcutaneous (s.c.) injections of either estradiol benzoate (E2), testosterone (T), or vehicle (Veh) which began five days prior to additional operant testing and lasted throughout the study. Rats were given a response requirement (RR) Monday-Thursday where they had 20-minutes to meet the required lever presses which resulted in 20-minute access to alcohol. Testing occurred over three weeks which resulted in 12 days of alcohol-drinking behavior. On Fridays, rats were given a 20-minute extinction session where number of lever presses were recorded which resulted in three days of alcohol-seeking behavior. Overall, females drank more alcohol than males in both training and testing. This was seen in both Veh and Sham rats. There were no sex differences in alcohol-seeking behavior. There was no effect of E2 or T in either sex as there were no differences in alcohol intake or lever presses during extinction compared to Veh groups. There were also no sex or group

differences in blood ethanol concentrations (BEC), but BEC did correlate with alcohol intake. This study is one of the first examining gonadal hormones in a selectively bred line of rat that prefers to drink alcohol and is unique in that it included both females and males in each of the treatment groups. The activational effects of gonadal hormones may have a limited impact on alcohol-related behaviors in P rats, but more research is needed to make definitive conclusions about their role.

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Analyzing the Effects of Gonadal Hormones on Alcohol Seeking and Drinking in Alcohol-Preferring P Rats

In humans, alcohol use disorder (AUD) has been more common among men than women, but that sex/gender gap is narrowing each year (Becker & Koob, 2016; Grant et al., 2017; Keyes et al., 2010; White et al., 2015). While there are likely many factors that are contributing to this convergence, it has become increasingly apparent that understanding alcohol-related behaviors in both females and males is vital. Sex differences have been seen in alcohol consumption in rodents with female rodents often drinking more than males (de la Torre et al., 2015; Li et al., 2019; McCane et al., 2018; Oberlin et al., 2011; Priddy et al., 2017; Sneddon et al., 2020). Clarifying the source of sex differences can lead to better understanding of behavior and the brain which can result in more translational research and novel interventions. One possible explanation for these disparities in rodents is differences in levels of circulating gonadal hormones. Female rodents have higher estrogen and progesterone levels compared to males, who in turn, have higher androgen levels. Several studies have used gonadectomies and hormone replacement to study the possible effects of gonadal hormones on alcohol seeking and drinking (e.g., Hilderbrand & Lasek, 2018; Satta et al., 2018), but many have used methodology which focuses on manipulating hormones within one sex (e.g., giving gonadectomized females estradiol replacement). The current study used "sex-typical" as well as "cross-sex" hormone treatments to study the effects of circulating gonadal hormones in an appetitive/consummatory alcohol operant paradigm.

Sex Differences in Alcohol Use

Sex and gender differences have been seen in motivation for drinking and time from the initiation of drinking to development of an AUD. Compared to men, women are more likely to report drinking alcohol to cope with negative emotions or psychological distress (Kuntsche & Muller, 2012) and this may, at times, result in a faster spiral from drug use to dependence (Becker et al., 2012). Similarly, differences have also been seen in the reasons for initiation of drinking, with boys tending to start to drink because of risk-taking behaviors and girls to relieve anxiety (Flores-Bonilla & Richardson, 2020). It is becoming increasingly important to understand not only any behavioral differences between women and men, but also be aware of any underlying sex differences in biological processes. Preclinical models using rodents provide a useful tool in uncovering these possible biological processes.

As noted above, female rodents often drink more alcohol than males. Females have also been shown to develop habit faster than males (Schoenberg et al., 2019) but cue reactivity may be more likely to predict alcohol-related behaviors in males (Barker & Taylor, 2019). In operant studies, female rats have been shown to lever press for alcohol more than males (Nieto & Kosten, 2017) and have greater reinstatement (Bertholomey et al., 2016; Bertholomey & Torregrossa, 2019). Although the direction of these sex differences may not be universal, as others have found that males have greater lever presses and reinstatement (Lorrai et al., 2019; Randall et al., 2017) or found no sex differences in alcohol seeking (Bianchi et al., 2018; Hernandez et al., 2020). Female rats showed a decrease in reinstatement for alcohol after footshock and abstinence compared to male rats (Campbell et al., 2021). Male mice given chronic intermittent ethanol (CIE)

exposure by vapor or air-exposed controls had reduced alcohol seeking in a conditioned place paradigm following footshock regardless of ethanol preexposure (Xie et al., 2019). In contrast, only female mice who underwent CIE, but not air-exposed controls, showed a similar reduction (Xie et al., 2019). There is mixed evidence in the differences between females and males in alcohol seeking paradigms.

Sex differences in alcohol seeking behaviors appear less consistent than alcohol drinking, although these results span across different tasks and species. One thing to note is that in most of the studies above, female animals consumed more alcohol than males, even when demonstrating less seeking behavior. For example, no sex differences in responding were found during training or reinstatement, but female rats drank more than males within these tests (Bianchi et al., 2018). Although alcohol drinking was measured during operant responding, this incongruency may highlight different systems underlying appetitive and consummatory behaviors (see Appetitive/Consummatory Behaviors). Overall, there appear to be behavioral differences between females and males that need to be further studied and alcohol-related behaviors within each sex should continue to be explored. Levels of gonadal hormones may provide one possible context to explain sex differences.

Strategies for Studying Gonadal Hormones

Gonadal steroid hormones are fat-soluble and include progestogens, androgens, and estrogens, the most common of which are progesterone, testosterone, and estradiol, respectively. As stated in Nelson and Kriegsfeld (2017), steroid hormones are synthesized in many organs including the gonads, the adrenal glands, and the brain, so while referred to as gonadal or sex hormones, it is important to note that they have

functions beyond the gonads and reproductive behaviors. Steroid hormones are derived from cholesterol. Progestogens and pregnanolone are precursors for androgens and estrogens, with androgens also being precursors for estrogens. In a seminal study by Phoenix et al., (1959), it was found that there are organizational and activational actions of gonadal steroid hormones. Organizational effects are defined by the authors, and in the context of mating behaviors, occur prenatally, arranging the brain in specific ways. Activational effects were defined as the impact hormones had on behavior in adulthood. There is evidence that suggests that activational effects of hormones can cause more permanent/long-lasting changes, blurring the lines of this strict dichotomy (Arnold & Breedlove, 1985). Organizational actions can also occur outside of critical periods in prenatal development, including during adolescence and at puberty (Schulz & Sisk, 2016). Differentiating between activational and organizational effects of gonadal hormones can help to determine their specific impact on the brain and behavior.

There are a few strategies that have been recommended when investigating the actions of gonadal hormones. As described in Becker et al. (2005) and McCarthy et al. (2012), the most common technique would be to gonadectomize (GDX) adult animals. Removing most of the circulating hormones in adult animals would help elucidate if there are activational effects of these hormones in adulthood causing differences in the behavior of interest. Comparisons can be made within the same sex of gonadectomized animals, with or without hormone replacement, across females and males if hormone levels are equalized, or by mimicking the hormone levels of one sex in both sexes. If a prior sex difference is not present in animals after gonadectomy, then the sex difference is most likely due to activational effects. If there is a difference, then organizational

effects of hormones that occurred during development could be the cause and may warrant further exploration. Organizational effects can be investigated using neonates treated with androgens or hormone antagonists. Timing of gonadectomies is also a useful tool in the exploration of organizational effects. If a behavior results from organizational effects during puberty, then animals gonadectomized pre-puberty should show differences compared to animals gonadectomized post-puberty. A useful first step in exploring the cause of sex differences in a behavior is to analyze possible gonadal hormone activational effects in adult rats. Hormones are often modulating sex differences (McCarthy et al., 2012) and adult hormone levels may be a reasonable conclusion in which further studies focusing on developmental hormonal effects may not be necessary.

The current study used some of the techniques described above to elucidate the effects of gonadal hormones on alcohol seeking and drinking. The study sought to analyze activational effects of estradiol and testosterone using gonadectomies and hormone treatments in adult alcohol-preferring rats. Treating gonadectomized females and males with either estradiol or testosterone helps to isolate whether either of these hormones are having an impact on alcohol-related behaviors in adulthood. It was hypothesized that female and male GDX rats given estradiol would increase their alcohol seeking and drinking and GDX females and males given testosterone would show a decrease.

Estrogen and Alcohol-Related Behaviors

Briefly, the effects of estradiol have been frequently studied in other addiction fields beyond alcohol. Female ovariectomized (OVX) Sprague-Dawley rats given estradiol self-administered higher levels of and displayed higher motivation for fentanyl

than OVX rats given vehicle (Towers et al., 2023). Estradiol may also help explain sex differences seen in cocaine behaviors. After extended access, females self-administered more cocaine than males and OVX females given estradiol infused more cocaine than OVX females given vehicle (Ramoa et al., 2013). Female rats also showed greater preference for cocaine over food reward than males and OVX females given vehicle showed lower cocaine preference than intact and OVX females given estradiol (Kerstetter et al., 2012). These studies highlight estradiol's relevance to addiction-like phenotypes and how it may be modulating sex differences seen in these behaviors. Estradiol may also be playing a role in alcohol-related behaviors.

In a study comparing four mouse lines, OVX only impacted C57 mice with OVX females drinking significantly less than their intact counterparts (Becker et al., 1985). OVX has also been shown to decrease alcohol drinking in female Long Evans rats and this effect was rescued with estradiol replacement (Ford et al., 2002a). Satta et al. (2018) saw a similar pattern between OVX C57 mice with and without estradiol replacement in a four-hour drinking-in-the-dark (DID) session. It was also found that intact female mice drank more than male mice during DID and that intake levels were similar for OVX females and males, but their blood ethanol concentrations (BECs) were not the same (Satta et al., 2018). This conflict between BEC and alcohol intake could mean there are more nuanced differences such as drinking patterns or alcohol tolerance that have yet to be explored. Table 1 was modified from Erol et al. (2019) and includes studies that analyzed alcohol intake specifically in rats and included some type of manipulation of estradiol or testosterone. These specific variables were chosen because they are of particular relevance to the current study. For an excellent recent review examining

alcohol use and gonadal hormones, including human and rodent studies, see Maddern et al. (2024).

Estradiol may also impact other alcohol-related behaviors as intact female Wistar rats demonstrated increased conditioned place preference for 1 g/kg ethanol compared to OVX rats (Torres et al., 2014). Increases in conditioned place preference for ethanol have been shown to require both α and β estrogen receptors (ER; Hilderbrand & Lasek, 2018). Conversely, increased drinking in two bottle choice was found after estradiol treatment in both ERα and ERβ null mice (Rajasingh et al., 2007), suggesting that the same receptor requirement may not be necessary for alcohol drinking. Intact Sardinian alcohol-preferring (sP) female rats consumed more alcohol than OVX females and males in two-bottle choice prior to any operant training, but no differences were seen in estimated alcohol self-administration during FR4 training, despite males pressing significantly more than both female groups (Lorrai et al., 2019). There were also no differences between OVX and intact females in breakpoints on a progressive ratio task (Lorrai et al., 2019). There are some inconsistencies found in the effects of estrogen and alcohol, with some studies finding only a small or no effect of OVX on alcohol drinking (Cailhol & Mormede, 2001; Vetter-O'Hagen & Spear, 2011). Despite these inconsistencies, it is possible that estradiol is, at least in part, causing females to drink more alcohol.

If these behaviors are driven solely by circulating estradiol, then castrated (CAST) males treated with estradiol would also show increased alcohol drinking. Although this type of study has rarely been conducted with alcohol-related behaviors, it has been shown that CAST males given estradiol enhanced their choice of cocaine over a food reward (Bagley et al., 2019). The authors report the dose of estradiol was chosen because it had

similar results in OVX females (Bagley et al., 2019; Kerstetter et al., 2012), suggesting that circulating estradiol influences cocaine reward regardless of gonadal sex.

Testosterone and Alcohol-Related Behaviors

Fewer studies have examined possible effects of testosterone on alcohol behaviors. Male rats castrated in adolescence that were then given ethanol injections into adulthood showed less motor and grooming behaviors in an open-field test (Yan et al., 2015). These results suggest that gonadectomy during adolescence has an impact on adult open-field behaviors and that testosterone may influence the long-term effects of adolescent exposure to alcohol. Female and male rats that were gonadectomized in adolescence also show differences in alcohol drinking behaviors in adulthood compared to their sham counterparts. GDX males drank more alcohol than sham males and GDX females drank less than sham females in 30-minute drinking sessions (Sherrill et al., 2011). It should be noted that the effect in OVX females was not present in animals that were exposed to alcohol in adolescence via ethanol injections (Sherrill et al., 2011), suggesting that gonadal hormones and adolescent exposure to alcohol have an impact on adult drinking behavior in a sex-specific way. It has also been shown that adult CAST rats given vehicle earned more reinforcers and drank more alcohol in an operant box than CAST rats given testosterone and sham males (Bertholomey & Torregrossa, 2019). Differences between CAST males and sham males demonstrate that testosterone may be playing a role in alcohol's impact on behavior and alcohol drinking and are further supported by testosterone replacement reversing that effect.

Spear et al. have examined testosterone's impact on alcohol drinking and other behaviors across several experiments. Male Sprague-Dawley rats gonadectomized at

postnatal day (PND) 23 or PND70 increased their alcohol consumption in adulthood (Vetter-O'Hagen & Spear, 2011). This effect was mimicked in a separate study that used only adult gonadectomized males, and testosterone treated males drank similar amounts of alcohol as sham and intact males (Vetter-O'Hagen et al., 2011). These results suggest that testosterone is playing a modulating role in alcohol intake in male Sprague-Dawley rats, although as reviewed in Varlinskaya et al. (2013), these effects may be modest. This research group has also found that gonadectomies at PND23 and PND70 had little influence on alcohol's impact on novelty-directed behaviors (Vetter-O'Hagen & Spear, 2012) and that the changes in alcohol drinking were not likely caused by changes to alcohol's social inhibitory (Morales et al., 2014) or aversive effects (Morales & Spear, 2013). Similarly, despite GDX males showing increased social anxiety-like behaviors compared to GDX females, there were no differences in GDX animals and their sham counterparts in their sensitivity to alcohol as measured by conditioned taste aversion (Kim & Spear, 2016). Taken together, there is evidence that testosterone impacts drinking behavior in male rats, but the precise mechanism of action remains unclear.

Testosterone may be playing a modulating role in alcohol-drinking behaviors. Male rodents may drink less alcohol than females due to their higher testosterone levels. Reducing circulating testosterone levels may lead to increased alcohol consumption in males and treating OVX females with testosterone may lead to decreased drinking.

Table 1

Alcohol Intake in Rats Adopted from Erol et al. (2019)

Paper	Strain	Sex	GDX and Hormone Details	Method	Findings
Almeida et al. (1998)	Wistar	F, M	Intact F, Intact M, neonatally-estrogenized F (NE), OVX+V, OVX+E2, CAST+V, CAST+DHT	2BC 2-12% EtOH increased over 8 days	Intact F > NE > Intact M; OVX+E2 < Intact F; CAST+DHT < Intact M, CAST+V
Bertholomey & Torregrossa (2019)	SD	F, M	OVX+V, OVX+E2, Sham F, CAST+V, CAST+T, Sham M	30-min operant self-administration 10% EtOH+0.1% saccharin	F > M; OVX+E2 > OVX+V, Sham F; CAST+V > CAST+T, Sham M
Cailhol & Mormede (2001)	WKY, WKHA, SHR	F, M	OVX, CAST, Sham F, Sham, M	2BC 6% EtOH	F > M; OVX decreased EtOH intake particularly in WKY rats which also showed reduction in overall fluid intake
Ford et al. (2002)	LE	F	OVX+V, OVX+E2, Sham+V, Sham+E2	2BC 10% EtOH	OVX+V decreased EtOH intake compared to baseline; OVX+E2 no difference from baseline after E2 treatment
Ford et al. (2004)	LE	F	Sham+V, OVX+V, OVX+E2 at 5 different doses	2BC 10% EtOH	OVX+V decrease in drinking compared to baseline, OVX+E2 dose-dependent increase
Forger & Morin (1982)	SD	F	OVX, Sham	2BC 4% EtOH	OVX < Sham
Juárez et al. (2002)	Wistar	M	CAST+E2, Sham	Forced access (FAC) 6% EtOH only liquid available. 2BC 6% EtOH or Water+2% sucrose	FAC: No diff between CAST and Sham 2BC: No diff between CAST and Sham E2 decreased intake compared to baseline in CAST males in 2BC
Juárez et al. (2005)	Wistar	M	CAST, E2 then Oil (EO), O then E2 (OE), E2 then E2 (EE) (6 days of treatment)	2BC 10% EtOH During second 6 days of treatment	EO > EE > OE
Lakoza & Barkov (1980)	Albino rats	M	CAST, CAST+T, CAST+E2	3-hour 2BC 8% EtOH	EtOH preference: CAST+T > CAST, CAST+E2
Lorrai et al. (2019)	sP	F, M	OVX F	2BC 10% EtOH; FR4 30-min operant 15% EtOH	2BC: F > OVX F, M FR4: No differences in alcohol intake between OVX F, F, or M
Marinelli et al. (2003)	Lewis, Wistar	F	Intact+V, Intact+EV 9 weeks prior to drinking	2BC 2-8% EtOH over 4 days (maintained at 8%)	EV > V; Wistar > Lewis
Quirarte et al. (2007)	Wistars	F	Intact+V, Intact+EV, OVX+EV	6-hour 2BC 12% EtOH+5% sucrose	Intact+EV, OVX+EV > Intact+V

Reid et al. (2002)	SD	F, M	Intact+EV, Intact+V Injected at differing times prior to alcohol	2-hour 2BC 12% EtOH+0.25% saccharin	F: Intact+EV > Intact+V 1-month prior injection, no differences in 2-month prior group M: Intact+EV < Intact+V 5-days post injection (similar in F)
Reid et al. (2003)	SD	F	Intact+V, Intact+Estradiol valerate (EV) 15 days prior to drinking	2BC 12% EtOH+0.25% saccharin	EV > V
Sandberg & Stewart (1982)	Wistar	F	OVX+E2, OVX+V	2BC 10% EtOH	OVX+E2 < OVX+V After 2 weeks, no difference between groups
Sherrill et al. (2011)	LE	F, M	OVX, CAST, Sham F, Sham M Performed at PND20	30-min homecage single bottle modified sucrose fading 5-20% EtOH (rats PND100)	Sham F > Sham M; OVX F < Sham F; CAST M > Sham M
Vetter-O'Hagen & Spear (2011)	SD	F, M	OVX, CAST, Sham F, Sham M, Intact F, Intact M at PND23 or PND70	2-Hour 2BC 6% EtOH+.01% saccharin (first 4 days) then 10% EtOH+0.1% saccharin (last 8 days)	F > M EtOH intake; CAST M > Sham M 10% EtOH intake in both PND23 and PND70 animals; No differences in F groups Effect of surgery
Vetter-O'Hagen et al. (2011)	SD	M	CAST, CAST+T, Sham, Intact	2-Hour 2BC 6% EtOH+.01% saccharin (first 4 days) then 10% EtOH+0.1% saccharin (last 8 days)	CAST > Sham

Note. LE: Long Evans; sP: Sardinian alcohol-preferring; WKY: Wistar Kyoto; SHR: Spontaneous hypertensive; WKHR: Wistar Kyoto Hyperactive; SD: Sprague-Dawley; OVX: ovariectomy; CAST: castration; E2: estradiol; EV: estradiol valerate; T: testosterone: V: vehicle; DHT: dihydrotestosterone; 2BC: 2-bottle choice; EtOH: ethanol

Appetitive/Consummatory Behaviors

Appetitive and consummatory sexual behaviors have been thoroughly researched. Networks of brain regions have been identified that uniquely contribute to either appetitive or consummatory sexual behaviors, and some regions that contribute to both (Jennings & de Lecea, 2020). In addition, sex differences within these brain networks have been demonstrated. Gonadal hormones also influence both behaviors. For example, ovarian hormones can impact female sexual maturation (Jennings & de Lecea, 2020). It is known that adult sexual behavior in rodents requires programming of the brain during development (McCarthy, 2023). For example, giving an adult male rat estradiol will not result in that male showing lordosis. While organizational effects of hormones are necessary for appetitive/consummatory sexual behaviors, it has not been thoroughly explored if organizational effects are necessary for appetitive/consummatory drug behaviors.

As stated above, giving CAST males estradiol led to an increase in cocaine choice (Bagley et al., 2019), suggesting that circulating hormones may modulate seeking behaviors without the necessity of organizational hormonal effects. A separation of appetitive and consummatory behaviors has been seen in alcohol-related behaviors. It is known that pharmacological manipulations can decrease seeking behavior without affecting intake (Czachowski et al., 2002) and vice versa (Czachowski et al., 2001). Social isolation also increased alcohol consumption but did not affect seeking behavior as measured by lever presses in extinction (McCool & Chappell, 2009). In an operant paradigm that uses a lickometer system to separate appetitive and consummatory behaviors, unique behavioral phenotypes of Drinkers, Responders, and Non-responders

were identified in male Long Evans, Wistar, and Sprague-Dawley rats (Patwell et al., 2021), demonstrating distinct behavioral profiles in alcohol seeking and drinking. An alcohol associated houselight cue induced greater seeking behavior in a paired group even though alcohol drinking was similar across paired and unpaired groups (Cofresi et al., 2019). These incongruencies in seeking and drinking suggest that different systems may be underlying appetitive and consummatory alcohol behaviors. Measuring appetitive behaviors using lever presses in an extinction trial was found to positively correlate with breakpoints in progressive ratio, suggesting extinction probe trials are a useful tool for measuring motivation and further supports that these are two separate systems (Ortelli & Weiner, 2024). Gonadal hormones may also be impacting these behaviors differently; however, fewer studies have analyzed gonadal hormones and seeking/appetitive behaviors. The current study analyzed how estradiol and testosterone treatments affect both appetitive and consummatory alcohol behaviors. It also provides a foundation for exploring if organizational effects of hormones are necessary by first isolating the circulating effects of these hormones.

Alcohol-Preferring (P) Rats

The studies discussed so far have used various species of rats and mice which may show strain-specific hormonal effects (Becker et al., 2005) and impact alcohol-specific behaviors (Priddy et al., 2017). Alcohol-preferring (P) rats have been selectively bred to consume high amounts of alcohol and prefer to drink alcohol over water (McBride & Li, 1998). Although they have fewer dopamine neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens compared to Wistars, they have been shown to have increased burst firing which may be a compensatory mechanism to

elevate dopamine levels (Morzorati, 1998). This highlights differences between strains in areas of the brain related to alcohol addiction. In addition, P rats demonstrate cognitive impulsivity (Beckwith & Czachowski, 2014) and motor impulsivity (Beckwith & Czachowski, 2016). A paradigm that separates alcohol seeking from alcohol drinking using a second-order reinforcement schedule found that seeking performance was similar in male P rats regardless of alcohol history (Giuliano et al., 2015). Possible differences between alcohol drinking and other alcohol-related behaviors have been noted in P rats and in other high alcohol-preferring rat lines. For example, high alcohol consumption did not directly indicate compulsive drinking in alcohol-preferring strains (Vengeliene et al., 2009), compulsive alcohol seeking was only found in a subset of male P rats (Giuliano et al., 2018), and P rats did not show increased impulsivity in a 5-choice serial reaction time task (Pena-Oliver et al., 2015). Conversely, in a 2-Way Conditioned Access Protocol it was found that P rats did demonstrate compulsive behavior when presented with quinine-adulterated alcohol compared to Wistar rats (Timme et al., 2022). This demonstrates the importance of examining alcohol-related behaviors in conjunction with and separate from alcohol drinking and that there may be differences in these behaviors in P rats.

It has been reported that the strength of genetic influences on alcohol-related outcomes are similar in females and males (Salvatore et al., 2017) and P rats offer a unique opportunity to continue to shed light on the intersection of sex differences and a genetic model of AUD. Female P rats have been shown to drink more alcohol than males in an operant paradigm similar to the procedure in the current study (McCane et al., 2018) and for up to six weeks in a continuous-access paradigm (Bell et al., 2011). It should be noted this may differ by methodology as an initial sex difference faded in a

three-bottle choice paradigm after 10 days (Moore & Lynch, 2015) and female P rats drank more than males in two-bottle choice after one week but males drank more after four weeks (Bell et al., 2006). Others have found that males drink more 10% alcohol in two-bottle choice than females (Rezvani et al., 2017). It has been shown that sP female rats drink more than male rats in an intermittent access two-bottle choice paradigm, but not in continuous access (Loi et al., 2014). Directly comparing across homecage and operant paradigms has previously been cautioned (Bell et al., 2006) and so the current study will expand on previous studies by analyzing possible differences between female and male P rats in an operant paradigm.

Operant responding for 15% alcohol and the amount of alcohol consumed were reduced in male P rats after injections of pregnanolone (Besheer et al., 2010), suggesting that neuroactive steroids may be modulating alcohol behaviors. As previously discussed, in a different line of alcohol-preferring rats (sP), OVX decreased drinking in female rats compared to intact females in two-bottle choice (Lorrai et al., 2019), demonstrating that gonadal hormones can influence alcohol drinking in selectively bred animals. To this author's knowledge, the current study is the first to manipulate gonadal hormones in P rats and will expand knowledge on the intersection between a family positive history of AUD and activational effects of hormones.

The Current Study

Aim 1

Investigate the role of gonadal hormones in alcohol-seeking and -drinking behaviors in female P rats.

Hypothesis 1A. Ovariectomized (OVX) female P rats will show diminished seeking and drinking compared to sham-gonadectomized (Sham) female P rats. OVX P rats given estradiol benzoate (E2) replacement will have increased drinking behaviors compared to OVX rats, similar to levels of Sham females. OVX has decreased alcohol intake in rats, an effect that was rescued by estradiol replacement (Ford et al., 2002a). Estradiol level may play a role in alcohol-drinking behaviors. For example, Marinelli et al. (2003) found that rats injected with estradiol valerate increased intake of 8% alcohol. Therefore, reducing estradiol levels should reduce alcohol drinking and replacing estradiol should rescue this effect.

Hypothesis 1B. OVX female P rats given testosterone (T) replacement will have diminished alcohol drinking and seeking behaviors compared to Sham and OVX females. While limited research has been conducted on T effects in female rodents, there are some studies which suggest T may have modulating effects on alcohol drinking in males (see Aim 2). Giving OVX females T will isolate the possible effects of the circulating hormone on alcohol seeking and drinking.

Aim 2

Investigate the role of gonadal hormones in alcohol-seeking and -drinking behaviors in male P rats.

Hypothesis 2A. Castrated (CAST) male P rats will show increased seeking and drinking behaviors compared to Sham male P rats. It has been shown that gonadectomized male rats consumed more alcohol than sham males (Sherrill et al., 2011; Vetter-O'Hagen & Spear, 2011). CAST males given T replacement will show decreased alcohol drinking compared to CAST males, similar to levels of SHAM males. It is known that testosterone can affect the brain and behaviors in male rodents by converting it to estradiol (Wallen & Baum, 2002). Therefore, it is possible that CAST male rats given T will show decreased alcohol drinking because of the aromatization of estradiol, and not because of a direct effect of the T. However, if that is the case then CAST males given E2 (below) will have similar results to CAST males given T. This effect would be similar for OVX females given T as well. If T is having an effect by being aromatized into estradiol, then OVX females given either T or E2 will show similar results.

Hypothesis 2B. CAST males given E2 replacement will have increased alcohol drinking and seeking compared to Sham and CAST males. Giving E2 to CAST males will isolate the circulating effects of this hormone on alcohol seeking and drinking. If E2 is driving increases in alcohol drinking, then we would expect to this effect in CAST males.

Aim 3

Explore sex differences and sex-specific effects in alcohol seeking and drinking in P rats.

Hypothesis 3. Sham female rats will drink more alcohol than Sham male rats. Many studies have found that female rodents consume more alcohol than male rodents including in high alcohol preferring mice (Oberlin et al., 2011), C57BL/6J mice (Sneddon et al., 2020), Wistar rats (de la Torre et al., 2015), and P rats (McCane et al., 2018). It is likely this sex differences trend will continue in the following study. Although slightly less consistent in the literature, female rats have been shown to lever press for alcohol more than males (Nieto & Kosten, 2017) and demonstrate greater reinstatement (Bertholomey et al., 2016). Therefore, it is hypothesized that Sham female rats will demonstrate more seeking behavior as measured by increased number of lever presses on extinction days compared to males.

It is likely that any sex differences in OVX and CAST rats will be diminished, pointing towards an activational role of gonadal hormones to explain these sex differences. However, it is possible that sex differences may be seen in gonadectomized animals which would imply that circulating gonadal hormones would not be able to fully explain the differences between females and males. There is also a possibility that sex differences will be seen, but in the opposite direction of the Sham animals such that OVX females will consume less alcohol than CAST males.

The current hypotheses in Aim 1 and Aim 2 suggest that E2 and T will have similar effects in both females and males (i.e., E2 will increase alcohol behaviors and T will decrease alcohol behaviors). An alternative outcome is that one or both hormones only show effects in one sex. For example, E2 may only show an increase in alcohol-related behaviors in females. This outcome would demonstrate that other factors (e.g.,

chromosomes, organizational hormone effects) are interacting with circulating levels of the hormone to influence seeking and drinking behavior.

Method

Subjects

67 adult P rats (34 female; 33 male) bred at Indiana University Purdue University Indianapolis (IUPUI) were used. Rats were between 8-10 weeks old at the start of the experiment where they were single housed. All rats were handled for three days prior to any procedures. Animals were on a 12-hour reverse light/dark cycle (lights on: 21:00 – 9:00) and had ad libitum access to food (Purina LabDiet #5001 Rodent Lab Pellet) and water except where otherwise noted. All experimental procedures took place during the dark cycle. Five female rats (3 OVX; 2 Sham) and one male rat (CAST) died post-surgery. One female and one male rat were removed from the study following surgery due to poor health outcomes, leaving the total of 59 (28 female; 31 male). Rats were tested across two cohorts (29 rats in cohort one; 30 rats in cohort two). All procedures were approved by the IUPUI School of Science Animal Care and Use Committee. See Table 2 for group sizes.

Table 2

Number of Animals Per Group Included in the Study

Females		Males	
Group	**n**	**Group**	**n**
Sham	6	Sham	8
E2	8	E2	7
T	7	T	7
Veh	7	Veh	9

Note. E2, T, and Veh animals all received gonadectomy surgery prior to hormone treatments. Sham animals received sham surgery.

Equipment

The operant chambers used were 30 x 30 x 24.5 cm from Med-Associates (East Fairfield, VT) and were equipped with a retractable lever, an illuminated house light, a fan, and a retractable sipper tube with a stainless-steel double ball bearing spout. Chambers were individually kept in sound-attenuating cabinets. Data from the operant chambers was collected using MED-PC software (Med-Associates).

Drugs

190 proof ethanol was diluted with tap water to make 20% ethanol (v/v) which was used in the operant chambers. Estradiol benzoate (E2; MilliporeSigma) dissolved in sesame oil at 0.05 mg/ml was given at 0.05 mg/kg based on prior studies (Bertholomey & Torregrossa, 2019; Larson, Roth, Anker, & Carroll, 2005; Souza et al., 2014). Testosterone (T; MilliporeSigma) dissolved in sesame oil at 2 mg/ml was given at 2 mg/kg (Bertholomey & Torregrossa, 2019; Chen et al., 2003). Both drugs were injected subcutaneously (s.c.) every day between 11:00-12:00 approximately 4 hours before operant testing and were injected at the same time on weekends. Vehicle (Veh) animals received s.c. injections of sesame oil at the same volume.

Procedure

Training

Rats were given access to 20% alcohol in the homecages for one hour on two consecutive days to avoid neophobia to alcohol in the operant chambers. Operant training and testing took place Monday-Friday. For the first four days of training, animals were water deprived (Monday – Thursday) and water was used as a reinforcer in the operant chambers. Rats were given access to water in their homecages for 2 hours following

operant-training sessions. On the first day of training rats were given approximately 200 free licks before the sipper tube was retracted and the lever was inserted into the box. Rats were then shaped on the lever on a fixed ratio (FR1) schedule. On the fifth day of training (Friday) the reinforcer was switched to 20% alcohol and animals had regular access to water in their homecages. Operant sessions during FR training were approximately 60-minutes long. During the second week of training, rats were moved from an FR1 to an FR4 schedule before switching to a response requirement (RR4) on Friday where rats had 20 minutes to give 4 lever responses which resulted in 20-minute access to alcohol. Once the response requirement was met the lever was retracted and rats received immediate access to alcohol. The response requirement then increased by 2 each day (except Mondays) for weeks three and four of training until an RR20 was reached. Week five was an additional week of RR20 training. If an animal failed to meet the RR then the lever was retracted and the session ended. In this event, the RR for the following day was adjusted to the number of responses given. Four animals failed to meet at least one RR during training and one female did not meet a RR during testing. See Figure 1 for a timeline of the study.

Surgery

Rats then underwent OVX, CAST, or Sham surgeries. The first cohort of animals received surgery across 10 days and the second cohort received surgery across 8 days. All animals received Carpofen (5 mg/kg @ 1.0 ml/kg) which was administered i.p. prior to surgery. Anesthesia was induced and maintained with isoflurane. Warm saline (10 ml/kg) was also given s.c. following surgery.

For ovariectomy, rats had both sides shaved and their skin disinfected with betadine and alcohol. With the animal on their side, an incision (1-2 cm) was made in the skin just below the ribs (posterior) followed by another incision in the muscle. The ovary was then externalized along with additional fatty tissue. The tissue between the ovary and uterus was clamped with a hemostat and the ovary was removed with scissors. After there was confidence no bleeding was occurring the tissue was gently placed back inside the abdomen. The muscle tissue and the skin were then each sutured using 4-0 absorbable sutures. The same procedure was repeated on the other side. Sham animals received the same surgical procedures without the removal of the gonads.

For castration, rats had their scrotum shaved and the skin was disinfected with betadine and alcohol. A 1-2 cm incision was made in the scrotum. On one side, a small incision was made in the tunica and one testis and epididymis was pushed out of the tunica. The testis was then ligated with 4-0 absorbable suture before being cut. After confidence there was no bleeding occurring, the remaining tissue was gently placed back into the tunica and the procedure was repeated on the other side. The skin incision was then sutured using 4-0 absorbable sutures. Sham animals received the same surgical procedures without the removal of the gonads.

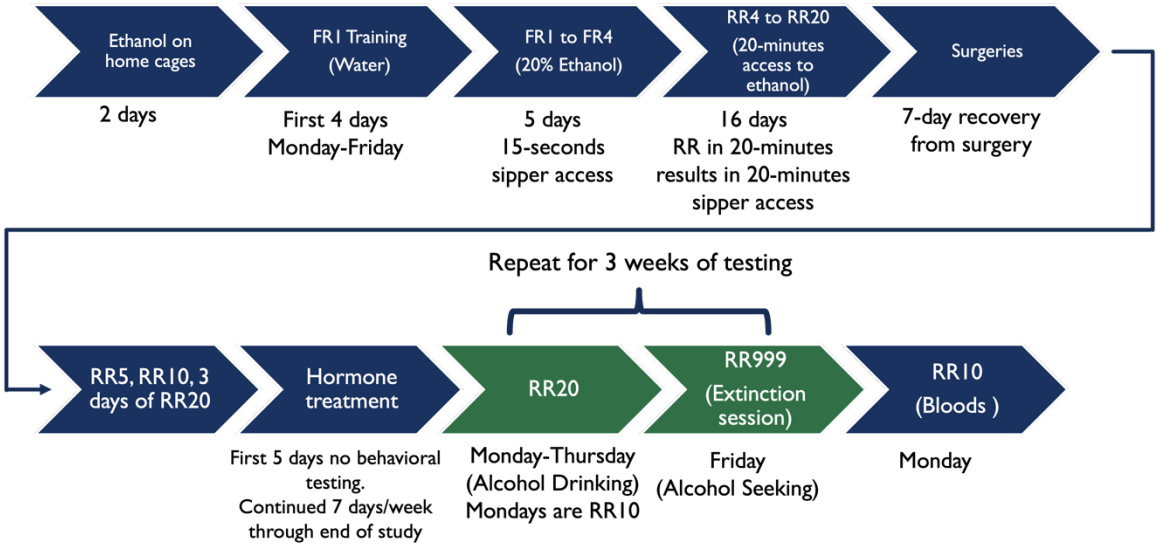
All rats were given 7 days to recover and underwent surgery checks daily. After recovery and prior to any injections, rats were given five operant sessions (RR5, R10, three days of RR20) to reacquaint them with the operant chambers. OVX and CAST rats were then given either E2, T, or Veh injections for three days prior to additional operant testing. Due to unforeseen delays in shipping, the first cohort animals received either E2 or Veh, and the second cohort animals received either T or Veh.

Testing

Rats continued to receive treatments and were given three weeks of testing in a seeking/consummatory operant paradigm (Samson et al., 2001; Samson et al., 1999). On Mondays, an RR10 resulted in 20-minutes of access to alcohol. On Tuesdays-Thursdays, an RR20 was implemented. These 12 days were used as a measure of drinking behavior. On Fridays, animals were given a 20-minute extinction session in which lever pressing did not result in access to alcohol. Bottles with alcohol were placed on cages for scent cues but did not enter the chambers at any point. These three extinction sessions were used as a measure of seeking behavior. The response requirement on Mondays was dropped to an RR10 to avoid any carryover effects from the extinction sessions on Fridays.

Figure 1

Timeline for Study



Blood Analyses

Blood Ethanol Concentration (BEC)

On the Monday following the last extinction session, animals underwent an additional RR10 operant session. Immediately following the end of the session blood samples were collected from a small tail snip. Blood samples were kept on ice until they could be centrifuged in a 4°C centrifuge at 14,000 rotations per minute for 5 minutes. Serum was then collected and stored at -80°C. BECs were analyzed using an Analox Ethanol Analyzer (Analox Instruments, Lunenburg, Massachusetts, United States).

Serum Hormone Levels

Estradiol and testosterone levels were analyzed in serum samples using competitive enzyme-linked immunosorbent assay (ELISA) kits from Arbor Assays (Ann Arbor, MI) to detect 17β-Estradiol or testosterone. Estradiol kits had a sensitivity of 2.21 pg/mL. Sample preparation required serum be diluted at least 1:20 with Assay Buffer which is the dilution all samples were run. Testosterone kits had a sensitivity 6.53 pg/mL. Sample preparation required serum to be diluted at least 1:36 with Dissociation Reagent and Assay Buffer which is the dilution female samples were run. Male serum levels were diluted to 1:108 as it was believed higher dilutions would be necessary to fit samples onto the standard curve for males. Plates were read on a plate reader capable of reading the optical density of each well at 450nm. The online tool from MyAssays was used to calculate data. Samples and standards for each assay were run in duplicates.

Analyses

Weight data were analyzed within each sex using a mixed-methods ANOVA that compared group weights across both training and test days. Changes in weights were

analyzed using a one-way ANOVA. Testosterone ELISA data were analyzed with a two-way chi-square (χ^2) test for each sex. Estradiol ELISA data compared groups using a one-way ANOVA for each sex. Alcohol intake during training was examined across days and sex. Based on the ELISA data, alcohol intake, licks, extinction lever presses during testing, and BEC data were compared using only Veh, E2, and T groups. Sham animals were analyzed separately in order to explore any possible sex differences using an independent samples *t*-test. Alcohol intake and number of licks were each first analyzed using a 12 (day) x 2 (sex) x 3 (group) mixed-methods ANOVA. Data were then averaged across the 12 days and analyzed with a 2 (sex) x 3 (group) one-way ANOVA. Lever presses during extinction were examined using a 3 (day) x 2 (sex) x 3 (group) mixed-methods ANOVA followed by a 2 (sex) x 3 (group) one-way ANOVA on lever presses averaged across the 3 days. A one-way ANOVA was used to compare intake on day 13 when blood was drawn. BEC and intake on day 13 were then correlated using a Pearson's correlation. BEC was also analyzed by group using a 2 (sex) x 3 (group) ANOVA. All mixed-methods ANOVAs included cohort as a covariate. When appropriate, data were collapsed across variables or analyzed split by sex. Bonferroni-corrected pairwise comparisons and Bonferroni post hoc tests were used.

Data were analyzed using IBM SPSS Statistics (International Business Machines Corporation, version 29.0.2.0) or GraphPad Prism (GraphPad Software, Inc. La Jolla, CA, version 10.2.3). Graphs were made using GraphPad Prism. Error bars on graphs represent standard error of the mean (SEM).

Results

Weight Across Training and Testing

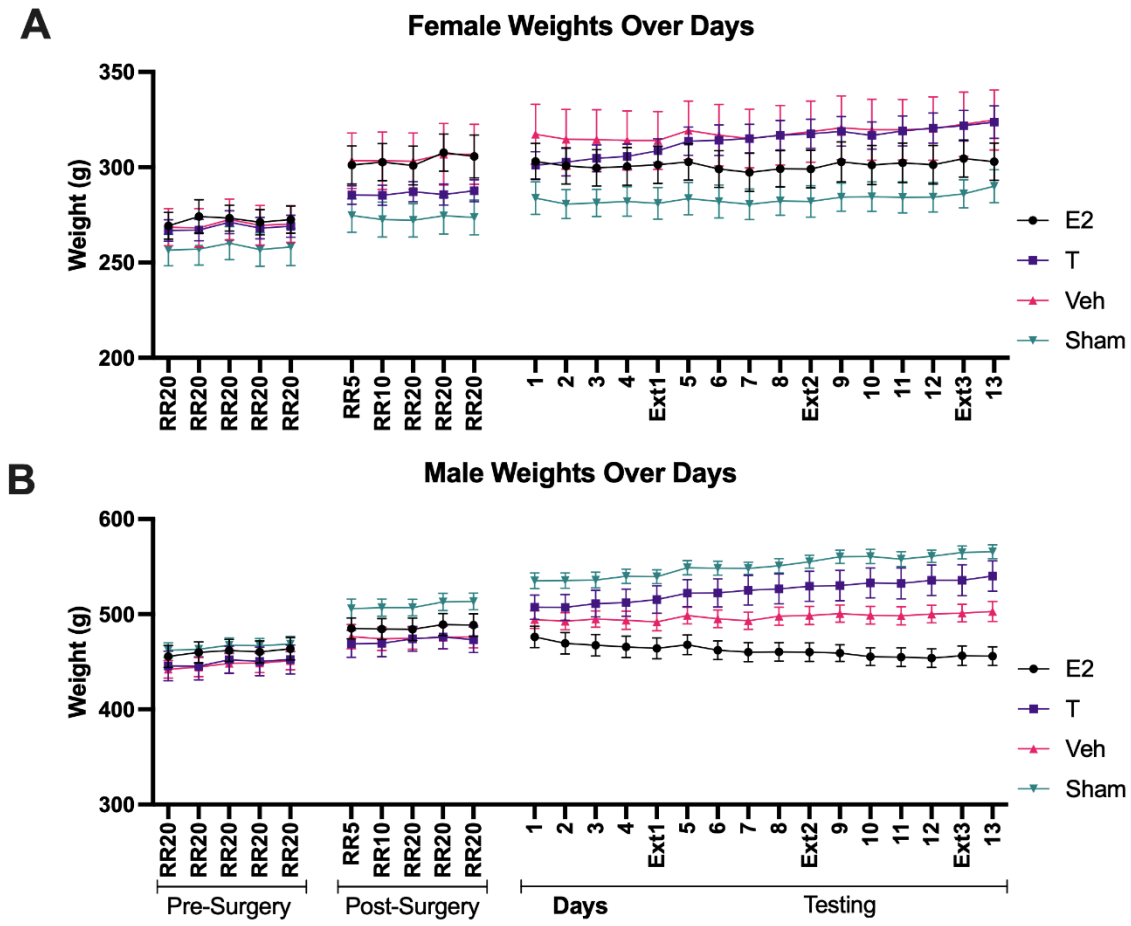
Due to known weight disparities between female and male P rats, weight was analyzed within each sex. A 26 (day) x 4 (group) ANOVA tested weight across training and testing days. The last five days of training prior to surgery, the five days of training post-surgery but pre-hormone treatment, and the 16 days of testing including the 12 drinking days, 3 extinction days, and the final drinking day where bloods were drawn were included in the analyses. See Figure 2 for female and male weights over days. In females, there was a significant effect of day, $F(25, 575) = 2.28$, $p < .001$, $\eta^2_p = .24$, and a significant interaction effect of day x group, $F(75, 575) = 3.84$, $p < .001$, $\eta^2_p = .33$. There was a main effect of cohort, $F(1, 23) = 6.32$, $p = .019$, $\eta^2_p = .22$, but no main effect of group. Pairwise comparisons of day x group showed that T and Sham weights differed starting on day 7 of alcohol drinking during testing and continued until the end of the study. On the last day of testing there was also a difference between E2 and T animals. In males, there was a significant effect of day, $F(25, 650) = 17.24$, $p < .001$, $\eta^2_p = .40$, and significant interaction effects of day x cohort, $F(25, 650) = 4.26$, $p < .001$, $\eta^2_p = .14$, and day x group, $F(75, 650) = 26.45$, $p < .001$, $\eta^2_p = .75$. There was a main effect of group, $F(3, 26) = 7.44$, $p < .001$, $\eta^2_p = .46$, but no main effect of cohort. Pairwise comparisons of day x group showed that starting on the first day of testing there were differences between E2 and T, E2 and Sham, and Veh and Sham animals which continued until the end of the study. On day 6 of alcohol drinking E2 and Veh rats were significantly different from each other also throughout the rest of the study. The significant effects of cohort in both the females and males are likely driven by the

treatment groups included within each cohort (e.g., E2 animals were only included in cohort 1, male E2 animals weigh less after hormone treatment than other groups, likely driving the interaction of cohort and day).

To examine the effects of hormone treatment on weight, changes in weights were calculated between last day of training pre-surgery and the last day of the study (day 13 of alcohol intake) and groups were compared with one-way ANOVAs. In females, there was a significant main effect of group, $F(3, 23) = 4.15$, $p = .017$, $\eta^2_p = .35$, but Bonferroni post hoc tests did not reveal any significant differences between the groups (Figure 3A). In males, there was also an effect of group, $F(3, 26) = 37.29$, $p < .001$, $\eta^2_p = .81$, and post hoc tests revealed that while Sham and T were not different from each other, all other groups were significantly different from each other (Figure 3B).

Figure 2

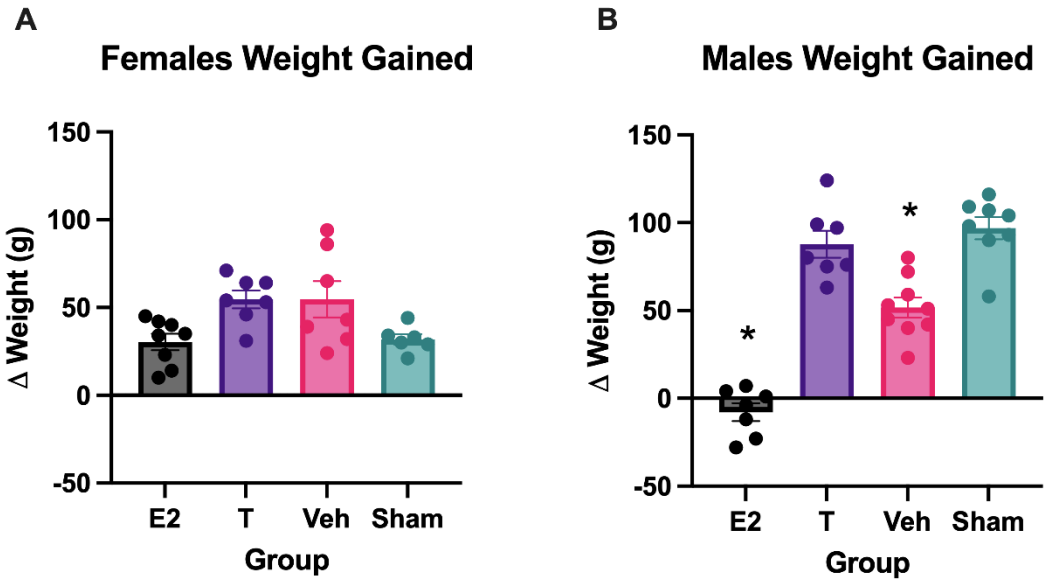
Weights Across Days for Females (A) and Males (B)



Note. A. In females, T and Sham animals differed starting on testing day 7. T and E2 were significantly different on the last day of the study. B. In males, on testing day 1, Sham animals were different from E2 and Veh groups. E2 was different from T starting on day 1 and from Veh starting on day 6.

Figure 3

Changes in Weight for Females (A) and Males (B)



Note. Weight gained from the last day of training pre-surgery to the last day of the study. E2, T, and Veh animals all received gonadectomy surgery then respective hormone treatment. Sham animals underwent sham surgery. A. There was a significant effect of group in the females but no significant post hoc group differences. Generally, it appears Sham and E2 groups had similar weight gains while T and Veh had similar changes in weight. B. Males had a significant effect of group. T and Sham groups had similar weight gains, while all other groups differed. *$p < .05$ compared to all other groups.

Serum Gonadal Hormone Levels

It was found that many animals did not show detectable levels of testosterone (Table 3) and therefore a two-way χ^2 test was used within each sex to compare the percentage of animals in each group that had detectable levels. All four treatment groups were included in these analyses. In females, T animals were more likely to have detectable testosterone levels compared to Sham, Veh, and E2 animals, $\chi^2 = 20.25$, $p < .001$, $V = .85$ (See Table 3 for percentages). A similar pattern was seen in males where T animals were more likely to have detectable levels than the other groups, $\chi^2 = 16.54$, $p < .001$, $V = .73$. Averaged testosterone levels for the T groups are reported in Table 3.

Estradiol levels were compared across groups in each sex using a one-way ANOVA. Only animals that had detectable estradiol levels were used which included 24 females and 28 males. In females, there was a significant main effect of group, $F(3, 20) = 4.75$, $p = .012$, $\eta^2_p = .42$. Bonferroni post hoc tests showed that E2 rats had higher estradiol levels compared to Sham rats. There was also a main effect of group in males, $F(3, 24) = 14.90$, $p < .001$, $\eta^2_p = .65$, with the E2 group having significantly higher estradiol levels than all other groups. See Table 3 for averaged estradiol levels. Surprisingly, there were no significant differences in estradiol levels between Sham female and male animals.

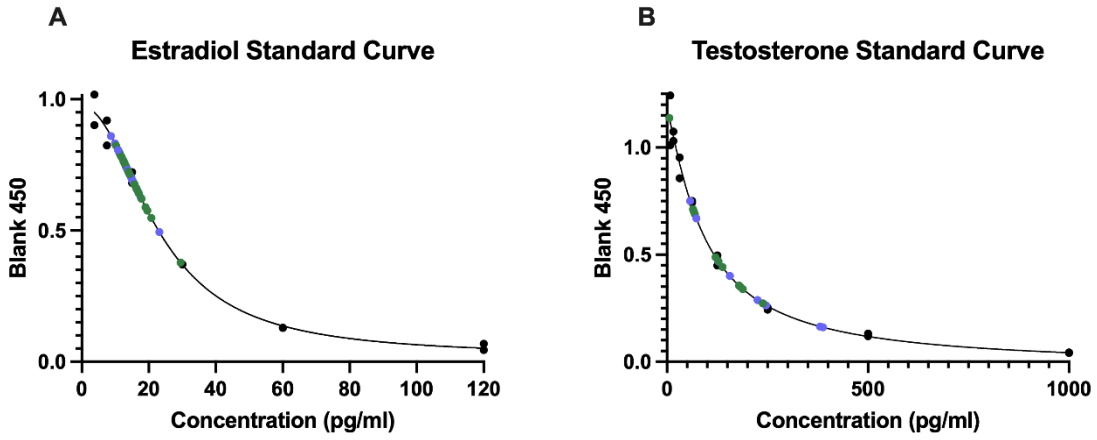
In order to provide further context to the ELISA results, the diluted samples were plotted onto the standard curves for each of the respective hormones. Figure 4A shows the estradiol standard curve and Figure 4B shows the testosterone standard curve. There is a possibility that where the samples fell on the standard curve could be leading to

inaccurate or inflated levels of hormones, especially once the dilution factor is taken into account.

Based on these ELISA data, there is evidence that testosterone and estradiol levels were increased in each of their respective treatment groups. However, there is not conclusive evidence that the Sham animals had different circulating hormone levels than the Veh group, meaning that it is unknown if GDX surgeries successfully manipulated gonadal hormone levels. Due to these inconclusive findings, the Sham group was not included in many of the analyses which instead compared only the GDX groups. Because Veh, E2, and T animals all received GDX surgery it removed surgical manipulation as an independent variable and focused analyses on hormone treatment alone (i.e., increasing either estradiol or testosterone).

Figure 4

Standard Curves of ELISA Assays with Diluted Samples



Note. Estradiol and Testosterone ELISA assays with plotted diluted samples. A. Many of the samples are on the lower end of the standard curve. 6 samples are not represented because they did not have detectable levels of estradiol. B. 38 samples are not represented on the standard curve as they did not have detectable levels of testosterone. Purple dots represent females, green dots represent males, black dots are the standards measured to create the standard curve.

Table 3

Hormone Levels Based on ELISA Assays

Number of Animals Testosterone Detected

Females				Males			
E2	**Veh**	**T**	**Sham**	**E2**	**Veh**	**T**	**Sham**
0/8	1/7	7/7	1/6	0/7	2/9	7/7	3/8
0%	14.28%	100%**	6.67%	0%	22.22%	100%**	37.5%
Average Testosterone (ng/ml)							
	1.84	8.24 ± 5.25	5.31		14.04 ± 7.91	15.75 ± 7.34	2.78 ± 4.07

Number of Animals Estradiol Detected

Females				Males			
E2	**Veh**	**T**	**Sham**	**E2**	**Veh**	**T**	**Sham**
6/7[#]	6/7	7/7	5/6	7/7	8/9	7/7	6/8
85.71%	85.71%	100%	83.33%	100%	88.89%	100%	75%
Average Estradiol (pg/ml)							
331.63 ±79.08*	254.53 ±32.09	263.37 ±23.38	223.66 ±49.48	410.46 ±84.47*	245.63 ±45.24	269.19 ±24.53	263.15 ±33.39

Note. Hormone levels presented as mean ± SEM.

[#]Not enough serum to run one animal.

***p* < .05 compared to all other groups, **p* < .05 compared to respective Sham group.

Alcohol Intake During Training

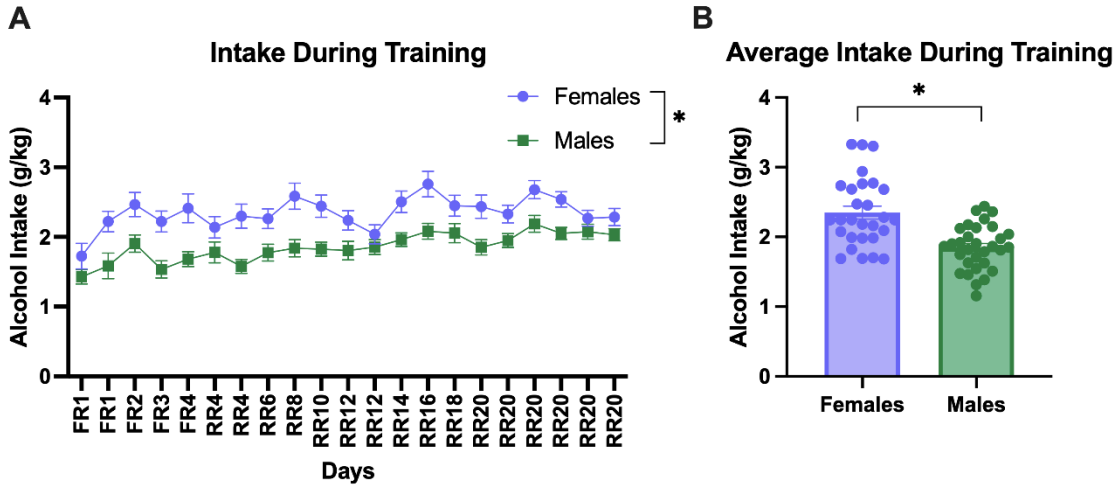
A 21 (day) x 2 (sex) mixed-methods ANOVA analyzed sex differences in intake during training. 26 females and 28 males were included (some animals not included due to missing data). A main effect of day, $F(20, 1020) = 2.25$, $p = .001$, $\eta^2_p = .04$ (Figure 5A) and an interaction of day x cohort, $F(20, 1020) = 2.06$, $p = .004$, $\eta^2_p = .04$, were found. Bonferroni pairwise comparisons showed that the first day of FR1 alcohol training was significantly different than several other days of training; however, generally drinking remained consistent across all training days. There was a main effect of sex, $F(1, 51) = 17.38$, $p < .001$, $\eta^2_p = .25$ where overall, females drank more than males.

When intakes were averaged across training days and analyzed in a one-way ANOVA comparing sex with cohort as a covariate, there was a main effect of sex, $F(1, 56) = 22.02$, $p < .001$, $\eta^2_p = .28$ (Figure 5B).

Intake on the last day of training before testing compared eventual treatment groups using a 2 (sex) x 4 (group) ANOVA with cohort as a covariate to assess any potential baseline differences between groups. Only 26 females were included in this analysis due to one female not meeting the response requirement and an error in measuring intake for another female. There was an expected main effect of sex, $F(1, 48) = 4.19$, $p = .046$, $\eta^2_p = .08$, in which females drank more than males, but no other significant effects, suggesting that baseline intake was similar across all of the groups.

Figure 5

Intake Across Training Days Indicates Females Drink More Than Males



Note. Alcohol intake during training across days and averaged across all training days. A. Females drank more than male rats. The first day of FR1 training was different than several other training days, but intake was relatively stable. B. Averaged intakes show females consumed more alcohol than males.

Alcohol Intake During Testing

Due to inconclusive evidence of a hormone level difference between Veh and Sham groups in both the females and males, the Sham animals were not included in the testing analyses in comparison to the GDX groups. Where noted, Sham animals were included in separate analyses for comparisons across sex.

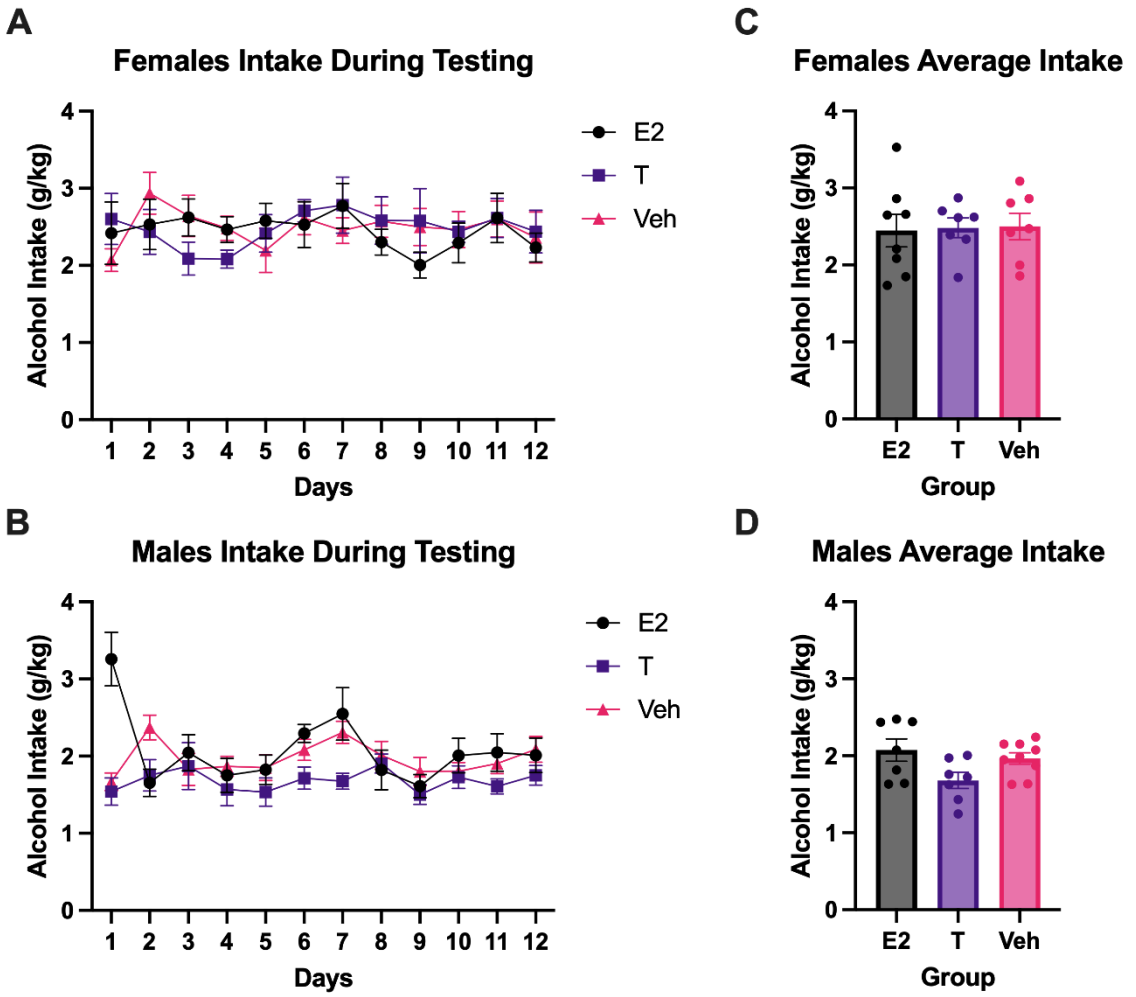
A 12 (day) x 2 (sex) x 3 (group) ANOVA analyzed intake across the 12 testing days. Two females were not included in analyses due to an error in intake measurement and one female not meeting a response requirement on one of the days. There was a significant main effect of sex, $F(1, 36) = 18.87$, $p < .001$, $\eta^2_p = .34$, where overall females

drank more than males (See Figure 6A for females). There was also a significant interaction of day x group, $F(22, 396) = 1.89$, $p = .009$, $\eta^2_p = .10$. Follow-up pairwise comparisons collapsed across sex found that on day 1, E2 animals drank significantly more than Veh and T animals. This effect is likely driven by the males which showed a similar pattern on day 1 of testing (Figure 6B). E2 animals also drank more than the T group on day 7. Across all the groups, intake appeared stable across all days of testing.

Intake was then averaged across the 12 days of testing and analyzed using a 2 (sex) x 3 (group) one-way ANOVA collapsed across the cohorts. There was a main effect of sex, $F(1, 39) = 22.89$, $p < .001$, $\eta^2_p = .37$, where overall females drank more than males. There was no effect of group or interaction of group x sex. Averaged intakes split by sex are represented in Figure 6 C and D. To test the hypothesis in Aim 3 and further explore any potential sex differences, Sham female and male rats' average intake was compared using a *t*-test. It was found that female Sham rats drank more than male sham rats, $t(14) = 2.68$, $p = .02$, $d = 1.45$ (Figure 9A). This finding is consistent with the finding of GDX females drinking more alcohol than GDX males.

Figure 6

Alcohol Intake Across Testing Days in Females and Males



Note. Alcohol intake during testing days by day and averaged across all 12 days. Overall, females drank more than males in 30-minute operant sessions with 20% ethanol. A. Females' intake across the testing days. B. Males' intake across the testing days. E2 rats drank more on the first day of testing compared to the other groups and to the other testing days. C. There were no differences between the female groups when the testing days were averaged. D. No differences between the male groups when the testing days were averaged together.

Licks During Testing

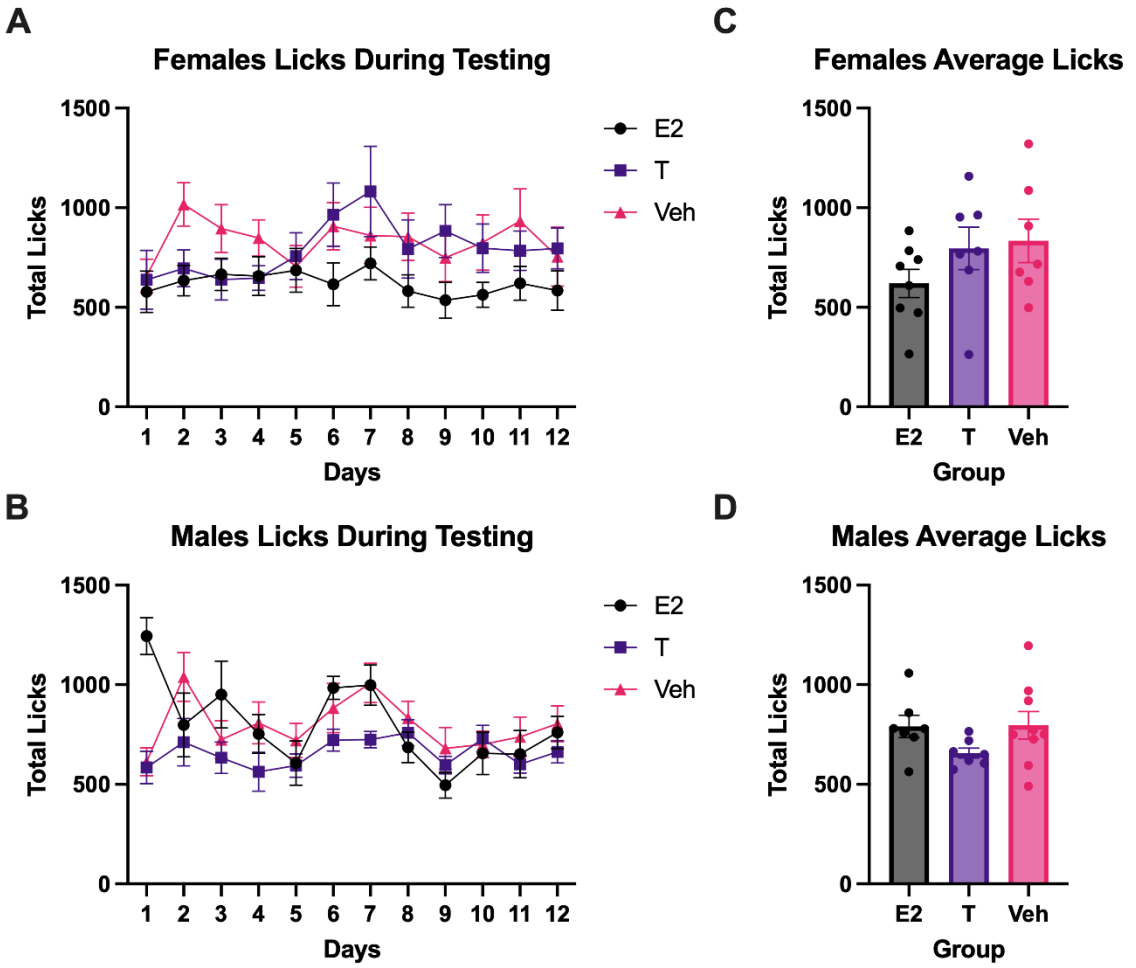
A 12 (day) x 2 (sex) x 3 (group) ANOVA compared licks in operant sessions during testing. One female and one male were not included in analyses due to missing data on one of the days. There were interactions of day x group, $F(22, 396) = 2.01$, $p = .01$, $\eta^2_p = .10$, day x sex x group, $F(22, 396) = 1.75$, $p = .020$, $\eta^2_p = .09$, and a trend of day x sex, $F(11, 396) = 1.82$, $p = .050$, $\eta^2_p = .05$. The three-way interaction was analyzed by running separate ANOVAs for each sex. In females, there was a main effect of day, $F(11, 198) = 2.26$, $p = .013$, $\eta^2_p = .11$. Pairwise comparisons did not show any significant differences between the days (Figure 7A). In males, there was a main effect of day, $F(11, 209) = 4.43$, $p < .001$, $\eta^2_p = .19$, and an interaction of day x group, $F(22, 209) = 2.76$, $p < .001$, $\eta^2_p = .23$ (Figure 7B). Follow-up pairwise comparisons showed that E2 males had significantly more licks than Veh and T groups on day 1. This increase on day 1 is in line with the increased alcohol intake on day 1 for the E2 group. There were also overall differences between days 5 and 7, and day 9 was different than days 6 and 7, but there were no other significant differences. Despite the significant main effects of day in females and males, licks appear to be relatively stable across the testing days.

Despite the increase in licks in the male E2 group on day 1, licks were averaged across the 12 testing days. Note that averages were also calculated excluding day 1 and were not statistically different from the averages including it; therefore, all 12 testing days were included. There were no significant main effects of sex or group and no significant interaction effect. Figure 6 C and D show average licks for both females and males. In order to test sex differences in the Sham animals, a separate *t*-test was conducted and found no significant difference (Figure 9B). The lack of significant sex

differences in licks in both the GDX and Sham groups implies that although females were consuming more g/kg alcohol, they were licking the same amount.

Figure 7

Number of Licks Across Testing Days in Females and Males



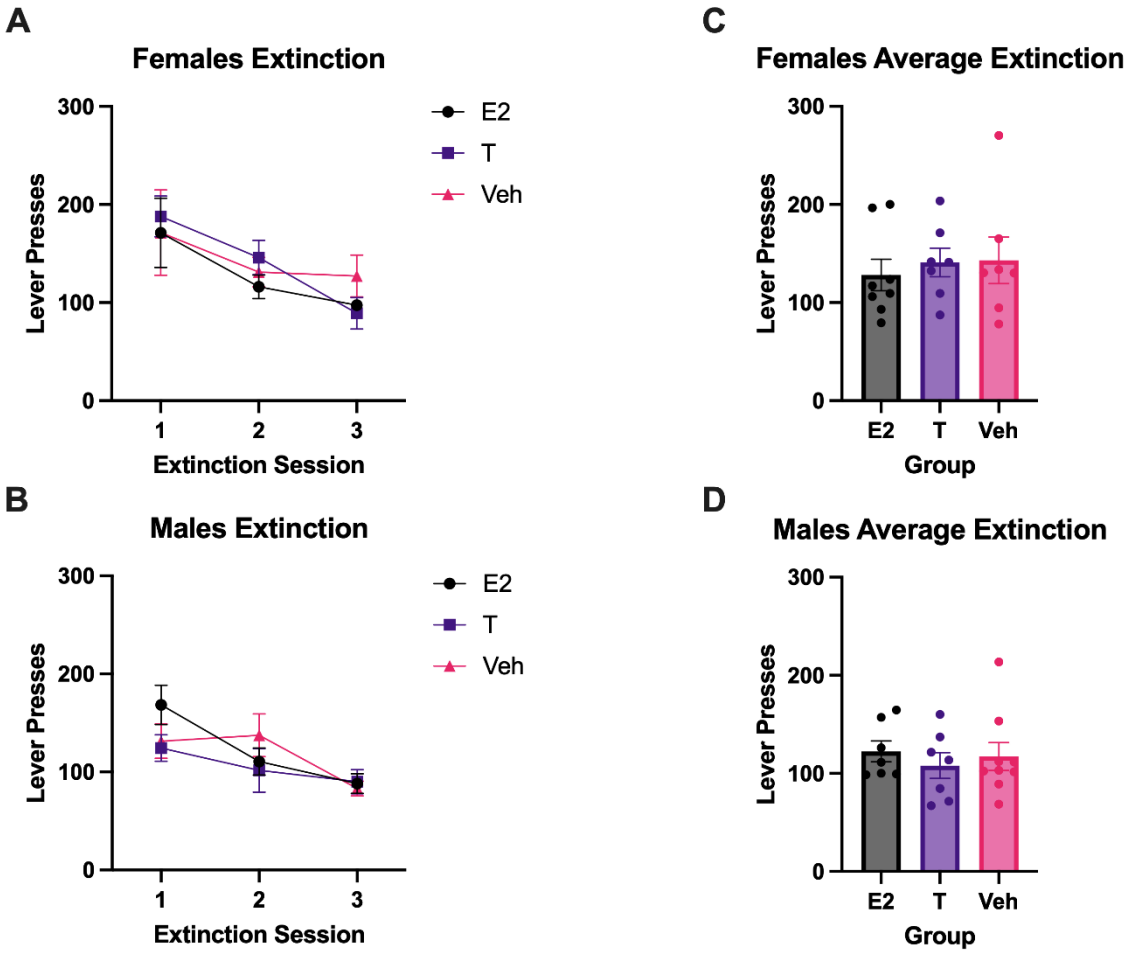
Note. Number of licks during testing days across days and averaged across all 12 days. A. Female licks across testing days. B. Male licks across testing days. The E2 group had more licks on day 1 of testing, consistent with this effect seen in alcohol intake on day 1. C. When licks were averaged across all days of testing there were no group differences in the females. D. No differences in the males between groups in average number of licks.

Lever Presses During Extinction

Lever presses were analyzed with a 3 (day) x 2 (sex) x 3 (group) ANOVA in order to examine alcohol-seeking behavior. There were no significant main effects of day, sex, or group and no interaction effects. Figure 8 A and B show female and male extinction sessions across the 3 sessions. Average lever presses across the 3 extinction days were analyzed and again there were no significant main or interaction effects. Average lever presses during extinction shown in Figure 8 C and D. Sham females and males average lever presses were compared and there was not a significant difference in number of lever presses (Figure 9C). Although females consume more alcohol than males, they demonstrate similar seeking behavior.

Figure 8

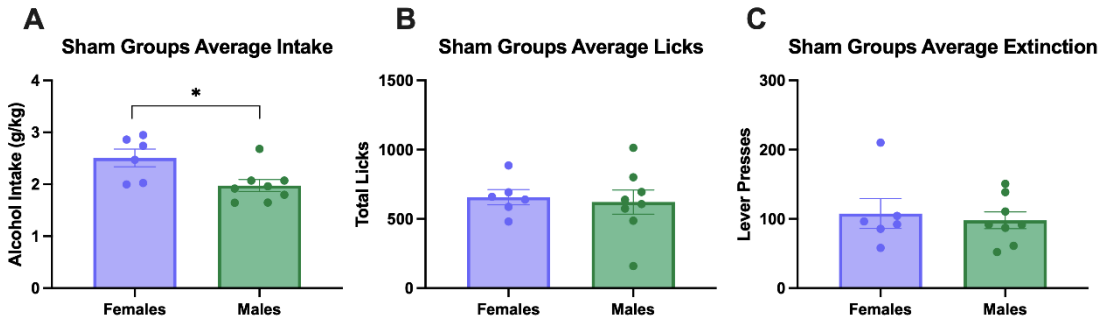
Number of Lever Presses During Extinction Sessions in Females and Males



Note. Number of lever presses during probing extinction sessions. Rats had no access to alcohol during these 30-minute operant sessions. A. Lever presses during the three extinction sessions in females. B. Lever presses during extinction sessions in males. C. There were no group differences in female rats when lever presses were averaged across the three sessions. D. No differences in averaged lever presses in the males.

Figure 9

Sex Differences Comparison Across Female and Male Sham Animals



Note. Sham female and male rats compared across average intake during testing, average licks during testing, and average lever presses during extinction. A. Female Sham rats drank more alcohol than male sham rats. B. There were no differences between females and males in average number of licks. C. There were no differences between females and males in average lever presses during extinction.

Within-Session Analyses

To explore possible differences in drinking patterns or lever presses within one operant session, cumulative licks were calculated for the first and last day of alcohol testing and cumulative lever presses were calculated for the last day of extinction. A slightly modified version of the front-loading code provided by Ardinger et al. (2022) was used to assess front-loading behavior on alcohol test Days 1 and 12 using number of licks across the sessions. The 20-minute sessions were analyzed across one-minute bins. Similarly, lever presses from Extinction Day 3 were also run. Because the original front-loading code was optimized for longer drinking sessions, for this study Criterion 3 was modified so that an animal was classified as a frontloader if the pre-changepoint slope was greater than the post-changepoint slope, but it did not have to be significantly

greater. This allows a comparison of the number of front-loaders across groups, changepoints, and slopes from 0 to the changepoint. Table 4 shows the number of animals classified as frontloaders or non-frontloaders on Days 1 and 12 of alcohol testing.

Separate χ^2 tests were run on females and males comparing E2, T, and Veh animals analyzing licks on Days 1 and 12 and analyzing lever presses on Extinction Day 3. On Day 1, there were no significant differences between the groups in the likelihood of animals being frontloaders. On Day 12, there were no significant differences between the groups in likelihood of animals being frontloaders. A McNemar test was run comparing Day 1 and Day 12 in change of group membership (i.e. frontloader or non-frontloader) and no significant difference was found. This suggests that while there were more non-frontloaders on Day 12 there was not a significant difference in animals classified as non-frontloaders between the two days. For Extinction Day 3 there were no differences between the groups as all animals were classified as frontloaders.

Changepoints and slopes were both analyzed using 2 (sex) x 3 (group) x 2 (frontloader) ANOVAs for Days 1, 12, and Extinction Day 3. Day 1 changepoint analysis had an expected significant difference between frontloaders and non-frontloaders, $F = 21.66$, $p < .001$, but there were no differences across sex or group and no significant interaction effects. A similar pattern was found when analyzing slopes for Day 1 where frontloaders and non-frontloaders showed a significant difference, $F = 4.27$, $p = .047$, but there were no other significant differences. There also were no significant differences between female and male Sham animals on Day 1 for slope or changepoints. It is expected that frontloaders, compared to non-frontloaders, would have earlier changepoints and higher slopes which is what was seen in the current study. See Figure

10 for females, males, and Sham animals' licks across Day 1. Note that due to the low number of non-frontloaders, data are presented collapsed across frontloaders and non-frontloaders. Although E2 males consumed more alcohol and had more licks overall on Day 1, their pattern of intake was similar to Veh and T animals.

Day 12 changepoints showed main effects of group, $F = 3.91$, $p = .030$, sex, $F = 7.45$, $p = .010$, and frontloaders, $F = 64.41$, $p < .001$, and had a significant sex x group interaction, $F = 3.68$, $p = .036$. To explore this significant interaction, separate ANOVAs were run for each sex. In females, there was a significant effect of frontloader, $F = 40.02$, $p < .001$, but no significant effect of group. In males, there was a significant effect of frontloader, $F = 31.371$, $p < .001$, and a significant effect of group, $F = 10.44$, $p < .001$. Post hoc analyses in the males showed that all groups were significantly different from one another with T males having the earliest changepoint, followed by Veh, then E2 males. All T males were frontloaders on Day 12 which may be skewing the results to an earlier changepoint as the other two groups average overall changepoints would also include non-frontloaders. However, when only frontloaders were included, there was still a significant effect of group, but post hoc analyses showed that while Veh and T males were not significantly different, E2 animals had later changepoints than both T and Veh groups. Day 12 slopes had a significant main effect of frontloaders, $F = 5.70$, $p = .023$, and a significant sex x group interaction effect, $F = 3.72$, $p = .035$. Follow-up ANOVAs run for both females and males showed that males had a significant effect of group, $F = 5.16$, $p = .017$. Post hoc tests showed that T males were significantly different from both E2 and Veh animals. When an additional analysis was run including only frontloaders, it was found that T males were only significantly different from E2 males. There were no

significant differences in changepoints or slopes between the Sham females and males. See Figure 11 for licks across the session for female, male, and Sham animals. Earlier changepoint and higher slope typically represent heavier front-loading in those animals. These results suggest that in the males while there is no difference between the number of frontloaders in each group, front-loading E2 animals may be front-loading in a different pattern (e.g., less intensely) compared to T animals on Day 12 as they had later a changepoint and a lower slope.

Changepoints and slopes were also calculated on Extinction Day 3 lever presses to possibly classify the rate of lever presses across the session. For E2, T, and Veh groups, all animals were classified as frontloaders and only 1 Sham female in all of the groups was classified as a non-frontloader. There were no significant differences between sex or group and no significant interaction of sex x group for either changepoint or slope analyses comparing E2, T, and Veh animals. There were also no significant differences in changepoint or slope between Sham females and males. See Figure 12 for lever presses across the session for female, male, and Sham animals. As an additional measure of seeking behavior, latency to first lever press during Extinction Day 3 was compared using a 2 (sex) x 3 (group) ANOVA which revealed no significant differences between sex or group and no significant interaction of sex x group. There were no differences between females and males or treatment groups in how fast they pressed the lever the first time.

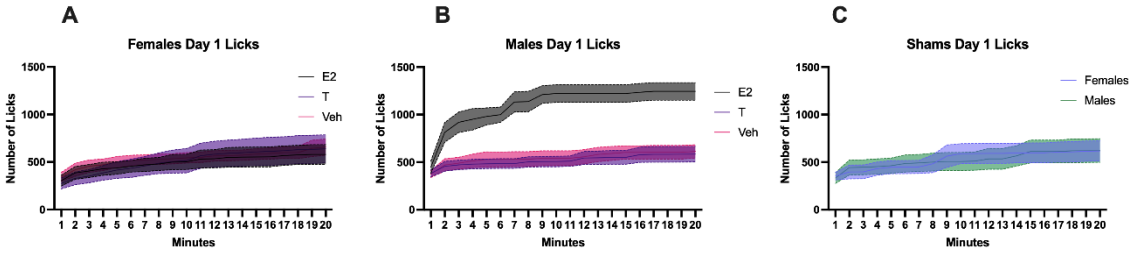
Table 4

Number of Frontloaders and Non-Frontloaders for Alcohol Testing Days 1 and 12

	Day 1							
	Females				Males			
	E2	**T**	**Veh**	**Sham**	**E2**	**T**	**Veh**	**Sham**
Frontloaders	7	5	5	6	7	5	7	4
Non-Frontloaders	1	2	1	0	0	2	2	4
	Day 12							
	E2	**T**	**Veh**	**Sham**	**E2**	**T**	**Veh**	**Sham**
Frontloaders	5	5	3	5	5	7	6	4
Non-Frontloaders	3	2	4	1	2	0	3	4

Figure 10

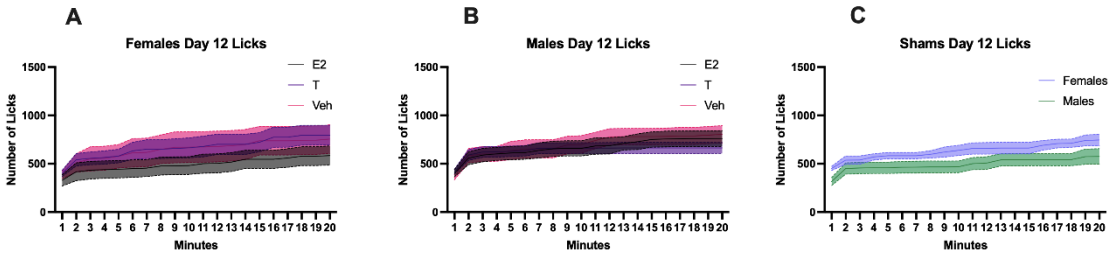
Cumulative Licks Across Session for Alcohol Testing Day 1



Note. 20-minute session shown in one-minute bins. Lines represent the average and shaded regions represent standard error of the mean. A. There were no differences between the groups in front-loading behavior in the females. B. Although the E2 males drank more alcohol, there were no differences in drinking patterns between the groups. C. There were no differences between the Sham females and males.

Figure 11

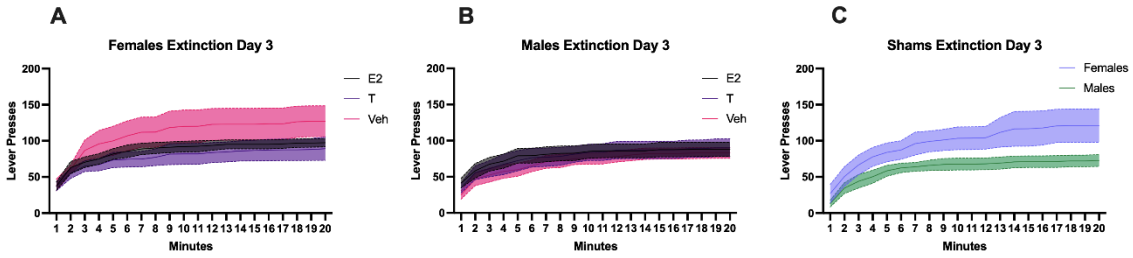
Cumulative Licks Across Session for Alcohol Testing Day 12



Note. 20-minute session shown in one-minute bins. Lines represent the average and shaded regions represent standard error of the mean. A. There were no differences between the groups in front-loading behavior in the females. B. E2 males had later changepoints compared to T and Veh males. T males had higher slopes compared to Veh and E2 males. E2 males may have demonstrated less intense front-loading behavior while T males may have demonstrated heavier front-loading. C. There were no differences between the Sham females and males.

Figure 12

Cumulative Lever Presses Across Session for Extinction Day 3



Note. 20-minute session shown in one-minute bins. Lines represent the average and shaded regions represent standard error of the mean. A. There were no differences between groups in rate of lever presses across the session in females. B. There were no differences between the groups in males. C. There were no significant differences between the Sham females and males in lever pressing behavior.

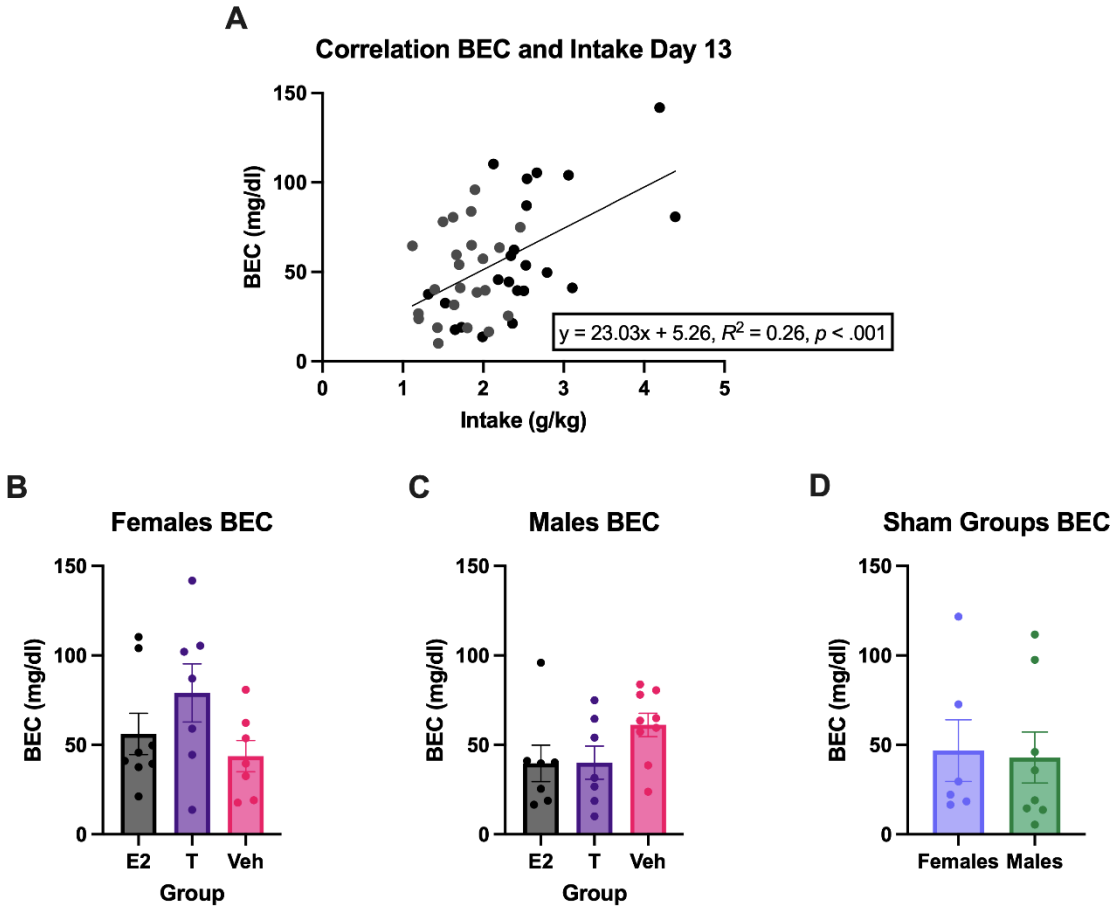
Blood Ethanol Concentration

Intake on day 13, the day of blood draws, was examined with a 2 (sex) x 3 (group) ANOVA. Similar to intakes across other testing days, there was a significant main effect of sex, $F(1, 39) = 17.74$, $p < .001$, $\eta^2_p = .31$, where overall females drank more than males, but there were no differences in alcohol intake between groups. In the E2, T, and Veh groups, as shown in Figure 13A, BEC and intake on day 13 were positively correlated, $R = .51$, $p < .001$, such that BEC increased as intake increased.

A 2 (sex) x 3 (group) ANOVA analyzing BEC levels found a significant interaction between sex x group, $F(2, 39) = 3.59$, $p = .037$, $\eta^2_p = .16$. However, when split by sex there was not a significant effect of group in either the females (Figure 13B) or the males (Figure 13C). A *t*-test comparison of female and male Sham animals did not show any significant differences between them (Figure 13D). Although females drank more alcohol than males, they have similar BEC levels.

Figure 13

Blood Ethanol Concentration



Note. Blood ethanol content (BEC) taken on day 13 after a 30-minute operant session with 20% ethanol. Overall, female rats drank more alcohol on day 13 than male rats. Males are represented by grey dots. A. There was a positive correlation of BEC with alcohol intake. Only E2, T, and Veh groups are included. B. There were no significant group differences in BEC levels in the female rats. C. There were no significant differences between the male rats. D. There were no significant differences between Sham female and Sham male rats.

Discussion

The current study investigated the role circulating gonadal hormones play in alcohol seeking and drinking in P rats. It was found that overall female rats consumed more alcohol than male rats, and this effect was found in both Sham and GDX animals. However, there were no differences seen between females and males in alcohol seeking. In addition, contrary to the hypotheses, there were also no differences between E2, T, and Veh groups in drinking or seeking behaviors. This study is one of the first investigating gonadal hormones in a selectively bred line of rats that prefer to drink alcohol and is unique in that it included both females and males in each of its hormone treatment groups. The activational effects of gonadal hormones may have limited impact on alcohol-related behaviors in P rats but further research is needed to explore possible organizational effects and other possible explanations for the sex difference in alcohol intake.

Sex Differences in Alcohol Use

In humans, women have been shown to demonstrate greater cognitive impairment and achieve higher BECs than men after the same amount of alcohol (Mumenthaler et al., 1999). It has also been reported that despite the higher prevalence of heavy drinking in men (at the time of the study), women that were heavy drinkers experienced more severe health outcomes including increased rates of mortality and liver cirrhosis (Jarque-Lopez et al., 2001). Female rodents often consume more alcohol than males (de la Torre et al., 2015; Li et al., 2019; Sneddon et al., 2020). It should be noted however that some have found no differences in alcohol intake (Albrechet-Souza et al., 2020; Fulenwider et al., 2019). The results of the current study found that females had higher alcohol intakes

during both training and testing. This sex difference persisted after hormone treatment and so elevating estradiol or testosterone did not impact female rats drinking more than male rats in the current study. Despite females drinking more than males on the final day of drinking there were no difference in BECs between the two sexes. This is not completely surprising at it is known females have a more rapid metabolism of alcohol compared to males (Maddern et al., 2024). The BEC levels were not quite as high as anticipated based on the g/kg intake data; however, the animals were run in the dark cycle so factors like food intake could be impacting these results. There were no sex differences in alcohol-seeking behavior as measured by lever presses in extinction sessions. Alcohol-seeking behaviors have been less consistent in finding sex differences in that some studies have found female animals show greater seeking behavior, such as demonstrating more habit-like behavior when looking at latency to lever press after conditioned taste-aversion (Haines & Czachowski, 2022), while others have found no differences (Randall et al., 2017). It is likely that there are different underlying processes driving drinking and seeking behaviors which may help explain the inconsistencies of sex differences being found. Different methodologies across studies also likely contribute to differences in findings.

The seeking/consummatory operant paradigm used in this study has been well established (Czachowski et al., 2001; Czachowski & Samson, 1999; Samson et al., 2001), but it has often only tested male subjects. McCane et al. (2018) used female and male Wistar and P rats and found that females showed higher drinking and seeking in both strains (although note this direct comparison was not statistically tested). However, in a similar paradigm that validated a probing extinction trial using Long Evans rats, no sex

differences were found in seeking behavior (Ortelli & Weiner, 2024). Additional studies comparing females and males, particularly P rats, in this paradigm would provide further evidence about sex differences in both operant alcohol drinking and seeking.

Both McCarthy et al. (2012) and Sanchis-Segura and Becker (2016) have put forth more clear operational definitions of different types of sex differences that may be encountered in research. The first type is sexual dimorphism (Becker: dichotomous sex differences) which would represent two separate endpoints of the sexes such that a behavior is present in one sex and not in the other. The second is sex difference (Becker: average sex differences) where a behavior exists on a continuum and the average is different between females and males. The third is sex convergence and divergence where a behavior may be the same in females and males but the causes and neuronal underpinnings for the behavior are different. Sanchis-Segura and Becker (2016) also have a fourth distinction called number sex differences in reference to the frequency females and males exhibit a behavior that they may both exhibit. These frameworks provide a useful foundation and important consideration for future studies. For example, the differences in intake levels in the current study represent a sex difference, but there may be a sex divergence in alcohol-seeking behavior such that although both females and males demonstrate it, different underlying processes could be contributing to alcohol seeking. Elucidating these mechanisms in female and male P rats could also uncover differences in how gonadal hormones may be modulating this behavior.

Along with the different strategies to consider how to study females and males in preclinical research there is also a growing movement to shift the conceptualization of "sex" away from a simple binary variable and move towards considering the sex

variables of importance (Massa et al., 2023). The variables of interest in the current study were estradiol and testosterone and their impact on alcohol seeking and drinking. By studying these possible effects in both females and males it helped to provide a more thorough and clearer picture of each hormone's actions. Future studies and analyses may find it beneficial to reframe the category of sex after GDX and hormone treatments which could provide further information about how these hormones impact behaviors across different genetic chromosomal backgrounds. For example, GDX females and males given E2 may have a similar profile of circulating gonadal hormones (i.e., high estradiol and low testosterone) and so it could be appropriate to group these animals together for analyses. The current study also used the same dose of each hormone for females and males, future studies might consider using different doses of these hormones across the two sexes for more accurate physiologically levels which could lead to different behavioral outcomes.

Activational/Organizational Effects of Gonadal Hormones

Hormone Levels

Based on the ELISA data in the current study there was inconclusive evidence that GDX reduced estradiol levels as the Veh group showed similar levels to the Sham group. Surprisingly, there were similar estradiol levels between females and males. Male rodents (and humans) often have lower estradiol levels compared to females (Nilsson et al., 2015). Adult female Wistar rats' estradiol levels have been reported to be as low as 35 pg/ml (Nilsson et al., 2015) and as high as 174 pg/ml (Montagnini et al., 2016) with other strains typically showing levels within that range, although also note that estradiol levels fluctuate across the estrous cycle. It is possible that differences in estradiol levels

across studies could be due to the techniques used to measure hormone levels (Morales & Spear, 2013). Estradiol levels shown in the current study for the Sham females are higher than those usually reported. It should be noted that in the current study, based on the dilution requirements of the assay, the serum samples fell on the lower end of the standard curve which may have led to inflated or inaccurate readings. Many studies that look at alcohol-related behaviors after manipulating gonadal hormones do not report estradiol levels. However, some have found a limited reduction of estradiol after OVX (Morales & Spear, 2013). It is known that E2 is produced by other organs in the body including the brain and the adrenal glands. The liver and kidneys also impact levels of gonadal hormones through metabolism and rate of excretion, respectively (de Vries & Forger, 2015). One possible explanation for the high estradiol levels in both the current OVX females and the Sham males is that P rats are producing estradiol at higher than usual rates from these other organs.

For testosterone, many of the groups did not have detectable levels except for T treated animals. The testosterone levels in the male T group were higher than typically reported in male rats (Heywood, 1980) and the females also had higher than typical physiological levels (Falvo et al., 1972). Male rats have been shown to have variable testosterone levels that cycle throughout the day and the range in Wistars has been reported to be between 0.58 ng/mL and 13.84 ng/mL (Heywood, 1980). Two of the male Veh animals also had detectable testosterone levels that when averaged appeared slightly supraphysiological. This detection of testosterone could imply that CAST was unsuccessful for those 2 animals, but the results did not change when they were removed from analyses and, therefore, they were kept in the study. Finally, the Sham males did not

all have detectable levels of testosterone, but those that did were in the range of typical male rat levels. The lack of detection in the entire Sham group of male animals may have been due to the dilution rate being too great which caused the levels to fall out of range for the standard curve. This factor may have worked in combination with it previously being noted that only males in an ultradian surge will have detectable testosterone levels (Dalla et al., 2024). The ELISA data cannot confirm that GDX reduced circulating gonadal hormones in either the females or the males (however, the change in weights across the experiment do somewhat replicate what would be expected of each of the treatment groups). Regardless, the ELISA data show that estradiol levels were higher in the E2 treated groups compared to Shams and that testosterone was more likely to be detected in the T treated groups suggesting that hormone treatments elevated respective levels of these gonadal hormones.

Estradiol

Females consumed more alcohol than males in the training phase. Post-surgery, Sham females drank more than males and this effect was also seen in the GDX groups during testing. Giving E2 did not increase drinking or seeking in female or male rats compared to Veh animals. Previous studies have concluded that E2 may be modulating female drinking behaviors by using OVX to reduce circulating estradiol which then reduced alcohol drinking (Sneddon et al., 2023). Some found this effect was reversed when OVX animals were given E2 treatment (Ford et al., 2002a; Satta et al., 2018) suggesting that circulating E2 contributes to alcohol drinking in female rodents. Circulating E2 levels have also been positively associated with alcohol consumption in women (Martel et al., 2017). It should be noted that others have found that OVX had a

limited impact on female's alcohol drinking (Cailhol & Mormede, 2001; Vetter-O'Hagen & Spear, 2011) and some have reported a decrease in drinking after OVX rats were given E2 (Almeida et al., 1998; Hilakivi-Clarke, 1996; Sandberg & Stewart, 1982). There may be a ceiling effect in females in which further elevating E2 does not impact drinking behavior. For example, one study found that increasing the dose of E2 to supraphysiological levels had no additional impact on alcohol drinking in Long Evans rats (Ford et al., 2004). The current study may be seeing a similar phenomenon. Others have found that an injection of estradiol valerate weeks prior to alcohol drinking in intact rats increased alcohol intake (Marinelli et al., 2003; Reid et al., 2003), an effect that was mimicked in OVX animals (Quirarte et al., 2007). However, exogenous E2 treatment does not appear to impact alcohol seeking or drinking in female P rats.

In male P rats, the current data would suggest that increasing E2 levels does not increase drinking or seeking behavior. There have been few studies conducted that have given E2 to males to examine alcohol-related behaviors. A previous study found that intact male mice given E2 treatment increased alcohol drinking (Hilakivi-Clarke, 1996), but others have found that E2 decreased alcohol intake in intact (Messiha, 1981) and CAST male rats (Juarez et al., 2002). Another study found opposite effects of E2 injections in CAST male rats depending on the timing of injections. It was found that animals treated with E2 for 6 days without access to alcohol then given an oil vehicle during 6 days of alcohol access drank more than their pretreatment alcohol drinking (Juarez et al., 2005). A similar pattern was seen in males injected with E2 over the whole 12 days but not in those given oil then E2 who demonstrated a decrease in intake (Juarez et al., 2005). Intact male rats given estradiol valerate showed a decline in drinking 5-days

post injection, but this trend was also seen in female rodents who then went on to show an increase in drinking 1-month post injection (Reid et al., 2002). More studies are needed to further investigate the role E2 is playing in alcohol drinking in male rodents. The time course and injection schedule may play an important role in the effects E2 is having in male rodents. For example, the current study showed that males given E2 showed an increase in alcohol drinking on the first day back in the operant boxes after hormone treatment. Future studies may want to narrow in the focus to further explore this possible first day effect in the males possibly by utilizing different injection schedules and analyzing more specific timepoints.

Testosterone

Testosterone levels in the current study were hard to interpret because as mentioned previously, generally only the T treatment group showed detectable levels. However, the T treatment group was more likely to show detectable levels of T suggesting that testosterone was likely elevated in those groups. In both females and males, T treatment did not significantly impact alcohol seeking or drinking. Fewer studies have examined the effects of testosterone on alcohol-related behaviors. While some have found that CAST did not impact males drinking behavior (Cailhol & Mormede, 2001) others have found that males increased alcohol drinking (Vetter-O'Hagen & Spear, 2011). In addition, T treatment after CAST has been found to reduce drinking back to similar levels of Sham and intact males (Bertholomey & Torregrossa, 2019; Sherrill et al., 2011; Vetter-O'Hagen et al., 2011) suggesting testosterone may be modulating alcohol drinking in males in the opposite direction estradiol is modulating this behavior in females. Although the interaction of sex x group was not significant for intake during testing, there

was a trend in the males when looking at average intake split by sex where the T group drank less than Veh and E2 groups ($p = .053$, Figure 4). This does leave room for the possibility that in the P rats T decreases alcohol drinking in a sex-specific manner. To the author's knowledge, no studies have examined the role testosterone may be having in alcohol-related behaviors in adult female rodents (although see below for manipulations in prenatal environment). Based on the current data, it seems that increasing testosterone levels in either the female or male P rats does not significantly change their alcohol drinking or seeking compared to Veh animals.

Within-Session Effects

To examine possible impacts estradiol and testosterone could be having on other alcohol-related behaviors, within-session analyses were conducted on the first and last alcohol testing day and the last extinction day. Front-loading behavior was assessed using criteria previously described in Ardinger et al. (2022). Although more specific to drinking patterns, front-loading analysis was also applied to lever presses during extinction. Overall, across all of the days measured there were no differences between groups in the number of frontloaders in either the females or the males. With the exception of the Sham males who had an equal number of frontloaders and non-frontloaders on days 1 and 12, a majority of the animals demonstrated front-loading behavior. When analyzing changepoints and slopes across the groups, there was a difference seen on Day 12 in the male rats. T males had the lowest changepoint and the highest slope, while E2 males demonstrated the opposite. This finding suggests that while there were no differences in the number of frontloaders between the two groups, the T animals may have demonstrated more intense front-loading behavior compared to E2 animals. Interestingly,

although E2 males had higher licks on Day 1 than the other two groups, there was not a significant difference in changepoints or slopes suggesting that the drinking patterns across the treatment groups did not differ. It appears that elevating testosterone or estradiol had a limited impact on overall front-loading behavior but may have had a more nuanced sex-specific effect on the level of front-loading, particularly later in alcohol testing. There has been limited research conducted on both females and males in front-loading behavior (Ardinger et al,, 2022) and more research is needed to further explore sex differences in this behavior and to establish what role gonadal hormones may be playing.

Extinction Day 3 showed that all animals, with the exception of one Sham female, frontloaded lever presses. Elevating estradiol or testosterone levels did not appear to impact changepoints or slopes in either sex. In addition, latency to first lever press was also not impacted by hormone treatment. Similar to the overall seeking behavior measured, neither estradiol nor testosterone treatment appear to impact the timing or rate of lever presses during extinction.

Activational/Organizational Effects

The current study would suggest that elevating gonadal hormone levels does not change alcohol-related behaviors. One possible explanation for this is that circulating gonadal hormones do not have a large influence on drinking behavior in the P rats and that more studies are needed to elucidate the cause of the differences seen between females and males. For example, although the activational effects of gonadal hormones may not be having a strong influence on drinking behavior, gonadal hormones may be having an impact via organizational effects. Future studies could adjust the timing of

gonadectomy to before puberty and/or during early development to examine possible organizational effects of gonadal hormones on the brain in P rats. It has been found that females that were neonatally estrogenized (manipulating organizational effects so they mimicked males) drank less alcohol than intact females, although not to the level of intact males (Almeida et al., 1998). In order to examine the effect of prenatal hormone environment on drinking behaviors, Huber et al. (2018) treated pregnant CD1 dams with dihydrotestosterone (DHT; an active T metabolite) or flutamide (an androgen receptor antagonist) and then tested their adult offspring for alcohol-drinking behavior. The authors concluded that prenatal androgen receptor activity impacts adult alcohol drinking such that activating androgen receptors in females increases alcohol drinking, while inhibiting androgen receptor activity increases drinking in males (Huber et al., 2018). Prenatal androgen exposure, quantified by a 2-digit to 4-digit ratio, was found to be higher in alcohol-dependent men compared to healthy men (B. Lenz et al., 2017). This may demonstrate an opposing effect of prenatal androgen exposure in rodents compared to humans, but it is also important to remember different processes of masculinization across species. Masculinization is thought to rely not only on testosterone but also on the aromatization of estradiol from testosterone (Wallen & Baum, 2002) in rodents. However, in rhesus macaques (and likely humans), masculinization relies solely on androgens (Thornton et al., 2009).

It is very likely that organization and activational effects of hormones are both contributing and interacting to influence alcohol behaviors. In the field of reproductive and sexual behavior these types of interactions have already been seen. Rats have a critical period of sexual differentiation that begins around embryonic day 18 and

continues until about PND10 (McCarthy, 2008). During this time females exposed to androgens can exhibit masculine sexual behaviors if exposed to androgens again in adulthood. This sensitivity to androgens is an example of organizational effects because it permanently changes the way the brain is organized and will have long-lasting impacts on how an animal responds to hormone levels. So while circulating hormones in adulthood are necessary for sexual behaviors (activational), their effects are dependent on gonadal hormone exposure during development (organizational; McCarthy, 2008). The hormonal milieu in development limits the response of the adult brain. For example, an adult male given female-typical hormones will have a much-reduced ability to show female sexual behavior because his brain was organized as male (Lenz & McCarthy, 2010). The sex difference that exists in alcohol drinking in P rats in the current study, even across the same treatment groups, may be explained by examining the timing and specific roles gonadal hormones are playing.

Other Hormonal Contributions

While testosterone and estradiol are often the focus when analyzing gonadal hormones, there are other hormones and neurosteroids that could also be influencing behaviors. For example, progestogen, including progesterone and allopregnanolone, have been correlated with alcohol liking and consumption (Peltier et al., 2021) and have been shown to influence habitual behavior for sucrose (Schoenberg et al., 2022). While progestogens have also been shown to have limited impact on alcohol drinking in female rodents, there has been a report of increased drinking in male rodents (Erol et al., 2019). Specifically in male P rats, ganaxolone, a synthetic neurosteroid, increased alcohol responding at a low dose but decreased responding for alcohol at a higher dose (Besheer

et al., 2010). Higher doses of pregnanolone also showed a similar pattern of decreased responding for alcohol (Besheer et al., 2010). Although not in P rats, a similar pattern of a biphasic effect of allopregnanolone was found in Long Evans rats where lower doses increased operant responding while higher doses depressed operant responding (Janak et al., 1998). Another study in P rats found that an acute injection of pregnanolone decreased alcohol intake and preference, but these results diminished after chronic treatment of 10 days (Rezvani & Levin, 2014). There may be an interaction with alcohol history and progestogens in the P rats as one study found that after a 2-week alcohol history, allopregnanolone increased alcohol consumption but reduced it after a 6-week alcohol history (Morrow et al., 2001). In female P rats, injections of allopregnanolone into the nucleus accumbens core reduced alcohol self-administration, but there were no significant differences when it was injected into the VTA (Ornelas et al., 2023). In another drug-related behavior, progesterone inhibits escalation of cocaine self-administration in female Wistar rats (Larson et al., 2007). Progestogens and their neurosteroid metabolites are likely acting on alcohol drinking behaviors and could provide a future avenue for possible treatment options of AUD (Finn, 2023). It is also known that females have higher basal levels of allopregnanolone that are estrous cycle dependent (Finn, 2023). The current study did not measure progestogen levels, but future studies may find interactions between estradiol, testosterone, and progestogens in the P rats that could elucidate causal mechanisms that could explain a sex difference in alcohol drinking.

Species considerations are likely necessary when attempting to translate preclinical findings into clinical outcomes. In humans, most circulating androgens and

estrogens are bound to transport proteins including sex hormone-binding globulin (SHBG) and albumin (Kovacs & Ojeda, 2012). Rodents do not have SHBG which could be an important factor to consider when extrapolating effects of gonadal hormones from rodents to humans. Differences in hormone levels were found between people with alcohol dependence and controls, but these were at times dependent on whether measuring total hormone levels of bioavailable levels (Ho et al., 2023). Furthermore, SHBG appeared to moderate gonadal hormone associations in both sexes (Ho et al., 2023). A mediating effect of SHBG would obviously not be detected in a rodent model and these considerations may prove important when creating future animal models.

Alcohol's Impact on Gonadal Hormones

An important consideration when discussing whether gonadal hormones influence alcohol-related behaviors is the inverse relationship of how alcohol may be impacting these hormones. It is known that alcohol can impact the brain, particularly drinking that occurs in adolescence (Jordan & Andersen, 2017; Meruelo et al., 2017). For example, girls who drank in adolescence may show changes in white matter (Bagley et al., 2019). However, recently others have found a lack of increase in adulthood alcohol consumption after adolescent exposure in both rats and mice which may suggest more nuanced interactions of variables and some that may be specific to humans (Sicher et al., 2024). Alcohol exposure impacts the HPA axis and influences stress hormones such as corticotropin releasing factor (Allen et al., 2011). Alcohol also impacts the hypothalamus-pituitary-gonadal (HPG) axis by inhibiting gonadotropin releasing hormone, which decreases testosterone secretion in males and estradiol in females (Devaud et al., 2006). Alcohol may also increase testosterone and estradiol levels in

women (Erol & Karpyak, 2015; Lenz et al., 2012) and some women who drink persistently stop menstruating (Devaud et al., 2006). Therefore, the directionality in which alcohol impacts these gonadal hormones in each of the sexes is not always consistent. The effects of alcohol on hormone levels and the HPG axis might create difficulties in establishing directionality in behavioral differences. This is an especially important consideration in studies using humans that correlate hormone levels with alcohol use.

There is additional evidence that alcohol impacts gonadal hormones. In male alcohol-preferring (AA) and non-preferring (ANA) rats, a higher dose ethanol injection (1.5 g/kg) decreased testosterone in both lines at both a morning and afternoon injection timepoint (Apter & Eriksson, 2003). Ethanol's effects on testosterone levels may not be related to selection based on alcohol preference since a decrease was seen in both preferring and non-preferring animals. Alcohol history may play a role as heavy drinking in men has also showed a decrease in testosterone levels, however in men without AUD, testosterone levels increased after 2-3 standard drinks (Sarkola & Eriksson, 2003). In select lines of mice, withdrawal seizure resistant (WSR) or withdrawal seizure prone (WSP), abstinence from alcohol revealed increases in testosterone levels in both sexes but only in WSR mice (Forquer et al., 2011) suggesting an interaction of sensitivity to withdrawal and testosterone levels. Higher androgen signaling activity in females could also be contributing to sex differences during withdrawal (Forquer et al., 2011). Binge-like drinking has also been shown to increase estrogen receptor 1 (Esr1) gene transcription in the VTA of both females and males, but only females showed an increase in the prefrontal cortex (Chen et al., 2022). There could be sex-specific and region-

specific effects of alcohol on steroid receptors. Alcohol is likely impacting gonadal hormone and receptor levels in rodents as well as humans in several different ways. In the current study, animals were trained with alcohol for several weeks prior to GDX and hormone treatments. Alcohol may have been impacting gonadal hormone levels, particularly in the Sham animals, although it is unclear what direction this effect would be in. The impact on alcohol on gonadal hormones has not been thoroughly investigated in the P rats and unique interactions might be occurring. The current study could have benefited from a group that had GDX prior to alcohol exposure. Future studies using P rats could more specifically examine the impact of alcohol on gonadal hormones and their respective receptors which would further the literature at the intersection of genetics, alcohol, and endocrinology and may be translationally relevant to an individual with a positive family history of AUD.

Gonadal Hormones and the Brain

Gonadal hormones are known to have specific roles in the brain and modulate various processes. There are various brain regions of note when examining the three stages of addiction (Koob & Volkow, 2016). These include the frontal cortex and hippocampus (preoccupation/anticipation stage), basal ganglia and mesocorticolimbic pathways (binge/intoxication stage), and the extended amygdala (negative affect/withdrawal stage). Sex differences exist within each of these stages and associated brain systems (Flores-Bonilla & Richardson, 2020) and gonadal hormones may also be impacting these brain regions of interest in alcohol-related behaviors. Estradiol and progesterone directly regulate GABAergic inhibition and interact with other factors such as stress in brain regions like the basolateral amygdala (Price & McCool, 2022). OVX

rats show a decrease in dopamine release in the nucleus accumbens, and estradiol infused directly into the nucleus accumbens resulted in an increase in dopamine release (Thompson & Moss, 1994). Estrogens can impact mesolimbic dopamine neurons, a pathway commonly studied with alcohol-related behaviors. Estradiol has been shown to enhance the response of dopamine neurons to alcohol in the VTA which could contribute to the enhanced rewarding effects of alcohol in female mice (Vandegrift et al., 2017). In addition, estradiol influences ethanol-stimulated dopamine release in the prefrontal cortex of female rats as OVX rats showed reduced dopamine release (Dazzi et al., 2007; Vandegrift et al., 2020). In the nucleus accumbens, ERα modulate miniature excitatory postsynaptic currents in medium spiny neurons female rats (Miller et al., 2023). Estradiol has also been shown to facilitate cocaine self-administration by interacting with mGluR5 (Martinez et al., 2016). Estrogens may also be regulating serotonergic neurons in the dorsal raphe nucleus by inhibiting ethanol-induced burst firing which could lead to higher binge drinking seen in female mice (Torres Irizarry et al., 2024). There may be behaviors more specific than overall alcohol intake that would be more sensitive to elevated gonadal hormones that were not captured in the current study.

While there are likely unique brain regions controlling the appetitive and consummatory behaviors studied in the current paradigm, the current study did not attempt to directly manipulate or measure any processes within the brain. Exploring specific pathways in the brain that relate to both alcohol seeking and drinking with and without hormone treatment could provide insight into changes within the brain that are not being captured with current operant behavior. When examining inputs to the VTA from the medial preoptic area it was found there was no sex difference in ERα expression

in the medial preoptic area or in ERα expressing GABAergic neurons (Martz et al., 2023). There were more androgen receptors in the medial preoptic area and more androgen expressing GABAergic neurons in male Sprague-Dawley rats (Martz et al., 2023). A mapping of steroid receptors in the P rat brain may show sex differences in particular brain regions that could change pre- and post-alcohol exposure. This could direct future studies in additional possible behaviors that may be impacted by changes in gonadal hormones.

Androgens, estrogens, and other steroid hormones are synthesized in the brain, including in the mesocorticolimbic system (Tobiansky et al., 2018). For example, estradiol and testosterone are synthesized locally in the hippocampus (Hojo et al., 2004; Munetsuna et al., 2009). There could be discrepancies between the amount of hormone measured in serum or blood and the amount present in certain brain regions. 6 weeks after GDX, GDX males had no detectable levels of testosterone in whole blood but did show detectable levels in the VTA, nucleus accumbens, and the medial prefrontal cortex (Tobiansky, Korol, et al., 2018). Furthermore, in Sham animals there were different levels of testosterone across whole blood and those same three brain regions (Tobiansky, Korol, et al., 2018; Tobiansky, Wallin-Miller, et al., 2018). When discussing how gonadal hormones influence the brain and behavior it may become increasingly important to note localized levels of the hormones of interest. Steroid hormones may still be moderating specific brain regions via local production even if circulating levels of that hormone are diminished.

Genetic Factors

Sex Chromosomes

There is increasing evidence that sexual differentiation is not quite as straightforward as sex chromosomes lead to differentiation of gonads resulting in different levels of gonadal hormones (McCarthy & Arnold, 2011). While many sex differences can be explained by gonadal hormones, as was the focus of the current study, there are also genetic influences that could explain these differences (Arnold, 2004). The interest in how chromosomes might be influencing behaviors has led to the creation of several mouse models attempting to isolate chromosomal effects (Arnold, 2009; Majdic & Tobet, 2011). The Four-Core-Genotype (FCG) mouse model has been used in several studies to isolate the effects gonadal hormones and from their sex chromosome complement. The FCG mouse model deletes the *Sry* gene from the Y chromosome and inserting an *Sry* transgene onto an autosome in the same mice (Arnold & Chen, 2009). Because testis determination is on an autosome, XX and XY mice can be gonadally female or male resulting in 4 possible mice (XXF, XXM, XYF, XYM) split on the factors of sex (gonadal male vs gonadal female) and sex chromosome complement (XX vs XY) (Arnold & Chen, 2009). The FCG mouse model has provided a useful tool in sex differences research and has helped discover certain behaviors that are mediated by chromosomes.

There are sex chromosomal influences over alcohol-related behaviors which have been discovered through the FCG mice. Habit-like behaviors are influenced by chromosomal sex regardless of gonadal phenotype as XY mice develop habit faster for alcohol than XX mice (Barker et al., 2010). Interestingly, in that same study gonadal

females drank more 10% alcohol than gonadal males and this effect persisted after GDX (Barker et al., 2010). It should be noted that an earlier study found that XX mice develop habit faster than XY mice for a sucrose reward (Quinn et al., 2007). Sex differences in habit are likely mediated by sex chromosome instead of gonadal hormones, although this effect may be reinforcer specific. Sneddon et al. (2022) found effects of gonadal phenotype and chromosomal sex in a two-bottle choice procedure using four different concentrations of ethanol. At ethanol concentrations of 5% and 20%, gonadal females drank more than gonadal males, but at concentrations of 10% and 15%, XX mice drank more than XY mice. This study suggests that hormonal and chromosomal influences on alcohol drinking may differ based on concentration of ethanol. Chromosome complement has also been implicated in binge-like alcohol drinking, aversion-resistant drinking, and concentration-dependent responding for alcohol in an operant paradigm (Sneddon, Masters, Ream, et al., 2023). In contrast to the results above, for binge-like drinking it was found that XXM mice consumed less 15% alcohol than XYM, but there were no differences in the gonadally female mice (Sneddon, Masters, Ream, et al., 2023). An interaction of gonadal phenotype and sex was also seen in progressive ratio for cocaine where XYM mice were higher than all other groups (Martini et al., 2020). CAST XYM acquired cocaine self-administration at a faster rate than CAST XXM and OVX XYF, and E2 treatment suppressed this cocaine vulnerability in XY males (Le et al., 2023). Again, this suggests a likely interaction between gonadal hormones and sex chromosome complement moderation self-administration. Sex chromosomes have also impacted motivation for sweetened condensed milk (Seu et al., 2014) nociception (Gioiosa et al., 2008). While it is nearly impossible to disentangle gonadal hormone levels from sex

chromosomes in P rats, there is a possibility that female P rats drink more alcohol than male P rats due to sex chromosome compliment. Based on some of the differences found in the mentioned studies possibly due to methodological differences, it could be of interest to see how the FCG mice would perform in the operant paradigm used in the current study to see if there are sex differences driven by hormones or chromosomes.

Genetics and Epigenetics

It is known that heritability of AUD is around 50% (Verhulst et al., 2015) and that children of parents with AUD have an increased risk of also developing the disorder (Eng et al., 2005; Schuckit & Smith, 1996). This relationship emphasizes the importance of examining potential genetic risk factors in alcohol use. In contrast to humans, rodent studies have found that paternal ethanol exposure 8 weeks prior to mating reduces alcohol operant self-administration (Nieto et al., 2022) and the rewarding effects of alcohol as measured by lever presses in progressive ratio and reinitiation (Nieto & Kosten, 2023) in offspring. The authors suggest that examining more specific behaviors in the paternal rodents, such as motivation for alcohol or possible lingering withdrawal symptoms impacting mating, could help better algin the rodent work with the human literature (Nieto & Kosten, 2023). The P rats allow researchers to study a positive family history of alcohol use without the necessity of prior alcohol exposure in either parents or offspring which could control for some of the potential confounds listed previously. Using summary statistics from genome-wide associations studies (GWAS) in humans it was found that there are likely genetic correlations with alcohol-use traits and sex hormone levels that may differ between females and males (Waller et al., 2024). Although some of the genetic correlations conflicted with previously reported

associations of alcohol-use and gonadal hormone levels (Waller et al., 2024). When examining alcohol consumption from adolescence to young adulthood in a twin study (participants born in Norway) it was found that genetic factors influenced alcohol drinking moderately while shared environmental factors had a moderate to high influence and that these were gender-specific (Seglem et al., 2016). Selectively bred animal lines could help expand this research using preclinical models to study genetics, gonadal hormones, and possible sex differences in a setting where environmental factors can be more controlled.

Limitations and Future Directions

The current study did not monitor estrous cycles in Sham females. Levels of estradiol fluctuate across the estrous and menstrual cycle (Kovacs & Ojeda, 2012). While female animals being housed in the same room as males should have limited the synchronization of Sham rats' estrous cycles, knowing which stage they were in could have provided more context to frame the current results. Previous studies have not found an effect of estrous cycle on overall alcohol intake (Ford et al., 2002b; Priddy et al., 2017), although drinking microstructure may be affected as bout frequency was greater during proestrus but bout size was smaller (Ford et al., 2002b). People who menstruate have been shown to have increased attention to alcohol-associated cues (Griffith et al., 2024) and greater alcohol-induced disinhibition as measured by proportion of errors on a go/no-go task (Griffith et al., 2023) in the late follicular phase (when estradiol levels are elevated). Impacts of the menstrual cycle on alcohol drinking have not been universally seen across the literature and determination of cycle phase has been done using inconsistent methodology from study to study (Warren et al., 2021). Remembering that

consideration, it has recently been found that in the late luteal phase (when progesterone-to-estradiol ratios are higher) women had a lower probability to binge drink and a higher progesterone-to-estradiol ratio was associated with a lower probability to binge drink in men (Hoffmann et al., 2024). Steroid hormone ratios are likely an important future direction in examining the effects of gonadal hormones on alcohol. The current study did not measure progesterone levels, and many animals did not show detectable testosterone levels therefore hormone level ratios were not calculated. It is possible that the relationship of these hormones is more important than their individual levels.

Changes in weights and hormone levels using ELISA assays were used as manipulation checks in the current study but were inconclusive in establishing if GDX successfully altered serum gonadal hormone levels. A possible contributing factor to this inconclusive evidence may have been low levels of the hormones in the serum samples which could have led to inaccurate readings. In the future, using extraction procedures on the serum samples may lead to better detection of both estradiol and testosterone even when it is present at low levels (Dighe & Sluss, 2004). In addition, future studies may want to include further manipulation checks such as postmortem dissections to confirm successful removal of the gonads or comparing uterine weights across females (Lemini et al., 2015). Although these dissections would only confirm if GDX was performed successfully and are not a direct measure of if gonadal hormone levels significantly changed. Having a clearer picture of how the hormones were specifically manipulated would provide better context to frame the behavioral results.

When looking at previous literature that examined the effects of gonadal hormone manipulation on alcohol intake in rats (see Table 1) most studies used a 2-bottle choice

homecage methodology instead of an operant paradigm and almost none used a concentration of alcohol as high as 20%. In a study using a different line of selectively bred alcohol preferring rats (sP), OVX reduced drinking in 2-bottle choice, but there were no differences in alcohol intake seen in an FR4 operant paradigm (Lorrai et al., 2019). The current study may not have shown the expected impact of elevated gonadal hormone levels due to the chosen methodology. Circulating gonadal hormones may be having an impact specifically in home cage drinking or at lower concentrations of alcohol. Future studies may want to adjust the concentration of alcohol used in the current paradigm to see if there are differences in the results and may also want to analyze the effect gonadal hormones could be having on home cage drinking in the P rats at different concentrations of alcohol. Table 1 had an intentional focus on rats specifically, as opposed to also in mice, as species differences are known to occur in other areas of sexual differentiation (Bonthuis et al., 2010). As the field grows to include more studies examining gonadal hormones and alcohol-related behaviors, it may become important to analyze and monitor any species differences.

This study analyzed circulating gonadal hormones and as such much of the focus has been on gonadal hormones and adjacent topics. There are of course numerous other possible explanations that could explain a sex difference between female and males that could be working independently of and interacting with gonadal hormones. As one example, a sex-specific effect of stress on relapse-like self-administration was found (Logrip & Gainey, 2020). There are likely sex-specific effects of factors such as stress that could be impacting the current results. In addition, interactions with other organs and the peripheral nervous system could be occurring that would provide alternative

explanations of the current and future results (de Vries & Forger, 2015). Sex differences in the brain and behavior result from several factors including gonadal hormones, chromosomes, neonatal, and early-life experiences (McCarthy et al., 2009). Future studies may want to incorporate these other possible explanations as they relate to gonadal hormones and beyond.

Conclusion

The current study examined the effects of estradiol and testosterone on alcohol seeking and drinking in a line of rats selectively bred to prefer alcohol. It utilized a unique study design where females and males both received either estradiol or testosterone hormone treatments in order to study the effects of these hormones within each sex. Female P rats consumed more alcohol than male P rats, an effect which was found in both Sham and GDX groups. There was no sex difference found in alcohol-seeking behavior as measured by lever presses during extinction. Elevating hormone levels did not appear to impact alcohol drinking or seeking as there were no group differences in either sex. Future studies may want to further expand on this research by exploring more about baseline gonadal hormone levels in P rats, adjusting injection schedules, or assessing these hormones in different behavioral contexts. It is possible there are limited activational effects of estradiol and testosterone in alcohol-related behaviors in the P rats, but more research is needed to fully understand the impact gonadal hormones are having on these behaviors.

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behaviour in adult male rats. *Neurosci Lett, 604*, 52-57.

doi:10.1016/j.neulet.2015.07.039

Curriculum Vitae

Kari M. Haines

Education

Nov 2024	**Ph.D. in Psychology, Addiction Neuroscience**
Indianapolis, IN	Indiana University
	Advisor: Dr. Cristine Czachowski
Dec 2020	**Master of Science in Addiction Neuroscience**
Indianapolis, IN	Indiana University-Purdue University Indianapolis
	Advisor: Dr. Cristine Czachowski
May 2017	**Master of Arts in Experimental Psychology**
Towson, MD	Towson University
	Advisor: Dr. Paul Pistell
May 2015	**Bachelor of Science in Psychology**
Towson, MD	Towson University
	Minor in LGBT (Lesbian, Gay, Bisexual, Transgender) Studies

Research Experience

Biopsychology:

2018 – Present	*Research Assistant / Graduate Student*
	Indiana University-Purdue University Indianapolis
	Mentor: Dr. Cristine Czachowski
	Research Focus: Sex differences, habit learning, aversion resistant drinking, and ethanol-seeking in selectively bred rats

2016 – 2018 *Oak Ridge Institute for Science and Education (ORISE) Fellow*

US Army Medical Research Institute of Chemical Defense (USAMRICD)

Aberdeen Proving Ground – Edgewood, MD

Mentor: Dr. John McDonough

Research Focus: Identifying novel treatments for nerve agent-induced seizures. As a research fellow with a master's degree, I was trusted with coordinating several NIH-funded projects. This experience allowed me to further develop my rodent surgical, data analysis, project management, and laboratory management skills.

2013 – 2017 *Research Assistant / Undergraduate and Graduate Student*

Behavioral Neuroscience Lab, Towson University

Mentor: Dr. Paul Pistell

Research Focus: Sex differences in learning and memory in models of aging

Sexual and Gender Identity:

2014 – 2018 *Research Team Member*

Gender Identity and Sexuality Lab, Towson University

Mentor: Dr. M. Paz Galupo

Research Focus: LGBTQ family microaggressions and assessment of differences in conceptualization of sex, gender, and sexual orientation across sexual minority groups

Publications

K.M. Haines, N. Smith, C. L. Czachowski (under preparation for resubmission). Examining aversion resistant drinking in female and male alcohol preferring and non-preferring rats. *Alcohol Clinical and Experimental Research*

6) **K.M. Haines**, C. L. Czachowski (2022). Evaluating habit formation across pairs of female and male selectively bred preferring and non-preferring rats. *Alcohol.* PMID: 35500755

5) H. S. McCarren, M. Eisen, D. Nguyen, P. Dubee, C. Ardinger, E. Dunn, **K.M. Haines**, A. Santoro, P. Bodner, C. Ondeck. C. Honnold, J. H. McDonough, P. Beske, P. McNutt. (2020). Characterization and treatment of spontaneous recurrent seizures following nerve agent-induced status epilepticus in mice. *Epilepsy Research.* PMID: 32182542

4) E. Dunn, L. Matson, **K.M. Haines**, K. Whitten, R. B. Lee-Stubbs, K. Berger, H. S. McCarren, C. Ardinger, C. Jackson-Piercy, S. M. Miller-Smith, J. H. McDonough. (2020). Evaluation of fosphenytoin, levetiracetam and propofol as treatments for nerve agent-induced seizures in pediatric and adult rats. *Neurotoxicology,* PMID: 32220603.

3) L. Matson, E. Dunn, **K.M. Haines**, S. Miller-Smith, R. Lee-Stubbs, K. Whitten, C. Ardinger, H. McCarren, J. H. McDonough. (2019). Evaluation of first line anticonvulsants to treat nerve agent-induced seizures and prevent neuropathology in adult and pediatric rats. *Toxicology*. PMID: 31362008

2) **K.M. Haines**, L. Matson, E. Dunn, C. Ardinger, D. Bibi, J. H. McDonough, M. Bialer (2018) Comparative efficacy of valnoctamide and sec-butylpropylacetamide (SPD) in terminating nerve agent-induced seizures in pediatric rats. *Epilepsia.* PMID: 30615805

1) **K. M. Haines**, C. R. Boyer, C. Giovanazzi, M.P. Galupo (2018). "Not a real family": Microaggressions directed toward LGBTQ families. *Journal of Homosexuality,*

PMID: 29144852

Leadership

2021-2023	Graduate Students Out in STEM
IUPUI	*Treasurer (2021), Vice President (2022), Secretary (2023)*
2021-2022	*Co-Chair*
IUPUI	Psychology Graduate Student Diversity Committee
2018-2022	*Addiction Neuroscience Representative and Founding Member*
IUPUI	Psychology Graduate Student Diversity Committee, IUPUI
2014-2015	*Vice President*
Towson University	Psi Chi, National Honors Society in Psychology